Evolution and biogeography of *Buxus* L. (*Buxaceae*) in Cuba and the Caribbean

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Summary

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Buxus L. is the largest genus of the family *Buxaceae*, with c. 100 species distributed in all continents except Australia and Antarctica. The centres of morphological and ecological diversity of *Buxus* are the Caribbean, East Asia and Africa including Madagascar. Cuba is the territory with the highest number of *Buxus* taxa in the world. In Cuba a total of 37 species and seven subspecies of this genus occur, 95% of which are endemic. Most Cuban *Buxus* are endemic to the serpentine outcrops in east, central and west Cuba while a smaller number of species inhabits limestone areas of the island. About 50% of the Cuban *Buxus* accumulate or hyperaccumulate nickel (Ni), being the only members of the family *Buxaceae* with such ability. The adaptation to growth on serpentines and their capacity to accumulate or hyperaccumulate Ni appeared among the factors triggering the evolution of *Buxus* and other groups in the Cuban flora. For the reasons exposed above the Cuban and Caribbean *Buxus* are an appealing group for biogeographical and evolutionary studies. This study uses them as a model to address the following questions: (1) Are the Cuban and the other Caribbean *Buxus* a monophyletic group? (2) When did *Buxus* arrive in the Caribbean and when occurred the diversification of the genus on the islands? (3) What are now the closest relatives and were then the ancestors of Cuban and Caribbean *Buxus* and where did they occur? (4) Are there specific migration routes of *Buxus* in the Caribbean? and (5) Which factors have triggered the speciation of *Buxus* in Cuba?

Following a detailed introduction that summarizes our current state of knowledge and reviews the existing literature, Chapter 2 of this thesis focuses on the phylogeny and biogeography of *Buxus* including a total of 53 species of *Buxus*, where 34 species and 5 subspecies are from Cuba (c. 90% of taxa known), several also from different localities, and also species from the Bahamas, Hispaniola, Jamaica, Mexico, Panama and Puerto Rico. *Buxus* representatives from Eurasia and Africa were also included. The outgroup includes all other genera of *Buxales* and representatives of

Trochodendrales, *Proteales*, *Sabiales* and *Ranunculales*. A combined matrix of plastid sequences (*trnL-F*, *petD* and *trnK-matK*) was analysed. The results reveal the existence of three major clades within a monophyletic genus *Buxus*: an African clade, an American clade (representing the neotropics) and an Eurasian clade. In the American clade (1 PP / 100% JK) two major clades are recovered, a Mexican clade (1 PP / 58% JK) and a Caribbean clade (1 PP / 75% JK). The Mexican clade, sister to the Caribbean clade, encloses the Mexican species and a Cuban species, *B. brevipes*, from western Cuba. The rest of the Cuban and Caribbean species form a well resolved clade, in which *B. jaucoensis* is placed in a position sister to three strongly supported subclades, the "Gonoclada"-clade (1 PP / 98% JK), the "Shaferi"-clade (1 PP / 100% JK) and the "Glomerata"-clade (1 PP / 100% JK). Using a relaxed molecular clock, three calibrations points in outgroup and ingroup nodes [*Buxales-Trochodendrales*, *Proteales-Sabiales*, Eurasian *Buxus* - *Pachysandra*/*Sarcococca*/*Styloceras*] and the combined plastid data set evidence is provided that *Buxus* started to radiate in the Caribbean during the Middle Miocene and the individual subclades diversified during the Pliocene. The ancestral *Buxus* in the Caribbean was likely a non-serpentine species from eastern Cuba. The adaptation of the Cuban *Buxus* to grow on serpentines and further evolution to accumulate and hyperaccumulate Ni, likely triggered the diversification of *Buxus* in Cuba, showed through the high diversification rate in the "Gonoclada"-clade (0.78 sp. Myr^{-1}). From east Cuba, *Buxus* migrated at least twice, once reaching central Cuba and a second time covering all areas in Cuba and other regions of the Caribbean. Migrations of *Buxus* within Cuba and to other regions of the Caribbean may have been favoured by hurricanes.

Three new species of *Buxus* endemic to Sierra de Nipe and Sierra del Cristal, northeastern Cuba, are described (Chapter 3). Morphological descriptions, including pollen and leaf anatomy are provided as well as sequences of the plastid *trnK-matK* and *trnL-trnF* regions, serving as molecular descriptions. DNA characters were evaluated to find suitable states for a molecular diagnosis that complements the morphological diagnosis. Using the newly described species of *Buxus* as an example, prospects and pitfalls of DNA characters to support species diagnosis are discussed. Furthermore, an assessment of the distribution, habitat, ecology, and conservation status of the three newly recognized endemic species is provided.

Chapter 4 entails a detailed analysis of the nuclear Internal Transcribed Spacer (ITS) region in the Caribbean and Cuban species of *Buxus*. This chapter aims to reconstruct a nuclear gene phylogeny and to compare it with a plastid tree in order to explore the existence of reticulate patterns (hybridization, introgression or incomplete lineage sorting) in the evolution of *Buxus* in Cuba and the Caribbean. The sampling of this study includes the same species used for the plastid analysis. The two internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene were used as a marker system. Large amount of samples were cloned because of polymorphic sites and obviously diverging paralogues. Sequences were classified into putative pseudogenes and putative functional ribotypes after an examination of their length, Guanine-Cytosine (GC) content and the visual analysis of five sequence motifs located in ITS1 (1) and in 5.8S (4), which have been reported as conserved in functional copies in plants. Most of cloned samples yielded different paralogues, which show intra-individual polymorphism, indicating that in *Buxus* the concerted evolution in this region has not been fully operational. A high portion of ITS sequences were classified as putative pseudogenic ribotypes (71%). MP and BI analyses were conducted in a matrix of 293 accessions included all ITS sequences obtained from direct sequencing of PCR products and from cloning. Based on the aggrupation of the divergent putative pseudogenes and functional ribotypes examples of stepwise pseudogenization could be detected for species of the "Gonoclada"-clade. On the other hand the inconsistent position of the putative pseudogenic and putative functional ribotypes of *B. shaferi* could reflect saturation caused by pseudogenization. Additionally, the incongruent topology of the phylogenetic trees show that in Cuba and the Caribbean the evolution of *Buxus* has been driven by reticulate patterns. For example *B. glomerata* is involved in several events of reticulation which suggest multiple ancient hybridizations.

Chapter 5 is a study of haplotypes of two Cuban endemic species of *Buxus*, *B. foliosa* and *B. shaferi*. In the plastid phylogeny carried out in the chapter 2, accessions of these two species enclosed in the "Shaferi"-clade, were not monophyletic. Such a pattern could either be a consequence of incomplete lineage sorting or hybridization or of hitherto overlooked cryptic species that are morphologically very similar. The plastid marker *trnK-matK* was amplified and sequenced from 49 samples of these two species and a network analysis was conducted. Eight different haplotypes were found (H1 to

H8), six of them exclusive of *B. shaferi*, one exclusive of *B. foliosa* and one shared by several samples of these two species collected in the same locality. The H8 of *B. shaferi*, from the southern slope of National Park Cristal (Sierra Cristal) is quite different from the other haplotypes of this species. This difference could be caused by geographic barriers limiting the action of the disperser agents. The existence of the same haplotype in samples of *B. foliosa* and *B. shaferi* from the locality of La Melba could be caused by incomplete lineage sorting or hybridization.

The main results of the present study led to suggestions and further questions regarding the phylogeny of *Buxus* in the world and in the Caribbean area. Further phylogenetic studies in the genus *Buxus* should include representatives of the species distributed in the southeastern part of Asia.

All the reticulation patterns detected within the Cuban and Caribbean *Buxus* should be investigated. For that it would be necessary to carry out cloning strategies of the ITS even with those samples, which yielded good pherograms from direct PCR products. Moreover it would be advisable to visit the relict populations of *B. jaucoensis*, *B. gonoclada* and *B. sclerophylla* in their *locus classicus* in order to collect more individuals. These new samples should be analysed to explore if their plastid and nuclear genomes support the hypothesis about hybridization suggested in the fourth chapter of this thesis. Another suggestion is to conduct a haplotypes study including other population of species included in the “Shaferi”-clade.

Zusammenfassung

González Gutiérrez, Pedro Alejandro. 2014. Evolution und Biogeographie von *Buxus* L. (*Buxaceae*) in Kuba und der Karibik. Doktorarbeit, Fachbereich Biologie, Chemie, Pharmazie. Institut für Biologie/Botanik, Freie Universität Berlin, Deutschland.

Buxus L. ist mit ca. 100 Arten die größte Gattung in der Familie der *Buxaceae*, und ist, außer in Australien und der Antarktis, auf allen Kontinenten verbreitet. Die Zentren der morphologischen und ökologischen Vielfalt von *Buxus* sind die Karibik, Ost-Asien, Afrika sowie Madagaskar. Kuba ist weltweit das Gebiet mit der größten Anzahl an *Buxus* Taxa. In Kuba existieren insgesamt 37 Arten und 7 Unterarten dieser Gattung, von denen 95% endemisch sind. Die meisten kubanischen *Buxus* Arten sind endemisch auf Serpentinen-Aufschlüssen in Ost-, Zentral- und West Kuba, während eine kleinere Anzahl von Arten auf Kalksteingebieten der Insel vorkommen. Über 50% der kubanischen *Buxus* Arten akkumulieren oder hyperakkumulieren Nickel (Ni). Sie sind die einzigen Mitglieder der Familie der *Buxaceae* mit diesen Fähigkeiten. Die Anpassung auf Serpentinenboden wachsen zu können, und ihre Fähigkeit Nickel zu akkumulieren oder hyperakkumulieren erschienen als die Faktoren, welche die Evolution von *Buxus* und anderen Gruppen der kubanischen Flora beeinflussten. Aus diesen oben genannten Gründen sind die kubanischen und karibischen *Buxus* Arten eine attraktive Gruppe für biogeographische und evolutionäre Studien. Diese Arbeit nutzt *Buxus* als Modell um folgende Fragen zu beantworten: (1) Bilden die kubanischen und die anderen karibischen *Buxus* Arten eine monophyletische Gruppe? (2) Wann gelangte *Buxus* in die Karibik, und wann hatte sich die Gruppe auf den Inseln verbreitet? (3) Wer sind die nähsten Verwandten und Vorfahren der kubanischen und karibischen *Buxus* und wo waren diese verbrietet? (4) Gibt es spezifische Ausbreitungssrouten von *Buxus* in der Karibik? (5) Welche Faktoren haben die diversifizierung von *Buxus* auf Kuba ausgelöst?

Nach einer ausführlichen Einleitung, die den aktuellen Wissensstand zusammenfast und einem Überblick über die vorhandene Literatur gibt, konzentriert sich die Arbeit des 2. Kapitels auf die Phylogenie und Biogeographie von *Buxus* (Buchsbäume), welche insgesamt 53 Arten beinhalten, wovon 34 Arten und 5 Unterarten aus Kuba stammen (c. 90% der bekannten Taxa), etliche aus verschiedenen

Lokalitäten sowie Arten von den Bahamas, Hispaniola, Jamaika, Mexiko, Panama und Puerto Rico. Ebenfalls enthalten sind *Buxus* Vertreter aus Eurasien und Afrika. Die Außengruppe umfasst alle anderen Gattungen der *Buxales* und Vertreter der *Trochodendrales*, *Proteales, Sabiales* und *Ranunculales*. Eine kombinierte Matrix aus Plastiden Sequenzen (*trnL-F*, *petD* und *trnK-matK*) wurde analysiert. Die Ergebnisse zeigen die Existenz von drei Großgruppen innerhalb der monophyletischen Gattung *Buxus*: Eine afrikanische Klade, eine amerikanische Klade (als Vertreter der Neotropis) und eine eurasische Klade. In der amerikanischen Klade (1 PP / 100% JK) wurden zwei Hauptkladen wiedergefunden, eine mexikanische Klade (1 PP / JK 58%) und eine karibische Klade (1 PP / 75% JK). Die mexikanische Klade, Schwestergruppe der karibischen Klade, umfasst die mexikanischen Arten sowie eine kubanische Art, *Buxus brevipes* aus West-Kuba. Der Rest der kubanischen und karibischen Arten bildet eine gut aufgelöste Klade, in welcher *Buxus jaucoensis* zu drei stark unterstützten Untergruppen, der Gonoclada"-Klade (1 PP / 98% JK), der "Shaferi"-Klade (1 PP / 100% JK) und der "Glomerata"-Klade (1 PP / 100% JK) eine Schwester-Position einnimmt. Unter Verwendung eines „relaxed molecular clock"-Modells, drei Kalibrierungspunkten in den Knoten der Außen- und der Innengruppe [*Buxales-Trochodendrales*, *Proteales-Sabiales*, Euroasian *Buxus* - *Pachysandra*/*Sarcococca*/*Styloceras*], sowie des kombinierten Plastiden Datensatzes, konnte gezeigt werden, dass *Buxus* seine Radiation in der Karibik während des mittleren Miozän begann und die individuellen Unterkladen sich während des Pliozäns verbreiteten. Der Vorfahre der heutigen *Buxus* Arten aus der Karibik war wahrscheinlich eine nicht-serpentinische Art aus Ost-Kuba. Die Anpassung des kubanischen *Buxus* auf Serpentinenboden zu wachsen und die weitere Entwicklung Nickel zu akkumulieren oder zu hyperakkumulieren, war wahrscheinlich der Auslöser für die Diversifikation der *Buxus* auf Kuba, welche durch die hohe Diversifizierungsrate im "Gonoclada"-Klade (0.78 sp. Myr^{-1}) gezeigt wird. Die *Buxus* Ausbreitung von Ost-Kuba aus, fand mindestens zweimal statt, einmal erreichte es Zentralkuba und ein zweites Mal alle Bereiche Kubas und andere Regionen der Karibik. Die Ausbreitung von *Buxus* in Kuba und anderen Regionen der Karibik könnte durch Wirbelstürme begünstigt worden sein.

Drei neue Arten von *Buxus*, die endemisch in der Sierra de Nipe und Sierra del Cristal, im Nordosten Kubas sind, werden im Kapitel 3 beschrieben. Morphologische

Beschreibungen, einschließlich Pollen- und Blattanatomie werden genauso wie Sequenzen der Plastiden-Regionen *trnK-matK* und *trnL-trnF*, die als molekulare Beschreibung dienen, bereitgestellt. Geeignete molekuare Merkmale (DNA Mutationen) wurden gesucht, um die morphologische Diagnose zu ergänzen. Mit den neu beschriebenen Arten von *Buxus* als Beispiel, werden die Perspektiven und Probleme der molekularen Diagnose als Bestandteil der Artbeschreibung diskutiert. Des Weiteren ist eine Bewertung der Verbreitung, des Habitats, der Ökologie und des Gefährdungsstatus bereitgestellt.

Kapitel 4 beinhaltet eine detaillierte Analyse über die nukleäre „Internal Transcribed Spacer“ (ITS) Region der karibischen und kubanischen Arten des *Buxus*. Das Ziel dieses Kapitels ist es, eine nukleäre Gen-Phylogenie zu rekonstruieren und mit einem Plastiden-Baum zu vergleichen, um die Existenz retikulärer Muster (Hybidisierung, Einkreuzung oder „Incomplete lineage sorting“) in der Evolution des *Buxus* in Kuba und der Karibik zu erkunden. Die Probennahme dieser Studie umfasst die gleichen Arten, welche auch für die Plastiden-Analyse genutzt wurden. Als Marker-System wurden die beiden „Internal transcribed spacers“ (ITS1 und ITS2) und das 5.8S Gen verwendet. Große Mengen an Proben wurden wegen polymorpher Stellen und offensichtlich divergierenden Paralogen kloniert. Die Sequenzen wurden nach einer Prüfung ihrer Länge, ihres Guanin-Cytosin (GC) Gehalts und der visuellen Analyse von fünf Sequenz-Motiven, die sich in ITS1 (1) und 5.8S (4) befinden, welche als funktionale Kopien in Pflanzen angezeigt werden, in mögliche Pseudogene und mögliche funktionelle Ribotypen klassifiziert. Die meisten der klonierten Proben ergaben unterschiedliche Paraloge, die intra-individuellen Polymorphismus zeigten, was darauf hindeutet, dass in *Buxus* die „Concerted Evolution“ in dieser Region noch nicht voll funktionsfähig ist. Ein großer Anteil an ITS-Sequenzen wurde als mögliche Pseudogen-Ribotypen (71%) eingestuft. Maximum Parsimony und Bayessche Analysen wurden mit einer Matrix aus 293 Akzessionen durchgeführt, welche alle ITS Sequenzen mit einbezogen und durch direkte Sequenzierung von PCR-Produkten und durch Klonierung erhalten wurden. Basierend auf der Gruppierung der divergenten putativen Pseudogene und funktionalen Ribotypen, konnten Beispiele der schrittweisen Pseudogenisierung für die meisten Arten der "Gonoclada"-Klade nachgewiesen werden. Auf der anderen Seite könnte die uneinheitliche Position der möglichen Pseudogene und

funktionalen Ribotypen von *B. shaferi* die Sättigung aufgrund von Pseudogenisation darstellen. Zusätzlich zeigt die inkongruente Topologie der phylogenetischen Bäume, dass in Kuba und der Karibik die Evolution von *Buxus* von retikuläre Muster vorkommen. Zum Beispiel ist *B. glomerata* an mehreren Retikulationen beteiligt, was auf mehrfache Hybridisierungen hindeutet.

Kapitel 5 beschäftigt sich mit einer Haplotypen-Studie von zwei kubanischen endemischen *Buxus*-Arten, *B. foliosa* and *B. shaferi*. Die Plastiden Phylogenie, welche im Kapitel 2 durchgeführt wurde, zeigt, dass diese zwei Arten, welche dem „Shaferi"-Klade beigefügt werden, nicht monophyletisch sind. Ein solches Muster könnte entweder die Folge von „incomplete lineage sorting" oder Hybridisierung sein, oder von bisher übersehenen kryptischen Arten stammen, die morphologisch sehr ähnlich sind. Die Plastiden-Marker *trnK-matK* von 49 Proben dieser zwei Arten wurden amplifiziert und sequenziert und damit eine Netzwerk-Analyse durchgeführt. Acht verschiedene Haplotypen wurden gefunden (H1 bis H8), sechs davon exklusiv von *B. shaferi*, eine exklusive von *B. foliosa* und eine geteilt von mehreren Proben dieser beider Arten, welche an dem gleichen Ort gesammelt wurden. Der Haplotyp (H8) von *B. shaferi*, vom Südhang des Nationalparks Cristal (Sierra Cristal) ist ganz anders als die anderen Haplotypen dieser Art. Diese Unterschiede könnten durch geographische Grenzen, welche die Ausbreitung limitieren, begründet sein. Die Existenz der gleichen Haplotypen in den Proben von *B. foliosa und B. shaferi* aus der Ortschaft La Melba könnte durch "incomplete lineage sorting" oder Hybridisierung verursacht werden.

Die wichtigsten Ergebnisse der vorliegenden Studie führten zu Anregungen und weiteren Fragen in Bezug auf die Phylogenie von *Buxus* in der Welt und in der Karibik. Weitere phylogenetische Studien in der Gattung *Buxus* sollten Vertreter der Art, welche im südöstlichen Teil Asiens verbreitet sind, umfassen.

Alle Retikulations-Muster, die in den kubanischen und karibischen *Buxus* entdeckt wurden, sollten untersucht werden. Dafür wäre es notwendig, für ITS eine Klonierungsstrategie zu entwickeln, sogar mit den Proben, welche ein gutes Pherogramm vom direkten PCR-Produkt ergaben. Darüber hinaus wäre es sinnvoll die Reliktpopulationen von *B. jaucoensis*, *B. gonoclada* und *B. sclerophylla* in ihrem Herkunftsort zu besuchen um mehr Individuen zu sammeln. Diese neuen Proben sollten

analysiert werden, um zu erkunden, ob ihr Plastiden- und Kerngenome die Hypothese über die Hybridisierung unterstützen, welche im vierten Kapitel dieser Arbeit diskutiert wurde. Ein weiterer Vorschlag wäre, eine Studie zu anderen Hyplotypen durchzuführen, welche andere Populationen von Arten des „Shaferi“-Klade umfassen.

Contents

Chapter 1

General introduction

Contents

1.1 Exploration history of the flora of Cuba and the Caribbean

Flowering plants are the largest and most diverse group in the plant kingdom. At present they comprise approximately 320 000 species, spanning and dominating most habitats on Earth and providing the vast majority of our food crops. This diversity and importance has led to a need to understand their origin and evolution (Borsch & al. 2003; Borsch 2012).

A significant percentage of the Earth´s known terrestrial biota is distributed on the islands of the Caribbean, which are considered a biodiversity hotspot (Santiago-Valentin & Olmstead 2004; Smith & al. 2004).

The Caribbean islands have been historically called as the West Indies. They comprise three main archipelagos: the Bahamas and the Turks and Caicos Islands, the Greater Antilles and the Lesser Antilles. The Bahamas and the Turks and Caicos Islands are comprised of hundreds of islands and keys for a total approximate area of 10 070 km^2. The Greater Antilles comprise the islands of Cuba, Hispaniola, Puerto Rico and Jamaica, plus their adjacent smaller islands, keys and islets for a total approximate area of 211 108 km^2. The Lesser Antilles belong to small volcanic arc with about 21 main islands and numerous adjacent islets with an approximate total area of 8 320 km^2 (Acevedo-Rodríguez & Strong 2008, 2012).

The islands of the Caribbean harbor c. 12 000 species of native plants and c. 8 000 of them are considered endemic (Santiago-Valentin & Olmstead 2004; Oleas & al. 2013). The complex geological history of the region has offered many opportunities for dispersal and vicariance to affect biotas (Hedges 2001). However disparity exists between the knowledge of the evolution and biogeography of plants relative to animals and most ideas on the history of the Caribbean biota have emerged from faunal data (Santiago-Valentin & Olmstead 2004).

The richness of the Caribbean floras has attracted the attention of botanists and naturalists since c. 500 years ago. Botanical records for the islands of the Caribbean date from the voyages of Christopher Columbus (Howard 1979). The first written reference

to the characteristics of the Cuban flora and vegetation were made by C. Columbus during his brief stay in the coastal locality of Bariay in October 1492. In his description he mentions the exuberance of the vegetation, and the characteristics of palms and other trees in comparison with species from the old world (Esquivel-Pérez & al. 2003). Other early references to Cuban plants were made by the Spanish historian Gonzalo Fernández de Oviedo in 1535 (León 1946).

The first contributions to the knowledge of the floras of the Caribbean islands emerged from the interests of colonial governments, supported by the administrators of the Caribbean possessions of England, France, Netherlands and Spain (Boldingh 1909; León 1946; Adams 1972; Howard 1979).

Current knowledge of the Caribbean floras is credited to the work of several botanists, who in the last three centuries patiently collected, preserved and described thousands of specimens from in the Caribbean islands. Among the most mentioned of these are: H. Sloane, O. Swartz, N. J. Jacquin, M. Catesby, A. Michaux, E. F. Poeppig, A. von Humboldt, A. Bonpland, W. Houston, J. A. de La Ossa, C. Wright, A. F. A. Eggers, R. P. Duss, E. L. Ekman, J. A. Shafer, N. L. Britton, P. Wilson, I. Urban, M. Fuertes, R. F. Moscoso, J. S. Sauget (brother León), M. Victorin, A. H. Liogier (brother Alain) and R. A. Howard. Their work and of many others made possible the publication of comprehensive floras for particular islands of the Caribbean such as the Netherlands Antilles (Boldingh 1909), Bahamas (Britton & Millspaugh 1920; Correll & Correll 1982), Jamaica (Adams 1972), Lesser Antilles (Howard 1989), Hispaniola (Urban 1920–1921; Liogier 2009) and Puerto Rico (1997).

In the case of Cuba the first descriptive flora was published in the “Historia física, política y natural de la isla de Cuba” (Sagra 1856). In the twentieth century, Joseph S. Sauget et Barbier (brother León), who was a member of the religious order La Salle, began his work on the Flora of Cuba (León 1946), which was published in five volumes and a supplement by him and Henri Eugene Liogier (brother Alain). In the foreword of the third volume of the Flora of Cuba, Alain (1953) warned about the difficulties in some genera because of the existence of little known species, suggesting that it was necessary to have more material of these species in order to have a clearer concept of all of them.

Professor Johannes Bisse, from the German University of Jena, started a new era in the study of the Cuban flora. He encouraged field expeditions all over the Cuban archipelago and at the same time promoted the publication of the new Flora de la República de Cuba (Anonymous 1985). The Flora de la República de Cuba is being published thanks to the coordinated effort of Cuban and German institutions: the National Botanical Garden of Cuba, Institute of Ecology and Systematics of Cuba and the Botanical Garden and Botanical Museum of Berlin-Dahlem. A product of this successful and fruitful cooperation is a taxonomic treatment of the family *Buxaceae* (Köhler 2014) which has been published in the nineteenth volume of the new Flora of Cuba.

1.2 The family *Buxaceae* and the phylogenetic position of *Buxus*

Buxaceae Dumort. is a small family of four genera and c. 120 species distributed on all continents except Australia and Antarctica. *Buxus* is the largest genus of *Buxaceae* including about 100 species. The rest of the genera of *Buxaceae* are: *Styloceras* Kunth *ex* A. Juss. with five South American species, *Pachysandra* Michx. with three species distributed in Asia and North America and *Sarcococca* Lindl. which has 11 species in eastern Asia (Balthazar & al. 2000). One species of *Sarcococca*, *S. conzattii* (Standley) I.M. Johnston, occurs in Mexico (Johnston 1938, 1939), although its affinities with the rest of the species of *Sarcococca* have been questioned and it was excluded from the last treatment of the genus (Sealy 1986).

A recent suggestion to include *Haptanthus* Goldberg & C. Nelson, with its unique species *Haptanthus hazlettii* Goldberg & C. Nelson, within *Buxaceae* (Shipunov & Shipunova 2011) would increase the number of species and genera for this family. *Haptanthus hazlettii* was described by Goldberg & Nelson (1989) without being assigned to a family and according to Doust & Stevens (2005) it is "an enigmatic broad-leaved angiosperm of uncertain affinities" best placed as an eudicot *incertae sedis*.

The position of *Buxaceae* in the plant kingdom has been controversial (Balthazar & al. 2000). Molecular studies suggest it is sister of *Didymelaceae* Leandri and together

they have been placed in the basal eudicots (Hoot & al. 1999; Worberg & al. 2007; Soltis & al. 2011). Thorne (2007) includes the family *Buxaceae* together with *Didymelaceae* in *Ranunculidae* Takht. *ex* Reveal, order *Buxales* Takht. *ex* Reveal.

All lineages of the basal eudicots emerged during the most recent stage of the Early Cretaceous (Anderson & al. 2005). The age of *Buxales* has been estimated from 117 Million years [My] (Anderson & al. 2005) to 121.1 My (Magallón & Castillo 2009). The oldest fossils referred to *Buxales* (*Lusistemon striatus*, *Lusicarpus planatus*, *Rutihesperipites*, *Striatopollis*), have been found in Vale de Agua (Portugal), and have been dated from the Late Aptian to Early Albian, c. 112 My (Pedersen & al. 2007).

Fossils of *Buxus* consisting of well preserved fruits and leaves from the Miocene have been found in western Bohemia (Kvaček & al. 1982). No buxaceous fossil records have been found in South America or the Caribbean in paleobotanical investigations carried out in areas where *Buxus* inhabits or could have inhabited in the past, such as Colombia (Hooghiemstra & al. 2006), Puerto Rico (Graham 1996) or Cuba (Graham & al. 2000).

Within *Buxaceae* the genera *Sarcococca, Pachysandra* and *Styloceras* are closely related and their relationships are supported by morphological characteristics, such as the occurrence of two (rarely three) carpels, the lack of interstylar nectaries, a micropyle formed by both integuments, attractive stamens in male flowers and fleshy fruits (Balthazar & Endress 2002). Molecular studies have also confirmed the affinities of these three genera (Balthazar & al. 2000; Jiao & Li 2009).

Balthazar & al (2000) found that the species of *Buxus* from Africa, America and Eurasia are enclosed in three independent clades, although the support of the African clade is weak as well as the support of the node linking the American and African clades. In spite of the particular characteristics of the pollen of *Notobuxus* described by Köhler & Brückner (1982), the molecular affinities of this genus with *Buxus* have been confirmed (Balthazar & al. 2000; Jiao & Li 2009) and nowadays the few African species of *Notobuxus* should be included within *Buxus* (Balthazar & al. 2000).

Buxus is not only the largest genus of *Buxaceae*, but also the most widespread since it occurs in continental Africa, in Madagascar, in Eurasia and in the Americas. In tropical America occur c. 50 species of *Buxus*, with a center of diversity in Cuba (Köhler 2014). About 70% of the Neotropical species are distributed in Cuba, c. 95% of the Cuban species are endemic.

1.2.1 Taxonomic history of *Buxus* with emphasis on the Cuban species

Buxus was described by Linnaeus (1753) and its type species is *B. sempervirens* L. Schreber (1791) described the genus *Tricera* Schreb. and Swartz (1797) placed within this a Jamaican species, *Tricera laevigata* Sw., which was later transferred to *Buxus* (Sprengel 1826). The first division of *Buxus* into two sections, *Eubuxus* Baill. and *Tricera* Schreb., was proposed by Baillon (1859).

Tieghem (1897) considered within *Buxus* only the Eurasian species and proposed the transference of the African species to three genera, *Buxanthus* Tiegh., *Buxella* Tiegh. and *Notobuxus* Oliv. The genus *Notobuxus* had been described by Oliver (1882). Tieghem (1897) also stated that the Antillean species of *Buxus* constituted a homogeneous group different from the other species from Eurasia and Africa and considered all them to be within the genus *Tricera*. This classification of Tieghem (1897) was mostly supported by morphological characteristics such as the presence/absence of meristems in the stem, presence/absence of fibrous fascicles in the petiole and presence/absence of nectaries.

Mathou (1940) suggested that *Tricera*, *Austrobuxus* Miquel, *Buxanthus* and *Buxella* should be retained as sections or subsections within *Buxus*, and proposed a new classification consisting in sections and subsections taking into account the characteristics of inflorescences, flowers and meristems. The new classification proposed by Mathou (1940) consisted in four sections and four subsections. The four sections are: *Eubuxus*, *Austrobuxus*, *Tricera* and *Probuxus* Mathou. The section *Eubuxus* encloses all the Euroasian species and most of those from Oceania. Within *Eubuxus* she included the subsections *Sessiliflorae* Mathou and *Pedicellatae* Mathou.

The section *Austrobuxus* only includes the species *B. nitidus* Hallier f. from Sumatra. In the section *Tricera* are included all the American species. The section *Probuxus* includes all the African species and is divided into two subsections, *Buxanthus* and *Buxella*. Mathou (1940) also accepted *Notobuxus* as a different genus of *Buxaceae*.

In a taxonomic work of the Asiatic species, Hatusima (1942) proposed a new section, *Eugeniobuxus* Hatusima, described new species, subspecies and varieties.

Friis (1989) proposed a new classification for the African *Buxus* consisting in three sections: *Buxella*, *Notobuxus* and *Tricera*. Within the section *Notobuxus* he included four African species, within *Buxella* he included four other species of southern Africa and Madagascar and in the section *Tricera* he placed *B. hildebrandtii* Baill., taking into account the similarities of this species with Caribbean species, specifically regarding its venation and pollen. Friis (1989) also considered *Buxanthus* as a synonym of *Tricera*.

Most of the Cuban species of *Buxus* were described by Grisebach (1860, 1865) and Britton (1915). The species published by August Heinrich Rudolf Grisebach and Nathaniel Lord Britton were originally placed in the genus *Tricera* and transferred later into *Buxus* by Urban (1908, 1923, 1925). Other changes and corrections were later published by Alain (1969).

During the second half of the twentieth century collaboration between Cuban and East-European botanic institutions was formed and this ushered in a new era in the history of Botany in Cuba. During this period the professor Johannes Bisse, from the University of Jena, and a young team of Cuban and European botanists (most of them from East Germany) carried out expeditions to localities in all the Cuban provinces to collect new plant material and rewrite the Flora of Cuba. Thousands of specimens were collected which led to the discovery and publication of new species. Borhidi & Muñiz (1973, 1977) published three new species of *Buxus* for Cuba. Between 1982 and 2013 (Köhler 1982, 1998, 2006; González-Gutiérrez & al. 2013), 12 new species were described. In the last revision of *Buxus* for Cuba (Köhler 2014), 37 species and seven subspecies are reported to the Cuban archipelago. All of these taxa, except *B. bahamensis* Baker and *B. glomerata* Müll. Arg., are considered endemic to Cuba. Two

Cuban species, *B. cubana* Baill. and *B. vaccinioides* (Britton) Urb., are only known through type specimens. Most of Cuban species of *Buxus* are known from unique populations or have been collected in one or few localities (Köhler 2014).

Worldwide many species of *Buxus* are considered rare or endangered (Adams 1972; Carrero-Rivera 2001; Schatz & Lowry 2002; González-Oliva & al. 2004). In Cuba, 42% of *Buxus* species are considered threatened (Berazaín-Iturralde & al. 2005).

In the National Botanical Garden of Cuba a living collection of Cuban *Buxus* has been established (Rankin-Rodríguez & al. 1999; Köhler 2001). This collection has allowed the conservation *ex situ* of c. 30 Cuban taxa and makes possible the study of them under controlled conditions. In this collection, the taxa collected in serpentines and specifically the nickel hyperaccumulators are difficult to maintain due to their specific ecologic requirements.

1.2.2 Characteristics of *Buxus* with emphasis on the Cuban species

The species of *Buxus* are monoic, evergreen shrubs or small trees, densely branched with opposite leaves. The leaves are shortly petiolated, leathery or thinly leathery, with bracts, with venation pinnate and margin entire, and in some species, also revolute.

In Cuba *Buxus* has a wide morphological diversity. This diversity includes small shrubs like *B. revoluta* (Britton) Mathou and *B. foliosa* (Britton) Urb., which normally grow less than 1–1.5 m high (Köhler, 2014), and also trees, 3–7 m high, like *B. koehleri* P. A. González & Borsch. There are species with large leaves such as *B. crassifolia* (10–12 cm long, 4–5 cm wide) and also species with extremely small leaves (1–1.5 cm long, less than 1 cm wide) such as *B. foliosa*, *B. revoluta* and *B. wrightii* Muell. Arg. In Cuba most species of *Buxus* have coriaceous leaves with margin revolute (Köhler 2014; Fig. 1.1 A).

In *Buxus* the inflorescences are axillar or terminal, racemose, with a terminal female flower surrounded by few or several lateral masculine flowers. The presence of

tepals instead of sepals and petals is a characteristic that *Buxus* shares with other basal angiosperms (Hansen & al. 2007). In Cuba and the Caribbean several species have small and inconspicuous green tepals like *B. revoluta* and *B. bahamensis* Baker, and other have relatively big and white tepals like *B. marginalis* (Britton) Urb. (Fig. 1.1 A, B, C). The feminine flowers have 6 tepals in two series. In some species the nectaries are well developed (Fig. 1.1 D) while in others these are rudimentary (Köhler 2014). The masculine flowers usually have a pedicel and 4 tepals in two series; the ovary has three locules and three separated styles (Köhler 2014).

The fruit of *Buxus* is an explosive capsule with the inner layer of pericarp separating from the outer layer and with three persistent horn-like styles (Fig. 1.1 E). *B. macrocarpa* Capuron, a Malagasy species, has large fruits, 20–30 mm long, with a 5–6 mm thick and fleshy endocarp (Schatz & Lowry 2002). In the Neotropics and specifically in Cuba the capsule of *Buxus* does not exceed c. 1 cm in length and can be even smaller (Köhler 2014). The capsules of *B. excisa* Urb. are angulose, in *B. bissei* Eg. Köhler have longitudinal ribs and in *B. brevipes* (Britton) Urb. are rugose (Fig. 1.1 F, G, H). The seeds of *Buxus* are trigonous, shiny black, with a fleshy and oily endosperm, the cotyledons are oblongate, thin and flat (Alain 1953; Köhler 2014).

The pollen of *Buxus* is 3–6-zonocolporate, 5–15-pantocolporate o 12–40-pantoporate (Köhler, 2014). Palynological studies show a high morphological diversity mostly in the African and Neotropical species (Köhler 1979, 1981; Köhler & Brückner 1982, 1989, 1990; Brückner 1993). These authors suggested evolutionary patterns from few-porate pollen grains to pantoporate forms (Köhler & Brückner 1990).

The basic chromosome number in *Buxus* is n = 14, and has been confirmed in some Cuban species such as *B. brevipes*, *B. leivae* Eg. Köhler, *B. triptera* Eg. Köhler and *B. yunquensis* Eg. Köhler (Köhler 2006, 2014). Investigations on cultivated species have shown the existence of triploid cultivars of *B. sempervirens* and *B. microphylla* Siebold & Zucc., and tetraploid cultivars of *B. hyrcana* Pojark., *B. harlandii* Hance and *B. microphylla* (Laere & al. 2011). The size of the plastid genome of *B. microphylla* is 159 010 bp, which is also rich in Adenine (A) + Thymine (T) [Hansen & al. 2007].

Fig. 1.1. Morphological variability of *Buxus* in Cuba. A- detail of the leaves, tepals and capsule of *B. revoluta* (Yamanigüey, Cuba); B- detail of leaves and flowers of *B. bahamensis* (Gibara, Cuba); C- flowers of *B. marginalis* (National Botanical Garden of Cuba); D- feminine flower with bulky nectaries of *B. gonoclada* ssp. *gonoclada* (Motembo, Cuba); E- open capsule of *B. marginalis* (National Botanical Garden of Cuba); F- capsule of *B. excisa* (Baracoa, Cuba); G- capsule of *B. bissei* (National Botanical Garden of Cuba); H- capsules of *B. brevipes* (National Botanical Garden of Cuba). Photographs by Kurt Zoglauer (A, F), Pedro A. González-Gutiérrez (B, D, G) and Rosa Rankin-Rodríguez (C, E, H). Scale bars = c. 1 cm.

1.2.3 Distribution, habitat and ecology of *Buxus* with emphasis on the Cuban species

Worldwide there are about 100 species of *Buxus* (Köhler 2014). In Africa and Eurasia there are c. 50 species, 9–10 of them on the African continent (Köhler & Brückner 1982; Friis 1989), nine in Madagascar and the Comoros Islands (Schatz & Lowry 2002), 17 species in China (Tianlu & Brückner 2008) and about 10–15 species grow in other regions of Eurasia such as western and southern Europe, the Caucasus, Korea, Japan, Iran, Pakistan, south Asia, Indonesia and the Philippines.

About 50 species of *Buxus* grow in tropical America. Of these 37 species and seven subspecies grow in Cuba (Köhler 2014), 4–5 species grow in Mexico, one of the Mexican species spreads into Guatemala and El Salvador, 4–5 grow in Jamaica (Adams 1972), one species grows in the Bahamas [*B. bahamensis* Baker (Correll & Correll 1982)], which also grows in Cuba and in the Cayman islands (Proctor 1984), one species grows in Hispaniola [*B. glomerata* Müll. Arg. (Liogier 1986)], which also occurs in Cuba, two species are endemic in Puerto Rico (Liogier 1988), one in Martinique (Howard 1989), and one in northern South America from Suriname to Panama (Gentry 1978).

The *Buxus* species occupy a wide diversity of habitats. Most species inhabit tropical areas of America, Africa and Asia, and a few grow in extratropical regions of Europe and Asia. The northern most species of *Buxus* are the European *B. serpervirens* and the Korean-Japanese *B. microphylla*. The common box, *B. sempervirens*, is widely present throughout southern and western and central Europe, north Africa and western Asia, usually in mesophyllous forests, mixed with deciduous species or forming pure populations (Roselló & al. 2007).

In Madagascar *Buxus* inhabits bushes and thickets, deciduous forests, sub-humid to montane evergreen forests and humid forests on diverse soils such as limestone and laterites (Schatz & Lowry 2002).

In continental south Asia, in some Indonesian islands and in the Philippines *Buxus* grows on a wide range of tropical types of vegetation, such as thickets, forests at low and high altitudes, in valleys and riversides (Merrill 1923; Tianlu & Brückner 2008; Backer & Brink 1965; Julius 2014).

The distribution of *Buxus* in continental America is not continuous. The genus is distributed in Mexico, Guatemala and El Salvador and also from Panama to Suriname, but there are no species of the genus in Honduras, Nicaragua and Costa Rica. *B. citrifolia* has been found in forests on limestone, c. 300 meters above sea level in Colombia (herbarium specimen: *Gentry & Cuadros 60117 MO*). This species inhabits humid forest areas near rivers in Panama (Carmen Galdames, Smithsonian Tropical Research Institute of Panama, personal communication). The Mexican species grow in dry and humid ecosystems. The most isolated Mexican species is *B. pubescens* Greenm., which is endemic to the Tres Marías archipelago in the Pacific Ocean (herbarium specimen: *Chiang & Flores 1131 IEB*). *B. mexicana* Brandegee grows in the south-central part of Mexico in thickets on limestone 2000–2200 meters above the sea level (herbarium specimen: *Tenorio 11206 MEXU*). Other two Mexican species, *B. bartlettii* Standl. and *B. moctezumae* Eg. Köhler, R. Fernández & Zamudio, grow close to basins of rivers and streams, 130–190 meters above sea level (Egon Köhler, personal communication).

A large number of Cuban species inhabit the commonly called “cuabales” and “charrascales”, which are dry lowland xeromorph serpentine shrubwoods and semiarid montane serpentine shrubwoods, respectively, according to classifications of Cuban vegetation (Capote & Berazaín-Iturralde 1984; Borhidi 1996). A smaller number of Cuban species grow on vegetation on limestone along the coasts or inland (Köhler 2014). In Cuba, *Buxus* is represented in the three phytogeographic subprovinces, which are defined according to the criteria of Borhidi (1996) [Fig. 1.2]. In the phytogeographic subprovince of west Cuba grow two species and a subspecies, in the phytogeographic subprovince of central Cuba grow other two species and a subspecies and in the phytogegeographic subprovince of east Cuba grow 34 species and five subspecies.

The Cuban *Buxus* growing on serpentine outcrops spread into the three Cuban phytogeographic subprovinces. In each of the most important Cuban serpentine outcrops

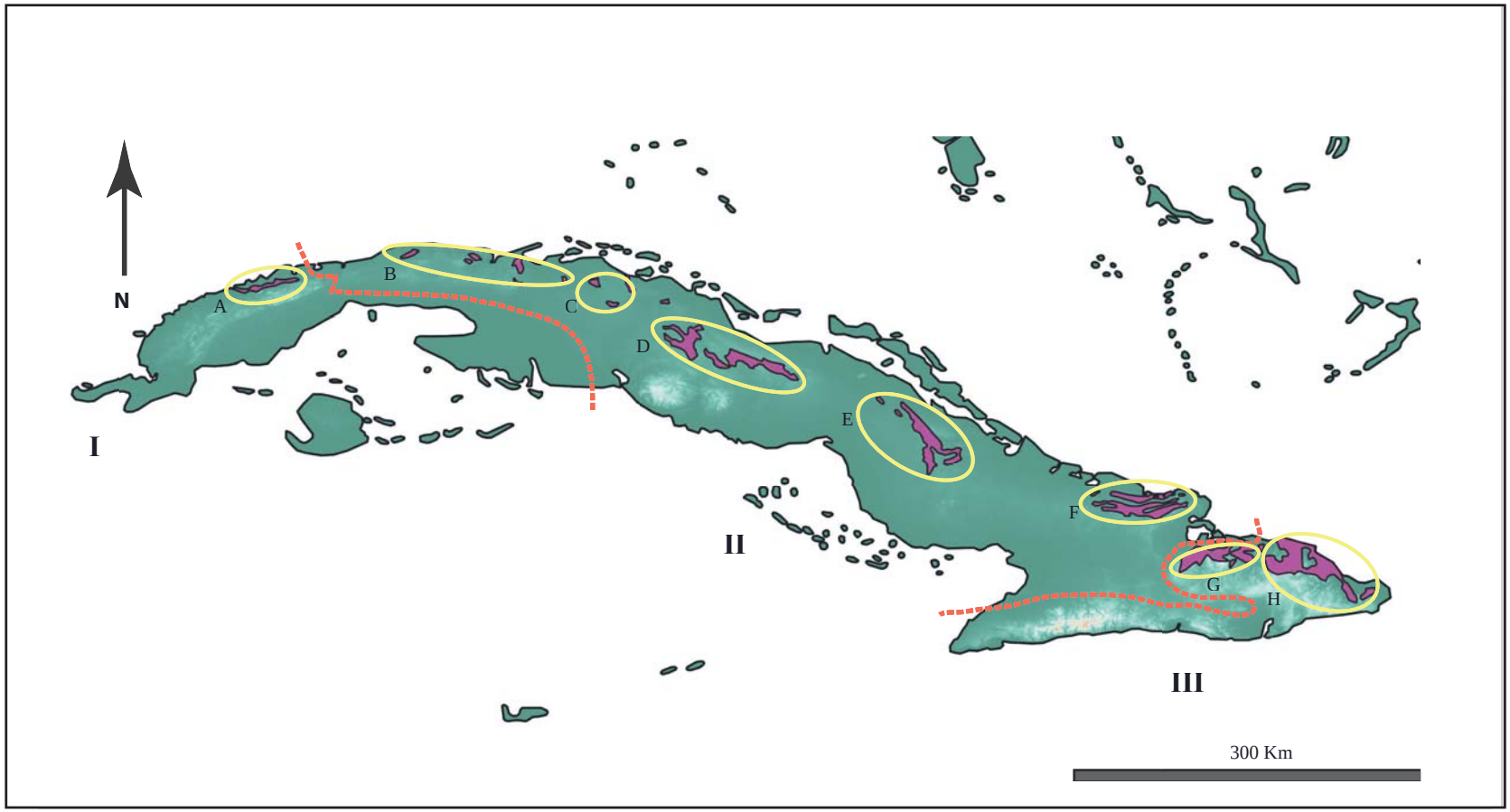


Fig. 1.2. Map of Cuba with a schematic representation of the three Cuban phytogeographic subprovinces (delimited by discontinuous red lines): west Cuba (I), central Cuba (II) and east Cuba (III). The most important serpentine outcrops are shown within yellow ovals: A- Cajálbana, B- Havana-Matanzas, C- Motembo, D- Santa Clara, E- Camagüey, F- Holguín, G- Sierra de Nipe-Sierra Cristal, H- Moa-Baracoa. The serpentine outcrops of Cuba have been adapted from Anonymous (1978).

grow at least one species or subspecies of *Buxus*, except in the serpentines of Camagüey, in which spite of its big area no species of the genus have been ever recorded.

In the phytogeographic subprovince of west Cuba, *B. wrightii* Müll. Arg. ssp. *wrightii* and *B. wrightii* ssp. *leonii* (Britton) Eg. Köhler, grow in thickets on serpentines, occasionally along the basins of rivers and streams of the Plateau of Cajálbana (Köhler 2014). *B. gonoclada* (Wright *ex* Griseb.) Müll. Arg. ssp. *gonoclada* grows in serpentine outcrops of the phytogeographic subprovince of central Cuba from Havana-Matanzas until the serpentines of Holguín (Köhler 2014).

In the mountains of Sierra de Nipe, Sierra Cristal and massif of Moa-Baracoa in the northeastern region of Cuba, grow c. 85% of all Cuban species of *Buxus* (Köhler 2014). In this region the *Buxus* species inhabit thickets on serpentines and rainforests. *B. acunae* Borhidi & Muñiz and *B. revoluta* are endemic to Yamanigüey (Fig. 1.3) and *B. pilosula* Urb. ssp. *pilosula* grows on the serpentine outcrops of Sierra de Nipe. Other species inhabit the understorey of the rainforests or the so called gallery forest on serpentines, very frequently near to the basin of mountain rivers and streams, among stones and rocks. Among the most common species in these habitats are *Buxus marginalis* (Britton) Urb. and *Buxus foliosa* (Britton) Urb. (Fig. 1.3). The serpentines of eastern Cuba are rich in nickel (Ni), iron (Fe), cobalt (Co) and other heavy metals. About 50% of the Cuban *Buxus* are Ni hyperaccumulators (Reeves & al. 1996; Berazaín-Iturralde 2004), only outnumbered by the Cuban species of *Leucocroton* and *Phyllanthus* (Reeves & al. 1996).

B. bahamensis and *B. glomerata* are the most common species growing in the Cuban coastal ecosystems. *B. bahamensis* grows on coastal thickets in the Bahamas (Correll & Correll 1982), in the Cayman Islands (Proctor 1984), and along the northern coast of Cuba (Köhler 2014). *B. glomerata* occurs in west, central and east Cuba, on coastal thickets on limestone, on inland hills of limestone and also a small population of c. 15 individuals grow on a serpentine outcrop of Bahía de Naranjo in the province of Holguín. In the island Hispaniola this species grows in vegetation on limestone and on serpentine outcrops (Liogier 1986).

Fig.1.3. A- Thickets on serpentines in Yamanigüey (Moa, Holguín), habitat of *B. acunae* and *B. revoluta*. B- Riverine vegetation along the way to La Melba (Moa, Holguín), habitat of *B. foliosa*.

B. sclerophylla Eg. Köhler grows on coastal thickets on limestone and *B. jaucoensis* Eg. Köhler grows on limestone cliffs. Both of these grow in the southeastern province of Guantánamo (Köhler 2014).

The Cuban species of *Buxus* are represented by isolated populations and for some species such as *B. aneura* Urb., *B. acunae*, *B. jaucoensis* and *B. sclerophylla* only a single population of a few plants is known. *B. shaferi* could be cited among the few exceptions showing an almost continuous distribution pattern in northeastern Cuba, from Sierra Cristal to the mountains of Moa and Baracoa (Köhler 2014).

In Puerto Rico, *B. vahlii* Baill. grows in dry forests on limestone (Carrero-Rivera 2001) and *B. portoricensis* Alain has been collected on serpentines and in vegetation on limestone (Liogier 1988). The endemic Jamaican species grow in vegetation on limestone between 500 and about 1000 meters above sea level (Adams 1972).

The reproduction biology of *Buxus* has been only studied in European species. It is known that *B. balearica* is ambophilous; it is pollinated by both wind and insects, although wind is the main vector dispersing the pollen long distances (Lázaro & Traveset 2005; Lázaro & Traveset 2006; Rosselló & al. 2007). May be other species of *Buxus* are ambophilous but there is no published information available. According to Egon Köhler (personal communication) the flowers of *Buxus* in Cuba are likely pollinated by very small insects that are not capable of flying long distances. During field work in Cuba, three different species of insects have been seen visiting the flowers of *Buxus*. The common bee (*Apis mellifera*) and a fly (aff. *Tachinidae*, *Diptera*), were seen in the flowers of *B. nipensis* Eg. Köhler & P. A. González, in the locality Río Piloto, Mayarí, province of Holguín (Fig. 1.4). The flowers of *B. bahamensis* have been seen visited by ants (aff. *Dolichoderinae*, *Hymenoptera*) in Gibara, province of Holguín, Cuba (Fig. 1.4). In Puerto Rico *Apis mellifera* and a species of the genus *Dolichoderus* have been seen visiting the flowers of *B. vahlii* (Carrero-Rivera 2001).

The capsules of *Buxus* are dehiscent (Fig. 1.1 E) and eject their seeds not far from the mother plant (Rosselló & al. 2007). Ants have been identified as predators and secondary dispersers of the seeds of *B. balearica*; however when they act as dispersers

the seeds are not dispersed long distances from the source plant (Lázaro & al. 2006; Rosello & al. 2007).

Some species grow close to rivers and streams, including a large percentage of Cuban species (Köhler 2014) and in these cases water could be an important vector of dissemination. The extreme meteorological events and specifically the hurricanes have been signalled among the most important dispersers of the Caribbean biotas (Borhidi 1996). The strong winds of the hurricanes and the frequent overflows of rivers after their impact could be among the most important dispersers of *Buxus* in the Caribbean region. The strong winds accompanying hurricanes could transport and disperse branches or whole plants of *Buxus* bearing fruits and seeds.

1.2.4 The economic botany of *Buxus*

Buxus is commonly used as an ornamental plant. The most cultivated species for this purpose are *B. sempervirens* and *B. microphylla*, although other species like *B. balearica*, *B. harlandii* Hance and *B. hyrcana* Pojark. are planted in some gardens. *Buxus* have been also used in the manufacture of musical instruments and for ritual, religious or medicinal purposes (Trier & Hermans 2007).

The genus *Buxus* is a rich source of alkaloids (Matochko & al. 2010). Steroidal alkaloids have been isolated from the roots of *B. sempervirens* and the leaves of *B. longifolia* (Rahman & al. 1992). An alkaloid isolated from *B. sempervirens* has antimycobacterial properties which have been proved effective against *Mycobacterium tuberculosis* (Tosun & al. 2004). *B. hildebrandtii* has antiviral elements, which have been proved effective against the Type A Influenza virus (Mothana & al. 2006). *B. hyrcana* has proven antimalarial properties (Esmaeili & al. 2009). Triterpenoidal alkaloids with acetylcholinesterase inhibitory properties have been isolated from *B. natalensis* (Oliv.) Hutch. (Matochko & al. 2010). *B. microphylla* is used for the treatment of cardiovascular diseases and to control hypertension (Yan Y-X & al. 2010).

In Cuba and the Caribbean no known practical use has been reported for *Buxus*; however some of the species of this region could be used as ornamental plants.

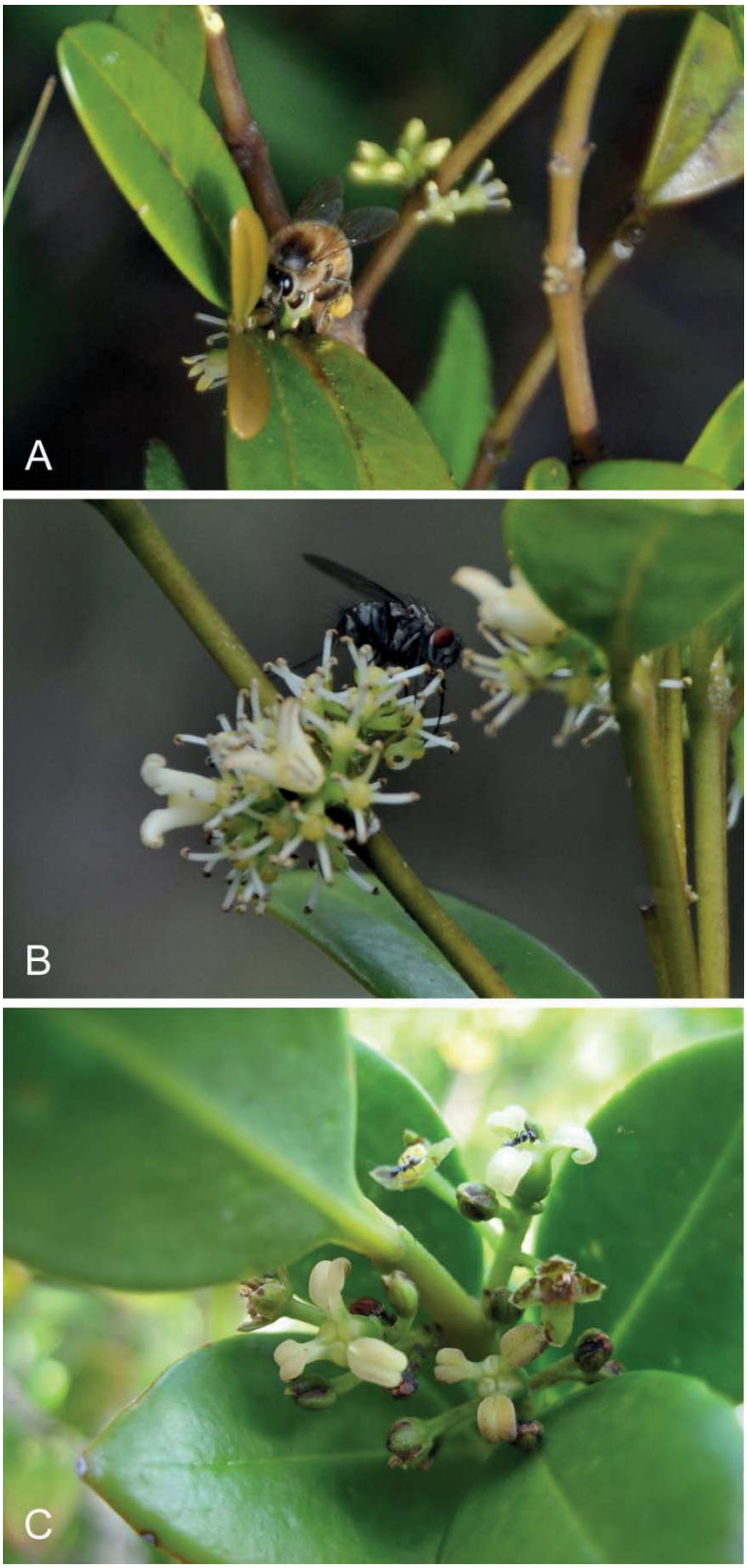


Fig. 1.4. A- Flowers of *B. nipensis* being visited by a bee (A). B- Flowers of *B. nipensis* being visited by a fly. C- Flowers of *B. bahamensis* being visited by ants. Photographs by Kurt Zoglauer (A and B) and Pedro A. González-Gutiérrez (C).

1.2.5 Current state of knowledge on *Buxaceae* with emphasis on the Cuban *Buxus*

In the last twelve years, three comprehensive systematic treatments of *Buxus* have been published, for Madagascar and the Comoros islands (Schatz & Lowry 2002), for China (Tianlu & Brückner 2008) and for Cuba (Köhler 2014). Nomenclatural changes and new species have been published for Malaysia and Thailand (Julius 2014; Soh & al. 2014). In Africa the most complete taxonomic work currently is the synopsis by Friss (1989).

The recent publication of *Buxaceae* for the Cuban flora (Köhler 2014) is the result of an extensive study carried out during about 40 years by Prof. Dr. Egon Köhler, who has undertaken palynological studies (Köhler 1979, 1981; Köhler & Brückner 1982, 1989, 1990), studies focused on the leave's nervature (Köhler 1984) and on the morphology and anatomy of the leaves (Köhler & Schirarend 1989).

Köhler (1981) investigated and described the pollen morphology of the Neotropical species of *Buxus* and placed them into eight different groups according to size, number of apertures and architecture of the exine. Köhler & Schirarend (1989) carried out a study of the anatomy of the leaves of Neotropical species of *Buxus* and proposed six morphologic groups. These authors also mention that there is evidence of parallel evolution in different small leaved groups of species.

Balthazar & al. (2000) conducted a molecular phylogenetic study of *Buxaceae* based on the nuclear DNA marker ITS (Internal Transcribed Spacer) and the plastid DNA marker *ndhF*. In their sampling these authors included 26 species of *Buxaceae*, 13 of which were representatives of *Buxus*. The most important results of their research were that the representatives of *Pachysandra* and *Sarcococca* were enclosed in two strongly supported clades and that *Buxus* is paraphyletic with *Notobuxus*. They also found that the species of *Buxus* are enclosed in three clades: an African clade, an American clade and a Eurasian clade. The study of Balthazar & al. (2000) is so far the most important published investigation of the molecular phylogeny of *Buxaceae*.

Roselló & al. (2007) carried out a study of ITS in isolated populations of *B. sempervirens* and *B. balearica*. They found intra-individual polymorphisms which suggest that in these species the concerted evolution has acted slowly.

Jiao & Li (2009) investigated the phylogeny and biogeography of the genus *Pachysandra* using *ndhF* and ITS. They also estimated the divergence time between *Styloceras* and *Pachysandra*, which resulted 23.5 ± 8.1 My.

The genetic diversity and relationships within a group of Eurasian *Buxus* was studied using AFLP (amplified fragment-length polymorphism), genome size analysis and chromosome counts (Laere & al. 2011). In this work an extensive discussion about the relationships among cultivars of *Buxus* is offered and the occurrence of hybrids between cultivated *Buxus* is documented.

1.3 Paleogeography of the Caribbean and the origin of Cuba

The paleogeographic history of the Caribbean and the origin of the islands of this region have been motive of discussion by several authors (Coney 1982; Draper & al. 1994; Coleman & Alexander 2004; Iturralde-Vinent 2006; Ali 2012). Iturralde-Vinent (2006) synthesises his paleogeographic and biogeographic findings about this topic in the Caribbean region.

The transformation of the Caribbean over time has created barriers as well as paths for the migration of the biotas. At the end of the Cretaceous (c. 70–65 Million years ago [Mya]) there was a maxim of emerged territories. In that time the future Greater Antilles were located between North America and South America and paleontological data support the exchange of terrestrial tetrapods between North America and South America. The impact of Chicxulub, 65 Mya, contributed to the extinction of most of the terrestrial and marine biota in the Caribbean. During the transition from the Palaeocene to the Eocene, 65–37 Mya, the emerged territories were not common and it is supposed that the future Greater Antilles were a group of isolated islands separated by marine channels of deep and shallow waters (Iturralde-Vinent 2006).

The Greater Antilles emerged in the Middle Eocene, c. 49 million years ago. The islands forming the archipelago of the Greater Antilles arose from the sea, except for portions of Cuba which were break-away fragments from the continent (Iturralde-Vinent 1981; Coney 1982), specifically from the Yucatan block (Perfit & Williams 1989). According to Draper & al. (1994) these islands were never connected to any continent but were available for colonization by biotic elements from the continents (Graham 2003; Iturralde-Vinent & MacPhee 1999). Iturralde-Vinent (2006) pointed out that during the Eocene-Oligocene transition (c. 35–33 Mya), a land bridge called GAARland, connected northern South America with the former Greater Antilles.

Borhidi (1996) mentioned that during the the "land bridge phase" (end Oligocene-end Pliocene), the Caribbean became connected to the continent via Honduras and Yucatan. The studies of Iturralde-Vinent (2006) do not support the existence of such connections.

Iturralde-Vinent (2006) suggested that in the Upper Oligocene sea levels rose up and terrestrial areas diminished. From the Miocene the emerged areas of land were isolated from one another in part because of the lateral drift of the Caribbean plate. In the Miocene-Pliocene the Caribbean plate continued its movement to the east. During the Early to Middle Miocene the former Cuban archipelago was composed by at least five emerged territories (see maps in Iturralde-Vinent 2006). During periods of low sea levels (glacial periods) there were short inhabited land connections among the islands but these did not connect the islands with the continents and were not as important as the connections of the Eocene-Oligocene periods for exchanges of biota (Iturralde-Vinent 2006).

Debates around hypotheses on the paleohistory of the Caribbean continue today and the hypothesis on the existence of GAARland has been also criticized (Ali 2012).

1.4 General characteristics of the current vegetation and flora of Cuba

The most recent classifications of vegetation in the Cuban archipelago (Capote & Berazaín-Iturralde 1984, Borhidi 1996) report a wide variety of forests, thickets,

herbaceous communities, complexes of vegetation and secondary vegetation. The distribution of these vegetation types is closely related to geologic, edaphic, geographic and climatic factors in Cuba.

Mangrove forests occur in the low littoral areas of the archipelago, mostly in river deltas of the southern coast and in swamps such as the Zapata swamp. Evergreen forests are common in coastal zones and in karstic elevations. Semi-deciduous forests occupy the plains of western, central and eastern Cuba and nowadays only isolated patches of these remain, showing high levels of antropization. Rainforests occupy areas in the mountains, mostly in east Cuba in zones with high levels of rainfall which are rich in endemic species (Martínez-Quesada 2009). Pine forests occur in western and eastern Cuba. Pine forests in western Cuba grow on white sands and slates areas whereas pine forests in eastern Cuba grow commonly on soils derived from ultramafic rocks (in the mountains of Nipe-Sagua-Baracoa), on soils derived from volcanic rocks (in the Sierra Maestra) and on limestone (in the region of Montecristo, province of Guantánamo). Thickets include the xeromorphic coastal thickets on limestone and the xeromorphic shrubwoods on serpentines ("charrascales" and "cuabales"). The "charrascales" and "cuabales" are rich in xerophytic and thorny shrubs and are the habitat of c. 920 endemic species of plants (Berazaín-Iturralde 2004).

Cuba has one of the richest insular floras in the world (Borhidi 1996; Santiago-Valentin & Olmstead 2004). In the Cuban archipelago occur about 6 850 species of vascular plants, c. 500 of which are pteridophytes and c. 6 350 are phanerogamic plants. One of the most relevant characteristics of the flora of Cuba is its high endemism, since 51.3 % of the phanerogamic Cuban flora is endemic. About 60% of Cuban species of plants are trees or shrubs and the remaining 40% are herbaceous species (Borhidi 1996). The flora of Cuba has 63 endemic genera (Berazaín-Iturralde 2008) mostly of *Asteraceae* (17 genera) and *Rubiaceae* (12 genera). Besides the high endemism, other remarkable features of the Cuban flora are the occurrence of disjunctions, vicariances, relicts and vulnerable taxa (Borhidi 1996).

In Cuba the distribution of the endemics is not homogeneous. Endemics are absent or poorly represented in mangrove forests and halophyte communities. Dry habitats on limestone and serpentine areas are rich in endemics. According to Borhidi

(1996) main factors influencing speciation in Cuba are the isolation, the insularity, the alternation of plains and mountains, the presence of serpentines and other ultramafic rocks, karstic rocks, acid white sands and slates. This author also mentioned that the mosaic of vegetal communities, alternation of humid and dry periods, mutagenic speciation, hybridogenic speciation, introgressions, and genetic drift could have played an important role in the speciation of Cuban plants groups.

In Cuba there is a mix of Laurasian and Gondwanan elements, although the second group is the most abundant. Among the Laurasian genera *Fraxinus*, *Clematis, Quercus* and *Salix* stand out, all represented by one species. On the other hand there is good representation of Gondwanan families such as *Annonaceae*, *Bignoniaceae* and *Sapotaceae*.

1.5 Phytogeography of Cuba

Chronologically, among the most important contributions to the phytogeography of Cuba are those by Alain (1958a, 1958b), Samek (1973), Berazaín-Iturralde (1976) and Borhidi (1996). The most extensive work about the Cuban phytogeography is that published by Borhidi (1996), which is based on critic revision of previous works and on personal studies of this author.

An important point conditioning the endemism in Cuba is the presence of ultramafic rocks and in general the diversity of geologic and edaphic conditions (Samek 1973; Borhidi 1996).

The Cuban phytogeography is distinguished by the polarity, due to the presence of several endemics on both extremes of the island, east and west, whereas the central region is relatively poor in endemics (Samek 1973). The diversity of natural conditions (geologic, edaphic and climatic) in western and eastern Cuba, contrast to the uniformity of these conditions in central Cuba. In Cuba differences among conditions between neighbouring habitats in some cases vary greatly (e.g. serpentine and limestone) and demand from the plants an extensive specialization (Berazaín-Iturralde 1976).

Borhidi (1996) considered Cuba a province within the Neotropical floristic kingdom based on its floristic richness and the high number of endemics in the island. At the same time like other authors (Alain 1958a, 1958b; Howard 1973; Samek 1973) he acknowledged the close relationship between the Cuban flora and the rest of the Antillean flora. Within Cuba he considers three phytogeographic subprovinces: west, central and east Cuba (Fig. 1.2); each of them with respective subdivisions for phytogeographic sectors, subsectors and districts.

Borhidi (1996) explained the development of the Cuban flora and vegetation in three main phases: "plate phase", "land bridge phase" and "archipelago phase". He stated that some primitive angiosperms (e.g. *Magnolia*, *Guatteria*) were present in Cuba from the "plate phase". During the "land bridge phase" (end Oligocene–end Pliocene) the Caribbean plate started to emerge and became connected to the continent via Honduras and Yucatan and later by the Lesser Antilles, and this is the period of large scale immigration of species into Cuba. Several genera migrated during this phase (e.g. *Bursera*, *Swietenia*, *Trichilia*).

The "archipelago phase" (end of Miocene to the current period) is characterized by the formation of stretches that separated Cuba, Hispaniola and Jamaica. In this time the Cuban flora and fauna were subjected to severe climatic and geological changes and internal migrations of taxa occurred (Borhidi 1996).

It is necessary to point out that Iturralde-Vinent (2006), in his synthetic work about the palaeography of the Caribbean did not mention any connection of Cuba with the continent via Honduras or other criteria supporting the hypothesized three phases mentioned by Borhidi (1996).

Borhidi (1996) postulated that the serpentine vegetation of "charrascales", pine-oak woodlands, coniferous-laurel forests, and the pine-*Dracaena* forests of Cuba are relicts left over from the southern portion of the Madrean-Tethyan vegetation and that the immigration of its sclerophyllous flora took place during the Early and Middle Miocene.

Migration paths via winds, hurricanes and oceanic currents between Africa, America and the Caribbean were suggested by Graham (2006a, 2006b). Main floristic migrations patterns within Cuba have been also proposed. The most important migrations of the serpentine flora occurred from east to central and west Cuba, while the migrations of the limestone flora mostly occurred from west to central and east Cuba (Samek 1973; Berazaín-Iturralde 1981; Borhidi 1996).

1.6 Ultramafic rocks (serpentines) and specifically adapted plants on Cuba

Ultramafic rocks and soils occupy c. 1% of the Earth´s surface (Proctor 1999), and are found in many parts of the world. They vary in the richness and uniqueness of the floras they support. Some ultramafic regions have outstanding numbers of endemic plant species (e.g. Cuba, New Caledonia and California) while others do not (Harrison & Safford 2004).

The ultramafic materials are those dominated by peridotite, which is composed of olivine and pyroxene, or by serpentinite, which is composed of serpentine. They occur from tropical to polar regions (Alexander 2004). Although mineralogically diverse, all ultramafic materials have high concentrations of magnesium (18–24% Mg, or 30–40% MgO) and of iron (6–9% Fe), and very low concentrations of calcium (1–4% Ca) [Alexander 2004]. They may also contain heavy metals such as nickel (Ni), cobalt (Co) and chrom (Cr) [Brooks 1987 in González-Torres & al. 2004; Proctor 1999]. These extreme soil features contribute to the principal characteristics of ultramafic vegetation: low productivity, distinct physiognomy, predominance of xerophytic species and high frequencies of ecotypes and endemic species (Brooks 1987 in González-Torres & al. 2004).

Apart from a trickle of descriptive papers, for many years the importance of the ultramafic areas of Cuba went unrecognized and unpublished (Proctor 2004), this was despite the fact that about 7% of the surface of the island is covered by ultramafics, which are distributed along the island and isolated from one another (Berazaín-Iturralde 1997, 2001; Vázquez-Glaría 2006).

The ultramafics have historically been erroneously called serpentines by biologists. Serpentine is technically a mineral, but the same word is often used for all ultramafic rocks, the soils that form from them, and the unique ecosystems that form on them (Harrison & Rajakaruna 2011). Proctor (1999) considers that although the term is incorrect, it has been used and repeated so long in scientific papers, congresses and conferences that to replace it would lead to more confusion and thus the name should remain in use.

The edaphic stress, insular spatial structure, and rarity of endemic plants on serpentine soils lead to several expectations regarding the origins and evolutionary consequences for these habitat specialists. If serpentine outcrops are truly island-like habitats due to their geographic isolation from one another, then their colonists may undergo adaptive radiations leading to increased diversification rates (Anacker & al. 2011).

The celebration of the Fourth International Conference on Serpentine Ecology in the National Botanical Garden of Cuba in April 2003 and the publication of the conference papers led to the hope of establishing Cuba as one of the leading areas in the world for the study of ultramafic vegetation (Proctor 2004).

The ultramafic soils of Cuba are warm or hot throughout the year and contain at least some water available for plants during most of the year. Soil moisture regimes are udic (moist for most of each year) in the regions of Cajálbana and eastern Cuba and either udic or ustic (seasonally dry) in the central region of Cuba (Coleman & Alexander 2004).

Richness of serpentine floras depends on the age and size of the serpentine region, the number of climatic changes, as well as on the specialization and richness of the surrounding flora Borhidi (1996). The estimated age of the old Cuban serpentines, the western region of Cajálbana and the eastern region of Nipe-Cristal-Moa-Baracoa, is about 10–30 My old and these hold most of endemic taxa, including endemic genera. The young regions are located in the central part of the island, are c. 1 My old, and have less floristic richness and no endemic genera (Borhidi 1992).

The Cuban serpentines are mostly covered by xeromorphic thickets (commonly known as “cuabales” and “charrascales”), pine forests and rainforests (Capote & Berazaín-Iturralde 1984; Borhidi 1996). The Cuban serpentine flora is rich in endemic genera and species. About 920 of the Cuban endemic taxa grow on serpentines, representing 15% of the Cuban endemic flora (Borhidi 1996; Berazaín-Iturralde 1997).

The specialization of trees and shrubs to serpentine rocks results in metabolic changes which are largely irreversible (Borhidi 1996). Little is known about the process by which a serpentine-tolerant population evolves. Studies concerning to genetic and adaptive differentiation in serpentine-tolerant and -intolerant species are necessary in order to reveal key innovations in the path to tolerance (Brady & al. 2005). Rajakaruna (2004) exposes evidence of prezygotic and postzygotic barriers affecting gene flow between edaphically divergent taxa. Plants growing on serpentines have developed mechanisms to adapt to the toxicity caused by the high heavy metal content either by exclusion or accumulation (Baker 1981; Kazakou & al. 2008).

1.7 The accumulation and hyperaccumulation of Nickel

There are plants with the ability to accumulate metals such as arsenic (As), cadmium (Cd), cobalt (Co), chrom (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb), selenium (Se) and zinc (Zn) in higher concentrations than the normal values (Reeves 2003; Reeves 2006).

In the case of Ni the normal concentrations in the leaves of plants are in the range 0.5–10 µg/g. In plants growing on serpentines or other ultramafic soils the concentration of Ni is often elevated, but in about 1–2% of species of plants growing on such ultramafic soils have a concentration of Ni in their leaf tissues of more than 1000 µg/g and therefore these are called Ni-hyperaccumulating plants (Brooks & al. 1977; Reeves & al. 1996). If the concentration of Ni is > 100 µg/g and does not reach 1000 µg/g the plant is considered a Ni accumulator (Brooks & al. 1977). Plants that hyperaccumulate heavy metals or not, depending on the characteristics of the soils where they grow, are considered facultative hyperaccumulators (Pollard & al. 2014).

Ni-hyperaccumulating plants are distributed on all the continents. The first studies on this topic carried out in Cuba took place 32 years ago. In that time few species were identified as accumulators or hyperaccumulators (Berazaín-Iturralde 1981). Ten years ago the number of Cuban plants identified as accumulators or hyperaccumulators of Ni had risen to 173 species (Berazaín-Iturralde 2004). The floras of Cuba and New Caledonia stand out worldwide for this characteristic (Reeves & al. 1999; Reeves 2003), although the ultramafic soils of Cuba host the largest number of Ni hyperaccumulators found in any one country (Proctor 1999; Reeves 2006). Plants with this curious ability are potentially useful for extraction of Ni from the soil and thus for remediation of Ni-contaminated soils (Reeves & al. 1999; Reeves 2003; Reeves 2006).

Ni-hyperaccumulating phenotypes occur in ferns, monocots and eudicots which suggest that it has evolved independently in multiple plant groups (Reeves & Baker 2000). About 368 species of plants belonging to 44 families are known as Ni hyperaccumulators (Reeves & Baker 2000; Burge & Barker 2010). Plant families with the highest number of members able to hyperaccumulate Ni in their tissues are *Euphorbiacaeae*, *Brassicaceae*, *Asteraceae*, *Salicaceae*, *Rubiaceae*, and *Buxaceae* (Burge & Barker 2010). *Buxaceae* is represented only by species of Cuban *Buxus*. Other genera of the Cuban flora with a relevant number of Ni-hyperaccumulating species are *Leucocroton* and *Phyllanthus* (Reeves & al. 1996; Berazaín-Iturralde 2004).

Studies of the metabolism of Ni hyperaccumulators are at an early stage and the biochemical processes by which Ni is absorbed, transported and sequestered are not well understood (Reeves & al. 1996; Seregin & Kozhevnikova 2006). The ecological meaning of Ni hyperaccumulation is not clear and has been a matter of discussion by Boyd & Martens (1992). They grouped the postulated functions of Ni hyperaccumulation into five hypotheses, (1) tolerance and disposal of metal from the plant, (2) drought resistance, (3) interference with neighbouring plants, (4) inadvertent uptake and (5) defence against herbivores or pathogens. These researchers state that evidences are in favour of the defence hypothesis.

The accumulation of Ni is not limited to plants. There are reports of insects with a preference for feeding on Ni-hyperaccumulating plants which consequently accumulate Ni in their bodies (Boyd 2002; Boyd & al. 2004). A species of beetle can

feed on Ni-hyperaccumulating plants and is able to eliminate excess Ni in its excreta (Mesjasz-Przybyłowicz & al. 2004). Pollard (2000) estimates that the elevated metal contents of hyperaccumulator plants create a chemically unusual food source for herbivores that has probably contributed to a plant-herbivore coevolution.

1.8 The plastid markers *trnL-F*, *petD* and *trnK-matK* and the nuclear marker ITS

During the last decades, DNA sequences have become the primary data for phylogenetic inference, due to their advantages upon morphological data in phylogenetic reconstruction (Linder & Rieseberg 2004). In comparison with morphological data, DNA sequences provide more information in relative little time. The majority of the first sequenced-based molecular phylogenies in plants were exclusively based on plastid genomes (Álvarez & Wendel 2003), but aware of the limitations of working with uniparentally inherited sequences (Doyle 1992), phylogeneticists started to include also sequences data from nuclear markers and among them the Internal Transcribed Spacer (ITS) has been the most extensively used (Liston & al. 1996; Álvarez & Wendel 2003; Bayly & Ladiges 2007).

In this study focused on the evolution and biogeography of *Buxus* in Cuba and the Caribbean, analyses are based in three plastid markers, *trnL-F*, *petD* and *trnK-matK* and in the nuclear ITS.

trnL-F and *trnK-matK* have provided adequate information to resolve species relationships in some taxa, but often provide little resolution at lower taxonomic levels and to obtain better phylogenetic resolution they are often coupled with other sequence data (Shaw & al. 2005).

The plastid *trnL-F* region has become one of the most widely used chloroplast markers for phylogenetic analyses in plants (Quandt & al. 2004) and has proved useful in molecular phylogenetic studies addressing diverse evolutionary questions from biogeographic history to character evolution in a broad range of plant groups (Pirie & al. 2007). It is among the most used plastid markers at different taxonomic levels. It has been used at the family and subfamily levels (Richardson & al. 2000; Jobson & al. 2003;

Verbylaité & al. 2006; Martín-Bravo & al. 2007; Pirie & al. 2007), in tribes (Rova & al. 2002; García & Olmstead 2003; Andersson & Antonelli 2005) and genera (Bakker & al. 1999; Brouat & al. 2001; Bytebier & al. 2007).

The plastid gene region *trnK-matK* has been considered a promising candidate region for phylogenetic reconstructions among early diverging land plants (Wicke & Quandt 2009). In a study of *Utricularia*, Müller & Borsch (2005a) found that the phylogenetic signal provided by the noncoding *trnK* intron partition of the data set is similar to that of the *matK* coding region, although the latter is twice as long. Hilu & al. (2008) found that combining *trnK* intron sequences with *matK* increases overall bootstrap support compared to analyses with *matK* alone. The phylogenetic information derived from *matK* has made of it a valuable gene for DNA barcoding, and for systematic and evolutionary studies (Hao & al. 2010). Hao & al. (2010) consider that *matK* cannot be regarded as a neutral marker. The *matK* gene, combined with other plastid markers and/or with the nuclear marker ITS, has been useful in phylogenetic studies of *Cinchoneae* [*Rubiaceae*] (Andersson & Antonelli 2005), *Disa* [*Orchidaceae*] (Bytebier & al. 2007) and *Primula* subg. *Auganthus* [*Primulaceae*] (Yan H-F & al. 2010).

Compared with *trnL-F* and *trnK-matK*, the use of the *petD* intron has not been extensive. However it was used for a phylogenetic analysis in basal angiosperms by Löhne & Borsch (2005) with good results since they proved its utility in tests of alternative hypotheses on the basal nodes of the angiosperm tree. Karehed & al. (2008) used *petD* among other plastid markers in their study of the tribe *Spermacoceae* (*Rubiaceae*), where it was the most phylogenetically informative. The combination *petD*, *trnL-F* and *trnK-matK* provided a fully resolved and well supported topology of a basal eudicot grade in a study undertaken by Worberg & al. (2007).

The ITS has been widely used for phylogenetic analyses. Its popularity is based on advantages such as its relative easy amplification with universal primers and the possibility to obtain good quality sequences even from herbarium specimens. In spite of its advantages the use of ITS can also lead to unexpected results such as the generation of sequences of contaminants (e.g. fungi) and pherograms with polymorphic sites (Álvarez & Wendel 2003). A common solution to generate pherograms without

polymorphic sites is cloning the products of PCR (polymerase chain reaction). The results of cloning can be a variable number of paralogous sequences, which can be classified as putative functional ribotypes or putative pseudogenic ribotypes (Liu & Schardl 1994; Liston & al. 1996; Razafimandimbison & al. 2004; Roselló & al. 2007; Harpke & Peterson 2008).

The comparative analyses of ITS and plastid phylogenies are useful to detect reticulation patterns (Fuentes-Bazan & al. 2012) and to identify some biologic phenomena associated to reticulate patterns such as incomplete lineage sorting and hybridization (Martín-Bravo & al. 2010; Pelser & al. 2010).

1.9 Organizational framework, overall goals and questions

The genus *Buxus* has been selected as a model to better understand the origin and evolutionary diversification of the flora of Cuba and the Caribbean. Contributing to this overall goal it constitutes an important study group in the context of a larger program carried out in collaboration between the Botanical Garden and Botanical Museum Berlin-Dahlem, Freie Universität Berlin, the National Botanical Garden of Cuba, Universidad de La Habana, and several other partners in Cuba and the Caribbean that aims at understanding the origin and diversification of the flora of Cuba and the Caribbean. This program is organized in line with the production of a new Flora de la República de Cuba. In the context of this collaboration agreement, a program of field expeditions integrated Cuban and German botanists started in 2010 (Borsch & al. 2012). Cuban classic localities have been already explored in eastern Cuba (e.g. mountains of Sierra de Nipe, Sierra Cristal and mountains of Moa-Baracoa), in central Cuba (e.g. Camagüey, Villa Clara) and in western Cuba (e.g. plateau of Cajálbana, Pan de Guajaibón) and about 2 700 specimens of the Cuban flora have been collected and are saved in the herbaria of the Botanical Garden and Botanical Museum of Berlin-Dahlem (B) and of the National Botanical Garden of Cuba (HAJB).

Buxus is a promising model group because it is distributed in almost all continents, however its highest morphological and ecological diversity occur in the

Caribbean, where c. 45% of all extant species of *Buxus* are distributed. In the Caribbean, the highest number of species occur in the mountains of the northeastern region of Cuba, where most of taxa are serpentine endemics growing on thickets and forests. Moreover only about 50% of the Cuban species of *Buxus* have been identified as Ni hyperaccumulators.

Considering *Buxus* as a model to better understand the origin and diversification of the flora of Cuba and the Caribbean, the following more specific questions appeared: (1) Are the Cuban and the other Caribbean *Buxus* a monophyletic group? (2) When did *Buxus* arrive in the Caribbean and when occurred the diversification of the genus on the islands? (3) What is the ancestral distribution of the Cuban and Caribbean *Buxus*? (4) Are there specific migration routes of *Buxus* in the Caribbean? and (5) Which factors have triggered the speciation of *Buxus* in Cuba?

A further major goal was to use the evolutionary analysis of *Buxus* in Cuba to evaluate species concepts and to contribute to a modern species-level treatment and diversity assessment.

Chapter 2

The biogeography of the Caribbean *Buxus* since the Miocene, modelled by serpentines and nickel hyperaccumulation

Contents

2.1 Introduction

With about 100 species, *Buxus* is the largest genus of *Buxaceae* and also the widest distributed in Africa, Madagascar, Eurasia and tropical America. *Buxus* is most diverse in Cuba. The treatment of *Buxaceae* for Cuba (Köhler 2014) reports 37 species and seven subspecies, which correspond to c. 40% of the whole genus.

The Cuban archipelago is the biggest of the Caribbean. It is composed of Cuba, with 105 007 km^2 and more than 4 000 smaller islands and keys (Nuñez-Jimenez 1982). Cuba is long and narrow along all its extension and like the other islands of the Caribbean, has a complex geologic history (Iturralde-Vinent 1982; Hedges 2001; Coleman & Alexander 2004; Iturrande-Vinent 2006).

Cuba has one of the richest insular floras in the world with c. 6 300 species of phanerogams, of which c. 51.3% are endemics. The Cuban flora has mostly a Gondwanan origin with influence of Laurasian elements, being the result of migrations south-north and north-south of South American and North American plants through the Caribbean islands (López-Almirall 1998). The insularity, the mixture of flat lands and mountains, the diversity of rocks and soils and the alternating wet and cold periods have been hypothesized as factors facilitating the speciation on Cuba (Borhidi 1996).

The most common bedrocks in Cuba are the limestone and the ultramafics and among the most abundant soils are those derived from them. In the case of the ultramafics are the serpentine soils (hereafter called serpentines). The serpentines contain low concentration of calcium (Ca), potassium (K) and sodium (Na), and high concentrations of magnesium (Mg) and heavy metals such as cobalt (Co), chrome (Cr), iron (Fe) and nickel (Ni) [Moores 2011]. These soil features contribute to the principal characteristics of ultramafic vegetation: low productivity, distinct physiognomy, predominance of xerophytic species and high frequencies of ecotypes and endemic species (Brooks 1987; González-Torres & al. 2004). The serpentines occupy around 1% of the Earth´s surface (Proctor 1999) and in Cuba they cover 7% of the territory, distributed as an archipelago of isolated serpentine outcrops (Berazaín-Iturralde 1997, 2001; Vázquez-Glaría 2006) across all three phytogeographic subprovinces east, central

and west Cuba (Borhidi, 1996) (Fig. 1.2 of chapter 1). The oldest Cuban serpentines located in the western plateau of Cajálbana and the eastern mountains of Nipe-Cristal-Moa-Baracoa, are estimated to be 10 to 30 Million years (My) old and hold most endemic species and genera (Borhidi 1992, 1996). The youngest serpentines, located in the central part of the island, are about 1 My old and have less floristic richness and no endemic genera (Borhidi 1992, 1996). The Cuban serpentines are recognized as sites of high plant speciation and diversification. Borhidi (1996) described them as intensive and successful workshops of plant speciation and diversification because one third of the Cuban endemic flora has developed on serpentine areas. *Buxus* is a good example of this since 84% of species, subspecies or ecotypes are exclusive of the Cuban serpentine outcrops.

Plants growing on serpentines have developed mechanisms to adapt to the toxicity caused by the high heavy metal content either by exclusion or accumulation (Baker 1981; Kazakou & al. 2008). Within the heavy metals accumulators, about 450 angiosperm species have been identified as hyperaccumulators (Rascio & Navari-Izzo 2011). Hyperaccumulation is the ability of some plants to sequester and accumulate certain elements such as aluminium (Al), arsenic (As), cadmium (Cd), Co, Cr, copper (Cu), manganese (Mn), Ni, lead (Pb), selenium (Se) and zinc (Zn) in higher concentrations than the normal values (Reeves 2003, 2006; Boyd 2004). A special case are the Ni hyperaccumulators which are defined as plants which have a Ni concentration of at least 1000 $\mu g\ g^{-1}$ in the dry matter of any above-ground tissue recorded in at least one specimen growing in its natural habitat (Proctor 1999). This phenomenon is quite rare and occurs only in around 2% of the serpentine species worldwide (Kazakou & al. 2008). The ultramafic soils of Cuba host the largest number of Ni-hyperaccumulating species found in any one country (Reeves & al. 1996, 1999; Proctor 1999; Burge & Barker 2010).

Phylogenetic studies with molecular markers including Cuban plant species are still scarce, particularly those addressing biogeographic questions. Examples are *Ginoria* [*Lythraceae*] (Graham 2010), *Spathelia* [*Rutaceae*] (Appelhans & al. 2012), *Leucocroton* [*Euphorbiaceae*] (Jestrow & al. 2012) and *Brunfelsia* [*Solanaceae*] (Filipowicz & Renner 2012a).

In Cuba, *Buxus* is one of the most outstanding lineages in terms of endemism. Ninety-five percent of the Cuban species are unique to the island with the majority of taxa or ecotypes found in forest and thickets on serpentines outcrops. Out of 44 Cuban taxa of *Buxus*, species and subspecies, 20 have been identified as Ni hyperaccumulators (Reeves & al. 1996; and updated in this study). Reeves & al. (1996) suggested that the presence of similar numbers of non-accumulators and hyperaccumulators among the serpentine endemics in *Buxus* should aid the tracing of evolutionary relationships among the species. The distribution and ecologic patterns of *Buxus* in Cuba offer an opportunity for better understanding of the natural history and the biogeography of Cuba and the Caribbean. *Buxus* has been selected as a study model to illuminate some factors that have spurred the origin of the Cuban flora and its endemism. In particular we investigate if the suggested radiation of *Buxus* has been conditioned by edaphic, ecologic or geologic factors.

The present study aims to reconstruct the phylogeny of *Buxus* on Cuba and other regions of the Caribbean and to test the monophyly of the genus in the Caribbean. Further the divergence time of each clade is estimated to explore correlations with geological events in the Caribbean and specifically in Cuba. In this sense, the ancestral distribution of *Buxus* in the Caribbean is estimated and the ancestral states of traits which could have triggered the speciation and high levels of endemism of the genus in this region such as the adaptation to grow on serpentines and the ability to hyperaccumulate or accumulate Ni, are also estimated.

2.2 Materials and methods

2.2.1 Taxon sampling and plant material

We included 105 samples of 53 species of *Buxus*, 34 species and 5 subspecies from Cuba (Köhler 2014), two species from Jamaica, two from Puerto Rico, samples of *B. bahamensis* and *B. glomerata* from Bahamas and Hispaniola, respectively, four species from Mexico, *B. citrifolia* from Panama, four Eurasian species and six from Africa, Madagascar and adjacent islands. As outgroup, we included sequences of all

other genera of *Buxales* and representatives of *Trochodendrales*, *Proteales*, *Sabiales* and *Ranunculales*. Samples included in this study were collected in the field, in living collections or were removed from herbarium specimens. In the case of the samples collected in the field or in living collections, few young and healthy leaves were selected and dried in silica gel and a herbarium voucher was made and preserved. Information about taxa, samples and their codes, localities, collectors and vouchers is shown in appendix 2.1.

2.2.2 DNA extraction, amplification and sequencing

Total DNA was isolated from silica-gel-dried leaves or herbarium specimens using a triple CTAB extraction method (Borsch & al. 2003) or the Nucleo Spin Plant II extraction kit (Macherey Nagel, Düren, Germany).

Three plastid markers (*trnK-matK*, *trnL-F* and *petD*) were amplified and sequenced in this study. The amplification of each marker was performed in reaction volumes of 50 µL, containing 2 µL of extracted DNA (with a concentration of 10–20 ng/µL), 14.7 µL of H_2O, 5 µL of 10× peqLab Taq. buffer S containing $MgCl_2$, 3 µL of $MgCl_2$ (25 mM), 10 µL of betaine monohydrate (5 M), 1 µL of BSA (10 ug/µl), 2 µL of forward primer (20 pm/µl), 2 µL of reverse primer (20 pm/µl), 10 µL dNTPs (each 0.25 mM) and 0.3 µL Taq polymerase 5 units/µl (PeqLab, Erlangen Germany).

The *trnL-F* region was amplified using the primers trnTc and trnTf (Taberlet & al. 1991). Polymerase Chain Reaction (PCR) conditions were: 30 cycles of denaturation (60 seconds at 96 ºC), annealing (60 seconds at 50ºC), and extension (120 seconds at 72 ºC). Sequencing was carried out with the primer trnTf and the additional internal primer trnTd (Taberlet & al. 1991). The *trnK-matK* region was amplified and sequenced in two fragments using the primer pairs trnK-F (Wicke & Quandt 2009) and BxmatK-1270R (5´- ATTCCAATTATGATACTCG-3´, designed for *Buxaceae* in this study), as well as BxmatK-467F (5´- TGTCAGATATACTAATACC-3,´ designed for *Buxaceae* in this study) and trnK-2R (Johnson & Soltis 1994). For some samples isolated from older herbarium specimens, the use of further internal primers, newly designed was necessary

(BxtrnK-779R, 5´-TAAATATACTCCTGAAAGAG-3´; BxtrnK-1750R, 5´-AATTTTCTAGCATTTGACTC-3´). The PCR program used was: 34 cycles of denaturation (60 seconds at 94 ºC), annealing (60 seconds at 50 ºC) and extension (120 seconds at 72 ºC).

The sequences of *petD* were generated using a similar methodology as Löhne & Borsch (2005). In some cases it was necessary the use of internal sequencing primers designed for this study: BxpetD500F (5´-ATTCATTTCCTCTGCATCG-3´) and BxpetD556R (5´- GTTACTAATATAGTCTAGCC-3´). All amplification products were purified by long gel electrophoresis. Sequencing was performed by Macrogen Inc., South Korea (http://www.macrogen.com).

2.2.3 Determining the concentration of nickel

Middle age leaves from 19 specimens of 12 taxa (8 species and 4 subspecies) of *Buxus* included in this study were removed from herbarium vouchers (Appendix 2.1). The samples were vacuum dried for two days before homogenization with plastic and ceramic instruments to avoid metal contamination and after that they were pulverized. Samples were digested in a closed vessel microwave system (MARS5 CEM Corp., Matthews, USA) using nitric and hydrochloric acids according to DIN (2001). Limit of detection (LOD) was calculated as the threefold standard deviation of instrument blank (acidified water). According to DIN (2004) the elemental analysis was performed with an inductively coupled plasma mass spectrometer (PQ exCell, Thermo Fisher Scientific Inc, UK). Calibrations were performed with mixed calibration samples which consisted of single and multi-element solutions (Bernd Kraft, Duisburg, Germany). Calibration validity was confirmed with digests of standard reference material (GBW7604, poplar leaves, China). The element concentration was derived in µg/l and later converted from µg/l into µg/g dry mass (Weiske & al. 2013).

2.2.4 Editing of sequences and alignment

Sequences were edited and aligned using PhyDE v.0 995 (Müller & al. 2007), following the criteria of Löhne & Borsch (2005). Regions of uncertain homology (mutational hotspots) were removed from the matrices prior to phylogenetic analyses. An inversion of 37 nucleotides found in the *trnK* 3' exon of *trnK-matK* sequences belonging to 9 samples was re-inverted and aligned. The indels were coded according the Simple Index Coding Method (Simons & Ochoterena 2000) with SeqState 1.40 (Müller 2005a).

2.2.5 Phylogenetic analyses

Maximum Parsimony (MP) and Bayesian inference (BI) analyses were made on a concatenated matrix of *petD*, *trnL-F* and *trnK-matK* including the coded indels. The MP analysis was done using the Parsimony Ratchet (Nixon 1999) as implemented in PRAP (Müller 2004) in combination with PAUP v. 4.0b10 (Swofford 1998). Ratchet settings were 200 ratchet iterations with 25% of the positions randomly up weighted (weight = 2) during each replicate and 10 random addition cycles. The command files generated with PRAP were then run in PAUP, using the heuristic search with the following parameters: all characters have equal weight, gaps are treated as ‘‘missing’’, TBR branch swapping, initial swapping on 1 tree already in memory, Maxtrees set to 100 (auto increased by 100) and branches collapsed actively if branch length is zero. The Jackknife (JK) support for branches was also performed in PAUP with 10 000 replicates, using a TBR branch swapping algorithm with 36.788% of characters deleted and one tree held during each replicate, following Müller (2005b).

The BI analysis was carried out using MrBayes 3.1 (Huelsenbeck & Ronquist 2001). The optimal nucleotide substitution models were determined following the Akaike Information criterion (AIC) in Modeltest 2.3 (Posada & Crandall 1998). The model selected was GTR + G for each marker. A binary (restriction site) model was implemented for the coded indels. All analyses were performed with four independent

runs of Markov Chains Monte Carlo (MCMC) each with four parallel chains. Each chain was performed for 1 million generations; saving one random tree every 100th generation. The burn in was set to 100 000 and a majority consensus tree was computed with the remaining trees.

2.2.6 Estimating divergence times, fossil calibration and speciation rates

The estimation of times of divergence was conducted with BEAST v1.8 (Drummond & Rambaut 2007). The original data set was reduced to 94 ascensions, among them 72 of *Buxus*, keeping only one sequence per species, subspecies or different ecotypes (e.g. *Buxus glomerata*, *B. yunquensis*). For taxa with more than one sample included in this study only different haplotypes were kept. Analyses were done assuming an uncorrelated relaxed clock model and a lognormal distribution of rate changes (Drummond & al. 2006). The analyses assumed a speciation Yule process, the most suitable tree prior for inferring relationships between species (Drummond & Rambaut 2007). Independent analyses of 20–50 × 10^6 generations were run in BEAST. When it was necessary the output files were combined with LogCombiner v1.8 (Drummond & Rambaut 2007). The performance of the analyses, convergence of the independent runs and, effective sample sizes, were evaluated using Tracer v1.4 (Rambaut & Drummond 2007). Mean and 95% highest posterior density (HPD) intervals of ages were then calculated from post burn-in trees using TreeAnnotator v1.8 (Drummond & Rambaut 2007).

Information about fossils consulted in this study appears in the table 2.1. A fossil species *Buxus egeriana* was documented by Kvaček & al. (1982). This is a macrofossil with good taxonomic resolution consisting of well-preserved fruits and leaves remains from the Lower/Middle Miocene (23.03–15.97/15.97–11.6 My) from western Bohemia. It has morphological affinities with species from China and Vietnam and according to Kvaček & al. (1982) a survey of the Tertiary *Buxus* records suggest that two European lineages evolved from a common stock which was replaced by small leaved species similar to *Buxus sempervirens*. We assumed a conservative age of 15.97 My for

constraining the stem node linking the Eurasian species of *Buxus* with *Pachysandra*, *Sarcococc*a and *Styloceras*.

Macrofossils (flowers) of *Lusistemon striatus* and *Lusicarpus planatus* and microfossils (pollen) of *Rutihesperipites* or *Striatopollis* have been found in Vale de Agua (Portugal) from Late Aptian and Early Albian (c. 112 My); and morphological studies suggest that such fossils are related to extant *Buxales* (Pedersen & al. 2007). These fossils were consulted for estimating the time of divergence between *Styloceras* and *Pachysandra* and the time of divergence of clades within *Pachysandra* (Jiao & Li 2009). The estimated age of the fossil found in Vale de Agua (c. 112 My) was applied here to constraint the node linking *Buxales* and *Trochodendrales*.

Fossil foliage, inflorescence and infrutescence of *Platanocarpus brookensis* (*Platanaceae*, *Proteales*) from the Early to Middle Albian (c. 110 My) have been found in Brooke, Virginia, USA (Crane & al. 1993). The age of these fossils was used to constrain the node linking *Proteales* and *Sabiales*.

The divergence age for *Styloceras* and *Pachysandra* (23.5 ± 8.1 My) was estimated by Jiao & Li (2009) and we used it as secondary calibration in one of our calibration scenarios. The most basal taxa included in this analyses are members of *Ranunculales*, thus in all cases the root was constrained to 121 My following Bell & al. (2010).

With the ages of fossils and already estimated ages exposed above we conceived seven calibration scenarios: (i) only the root was constrained, (ii) only the node linking *Buxales* and *Trochodendrales* was constrained, (iii) only crown nodes of the outgroup [*Buxales-Trochodendrales* and *Proteales-Sabiales*] were constrained, (iv) only the ingroup node linking Eurasian *Buxus* with *Pachysandra/Sarcococca/Styloceras* was constrained, (v) three outgroup and ingroup nodes [*Buxales-Trochodendrales*, *Proteales-Sabiales*, Eurasian *Buxus- Pachysandra/Sarcococca/Styloceras*] were constrained, (vi) the node *Buxales-Trochodendrales* was constrained, as well as the node linking *Pachysandra* and *Styloceras* with the age estimated by Jiao & Li (2009), (vii) three outgroup and ingroup nodes [*Buxales-Trochodendrales*, *Proteales-Sabiales*,

Table 2.1. Fossils data used in this study

Fossil	**Structures**	**Locality**	**Age**	**References**	**Calibrated node**
Buxus egeriana	Fruits and leaves	Western Bohemia, Czech Republic	Lower/Middle Miocene (c. 15.97 My)	Kvaček & al. (1982)	Node linking the Eurasian *Buxus* and the clade *Pachysandra-Sarcococca-Styloceras.*
Lusistemon striatus, *Lusicarpus planatus*, *Rutihesperipites* or *Striatopollis*	Staminate flowers, pistillate flowers and pollen	Vale de Agua, Portugal	Late Aptian/Early Albian (c. 112 My)	Pedersen & al. (2007)	Node linking *Buxales* and *Proteales*
Platanocarpus brookensis	Inflorescences and infrutescences	Brooke, Virginia, USA	Early to Middle Albian (c. 110 My).	Crane & al. (1993); Anderson & al. (2005); Doyle & Endress (2010).	Node linking *Proteales* and *Sabiales*

Eurasian *Buxus-Pachysandra/Sarcococca/Styloceras*] as well as the node linking *Pachysandra* and *Styloceras* were constrained.

Species diversification rates (SR), assuming an equal rate of random speciation Yule model, were calculated using the formula $SR= [(\ln(N)–\ln(N_0)]/T$, where N is the number of extant species, N_0 is the initial species diversity (=1 in a monophyletic group), and T is the age of a clade in years (Magallón & Sanderson 2001; Hughes & Eastwood 2006; Guzmán & al. 2009; Madriñán & al. 2013). This analysis was based only on the number of taxa included in this study.

2.2.7 Reconstruction of ancestral areas and ancestral traits

In both analyses the input file was a consensus tree generated by MrBayes for a reduced matrix of 73 individuals of *Buxus* (72) and *Pachysandra terminalis* as root. The ancestral area reconstruction was carried out with RASP 2.0 Beta (Yu & al. 2010). A Bayesian Binary Analysis was run, adjusting a maximum of four areas per node, the among-site rate variation was set to Gamma (+G) and the rest of parameters with default settings.

Species distributions were compiled from herbarium specimens and the literature (Adams 1972; Gentry 1978; Correll & Correll 1982; Liogier 1986, 1988; Schatz & Lowry 2002; Mabberley 2008; Köhler 2014). For the regionalization of distribution of Cuban species, the phytogeographical subdivision of Cuba (Borhidi 1996) was followed. Eight geographical areas were defined: east Cuba (A), central Cuba (B), west Cuba (C), other islands of the Caribbean [Bahamas, Hispaniola, Jamaica, and Puerto Rico] (D), South America and Panama (E), North America and Central America until El Salvador (F), Eurasia including the Mediterranean islands and North Africa (G) and Africa (south of Sahara) and adjacent islands of the Indic Ocean: Madagascar, Comoro islands, Socotra (H) [Appendix 2.1].

The reconstruction of ancestral traits was conducted with Mesquite v.2.75 (Maddison & Maddison 2011). Two traits associated with the evolution of the Cuban

Flora and particularly with *Buxus* were analysed, (i) the adaptation to grow on serpentines (Berazaín-Iturralde 1986; Borhidi 1996) and (ii) the ability to accumulate or hyperaccumulate Ni (Reeves & al. 1996). In Mesquite, the tool Trace Character History / Parsimony Ancestral States was selected.

Regarding the trait adaptation to grow on serpentines, the criteria of Reeves & al. (1999) and Kazakou & al. (2008) were considered. The species of *Buxus* were classified as serpentine-endemic [SE] and no serpentine-endemic [NS] (Appendix 2.1). This classification is based on field annotations and on the experiences accumulated during the establishment of the living collection of *Buxus* in the National Botanical Garden of Cuba (Rankin-Rodríguez & al. 1999). In the case of the capacity to accumulate and hyperaccumulate Ni, the taxa were classified in Ni hyperaccumulators [NiH], Ni accumulators [NiA], not Ni accumulators or hyperaccumulators [NAH] and ambiguous [NiA, NAH], based on data generated by Reeves & al. (1996) and the new analysis conducted as part of this study. A condition to hyperaccumulate Ni is to grow in Ni rich soils; thus the taxa which do not grow on serpentines, like *B. bahamensis* and the Mexican species are considered not hyperaccumulators. In the case of *B. shaferi*, with four accessions included in this study, we classified them according to the real concentrations found in each sample.

2.3 Results

A total of 332 new sequences of species of *Buxus* and other members of *Buxales* were generated, 111 sequences of *petD*, 111 of *trnL-F* and 110 of *trnK-mat*K (Appendix 2.1).

The Ni concentration of seven species (*B. braimbridgeorum*, *B. cristalensis*, *B. ekmanii*, *B. glomerata*, *B. nipensis*, *B. triptera*, *B. yunquensis*) and four subspecies (*B. ekmanii* ssp. *woodfredensis*, *B. gonoclada* ssp. *orientensis*, *B. gonoclada* ssp. *toldoensis*, *B. pilosula* ssp. *cacuminis*) was determined the first time in this study. In eight samples of four species (*B. braimbridgeorum, B. cristalensis, B. nipensis, B. triptera*) and three subspecies (*B. gonoclada* ssp. *orientensis*, *B. gonoclada* ssp. *toldoensis*, *B. pilosula* ssp.

cacuminis) were found concentrations of Ni higher than 1000 μg g^{-1} pointing these taxa as Ni hyperaccumulators (Appendix 2.2). In two samples of *B. shaferi* concentrations of Ni which are in the range 100–1000 μg g^{-1} were detected, thus these were considered Ni accumulators. The remaining eight samples of five species and a subspecies (*B. ekmanii*, *B. ekmanii* ssp. *woodfredensis*, *B. glomerata*, *B. olivacea*, *B. shaferi*, *B. yunquensis*), had Ni concentrations < 100 μg g^{-1} and are regarded as no Ni accumulators (Appendix 2.2).

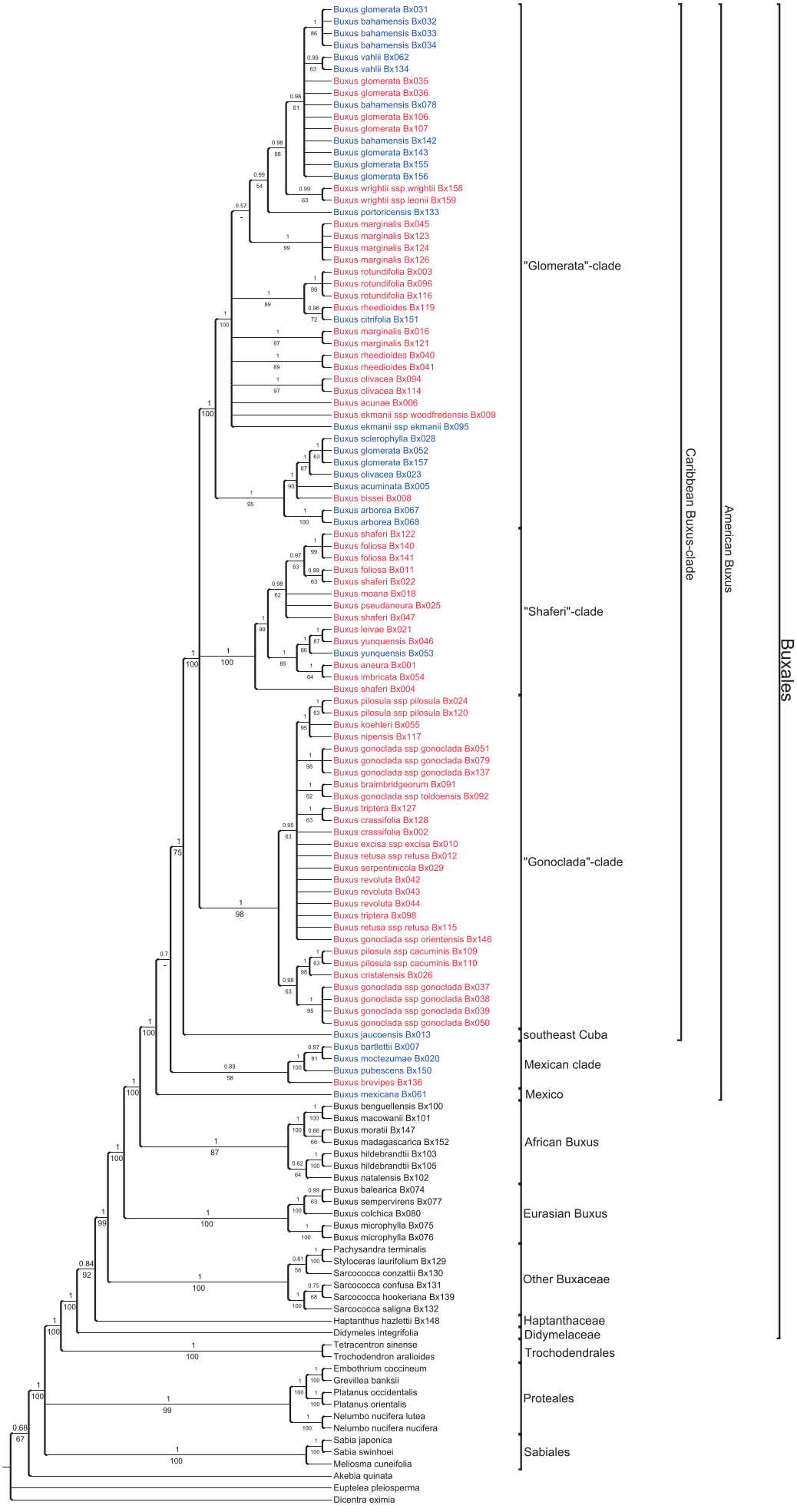
2.3.1 Phylogeny of *Buxales*

The combined final matrix of 126 individuals, without mutational hotspots and including coded indels, comprised 5807 characters (5386 plastid nucleotides and 421 indels) of which 1417 (24.4%) were parsimony informative. The MP search resulted in 258 shortest trees (L = 4088, CI = 0.724, RI = 0.874 and RC = 0.633). The topologies of the strict consensus trees derived from MP and the majority rule tree from BI analyses are the same. The BI tree with braches supports [Bayesian posterior probabilities (PP) and Jackknife (JK)] is shown in Fig. 2.1. The *Buxales* are maximally supported as monophyletic and also most of the internal nodes receive high support. *Didymeles integrifolia* (*Didymelaceae*, Madagascar-Comoro islands) and *Haptanthus hazlettii* (*Haptanthaceae*, Honduras) are in the most basal position of *Buxales*. The clade formed by *Pachysandra*, *Sarcococca* and *Styloceras* is well supported too (1 PP/ 99% JK). Within this clade, the three Asiatic species of *Sarcococca* form a highly supported clade (1 PP/ 100% JK). The position of the Mexican *Sarcococca conzattii* as sister to *Styloceras laurifolium* and *Pachysandra terminalis* is not well supported (0.81 PP / 58% JK). A *Buxus* clade is maximally supported (Fig. 2.1).

2.3.2 Phylogeny of *Buxus*

Based on the MP and BI reconstructions, *Buxus* is monophyletic with maximal support (Fig. 2.1). Within *Buxus* three clades (African-clade, American-clade and Eurasian-clade) are resolved with high supports.

Fig.2.1. Bayesian majority rule tree based on the concatenated matrix *trnK-matK+petD+trnL*-F. Bayesian posterior probabilities (PP) shown above branches and Jackknife values (JK) below branches. In the American Buxus clade the taxa and ecotypes growing on serpentines (serpentine endemics) appear in red, and those growing on other soils in blue.(next page).


Buxus glomerata Bx031
Buxus bahamensis Bx032
Buxus bahamensis Bx033
Buxus bahamensis Bx034
Buxus vahlii Bx062
Buxus vahlii Bx134
Buxus glomerata Bx035
Buxus glomerata Bx036
Buxus bahamensis Bx078
Buxus glomerata Bx106
Buxus glomerata Bx107
Buxus bahamensis Bx142
Buxus glomerata Bx143
Buxus glomerata Bx155
Buxus glomerata Bx156
Buxus wrightii ssp wrightii Bx158
Buxus wrightii ssp leonii Bx159
Buxus portoricensis Bx133
Buxus marginalis Bx045
Buxus marginalis Bx123
Buxus marginalis Bx124
Buxus marginalis Bx126
Buxus rotundifolia Bx003
Buxus rotundifolia Bx096
Buxus rotundifolia Bx116
Buxus rheedioides Bx119
Buxus citrifolia Bx151
Buxus marginalis Bx016
Buxus marginalis Bx121
Buxus rheedioides Bx040
Buxus rheedioides Bx041
Buxus olivacea Bx094
Buxus olivacea Bx114
Buxus acunae Bx006
Buxus ekmanii ssp woodfredensis Bx009
Buxus ekmanii ssp ekmanii Bx095
Buxus sclerophylla Bx028
Buxus glomerata Bx052
Buxus glomerata Bx157
Buxus olivacea Bx023
Buxus acuminata Bx005
Buxus bissei Bx008
Buxus arborea Bx067
Buxus arborea Bx068
Buxus shaferi Bx122
Buxus foliosa Bx140
Buxus foliosa Bx141
Buxus foliosa Bx011
Buxus shaferi Bx022
Buxus moana Bx018
Buxus pseudaneura Bx025
Buxus shaferi Bx047
Buxus leivae Bx021
Buxus yunquensis Bx046
Buxus yunquensis Bx053
Buxus aneura Bx001
Buxus imbricata Bx054
Buxus shaferi Bx004
Buxus pilosula ssp pilosula Bx024
Buxus pilosula ssp pilosula Bx120
Buxus koehleri Bx055
Buxus nipensis Bx117
Buxus gonoclada ssp gonoclada Bx051
Buxus gonoclada ssp gonoclada Bx079
Buxus gonoclada ssp gonoclada Bx137
Buxus braimbridgeorum Bx091
Buxus gonoclada ssp toldoensis Bx092
Buxus triptera Bx127
Buxus crassifolia Bx128
Buxus crassifolia Bx002
Buxus excisa ssp excisa Bx010
Buxus retusa ssp retusa Bx012
Buxus serpentinicola Bx029
Buxus revoluta Bx042
Buxus revoluta Bx043
Buxus revoluta Bx044
Buxus triptera Bx098
Buxus retusa ssp retusa Bx115
Buxus gonoclada ssp orientensis Bx146
Buxus pilosula ssp cacuminis Bx109
Buxus pilosula ssp cacuminis Bx110
Buxus cristalensis Bx026
Buxus gonoclada ssp gonoclada Bx037
Buxus gonoclada ssp gonoclada Bx038
Buxus gonoclada ssp gonoclada Bx039
Buxus gonoclada ssp gonoclada Bx050
Buxus jaucoensis Bx013
Buxus bartlettii Bx007
Buxus moctezumae Bx020
Buxus pubescens Bx150
Buxus brevipes Bx136
Buxus mexicana Bx061
Buxus benguellensis Bx100
Buxus macowanii Bx101
Buxus moratii Bx147
Buxus madagascarica Bx152
Buxus hildebrandtii Bx103
Buxus hildebrandtii Bx105
Buxus natalensis Bx102
Buxus balearica Bx074
Buxus sempervirens Bx077
Buxus colchica Bx080
Buxus microphylla Bx075
Buxus microphylla Bx076
Pachysandra terminalis
Styloceras laurifolium Bx129
Sarcococca conzattii Bx130
Sarcococca confusa Bx131
Sarcococca hookeriana Bx139
Sarcococca saligna Bx132
Haptanthus hazlettii Bx148
Didymeles integrifolia
Tetracentron sinense
Trochodendron aralioides
Embothrium coccineum
Grevillea banksii
Platanus occidentalis
Platanus orientalis
Nelumbo nucifera lutea
Nelumbo nucifera nucifera
Sabia japonica
Sabia swinhoei
Meliosma cuneifolia
Akebia quinata
Euptelea pleiosperma
Dicentra eximia
"Glomerata"-clade
"Shaferi"-clade
"Gonoclada"-clade
southeast Cuba
Mexican clade
Mexico
African Buxus
Eurasian Buxus
Other Buxaceae
Haptanthaceae
Didymelaceae
Trochodendrales
Proteales
Sabiales
Caribbean Buxus-clade
American Buxus
Buxales

The most complex grouping is shown in the American *Buxus* (Fig. 2.1) which is the most densely sampled. In the basal position of the American clade is *B. mexicana*. The node linking this species with the rest is poorly supported (0.7 PP/ < 50% JK). Branching next is the Mexican-clade with low support (0.89 PP/ 58% JK) enclosing three Mexican species: *B. bartlettii*, *B. moctezumae*, *B. pubescens* (1 PP/ 100% JK), which are related to the west Cuban *B. brevipes* (Fig. 2.1).

The next related clade encloses the Caribbean species of *Buxus* (Caribbean *Buxus*-clade), in which two lineages are resolved; one is the lineage of *B. jaucoensis*, a strict endemic of the locality of Jauco in southeastern Cuba, which is sister to the rest of the Cuban and Caribbean species. Three highly supported subclades are recovered forming a polytomy within the Caribbean *Buxus*-clade: the "Gonoclada"-clade, the "Shaferi"-clade and the "Glomerata"-clade (Fig. 2.1).

The "Gonoclada"-clade (1 PP/ 98 % JK) encloses 12 species and three subspecies, all of them serpentine endemics, mostly from the serpentine outcrops of the phytogeographic subprovince of east Cuba, with the exception of *B. gonoclada* ssp. *gonoclada* which is distributed in the serpentine outcrops of the phytogeographic province of central Cuba from Holguín to Havana-Matanzas (Fig. 2.2). Almost all species of this clade are Ni hyperaccumulators with the sole exception of *B. koehleri*, of which the concentration of Ni is unknown (Appendix 2.1).

The "Shaferi"-clade (1 PP/ 100% JK) encloses eight species, restricted to the mountains of northeastern Cuba. Most of them are serpentine endemics with the sole exception of a limestone ecotype of *B. yunquensis* (Fig. 2.3). Five species of this clade are Ni hyperaccumulators (Appendix 2.1).

The "Glomerata"-clade (1 PP/ 100% JK) encloses 17 species nested in two well supported subclades (Fig. 2.1). The smaller subclade (1 PP/ 95% JK, Fig. 2.1) encloses mostly non serpentine species or ecotypes, *B. sclerophylla* and *B. glomerata* (Bx052, Bx157) from southeast Cuba, the serpentine endemic *B. bissei*, and also two Jamaican species, *B. arborea* and *B. purdieana*. The position of *B. purdieana* is known after a MP analysis (not shown) of a combined matrix of *trnL-F* and *petD*, since from the sample of

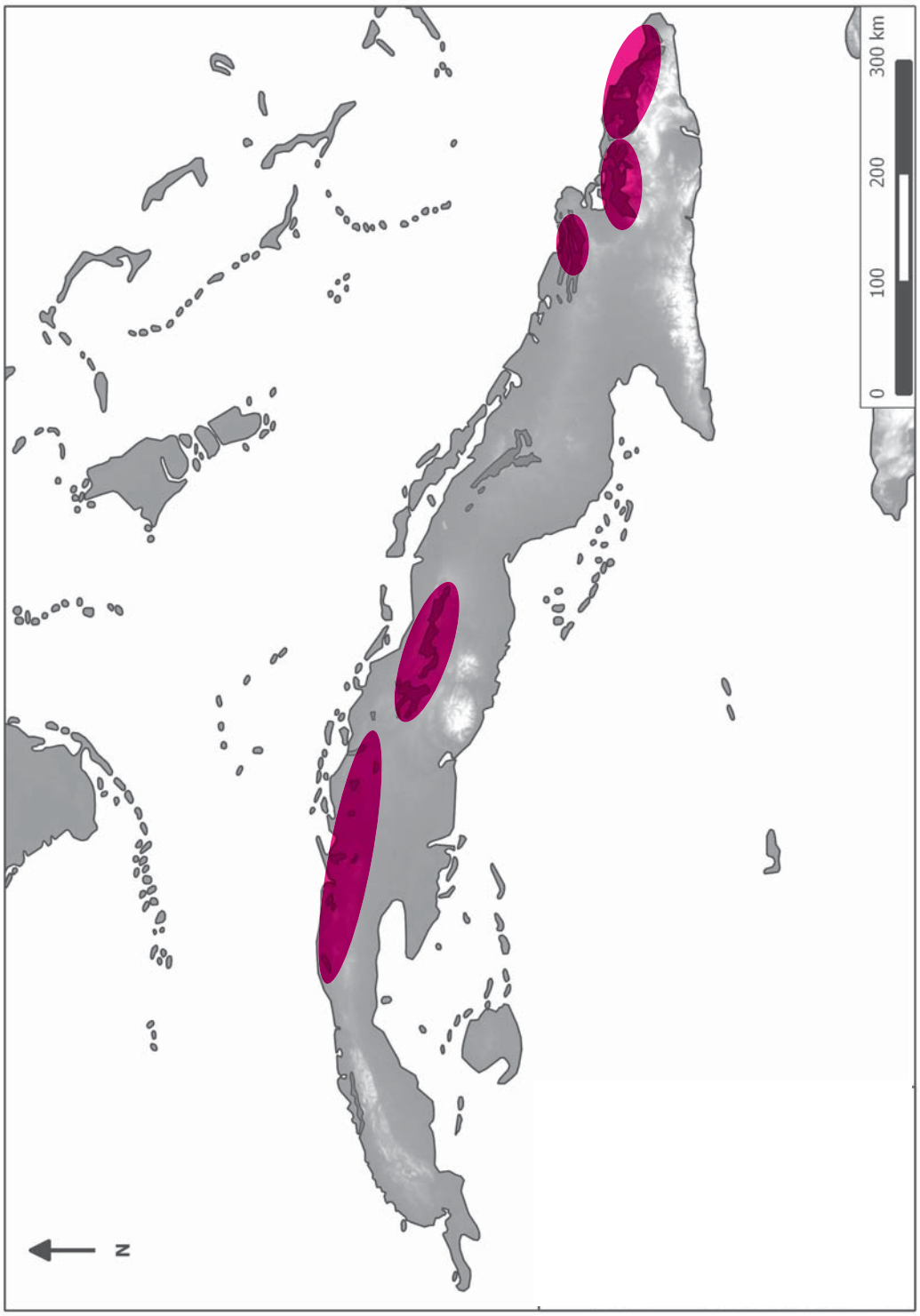


Fig. 2.2. Distribution of the species enclosed in the "Gonoclada"-clade.

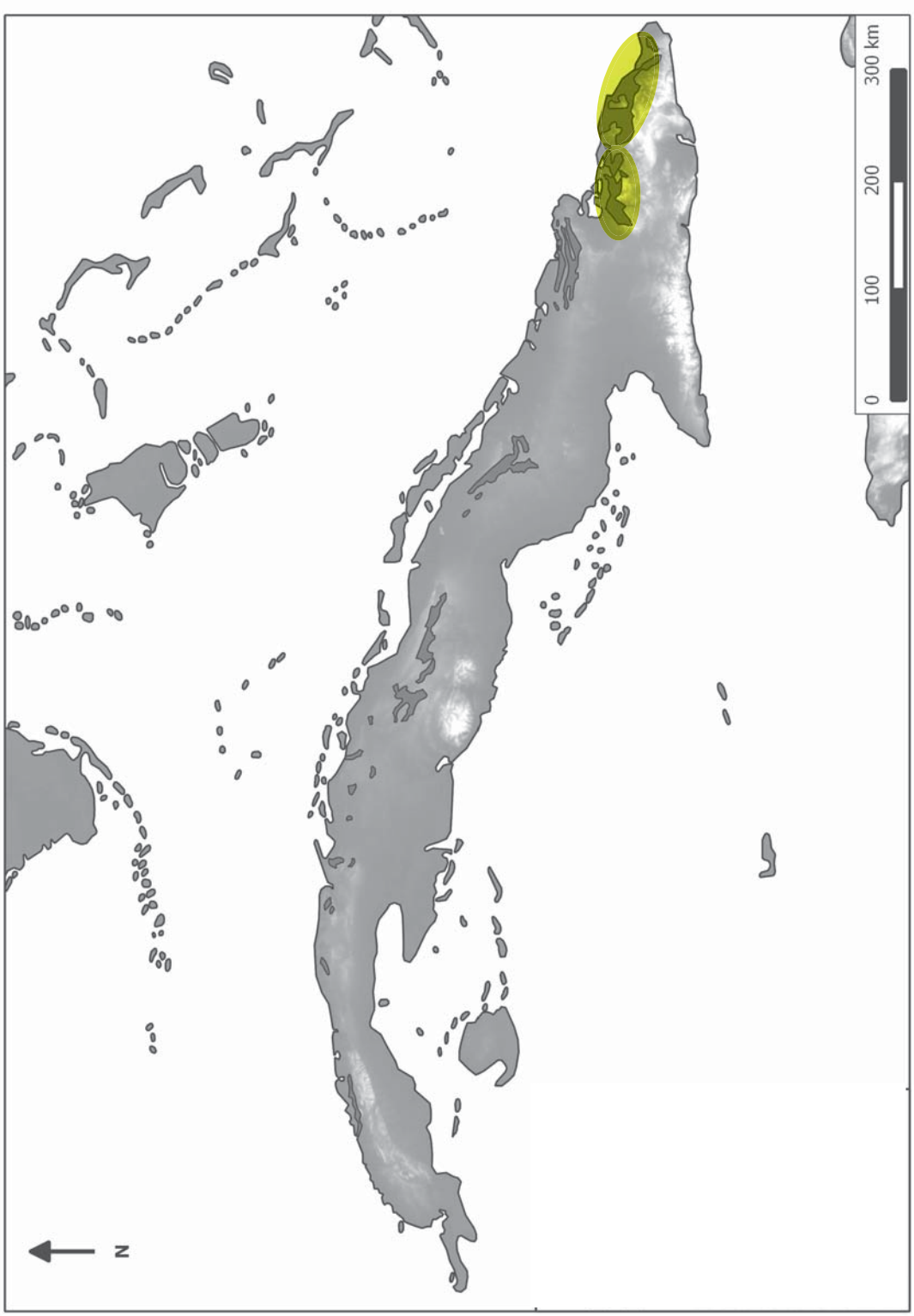


Fig. 2.3. Distribution of the species enclosed in the "Shaferi"-clade.

this species only sequences of these markers could be generated. The larger subclade (1 PP/ 100% JK) encloses most of taxa such as serpentine ecotypes of *B. glomerata* from Cuba and Hispaniola (Bx035, Bx036, Bx106 and Bx107), limestone ecotypes of the same species from Cuba and Hispaniola (Bx031, Bx143, Bx155, Bx156), *B. portoricensis* and *B. vahlii* from Puerto Rico and *B. citrifolia*, the unique species of *Buxus* distributed from Suriname to Panama (Fig. 2.4). The "Glomerata"-clade includes species from the three phytogeographic subprovinces of Cuba and from other regions of the Caribbean (Fig. 2.4). Among the serpentine endemics in the "Glomerata"-clade, none is a Ni hyperaccumulator, and only samples of *B. marginalis* have been reported as accumulators or no accumulators (Reeves & al. 1996) [Appendix 2.1].

Within the Caribbean *Buxus*-clade, some taxa represented by more than one sample are not monophyletic. In the "Glomerata"-clade, samples of *B. glomerata* from the locality Los Ciguatos (Guantánamo, southeastern Cuba) are nested with *B. sclerophylla* from the same region (1 PP/ 63% JK); whereas other samples of *B. glomerata* from central Cuba and Hispaniola are placed near to samples of *B. bahamensis* and *B. vahlii*. In the "Shaferi"-clade, *B. shaferi* is not monophyletic; e.g. the accession Bx122 and Bx022 of *B. shaferi* are related to accessions Bx140, Bx141, and Bx011 of *B. foliosa*, respectively, mostly with high supports (1 PP/ 99% JK and 0.99 PP/ 63% JK; Fig. 2.1). In this case samples of both species were collected in the same site (along the way to La Melba, Moa, northeastern Cuba). In order to explore the causes of this further studies are necessary.

2.3.3 Divergence times and diversification rates

The times of divergence estimated for clades including the Cuban and other Caribbean *Buxus*, under all seven scenarios are very similar (Table 2.2). The times of divergence estimated under the scenarios i and iv are slightly younger than the rest, but still within the confidence intervals of the ages estimated under the other scenarios. Further discussions about divergence times in this study are based on the times of divergence estimated with scenario v (Fig. 2.5). The Caribbean *Buxus*-clade originated in the Middle to Late Miocene (c. 12.3 Mya) and the three internal clades have diverged

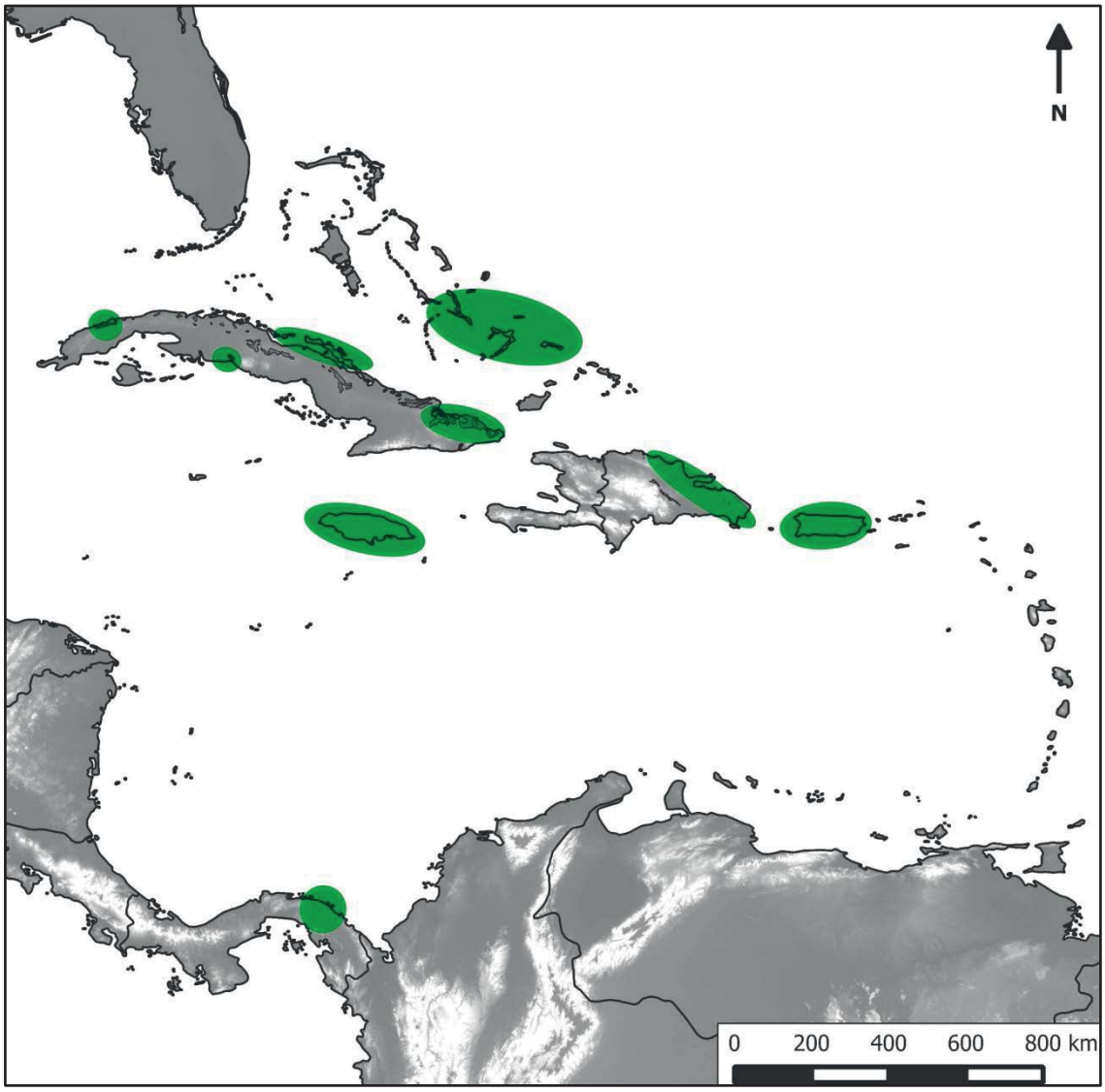


Fig. 2.4. Distribution of the species enclosed in the “Glomerata”-clade.

during the Latest Miocene and the Pliocene ["Glomerata"-clade (c. 5.3 Mya), "Shaferi"-clade (c. 4.8 Mya) and "Gonoclada"-clade (c. 3.2 Mya)] (Table 2.2; Fig. 2.5). The clades with the highest diversification rates are "Gonoclada"-clade and "Glomerata"-clade with 0.78 sp. Myr^{-1} and 0.53 sp. Myr^{-1}, respectively (Table 2.3).

2.3.4 Ancestral areas and ancestral traits

The Bayesian Binary MCMC analysis points to eastern Cuba as the ancestral area of *Buxus* in the Caribbean. The ancestors of the clades "Gonoclada", "Shaferi" and "Glomerata" also originated in eastern Cuba. Only the ancestor of an internal node within the "Glomerata"-clade was probably a plant from other Caribbean island, likely from Puerto Rico or Hispaniola (Fig. 2.6).

The ancestor of the Caribbean *Buxus* was most likely a plant growing on other soils than serpentines. The ancestors of the clades "Glomerata", "Gonoclada" and "Shaferi" were already serpentine endemics (Fig. 2.7). The serpentine ecotype of the west Cuban *B. brevipes* and, within the "Glomerata"-clade, the serpentine ecotypes of *B. glomerata* and *B. bissei* could represent independent attempts to establish in serpentine habitats.

Within the "Glomerata"-clade none of the serpentine taxa are Ni hyperaccumulator and only *B. marginalis* behaves as accumulator and no accumulator (Reeves & al. 1996), for this reason this species could be considered a facultative accumulator following the criteria of Pollard & al. (2014) and was classified as "ambiguous" in the reconstruction of this character. The ancestor of the "Glomerata"-clade was a plant without the capacity to accumulate or hyperaccumulate Ni. The reconstruction does not show clearly which was the characteristic of the ancestor of the "Shaferi"-clade. However the reconstruction allows saying that it was most probably a plant with the capacity to accumulate or hyperaccumulate Ni. The Ni hyperaccumulation evolved in ancestors of intern nodes of the "Shaferi"-clade at least twice. In the case of the ancestor of the "Gonoclada"-clade the analysis points that it was Ni hyperaccumulator (Fig. 2.8).

Table 2.2. Divergence times of Caribbean clades of *Buxus* estimated under the conditions of seven calibration scenarios

Calibration scenarios	**Clade / Crown age median** [95% confidence interval] (Myr)			
	Caribbean *Buxus*-clade	"Gonoclada"-clade	"Shaferi"-clade	"Glomerata"-clade
i	11.8 [9.0–14.9]	3.0 [1.8–4.8]	4.6 [3.2-6.5]	5.2 [3.9-6.8]
ii	12.4 [9.4–15.6]	3.2 [2.0–5.0]	4.8 [3.3-6.7]	5.4 [4.0-7.0]
iii	12.3 [9.5–15.6]	3.1 [1.9–4.9]	4.7 [3.2-6.7]	5.4 [3.9-7.2]
iv	11.8 [9.1–15.0]	3.0 [1.8–4.8]	4.6 [3.1-6.2]	5.1 [3.7-6.8]
v	**12.3** [9.2–15.8]	**3.2** [1.8–4.9]	**4.8** [3.1-6.8]	**5.3** [3.8-7.0]
vi	12.6 [9.5–15.8]	3.2 [2.0–5.0]	4.8 [3.4-6.8]	5.5 [4.0-7.2]
vii	12.6 [9.7–16.0]	3.2 [2.0–5.0]	4.8 [3.2-7.0]	5.5 [3.8-7.3]
Average ages	12.2 [9.3–15.5]	3.1 [1.9–4.9]	4.7 [3.2–6.7]	5.3 [3.9–7.0]

Table 2.3. Clades, number of species, divergence times and speciation rates in each clade

Clades	**Number of species**	**References**	**Divergence times** (Myr)	**Speciation rates [SR]** (sp. Myr^{-1})
Caribbean *Buxus*-clade	(c. 45), 39 included in this study	Adams (1972), Correll & Correll (1982), Liogier (1986), Liogier (1988), this study	12.3	0.30
"Shaferi"-clade	8	Köhler (2014)	4.8	0.43
"Gonoclada"-clade	12	Köhler (2014)	3.2	**0.78**
"Glomerata"-clade	17	Adams (1972), Correll & Correll (1982), Liogier (1986), Liogier (1988), Köhler (2014), this study	5.3	0.53

2.4 Discussion

2.4.1 Relationships within *Buxales*

Our results show that the relationships of *Haptanthus* are stronger with *Didymeles* than with the genera of *Buxaceae*, suggesting that it should be kept in *Buxales* as a monotypic genus of *Haptanthaceae*, near to *Buxaceae* supporting the suggestion of Doust & Stevens (2005) based on flower anatomy. In contrast the inclusion of *Haptanthus* into *Buxaceae* as proposed by Shipunov & Shipunova (2011) is not supported.

Within *Buxaceae*, the genera *Pachysandra*, *Styloceras* and *Sarcococca* are enclosed in a strong supported clade (Fig. 2.1). This close relationship was previously suggested by Mathou (1940) based on anatomical and morphological characters and was supported by the study of Balthazar & al. (2000) based on the molecular markers *ndhF* and ITS.

Within *Buxus*, the three resolved clades (Eurasian, African and American) support the sections *Eubuxus*, *Tricera* and *Probuxus* proposed by Mathou (1940). These three clades were previously found by Balthazar & al. (2000), although in their study the African clade was poorly supported.

The Mexican species of *Buxus* are related to the west Cuban *B. brevipes* but with low supports (Fig. 2.1). Anatomical studies (Köhler & Schirarend 1989) showed affinities of *B. brevipes* with the Mexican species.

B. jaucoensis, a relict endemic from the limestone cliffs of Jauco in the southeastern part of Cuba, is basally placed as an independent lineage, whereas the rest of the Cuban and Caribbean species are nested in three internal clades (Fig. 2.1). *B. jaucoensis* has anatomical affinities with other Cuban species such as *B. shaferi*, *B. obovata*, *B. moana*, *B. foliosa*, *B. glomerata* and *B. vahlii*, grouped in the *B. obovata* type by Köhler & Schirarend (1989) and has palynological affinities with *B. glomerata*

(Köhler 1982); however *B. jaucoensis* is the only American species in which the masculine flowers can lack of tepals.

All other species of Cuban and Caribbean *Buxus* constitute a monophyletic group, which is divided in three internal clades (Fig. 2.1). The "Gonoclada"-clade encloses species from east and central Cuba, all taxa of this clade share the synapomorphies of being serpentine endemics and Ni hyperaccumulators. The "Shaferi"-clade encloses species only distributed in northeastern Cuba, which inhabit the understorey of rainforests and along the water courses or rivers. The serpentine endemism is a synapomorphic character in this clade with the sole exception of a limestone ecotype of *B. yunquensis*. In this clade five species are Ni hyperaccumulators and *B. shaferi* may accumulate this metal or not. The "Glomerata"-clade is distributed in the three phytogeographic subrprovinces of Cuba, in four other Caribbean islands or archipelagos and in northern South America up to Panama. The species of the "Glomerata"-clade inhabit a wide range of ecological conditions on limestone or serpentines, from the coasts to the mountains. The number of serpentine endemics and non-serpentine endemics is similar. A facultative Ni accumulator (*B. marginalis*) is enclosed in this clade and no Ni hyperaccumulators are reported.

2.4.2 Diversification of *Buxus* in the Cuban serpentines

Our results point eastern Cuba as the most ancestral area of *Buxus* in the Caribbean (Fig. 2.6). Eastern Cuba was the biggest emerged Cuban territory with the highest elevations during the Miocene (Iturralde-Vinent 2006). Currently, this region has the mightiest rivers, highest diversity of rocks and soils (e.g., serpentines, limestone) compared to nearby regions. Additionally, it has also localities with the highest levels of rainfalls and other ones with the driest conditions (Nuñez-Jimenez 1982; Anonymous 1989). Furthermore in eastern Cuba occur the highest mountains of the island (600–c. 2000 meters above sea level), enhancing thus the diversity of habitats. Some of these characteristics could have occurred in the Miocenic eastern Cuba. The existence of such diverse conditions offered a wide range of available habitats to plants which could arrive

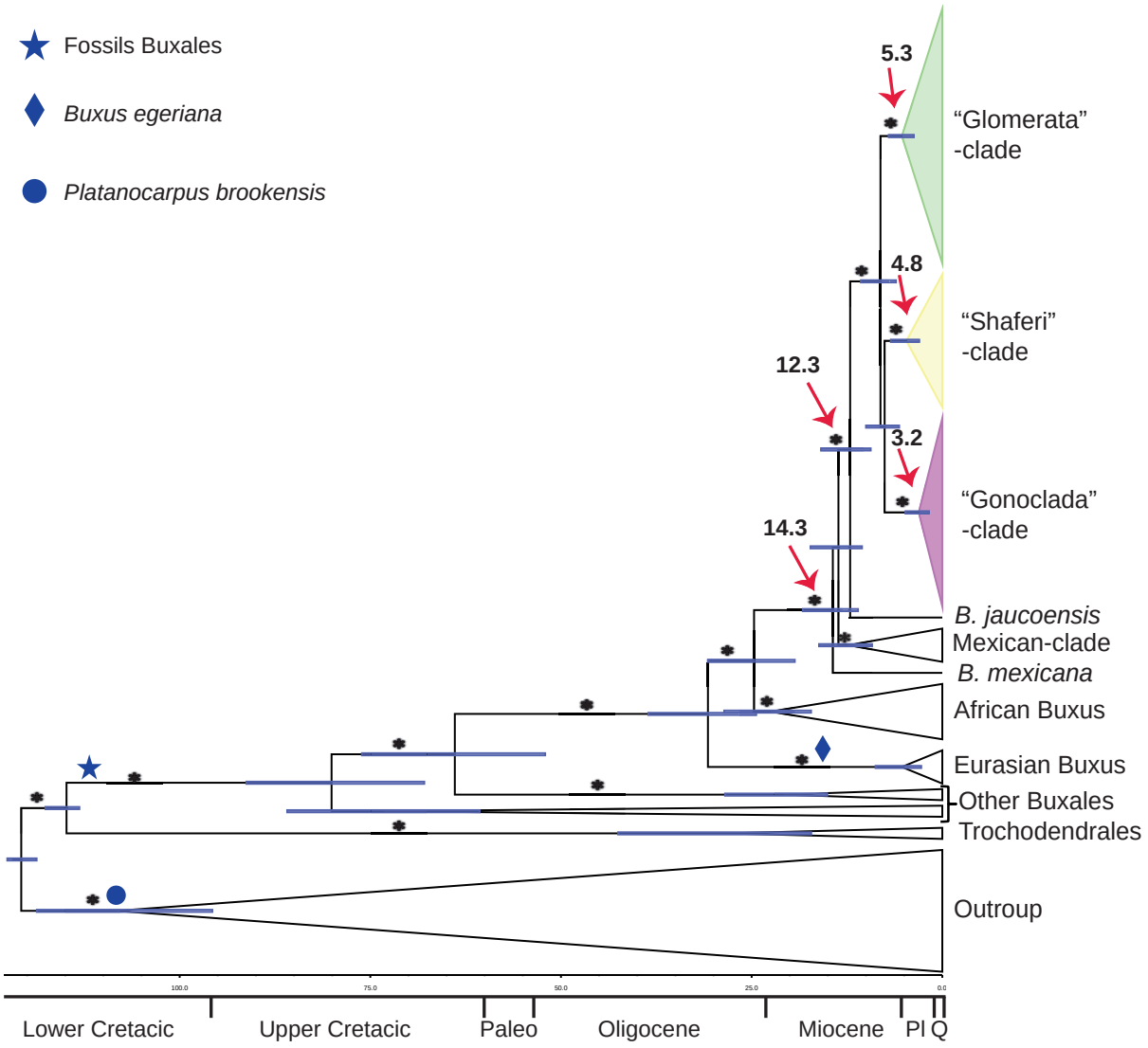


Fig. 2.5. BEAST tree showing the times of divergence of the Caribbean clades of *Buxus*. Grey bars at nodes show 95% confidence intervals. Paleo- Paleocene, Pl-Pliocene, Q- Quaternary. Asterisks indicated nodes with posterior probabilities = 0.99-1. The ages of the clades (in Million years) are indicated with red arrows.

from neighbouring regions. Some of these characteristics have been pointed as facilitating the speciation of endemics in Cuba (Borhidi 1996).

The ancestor of the Caribbean *Buxus* in eastern Cuba was most probably a non-serpentine plant, but a plant growing on other kind of soils, may be on limestone areas (Fig. 2.7). This is consistent with the hypothesis that the Cuban serpentine flora originated from non-serpentine ancestors exposed by Borhidi (1996). Jestrow & al. (2012) suggested that the ancestors of the *Leucocroton* alliance probably occurred on limestone soils.

The divergence age of the clade enclosing all Caribbean *Buxus* is placed in the Miocene (Fig. 2.5) like another emblematic Caribbean genus, *Spathelia* (8.7 My, Appelhans & al. 2012). Like in *Buxus*, most Cuban *Spathelia* (four out of five) grow on the serpentine outcrops of northeastern Cuba. The biggest and oldest serpentine outcrops of Cuba are located in the mountains of Nipe, Cristal, Moa and Baracoa in northeastern Cuba. The size and the age of the serpentine outcrops, and the richness of the surrounding flora have been pointed as factors influencing in the evolution of the serpentine flora (Borhidi 1996). The oldest east Cuban serpentine outcrops are 10–30 My (Borhidi 1996), so they were already among the available habitats when groups like *Buxus* and *Spathelia* arrived to east Cuba.

In Cuba, 84% of species, subspecies or ecotypes of *Buxus* are exclusive on serpentines. These habitats with extreme and stressing conditions (Berazaín-Iturrande 2004; Coleman & Alexander 2004) force the plants to change biotypes (Borhidi 1996). The colonization of the serpentines is one of the most important factors triggering the evolution of Cuban flora (Borhidi 1996). There is lack of information about the genetics of adaptation to serpentines and the adaptive mechanisms that confer to plants tolerance to serpentines are still not well understood. May be these mechanisms are involved with ion uptake discrimination at the root level, ion translocation properties, and/or chelation (Brady & al. 2005). Serpentine soils can contribute to speciation in two ways: the adaptation to serpentine soils can contribute indirectly to pre- or postzygotic reproductive barriers that genetically isolate serpentine populations from non-serpentine relatives and the patchy distribution of serpentine can contribute to the geographic

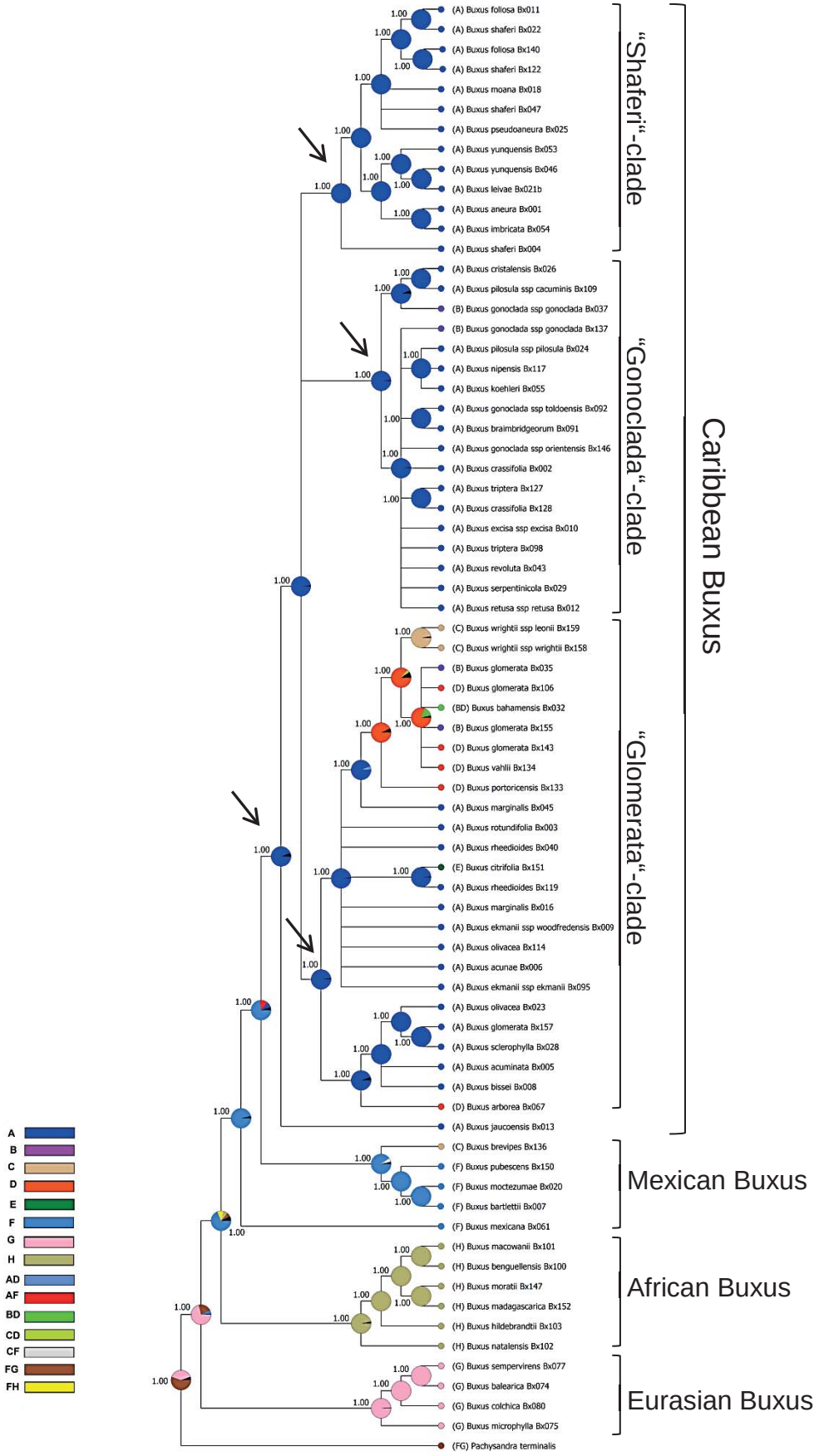


Fig. 2.6. Reconstruction of ancestral areas of *Buxus* using RASP. (A) east Cuba, (B) central Cuba, (C) west Cuba, (D) other islands of the Caribbean [Bahamas, Hispaniola, Jamaica, and Puerto Rico], (E) South America–Panama, (F) North America and Central America until El Salvador, (G) Eurasia including the Mediterranean islands and Mediterranean areas of North Africa, (H) Africa South of Sahara and adjacent islands of the Indic Ocean [Madagascar, Comoro islands, Socotra]. Arrows are showing the respective ancestral area of the clades of interest (see discussion).

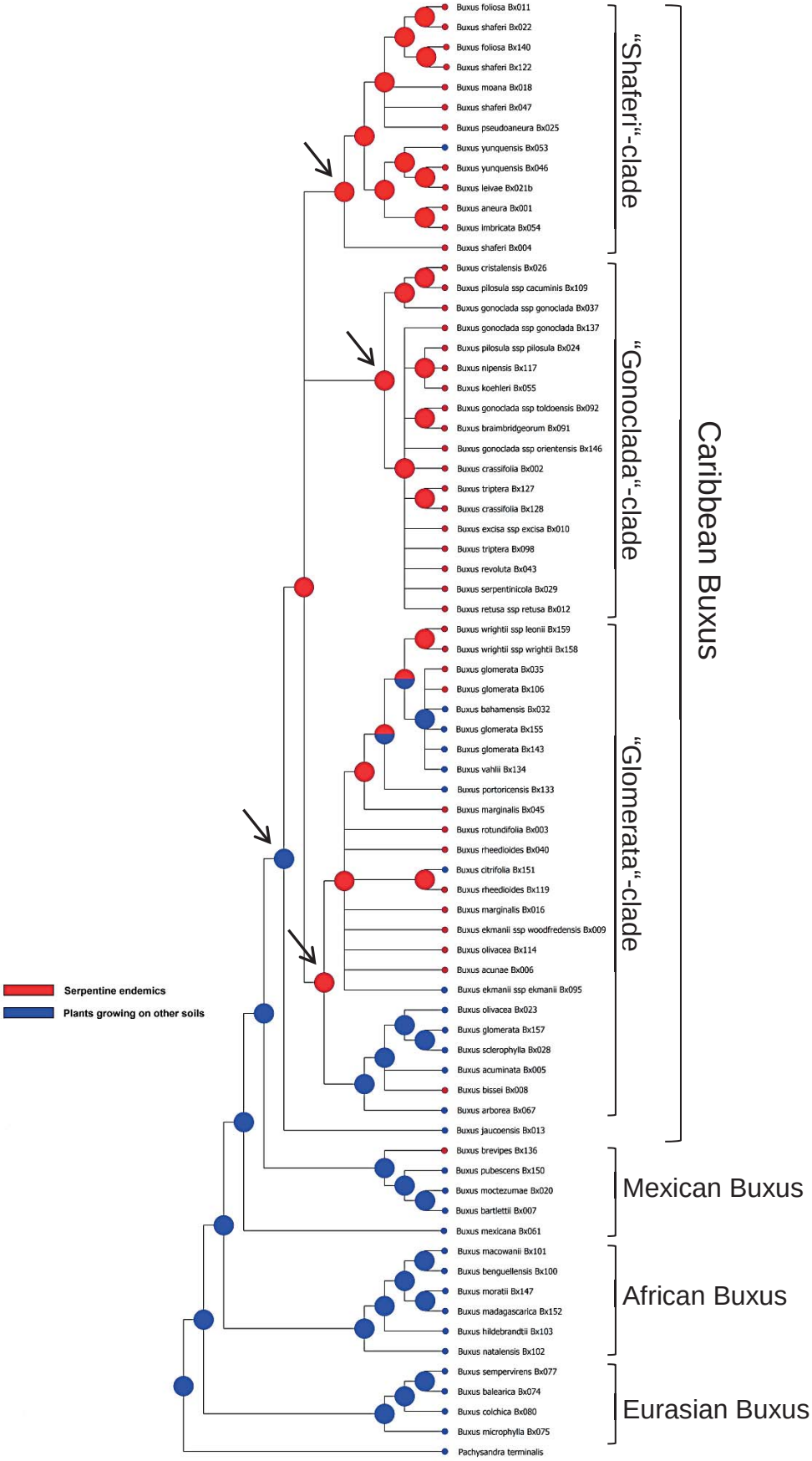


Fig. 2.7. Parsimony optimization of the trait: adaptation to grow on serpentines (serpentine endemism) or on other soils. Arrows are showing the respective ancestral state of the clades of interest (see discussion).

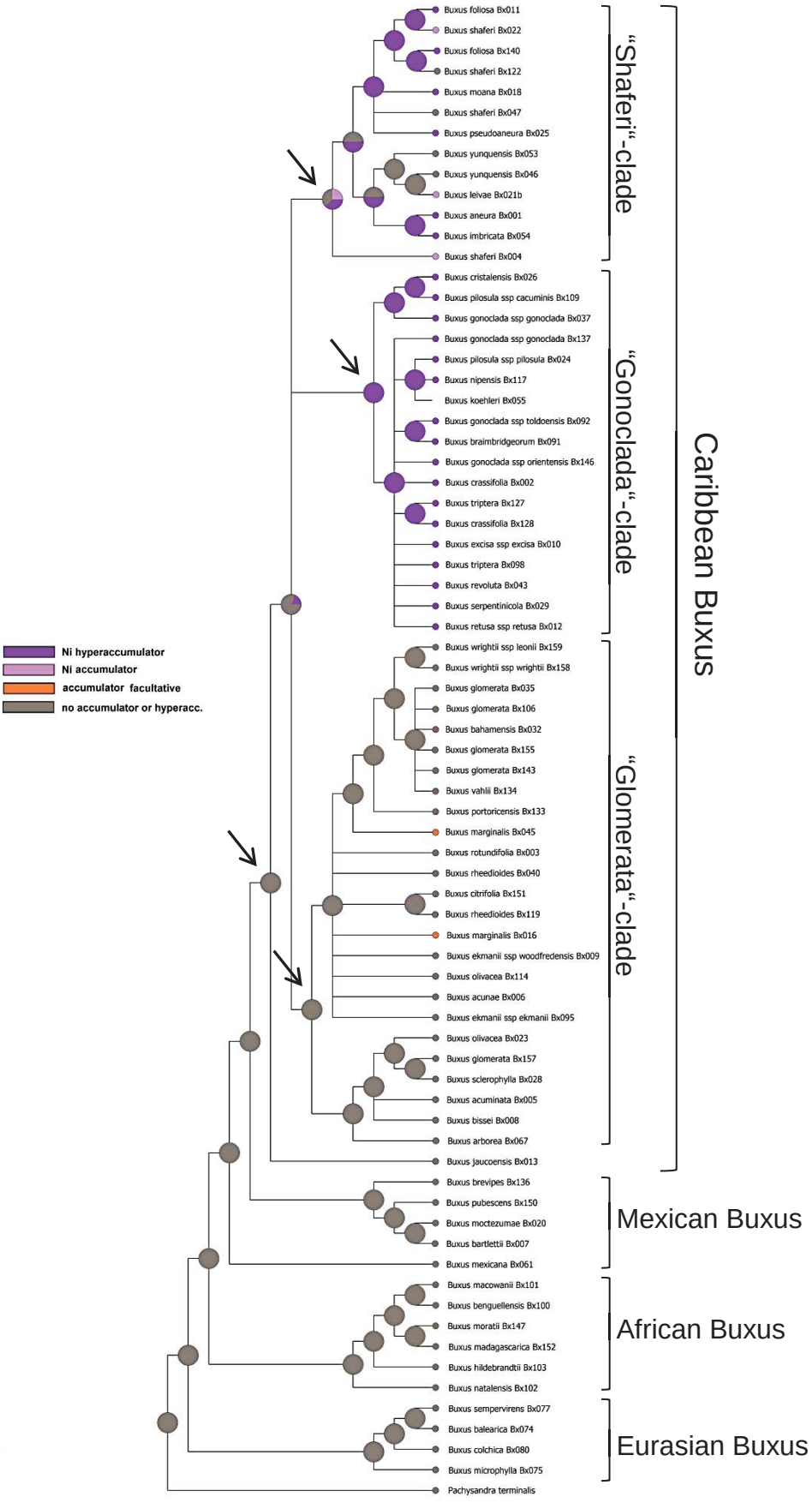


Fig. 2.8. Parsimony optimization of the trait hyperaccumulation or accumulation of Ni. Arrows are showing the ancestral state of the clades of interest (see discussion).

isolation of populations (Rajakaruna 2004; Kay & al. 2011). The adaptation of *Buxus* to the serpentines and its speciation could have happened as exposed above. The development of the strategies for the evolution of the first serpentine endemic *Buxus* in east Cuba likely needed a long period, like the c. 7 My from the time of divergence of the ancestor of the Caribbean *Buxus* (c. 12.3 My) to the divergence age of the oldest clade including serpentines endemics, the "Glomerata"-clade (c. 5.3 My) [Fig. 2.5]. Once that the serpentine biotype was achieved in *Buxus* the fastest speciation rates so far reported for shrubs and trees in the insular Caribbean took place in each one of the three intern clades (Table 2.3).

Each plant group growing on serpentines behaves in a special way with respect to each chemical factor of the serpentine substrate (Babalonas & al. 1984). Two basic strategies of plants dealing with the high concentrations of heavy metals in serpentines soils have been suggested: accumulation and exclusion (Baker 1981; Kazakou & al. 2008). The Cuban serpentine endemic seems to have followed different evolutionary paths. The low concentrations of Ni found in the leaf tissues of the serpentines endemics enclosed in the "Glomerata"-clade point that the exclusion or limitation of Ni uptake was the evolutionary strategy of this clade, which was successful with a SR of 5.3 sp. Myr^{-1}, the second highest within the Caribbean *Buxus*. The "Glomerata"-clade is the oldest among the Caribbean *Buxus*, thus the exclusion of Ni seems to be the first strategy developed by the Cuban serpentine *Buxus*. With the opposite strategy, accumulation or hyperaccumulation, the plants developed the ability to translocate and accumulate high concentrations of heavy metals. The processes and the genetic basis of the accumulation and hyperaccumulation are not well understood (Seregin & Kozhevnikova 2006). Some genes involved in stages of the hyperaccumulation process have been identified considering their overexpression in specific organs (Rascio & Navari-Izzo 2011). The capacity to accumulate Ni likely was a characteristic of the ancestor of the "Shaferi"-clade, c. 4.8 Mya, which was reinforced to Ni hyperaccumulation being a current feature of most taxa of this clade. The most remarkable evolutionary consequences of Ni hyperaccumulation in the Caribbean are shown in the "Gonoclada"-clade, were it is a characteristic of almost all taxa, and has triggered a SR of 0.78 sp. Myr^{-1}, the highest so far documented for a group of serpentine plants in the Caribbean region (Table 2.3). The ecologic role of the hyperaccumulation

of heavy metals is controversial. Boyd & Martens (1992) discussed five main hypotheses with examples and concluded that the strongest evidences suggest as a defence mechanism against herbivores. This topic has been discussed in different groups of plants as defence against herbivores (Boyd & al. 2004), against pathogens (Boyd & Shaw 2004) and as protection versus environmental stress such as UV irradiation (Berazaín-Iturralde & al. 2007). In the case of Cuban *Buxus* the studies of Ni concentration have been conducted only on leaves. The most plausible roles of the Ni hyperaccumulation in *Buxus* could be as protection against herbivores or as a natural barrier for avoiding the excessive loss of water in the stressing conditions of the serpentines. The mesophyll and epidermis cells of Cuban Ni hyperaccumulating species are quite darker than in other species (Köhler & Schirarend 1989), what suggests that Ni could be stored in these cells.

The values of SRs here recorded for the Caribbean clades of *Buxus* are low compared with those estimated for big radiations in other groups of plants such as *Cistus* (Guzmán & al. 2009), *Lupinus* (Drummond & al. 2012) and *Dianthus* (Valente & al. 2010); however the diversification rates of the Cuban and Caribbean *Buxus* are higher than the values reported for *Buxales* (Magallón & Sanderson 2001) and are remarkable considering the slow growth of the plants of the genus *Buxus* (Roselló & al. 2007), what implies a wider period of time among the generations and thus a deceleration in speciation.

2.4.3 Dispersal in east, from east to central and west Cuba and the rest of the Caribbean

In spite of the low supported relationships among the Cuban and Mexican species, probably *Buxus* migrated from Mexico to Cuba. The divergence time of the node linking *B. mexicana* with the other American *Buxus* (c. 14 My, Fig. 2.5), which is older than the Caribbean clade (c. 12 My, Fig. 2.5), and the morphological and anatomical affinities of the west Cuban *B. brevipes* with Mexican species support such hypothesis. The geology of the Caribbean is complex and there are few consensuses among the criteria about it (Iturralde-Vinent 1982; Hedges 2001; Coleman & Alexander

2004). Among the most recent and critic contributions on this topic is the work of Iturralde-Vinent (2006). This author does not mention the existence of a land bridge connecting Cuba and Yucatan which could have allowed the migration of *Buxus* from Mexico to Cuba and in his work the only stable connection of the Greater Antilles with the continent was during the transit Eocene-Oligocene, 35–32 My ago. This does not match with the age estimated for the node linking the Cuban and Mexican *Buxus* (13.6 My). On the other hand such a land bridge called GAARlandia should have connected the former Greater Antilles with northern South America and not with the former Mexico. *Buxus* seeds are primarily dispersed passively, ballistically and occasionally by ants (Lázaro & al. 2006) and there are no records of seed dispersal by birds or bats. Because the seeds and capsules of *Buxus* are too heavy to be transported by common wind the most likely mode of colonization in the Caribbean area were extreme meteorological events like hurricanes. The hurricanes have been suggested to be an irregular but significant dispersal vector for the Caribbean biotas (Iturralde-Vinent 1982; Borhidi 1996). The hurricanes winds were stronger in the past (Graham 2006a, 2006b). Nathan & al. (2008) point that plants are probably dispersed by extreme meteorological events irrespective of their taxonomy and dispersal morphology.

In Cuba, the serpentine outcrops are distributed like an archipelago from east to west (Fig. 1.2 of chapter 1). This distribution pattern of the Cuban serpentine outcrops has probably favoured the allopatric speciation in the genus after the evolution of the serpentine adaptation. Considering the restricted dispersal potential of *Buxus*, the migration in east Cuba, from east Cuba to central and west Cuba, to the rest of the Caribbean islands and northern South America must have been a slow and highly stochastic process.

The species of the “Shaferi”-clade are distributed in localities of the mountains of northeastern Cuba. Since most species in this clade inhabit rainforest or are confined to riverine vegetation they seem to be adapted to more humid conditions. Its dispersal has probably depended on rivers and their overflows. Northeastern Cuba has the highest records of rains in the island (Anonymous 1989).

The dispersal of the serpentine species belonging to the clades “Gonoclada” and “Glomerata”, which are present in the central and west regions of the island, probably

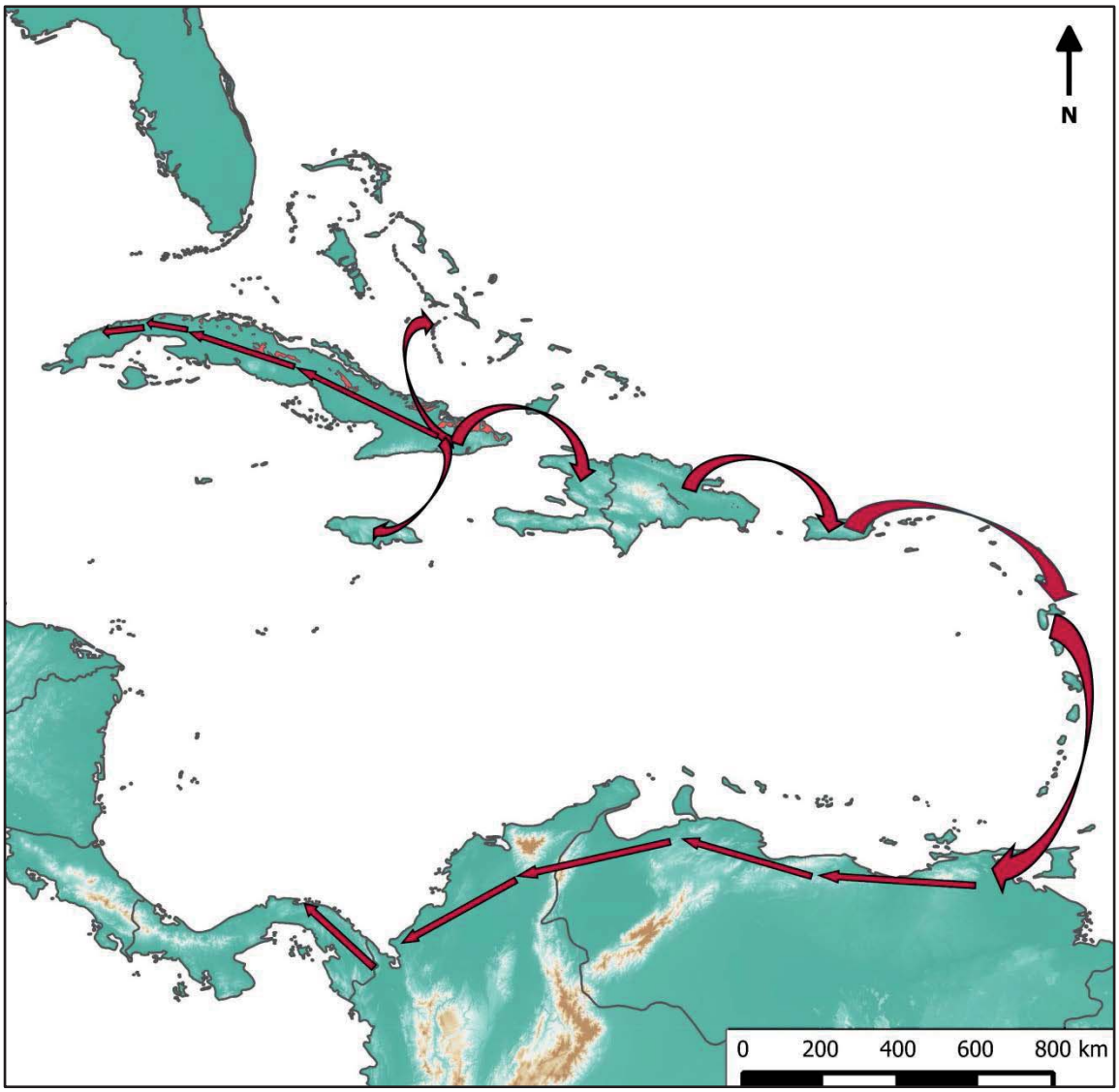


Fig. 2.9 Red arrows show the hypothetical migrations routes of *Buxus* in the Caribbean.

occurred following the migration routes of the serpentine flora, from the eastern to central and west Cuba (Fig. 2.9; see Borhidi 1996), probably facilitated by the strong winds and inundations associated to hurricanes. Species growing on riverine vegetation are also enclosed in the clades "Glomerata" and "Gonoclada"; in such cases the dispersal could have happened as suggested for the species of the "Shaferi"-clade.

The age of the "Glomerata"-clade (5.3 My), including the species distributed in the rest of the Caribbean islands and northern South America is not congruent with the existence of any important connection among islands. During the Pliocene seas descents could have allowed short connections among islands, but the most important islands (Bahamas, Cayman, Cuba, Hispaniola, Jamaica and Puerto Rico) were not in contact (Iturralde-Vinent 2006). In such Caribbean paleogeographic scenario the most recurrent vector for the dispersion of *Buxus* are the hurricanes, which probably also have driven the long distance dispersal of *Spathelia* (Appelhans & al. 2012) and other groups of the Caribbean biotas (Presley & Willig 2008) [Fig. 2.9].

B. citrifolia, the only species of *Buxus* distributed in northern South America (Suriname, Venezuela, Colombia and Panama) is included in the "Glomerata"-clade. The dispersal of the ancestor of this species probably was along the arc of the Lesser Antilles favoured by the hurricanes. The species *B. subcolumnaris* (not included in this study) endemic to the island of Martinique has morphological affinities with other species of the "Glomerata"-clade like *B. portoricensis* and *B. citrifolia* and may therefore represent a relict species of the migrations of the genus from the Greater Antilles to the Lesser Antilles and from there to South America (Fig. 2.9).

2.5 Main conclusions

Our study demonstrated that the Caribbean *Buxus* are monophyletic. The evolution of the genus in Cuba was probably triggered by the adaptation to the serpentine habitats which were already available when the ancestor of this group arrived to the eastern part of Cuba. The combination of analysis of traits related to adaptation to ultramafic soils, the accumulation or hyperaccumulation of Ni, with phylogeographic

analysis allowed elucidating major evolutionary steps within this genus. Our data suggest that the adaptations to the harsh conditions of the serpentine soils triggered a remarkable radiation within this genus and underline that speciation is not a regular process. The adaptation of *Buxus* to the Cuban serpentines was a gradual process, which included the exclusion of heavy metals like Ni as well as the accumulation and hyperaccumulation of Ni. Our results also show that speciation rates within clades can be slow for millions of years and then can increase by a major environmental adaptation such as adaptation to grow on serpentines and the Ni hyperaccumulation. Our study suggests that the Caribbean *Buxus* depended for dispersal on stochastic dispersal events such as hurricanes.

Appendix 2.1. Taxa, samples codes, herbarium vouchers, localities, GenBank accessions codes and complementary data about geographic distribution, serpentines endemics or plants growing on other soils, capacity to accumulate, hyperaccumulate Ni or not.

Taxon	Sample code	Family	Collector, herbarium vouchers	Origin: Country/ locality	trnK-matK	petD	trnL-F	Distribution	SE or NS	NiA, NiH or NAH
Akebia quinata (Thunb. ex Houtt.) Decne.		Lardizabalaceae	T. Borsch 3412 (BONN)	Botanical Garden Bonn	AF542587	AM396526	AM397152			
Buxus acuminata (Griseb.) Müll. Arg.	Bx 005*	Buxaceae	R. Berazaín & al. HFC 71535 (HAJB)	Cuba: Guantánamo, Baracoa, Yunque de Baracoa. Limestone. Cultivated JBN.	this study	this study	this study	A	NS	NAH (Reeves & al. 1996)
Buxus acunae Borhidi & O. Muñiz	Bx 006*	Buxaceae	T. Borsch & al. 4260 (B, HAJB, ULV)	Cuba: Holguín, Moa, Yamanigüey. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NAH (Reeves & al. 1996)
Buxus aneura Urb.	Bx 001*	Buxaceae	J. Gutiérrez & al. HFC 78357 (B, HAJB)	Cuba: Holguín, Mayarí, Rosa Castillo. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus arborea Proctor	Bx 067*	Buxaceae	E. Köhler 85 (B)	Jamaica: Parish of Trelawny, Island View Hill.	this study	this study	this study	D	NS	NAH
Buxus arborea Proctor	Bx 068	Buxaceae	Braimbridge sn (B)	Jamaica	this study	this study	this study			
Buxus bahamensis Baker	Bx 032*	Buxaceae	P. A. González HFC 85861 (B, HAJB)	Cuba: Holguín, Gibara, Los Cañones. Limestone.	this study	this study	this study	BD	NS	NAH
Buxus bahamensis Baker	Bx 033	Buxaceae	P. A. González HFC 85862 (B, HAJB)	Cuba: Holguín, Gibara, Los Cañones. Limestone.	this study	this study	this study			
Buxus bahamensis Baker	Bx 034	Buxaceae	P. A. González HFC 85863 (B, HAJB)	Cuba: Holguín, Gibara, Los Cañones. Limestone.	this study	this study	this study			
Buxus bahamensis Baker	Bx 078	Buxaceae	W. Greuter & al. 26957 (B)	Cuba: Villa Clara, Caibarién, Cayo Santa María. Limestone.	this study	this study	this study			
Buxus bahamensis Baker	Bx 142	Buxaceae	G. R. Proctor & W. T. Gillis 33161 (B)	Bahamas: Mayaguana.	this study	this study	this study			
Buxus balearica Lam.	Bx 074*	Buxaceae	collector? (B#13477)	Spain. Cultivated in Botanical Garden Berlin-Dahlem.	this study	this study	this study	G	NS	NAH
Buxus bartlettii Standl.	Bx 007*	Buxaceae	E. Köhler 75 (B)	Mexico: Oaxaca, Chiltepec, carretera Chiltepec-Tuxtepec.	this study	this study	this study	F	NS	NAH
Buxus benguellensis Gilg	Bx 100*	Buxaceae	J. Gutiérrez & Maiato 85996 (HAJB)	Angola, Lubango.	this study	this study	this study	H	NS	NAH
Buxus bissei Eg. Köhler	Bx 008*	Buxaceae	J. Gutiérrez & al. HFC 77565-A (HAJB)	Cuba: Holguín, Moa, entre Alto de La Calinga y Revuelta de Los Chinos. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NAH (Reeves & al. 1996)
Buxus braimbridgeorum Eg. Köhler	Bx 091*	Buxaceae	J. Gutiérrez & al. HFC 83347 (B, HAJB)	Cuba: Holguín, Moa, Ascenso al Toldo. Serpentine.	this study	this study	this study	A	SE	NiH (this study)
Buxus brevipes (Müll. Arg.) Urb.	Bx 136*	Buxaceae	R. Rankin HFC 87054 (B, HAJB)	Cuba, Pinar del Río, La Palma, orillas del río San Juán. Cultivated JBN.	this study	this study	this study	C	SE	NAH (Reeves & al. 1996)
Buxus citrifolia Spreng.	Bx 151*	Buxaceae	C. Galdames 6848 (B)	Panamá: Distrito de Panamá, Cerro Azul. Alt. 750 m.	this study	this study	this study	E		
Buxus colchica Pojark.	Bx 080*	Buxaceae	Borsch & al. 3970a (B)	Georgia	this study	this study	this study	G	NS	NAH
Buxus crassifolia (Britton) Urb.	Bx 002*	Buxaceae	R. Berazaín & al. HFC 74056 (B, HAJB)	Cuba: Holguín, Moa, río Limones. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus crassifolia (Britton) Urb.	Bx 128*	Buxaceae	M. Ackermann & al. 1001 (B, HAJB, ULV)	Cuba: Guantánamo, Baracoa, Santa María, Alto de Iberia. Serpentine.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus cristalensis Eg. Köhler & P. A. González	Bx 026*	Buxaceae	J. Gutiérrez & al. HFC 75386 (HAJB)	Cuba: Santiago de Cuba, Segundo Frente, entre El Halcón y Batista. Serpentine. Cultivated JBN.	HG004434	this study	HG004429	A	SE	NiH (this study)
Buxus ekmanii Urb. subsp. *ekmanii*	Bx 095*	Buxaceae	J. Gutiérrez & al. HFC 79695 (HAJB)	Cuba: Guantánamo, Baracoa, Yunque de Baracoa. Limestone.	this study	this study	this study	A	NS	NAH (this study)
Buxus ekmanii subsp. *woodfredensis* Eg. Köhler	Bx 009*	Buxaceae	J. Gutiérrez & al. HFC 75432 (B, HAJB)	Cuba: Holguín, Mayarí, Sierra Cristal, suroeste de Mandinga, al sur de la Mina José Martí. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NAH (this study)
Buxus excisa Urb. subsp. *excisa*	Bx 010*	Buxaceae	J. Gutiérrez & al. HFC 77577 (HAJB)	Cuba: Holguín, Moa, Alto de La Calinga. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus foliosa Urb.	Bx 011*	Buxaceae	R. Berazaín & al. HFC 74072 (HAJB)	Cuba: Holguín, Moa, camino a La Melba, alrededores del antiguo aserrío. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus foliosa Urb.	Bx 140*	Buxaceae	T. Borsch & al. 4315 (B, HAJB, ULV)	Cuba: Holguín, Moa, Entre los kilometros 22 y 26 del camino a La Melba. Serpentine.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus foliosa Urb.	Bx 141	Buxaceae	M. Ackermann & al. 955 (B, HAJB, ULV)	Cuba: Holguín, Moa, entre el km 26 km y las Comadres, en el camino a La Melba. Serpentine.	this study	this study	this study			
Buxus glomerata (Griseb.) Müll. Arg.	Bx 031	Buxaceae	P. A. González HFC 85860 (B, HAJB)	Cuba: Holguín, Gibara, Floro Pérez, cerro de San Marcos. Limestone.	this study	this study	this study			
Buxus glomerata (Griseb.) Müll. Arg.	Bx 035*	Buxaceae	P. A. González HFC 85864 (B, HAJB)	Cuba: Holguín, Rafael Freire, Bahía de Naranjo. Serpentine.	this study	this study	this study	B	SE	NAH (this study)

Appendix 2.1. Continued

Taxon	Sample code	Family	Collector, herbarium vouchers	Origin: Country/ locality	trnK-matK	petD	trnL-F	Distribution	SE or NS	NiA, NiH or NAH
Buxus glomerata (Griseb.) Müll. Arg.	Bx 036	Buxaceae	P. A. González HFC 86132 (B, HAJB)	Cuba: Holguín, Rafael Freire, Bahía de Naranjo. Serpentine.	this study	this study	this study			
Buxus glomerata (Griseb.) Müll. Arg.	Bx 052	Buxaceae	J. Gutiérrez & al. HFC 83461 (B, HAJB)	Cuba: Guantánamo, Los Ciguatos. Limestone.	this study	this study	this study			
Buxus glomerata (Griseb.) Müll. Arg.	Bx 143*	Buxaceae	R. García & al. 4839 (B)	Dominican Republic: Sierra de Bahoruco, Barahona. Limestone.	this study	this study	this study	D	NS	NHA
Buxus glomerata (Griseb.) Müll. Arg.	Bx 155*	Buxaceae	P. A. González 1108-2 HFC 87215 (B, HAJB)	Cuba: Cienfuegos, Jagua. Limestone.	this study	this study	this study	B	NS	NAH
Buxus glomerata (Griseb.) Müll. Arg.	Bx 156	Buxaceae	P. A. González 1108-3 HFC 87216 (B, HAJB)	Cuba: Cienfuegos, Milpa. Limestone.	this study	this study	this study			
Buxus glomerata (Griseb.) Müll. Arg.	Bx 157*	Buxaceae	R. Berazaín & al. HFC 72281 (HAJB)	Cuba: Guantánamo, Los Ciguatos. Limestone.	this study	this study	this study	A	NS	NAH
Buxus glomerata (Griseb.) Müll. Arg.	Bx 106*	Buxaceae	A. H. Liogier 16559 (NY)	Dominican Republic: Arroyo Francés, 4 miles W of Puerto Plata. Serpentine.	this study	this study	this study	D	SE	NiH (this study)
Buxus glomerata (Griseb.) Müll. Arg.	Bx 107	Buxaceae	A. H. Liogier 16560 (NY)	Dominican Republic: Arroyo Francés, 4 miles W of Puerto Plata. Serpentine.	this study	this study	this study			
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*	Bx 037*	Buxaceae	P. A. González HFC 85866 (B, HAJB)	Cuba: Holguín, Rafael Freire, Bahía de Naranjo. Serpentine.	this study	this study	this study	B	SE	NiH (Reeves & al. 1996)
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*	Bx 038	Buxaceae	P. A. González HFC 85867 (B, HAJB)	Cuba: Holguín, Rafael Freire, Bahía de Naranjo. Serpentine.	this study	this study	this study			
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*	Bx 039	Buxaceae	P. A. González HFC 86131 (B, HAJB)	Cuba: Holguín, Rafael Freire, Bahía de Naranjo. Serpentine.	this study	this study	this study			
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*	Bx 079	Buxaceae	J. Matos sn (B)	Cuba: Villa Clara, Santa Clara. Serpentine.	this study	this study	this study			
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*	Bx 137*	Buxaceae	J. Bisse & al. HFC 40211 (HAJB)	Cuba: Mayabeque, Canasí, Lomas de Galindo. Serpentine.	this study	this study	this study	B	SE	NiH (Reeves & al. 1996)
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*	Bx 050	Buxaceae	W. Carmenate & P. A. González HFC 86133 (B, HAJB)	Cuba: Holguín, Rafael Freire, Ceja de Melones. Serpentine.	this study	this study	this study			
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*	Bx 051	Buxaceae	E. Köhler sn (B)	Cuba: Villa Clara, Santa Clara. Serpentine.	this study	this study	this study			
Buxus gonoclada subsp. *orientensis* Eg. Köhler	Bx 146*	Buxaceae	J. Gutiérrez & al. HFC 80656 (B, HAJB)	Cuba: Guantánamo, Yateras, loma del Mulo. Serpentine.	this study	this study	this study	A	SE	NiH (this study)
Buxus gonoclada subsp. *toldoensis* Eg. Köhler	Bx 092*	Buxaceae	J. Gutiérrez & al. HFC 83339 (B, HAJB)	Cuba: Holguín, Moa, El Toldo. Serpentine.	this study	this study	this study	A	SE	NiH (this study)
Buxus hildebrandtii Baill.	Bx 103*	Buxaceae	N. Kilian & al. YP 2033 (B)	Yemen: Socotra.	this study	this study	this study	H	NS	NAH
Buxus hildebrandtii Baill.	Bx 105	Buxaceae	Skipka YP 2144 (B)	Yemen: Socotra.	this study	this study	this study			
Buxus imbricata Urb.	Bx 054*	Buxaceae	J. Gutiérrez & al. HFC 75300 (HAJB)	Cuba: Holguín, Mayarí, Pico Cristal. Serpentine.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus jaucoensis Eg. Köhler	Bx 013*	Buxaceae	R. Berazaín & al. HFC 72333 (B, HAJB)	Cuba: Guantánamo, Maisí, Paredones del Río Jauco. Limestone. Cultivated JBN.	this study	this study	this study	A	NS	NAH (Reeves & al. 1996)
Buxus koehleri P. A. González & Borsch	Bx 055*	Buxaceae	T. Borsch & al. 4091 (B, HAJB, ULV)	Cuba: Holguín, Mayarí, Sierra de Nipe, sendero del río Guayabo. Serpentine.	HG004433	this study	HG004428	A	SE	?
Buxus leivae Eg. Köhler	Bx 021*	Buxaceae	T. Borsch & al. 4317 (B, HAJB, ULV	Cuba: Holguín, Moa, entre los kilometros 22 y 26 del camino a La Melba. Serpentine.	this study	this study	this study	A	SE	NiA (Reeves & al. 1996)
Buxus macowanii Oliv.	Bx 101*	Buxaceae	P. A. González sn (B#100507478)	South Africa. Cultivated in Baumschulenweg, Berlin.	this study	this study	this study	H	NS	NAH
Buxus madagascarica Baill.	Bx 152*	Buxaceae	G. McPherson & Rabenantoandro 18322 (MO)	Madagascar	this study	this study	this study	H	NS	NAH
Buxus marginalis (Britton) Urb.	Bx 016*	Buxaceae	R. Berazaín & al. HFC cf. 73923 (HAJB)	Cuba: Holguín, Moa, km 8 del camino a La Melba. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NiA, NAH [ambiguous] (Reeves & al. 1996)
Buxus marginalis (Britton) Urb.	Bx 045*	Buxaceae	P. A. González HFC 85877 (B, HAJB)	Cuba: Guantánamo, Baracoa, Santa María. Serpentine.	this study	this study	this study	A	SE	NiA, NAH [ambiguous] (Reeves & al. 1996)
Buxus marginalis (Britton) Urb.	Bx 121	Buxaceae	T. Borsch et al 4312 (B, HAJB, ULV)	Cuba: Holguín, Moa, entre km 22-26 del camino a La Melba. Serpentine.	this study	this study	this study			
Buxus marginalis (Britton) Urb.	Bx 123	Buxaceae	M. Ackermann & al. 971 (B, HAJB, ULV)	Cuba: Holguín, Moa, Cayoguan. Serpentine.	this study	this study	this study			
Buxus marginalis (Britton) Urb.	Bx 124	Buxaceae	M. Ackermann & al. 859 (B, HAJB, ULV)	Cuba: Guantánamo, Baracoa, río Baez. Serpentine.	this study	this study	this study			

Appendix 2.1. Continued

Taxon	Sample code	Family	Collector, herbarium vouchers	Origin: Country/ locality	trnK-matK	petD	trnL-F	Distribution	SE or NS	NiA, NiH or NAH
Buxus marginalis (Britton) Urb.	Bx 126	Buxaceae	M. Ackermann & al. 925 (B, HAJB, ULV)	Cuba: Holguín, Moa, km 7-9 del camino a La Melba. Serpentine.	this study	this study	this study			
Buxus mexicana Brandegee	Bx 061*	Buxaceae	E. Köhler sn (B)	Mexico	this study	this study	this study	F	NS	NAH
Buxus microphylla Siebold & Zucc.	Bx 075*	Buxaceae	M. Ackermann & P. A. González sn (B#47533)	Korea and Japan. Cultivated in Botanical Garden Berlin-Dahlem.	this study	this study	this study	G	NS	NAH
Buxus microphylla Siebold & Zucc.	Bx 076	Buxaceae	M. Ackermann & P. A. González sn (B#47532)	Korea and Japan. Cultivated in Botanical Garden Berlin-Dahlem.	this study	this study	this study			
Buxus moana Alain	Bx 018*	Buxaceae	R. Berazaín & al. HFC 74025 (B, HAJB)	Cuba: Holguín, Moa, orillas del río Yagrumaje. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus moctezumae Eg. Köhler, R. Fernández & Zamudio	Bx 020*	Buxaceae	E. Köhler sn (B)	Mexico	this study	this study	this study	F	NS	NAH
Buxus moratii G. E. Schatz & Lowry	Bx 147*	Buxaceae	Barthelat 474 (B)	Mayotte: Grande Terre, Chiconi.	this study	this study	this study	H	NS	NAH
Buxus natalensis (Oliv.) Hutch.	Bx 102*	Buxaceae	P. A. González sn (B#100507477)	South Africa. Cultivated in Baumschulenweg, Berlin.	this study	this study	this study	H	NS	NAH
Buxus nipensis Eg. Köhler & P. A. González	Bx 117*	Buxaceae	T. Borsch & al. 4164 (B, HAJB, ULV)	Cuba: Holguín, Mayarí, Sierra de Nipe, Cabezadas del río Piloto. Serpentine.	HG004436	this study	HG004431	A	SE	NiH (this study)
B. olivacea Urb.	Bx 023*	Buxaceae	R. Berazaín & al. HFC cf. 72162 (HAJB)	Cuba: Guantánamo, Yateras, entre Diamante y Montecristo. Limestone. Cultivated JBN.	this study	this study	this study	A	NS	NAH (Reeves & al. 1996)
B. olivacea Urb.	Bx 094	Buxaceae	J. Gutiérrez & al. HFC 75433 (B, HAJB)	Cuba: Holguín, Mayarí, suroeste de Mandinga, al sur de la Mina José Martí. Serpentine.	this study	this study	this study			
B. olivacea Urb.	Bx 114*	Buxaceae	R. Berazaín & al. HFC 72260 (B, HAJB)	Cuba: Guantánamo, Yateras, charrascos entre el Alto de la Clarita y Montecristo. Serpentine.	this study	this study	this study	A	SE	NAH (this study)
Buxus pilosula subsp. *cacumini* s Eg. Köhler	Bx 109*	Buxaceae	J. Gutiérrez & al. HFC 75299 (B, HAJB)	Cuba, Santiago de Cuba, Segundo Frente, subida y firme del Pico Cristal. Serpentine.	this study	this study	this study	A	SE	NiH (this study)
Buxus pilosula subsp. *cacumini* s Eg. Köhler	Bx 110	Buxaceae	A. Álvarez & al. HFC 57245 (B, HAJB)	Cuba, Santiago de Cuba, Segundo Frente, subida al Pico Cristal. Serpentine.	this study	this study	this study			
Buxus pilosula Urb. subsp. *pilosula*	Bx 024*	Buxaceae	J. Gutiérrez & al. HFC 78358 (HAJB)	Cuba: Holguín, Mayarí, Pinares de Mayarí, charrascal La Cueva. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus pilosula Urb. subsp. *pilosula*	Bx 120	Buxaceae	T. Borsch & al. 4185 (B, HAJB, ULV)	Cuba: Holguín, Mayarí, Pinares de Mayarí, charrascal La Cueva. Serpentine.	this study	this study	this study			
Buxus portoricensis Alain	Bx 133*	Buxaceae	A. Areces 6873 (NY)	Puerto Rico: bosque estatal de Guajataca. Limestone.	this study	this study	this study	D	NS	NAH
Buxus pseudaneura Eg. Köhler	Bx 025*	Buxaceae	J. Gutiérrez & al. HFC 78267 (B, HAJB)	Cuba: Guantánamo, Parque Nacional Alejandro de Humboldt, Cayo Fortuna. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus pubescens Greenm.	Bx 150*	Buxaceae	F. Chiang & Flores 1131 (IEB)	Mexico: Mexico, Nayarit, isla María Madre, antena de telecomunicaciones, 600 msm.	this study	this study	this study	F	NS	NAH
Buxus purdieana Baill.	Bx 066	Buxaceae	H. Stenzel 923 (B)	Jamaica		this study	this study			
Buxus retusa Müll. Arg. subsp. *retusa*	Bx 012*	Buxaceae	J. Gutiérrez & al. HFC 77583 (B, HAJB)	Cuba: Holguín, Moa, camino entre el Alto de La Calinga y subida a El Toldo por la ladera sureste. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus retusa Müll. Arg. subsp. *retusa*	Bx 115	Buxaceae	J. Gutiérrez & al. HFC 78303 (HAJB)	Cuba: Guantánamo, Baracoa, río Baez. Serpentine.	this study	this study	this study			
Buxus revoluta (Britton) Mathou	Bx 042	Buxaceae	P. A. González HFC 85873 (B, HAJB)	Cuba: Holguín, Moa, al Este de Yamanigüey. Serpentine.	this study	this study	this study			
Buxus revoluta (Britton) Mathou	Bx 043*	Buxaceae	P. A. González HFC 85874 (B, HAJB)	Cuba: Holguín, Moa, al Este de Yamanigüey. Serpentine.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus revoluta (Britton) Mathou	Bx 044	Buxaceae	P. A. González HFC 85875 (B, HAJB)	Cuba: Holguín, Moa, al Este de Yamanigüey. Serpentine.	this study	this study	this study			
Buxus rheedioides Urb.	Bx 040*	Buxaceae	P. A. González HFC 85871 (B, HAJB)	Cuba: Holguín, Mayarí, charrascos del camino a La Caridad. Serpentine.	this study	this study	this study	A	SE	NAH (Reeves & al. 1996)
Buxus rheedioides Urb.	Bx 041	Buxaceae	P. A. González HFC 85872 (B, HAJB)	Cuba: Holguín, Mayarí, charrascos del camino a La Caridad.	this study	this study	this study			
Buxus rheedioides Urb.	Bx 119*	Buxaceae	TBorsch & al. 4166 (B, HAJB, ULV)	Cuba: Holguín, Mayarí, río Piloto. Serpentine.	this study	this study	this study	A	SE	NAH (Reeves & al. 1996)
Buxus rotundifolia (Britton) Mathou	Bx 003*	Buxaceae	J. Gutiérrez & al. HFC 77439 (HAJB)	Cuba: Holguín, Moa, subida a Pico el Toldo. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NAH (Reeves & al. 1996)
Buxus rotundifolia (Britton) Mathou	Bx 096	Buxaceae	J. Gutiérrez & al. HFC 83327 (B, HAJB)	Cuba: Holguín, Moa, ascenso al Toldo. Serpentine.	this study	this study	this study			

Appendix 2.1. Continued

Taxon	Sample code	Family	Collector, herbarium vouchers	Origin: Country/ locality	trnK-matK	petD	trnL-F	Distribution	SE or NS	NiA, NiH or NAH
Buxus rotundifolia (Britton) Mathou	Bx 116	Buxaceae	R. Berazaín & al. HFC 63382 (HAJB)	Cuba: Holguín, Moa, falda sur del Pico el Toldo. Serpentine.	this study	this study	this study			
Buxus sclerophylla Eg. Köhler	Bx 028*	Buxaceae	R. Berazaín & al. HFC 72282 (HAJB)	Cuba: Guantánamo, San Antonio del Sur, Los Ciguatos. Limestone. Cultivated JBN.	this study	this study	this study	A	NS	NAH (Reeves & al. 1996)
Buxus sempervirens L.	Bx 077*	Buxaceae	M. Ackermann & P. A. González sn (B#47534)	Greece. Cultivated in Botanical Garden Berlin-Dahlem.	this study	this study	this study	G	NS	NAH
Buxus serpentinicola Eg. Köhler	Bx 029*	Buxaceae	R. Berazaín & al. HFC 72288 (HAJB)	Cuba: Guantánamo, Maisí, Peladero de Jauco. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NiH (Reeves & al. 1996)
Buxus shaferi Urb.	Bx 004*	Buxaceae	J. Gutiérrez & al. HFC 75297 (B, HAJB)	Cuba: Santiago de Cuba, Segundo Frente, subida y firme del Pico Cristal. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NiA (this study)
Buxus shaferi Urb.	Bx 022*	Buxaceae	J. Gutiérrez & al. HFC 77598 (B, HAJB)	Cuba: Guantánamo, Baracoa, orillas del río Báez. Serpentine. Cultivated JBN.	this study	this study	this study	A	SE	NAH (this study)
Buxus shaferi Urb.	Bx 047*	Buxaceae	P. A. González HFC 85879 (HAJB)	Cuba: Guantánamo, Baracoa, Alto de Iberia. Serpentine.	this study	this study	this study	A	SE	NAH (this study)
Buxus shaferi Urb.	Bx 122*	Buxaceae	T. Borsch & al. 4319 (B, HAJB, ULV)	Cuba: Holguín, Moa, entre los kilometros 22 y 26 del camino a La Melba. Serpentine.	this study	this study	this study	A	SE	NAH (this study)
Buxus triptera Eg. Köhler	Bx 098*	Buxaceae	J. Gutiérrez & al. HFC 83396 (B, HAJB)	Cuba: Holguín, Moa, km 26 del camino a la Melba. Serpentine.	this study	this study	this study	A	SE	NiH (this study)
Buxus triptera Eg. Köhler	Bx 127*	Buxaceae	M. Ackermann & al. 956 (B, HAJB, ULV)	Cuba: Holguín, Moa, Entre el km 26 km y las Comadres, en el camino a La Melba. Serpentine.	this study	this study	this study	A	SE	NiH (this study)
Buxus vahlii Baill.	Bx 062	Buxaceae	E. Köhler & G. R. Proctor 111 (B)	Puerto Rico. Limestone	this study	this study	this study			
Buxus vahlii Baill.	Bx 134*	Buxaceae	A. Areces 6874 (NY)	Puerto Rico: Mogote del Parque de Las Ciencias, Bayamón, San Juán. Limestone.	this study	this study	this study	D	NS	NAH
Buxus wrightii subsp. *leonii* (Britton) Eg. Köhler	Bx 159*	Buxaceae	A. Álvarez de Zayas & al. HFC 43556 (HAJB)	Cuba: Artemisa, Las Pozas. Cultivated JBN. Serpentine.	this study	this study	this study	C	SE	NAH (Reeves & al. 1996)
Buxus wrightii Müll. Arg. subsp. *wrightii*	Bx 158*	Buxaceae	P. A. González & R. Rankin HFC 86142 (HAJB)	Cuba, Pinar del Río. Cultivated JBN. Serpentine.	this study	this study	this study	C	SE	NAH (Reeves & al. 1996)
Buxus yunquensis Eg. Köhler	Bx 046*	Buxaceae	P. A. González HFC 85878 (B, HAJB)	Cuba: Guantánamo, Baracoa, Santa María. Serpentine.	this study	this study	this study	A	SE	NAH (this study)
Buxus yunquensis Eg. Köhler	Bx 053*	Buxaceae	J. Gutiérrez & al. HFC 79696 (B, HAJB)	Cuba: Guantánamo, Baracoa, subida al Yunque de Baracoa. Limestone.	this study	this study	this study	A	NS	NAH (this study)
Dicentra eximia (Ker Gawl.) Torr.		Papaveraceae	T. Borsch 3468 (BONN)	Botanical Garden Bonn	DQ182345	AY590835	AY145361			
Didymeles integrifolia J. St.-Hil.		Didymelaceae	J. Rabenantoandro & al. 916 (MO)	Madagascar	AM396505	AM396540	AM397166			
Embothrium coccineum Forst.		Proteaceae	A. Worberg 004 (BONN)	Botanical Garden Bonn	AM396515	AM396536	AM397162			
Euptelea pleiosperma Siebold & Zucc.		Eupteleaceae	A. Worberg 003 (BONN)	Botanical Garden Bonn	AM396510	AM396525	AM397151			
Grevillea banksii R. Br.		Proteaceae	T. Borsch 3413 (BONN)	Botanical Garden Bonn	AF542583	AM396537	AM397163			
Haptanthus hazlettii Goldberg & C. Nelson	Bx 148*	Haptanthaceae	House & Vega 5397 (TEFH)	Honduras, Atlántida, Refugio de vida silvestre Texiguat.	this study	this study	this study	F		
Meliosma cuneifolia Franch.		Sabiaceae	A. Worberg 001 (BONN)	Botanical Garden Bochum	AM396513	AM396534	AM397160			
Nelumbo nucifera subsp. *lutea* (Willd.) Borsch & Barthlott		Nelumbonaceae	T. Borsch and Summers 3220 (FR)	USA, Missouri	AF543740	AY590836	AY145359			
Nelumbo nucifera Gaertn. subsp. *nucifera*		Nelumbonaceae	A. Worberg s.n. (BONN)	Botanical Garden Bonn	AM396514	AM396535	AM397161			
Pachysandra terminalis Siebold & Zucc.	*	Buxaceae	T. Borsch 3407 (BONN)	Botanical Garden Bonn.	AF542581	AM396541	AM397167	FG	NS	NAH
Platanus occidentalis L.		Platanaceae	Slotta s.n. (VPI)	USA, Virginia	AF543747	AY590834	AY145358			
Platanus orientalis L.		Platanaceae	A. Worberg 005 (BONN)	Botanical Garden Bonn	AM396503	AM396538	AM397164			
Sabia japonica Maxim.		Sabiaceae	Y-L. Qiu 91025 (NCU)	not available	AM396512	AM396533	AM397158			
Sabia swinhoei Hemsl.		Sabiaceae	Y-L. Qiu 99003 (NCU)	not available	HE651034	Barniske & al. (2012)	Barniske & al. (2012)			

Appendix 2.1. Continued

Taxon	Sample code	Family	Collector, herbarium vouchers	Origin: Country/ locality	trnK-matK	petD	trnL-F	Distribution	SE or NS	NiA, NiH or NAH
Sarcococca confusa Sealy	Bx 131*	Buxaceae	Ra 280 (B)	Asia. Cultivated in Botanical Garden Berlin-Dahlem.	this study	this study	this study	G		
Sarcococca conzattii (Standl.) I.M.Johnst.	Bx 130*	Buxaceae	M. Chazaro & R. Sánchez 9759 (B, XAL)	Mexico: Jalisco, Ayutia County, Sierra of Cacoma.	this study	this study	this study	F		
Sarcococca hookeriana Baill.	Bx 139*	Buxaceae	Schwerdtfeger 22670 (B)	Asia. Cultivated in Botanical Garden Berlin-Dahlem.	this study	this study	this study	G		
Sarcococca saligna Müll. Arg.	Bx 132*	Buxaceae	Schwerdtfeger 21283 (B)	Asia. Cultivated in Botanical Garden Berlin-Dahlem.	this study	this study	this study	G		
Styloceras laurifolium Kunth	Bx 129*	Buxaceae	J. L. Clark 7721 (US)	Ecuador: Ecuador, Tungurahua, Cantón Baños.	this study	this study	this study	E		
Tetracentron sinense Oliver		Trochodendraceae	T. Borsch 3494 (BONN)	Botanical Garden Freiburg	AM396504	AM396539	AM397165			
Trochodendron aralioides Siebold & Zucc.		Trochodendraceae	T. Borsch 3478 (BONN)	Botanical Garden Bonn	AF543751	AY590833	AY145360			

Note-Abbreviations: JBN– Jardín Botánico Nacional de Cuba (National Botanical Garden of Cuba); HFC– series of collection numbers for Herbarium Florae Cubensis. Distribution: A– east Cuba, B– central Cuba, C– west Cuba, D– other islands of the Caribbean [Bahamas, Hispaniola, Jamaica, and Puerto Rico], E– South America–Panama, F– North America and Central America until El Salvador, G– Eurasia including the Mediterranean islands and Mediterranean areas of North Africa, H– Africa and adjacent islands of the Indic Ocean [Madagascar, Comoro islands, Socotra]. SE– serpentine endemic, NS– no serpentine endemic. NiA– Ni accumulator, NiH– Ni hyperaccumulator, NAH no Ni accumulator/no Ni hyperaccumulator, ?– unknown data. *– represents samples included in the 73 individuals matrix for analyses of ancestral distribution and ancestral traits.

Appendix 2.2. Taxa, sample codes and nickel concentrations

Taxa (sample code)	**Nickel concentration ($\mu g\ g^{-1}$)**
Buxus braimbridgeorum (Bx091)	5258
Buxus cristalensis (Bx026)	28447
Buxus ekmanii (Bx095)	8
Buxus ekmanii subsp. *woodfredensis* (Bx009)	36
Buxus glomerata (Bx106)	15.8
Buxus glomerata (Bx035)	10
Buxus gonoclada subsp. *orientensis* (Bx146)	12662
Buxus gonoclada subsp. *toldoensis* (Bx092)	12052
Buxus nipensis (Bx117)	30759
Buxus olivacea (Bx114)	9
Buxus pilosula subsp. *cacuminis* (Bx109)	7775
Buxus shaferi (Bx122)	67
Buxus shaferi (Bx004)	668
Buxus shaferi (Bx022)	153
Buxus triptera (Bx127)	7025
Buxus triptera (Bx098)	9082
Buxus yunquensis (Bx053)	19
Buxus yunquensis (Bx046)	35

Chapter 3

New species of *Buxus* (*Buxaceae*) from northeastern Cuba based on morphological and molecular characters, including some comments on molecular diagnosis

Contents

3.1 Introduction

The genus *Buxus* is the largest of the family *Buxaceae* and comprises c. 100 species distributed in all continents except Australia and Antarctica. About 40 % of these species occur on Cuba. The centres of morphological and ecological diversity of *Buxus* are the Caribbean (c. 50 sp.), East Asia (c. 40 spp.) and Africa and Madagascar (c. 15 spp.) (Köhler & Brückner 1990; Schatz & Lowry 2002).

In Alain's (1953) treatment of *Buxaceae* for the Flora de Cuba, 27 *Buxus* species were recognized. During the second half of the 20th Century, in the context of collaboration between Cuban and East European botanical institutions, Borhidi & Muñiz (1973, 1977) published three further species. Starting in the seventies, Prof. Johannes Bisse and a young team of Cuban and East German botanists made collecting expeditions to all Cuban provinces in order to collect new plant material for the elaboration of a modern, critical Flora of Cuba. As a result of this, Köhler (1982, 1998, 2006) published nine new Cuban species of *Buxus*, raising their total number to c. 40. Recent field work, as well as morphological, palynological and anatomical studies and molecular phylogenetic analysis of the whole genus, led to the discovery of the three additional new species described below.

Buxus represents one of the most important species radiations in the flora of Cuba and also shows an interesting distribution pattern in the Caribbean with a few species on various islands and also in México and Central America. The genus was therefore chosen as a model group to reconstruct its phylogeny at the species level and to test hypotheses concerning the origin and evolution of the Cuban flora (González & al., work underway). So far only few groups of the Cuban flora have been investigated using phylogenetic techniques, such as *Croton* (Ee & al. 2008), *Pachyanthus* (Bécquer-Granados & al. 2008), *Ginoria* (Graham 2010), *Spathelia* (Appelhans & al. 2011, 2012), *Leucocroton* (Jestrow & al. 2012) and *Brunfelsia* (Filipowicz & Renner 2012). However, sampling at the species level was often not complete and taxonomic questions including analyses of species limits were not part of these works although this is of high importance in lineages that had previously just been treated with alpha-taxonomic approaches.

In the course of our analysis of the evolutionary diversification of *Buxus* on Cuba and in the Caribbean region, all previously described taxa as well as known morphologically deviant and geographically isolated populations are currently being included into molecular and morphological data sets. Molecular trees thereby facilitate the analysis of often more homoplastic phenotypic character states (e.g. flower morphology, pollen morphology, leaf anatomy) in a speciose clade. Phylogenetic analysis revealed well-resolved species-level trees (González & al., unpubl. data) and indicated several of the deviating specimens to belong to distinct sub-lineages within the Cuban radiation of *Buxus*. The goal of this paper is to formally describe three new species of *Buxus* that can be unambiguously recognized by both phenotypic and sequence data. This study will thereby also serve as a basis for the preparation of the treatment of the genus in the Flora de Cuba (Köhler & González, ongoing work).

3.2 Materials and methods

3.2.1 Plant material

The mountains of Nipe-Sagua-Baracoa have been visited repeatedly during the last 15 years in order to collect *Buxus* material. Material from these expeditions also allowed the establishment of a living collection of *Buxus* at the National Botanical Garden of Cuba, University of La Habana (Rankin-Rodríguez & al. 1999; Köhler 2001) with well-documented plants that could be studied at various stages of flowering and fruiting, and also served this study. The material included in this study is listed in both the information on types and the respective paragraphs on “additional specimens seen”. For those samples sequenced, the corresponding DNA samplecodes (Bx026, Bx055, Bx117, Bx162, Bx163, Bx164 and Bx165) that are used for all *Buxus* samples in the ongoing evolutionary analysis of the genus are added.

3.2.2 DNA isolation, sequencing, annotation and analysis

Sequences of the plastid *matK-trnK* and the *trnL-F* regions were generated for seven and five samples, respectively. Total DNA was isolated from silica-gel-dried leaf tissue or herbarium specimens using a triple CTAB extraction method (Borsch & al. 2003) or the Nucleo Spin Plant II extraction kit (Macherey Nagel, Düren, Germany). The amplification of each marker was performed in reaction volumes of 50 µL, containing 2 µL of extracted DNA (with a concentration of 10-20 ng/µL), 14.7 µL of H_2O, 5 µL of 10× peqLab Taq.Buffer S containing $MgCl_2$, 3 µL of $MgCl_2$ (25 mM), 10 µL of Betaine monohydrate (5 M), 1 µL of BSA (10 ug/µl), 2 µL of forward primer (20 pm/µl), 2 µL of reverse primer (20 pm/µl), 10 µL dNTPs (each 0.25 mM) and 0.3 µL Taq polymerase 5 units/µl (PeqLab, Erlangen Germany).

The *trnL-F* region was amplified using the primers trnTc and trnTf (Taberlet & al. 1991). The Polymerase Chain Reaction (PCR) program was: 30 cycles of denaturation (60 seconds at 96 ºC), annealing (60 seconds at 50ºC), extension (120 seconds at 72 ºC). Sequencing was carried out with the primer trnTf and the additional internal primer trnTd (Taberlet & al. 1991). The *trnK-matK* region was amplified and sequenced in two fragments using the primer pairs trnK-F (Wicke & Quandt 2009) and BxmatK-1270R (5´- ATTCCAATTATGATACTCG-3´, designed for *Buxaceae* in this study), as well as BxmatK-467F (5´- TGTCAGATATACTAATACC-3,´ designed for *Buxaceae* in this study) and trnK-2R (Johnson & Soltis 1994). For the samples Bx164 and Bx165, isolated from older herbarium specimens, the use of further internal primers for amplification and sequencing was necessary that were either newly designed (BxtrnK-779R, 5´-TAAATATACTCCTGAAAGAG-3´; BxtrnK-1750R, 5´-AATTTTCTAGCATTTGACTC-3´), or taken from Müller & Borsch (2005b, ACmatK-105F). The PCR program used was: 34 cycles of denaturation (60 seconds at 94 ºC), annealing (60 seconds at 50 ºC) and extension (120 seconds at 72 ºC).

In all cases the amplification products were purified by electrophoresis in a 1.2 % NEEO agarose gel (Carl Roth, Germany) running during 3 hours at 100 Volts. The gel extraction was performed using the AveGene Gel/PCR DNA Fragments Extraction Kit (Avegene life science Corporation), following the protocol provided by the

manufacturers. The concentration of the purified PCR products were measured with a NanoDrop spectrophotometer (ND-1000, PeqLab, Erlangen, Germany). Cycle sequencing, fragment purification and sequencing were performed by Macrogen Inc., South Korea (http://www.macrogen.com). The sequences were edited and manually aligned with a motif alignment approach (Löhne & Borsch 2005, Morrison 2009) using PhyDE v.0 995 (Müller & al. 2007). Boundaries of the genomic regions studied were annotated using a multiple sequence alignment in comparison with completely sequenced and annotated plastid genomes of *Nicotiana tabacum* (Z00044; Shinozaki & al. 1986) and *Buxus microphylla* (NC009599; Hansen & al. 2007). The respective character positions were then determined with reference to each specific sequence because of length variability within the genomic regions.

3.2.3 Micromorphology and anatomy

The palynological and anatomical methods applied in this study, as well as the technical terms employed in the discussion are those set out in Köhler (1981, 1982, 1998, 2006) and Köhler & Schirarend (1989).

3.3 Results

Buxus nipensis Eg. Köhler & P. A. González, **sp. nov.** – Fig. 3.1–7.
Holotype: Cuba, province Holguín, Mayarí, Cabezadas del río Piloto, en la zona de las cascadas, 20°27' 44"N, 75° 48' 59" W, 8 Mar 1998, 500–700 m, *J. Gutiérrez, E. Köhler, A. Leiva, R. Rankin & I. Silva HFC 75468* (HAJB; isotypes: B, BHU, JE) [= Bx165].

Morphological diagnosis — Leaves oblong to narrowly elliptic, apex retuse to emarginate and mucronulate. Male tepals broadly ovate to suborbicular, adaxially glabrous, margin narrowly membranous, apex apiculate. Ovary rounded-trigonous, dorsal veins sunken at edges; nectaries well-developed, angular; styles obliquely erect. Capsule ellipsoid-globose, dorsal veins scarcely prominent; nectaries prominent, rounded; styles erect then recurved.

Molecular diagnosis — Nucleotide character state "A" in position 343 and "G" in position 448 of *matK* coding sequence.

Morphological description — *Shrub or tree* to 5 m tall; branchlets angular; internodal folds narrow with slightly prominent ribs, dorsally ± keeled or variably keeled on each side; internodes 2–5(–8) cm long, glabrous. *Leaves* dimorphic; *normal-sized leaves* with petiole 4–8 mm long, blade greenish-yellow and slightly shiny adaxially, paler and dull abaxially, oblong to narrowly elliptic, 4–8 × 2–4 cm, coriaceous, glabrous, base broadly cuneate to shortly narrowed, apex obtuse, retuse to emarginate and mucronulate, midvein progressively sunken adaxially toward base, raised abaxially, secondary veins in 12–18 pairs, anastomosing in an adaxially prominent intramarginal vein 1–1.5 mm from revolute margin; *smaller decussate leaves* interspersed between normal ones, linear-lanceolate, 5–7 mm long, apex acute. *Inflorescences* sessile, 4–7 mm long, glabrous; bracts ovate-triangular to suborbicular, 1–1.8 mm long, margin scarcely ciliate, apex acute to apiculate with a bright membraneous tip. *Male flowers* with pedicel 1.5–3 mm long; tepals broadly ovate to suborbicular, 1.2–2 mm long, adaxially glabrous, margin narrowly membranous, scarcely ciliate, apex apiculate; stamens 2.5–4 mm long, filaments slightly flattened, anthers c. 1.2 mm long, with a prominent black tip; pistillode hemispherical, wrinkled. *Female flowers* with tepals apiculate with a bright membranous tip; ovary rounded-trigonous, c. 2.5 × 2.5 mm, glabrous, dorsal veins sunken at edges, commissures narrowly protruding between collateral furrows, continued into angled nectaries; styles obliquely erect, apically curved, 2–3 mm long; stigmas narrowly folded, 2–3 mm long. *Capsule* brownish, ellipsoid-globose, 7 × 5–7 mm, glabrous, with scarcely prominent dorsal veins, crowned by c. 4 mm long erect then recurved styles; nectaries forming prominent rounded knobs. *Seeds* black, shining, rounded-trigonous, 4 × 2.2 mm.

Molecular description — Sequences describe the type specimen (code Bx165) and are available in EMBL/GenBank/DDBJ under accession numbers HG004439 (*matK-trnK*) and HG004432 *trnL-trnF*. Further sequences describe paratype specimens (codes Bx117, Bx162, Bx163) and are available in EMBL/GenBank/DDBJ under

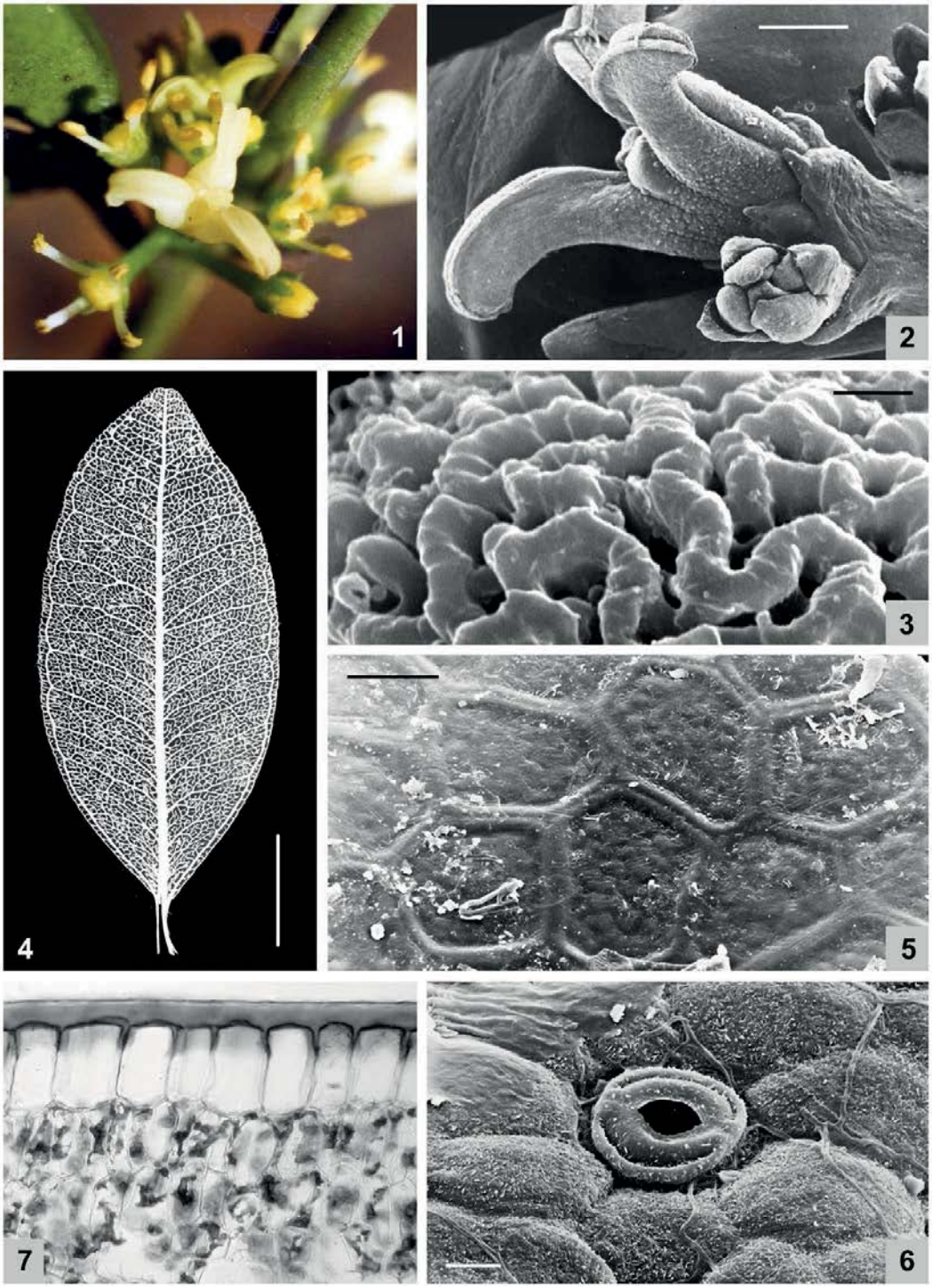


Fig. 3.1-7. *Buxus nipensis* – 1: inflorescence, female flower, recurved white stylodia, interstylary nectaries; 2: female flower, male flower before anthesis, scale bar = 1 mm; 3: exine detail, reticulate heterobrochate, with broad crenulate muri, 11 000×; 4: brochidodromous leaf venation pattern, scale bar = 1 cm; 5: adaxial leaf epidermis, reticulately raised anticlinal walls, sunken anticlinal borders, finely knobbed periclinal wall, 600×; 6: abaxial leaf epidermis, stoma with a peristomal rim, 1100×; 7: leaf cross-section, adaxial epidermis cells with light-line, absence of secretory cells. – 1, 2, 4, 5 from specimen *HFC 75431* (B); 3, 6 from *HFC 75468* (HAJB, holotype); 7 from specimen *HFC 80900* (B).

accession numbers HG004436, HG004437 and HG004438 (*matK-trnK*) and HG004431 *trnL-trnF* (only Bx117).

Pollen morphology — Pollen (3-) or 4(–6)-colporate, colpi 3- or 4-orate, reticulate, heterobrochate, higher murus segments broader than the lower ones, crenulated (conspicuously ribbed), bounding lumina of different size.

Leaf anatomy — *Buxus nipensis* is characterized by the absence of secretory cells. The adaxial epidermis consists of high, thin-walled cells, with anticlinal walls only slightly thickened in the apical part. The palisade parenchyma is composed of 2 or 3 layers of scarcely differentiated cells. The adaxial epidermis has a reticulate pattern of protruding anticlinal walls with sunken anticlinal borders (as in *B. retusa* Müll. Arg.) and slightly undulate periclinal walls. The stomata have a peristomal rim.

Etymology — The specific epithet alludes to the distribution area of this species, Sierra de Nipe, in the northeastern region of Cuba.

Distribution — *Buxus nipensis* is endemic to the Sierra de Nipe, municipality of Mayarí, current province of Holguín. In Sierra de Nipe it has been collected near to Woodfred, Brazo Dolores, La Casimba, La Plancha, loma de La Bandera, loma de La Estrella, río Piloto, Loma Mensura and near to Estación de Investigaciones de la Montaña. However during the last decade it has been refound only in the last three localities.

Habitat and ecology — *Buxus nipensis* grows on serpentines in subspiny xeromorphic thickets known in Cuba as “charrascal” or “charrascos”, in forest of *Pinus cubensis* Griseb., and in riverine rainforest along mountain brooks and rivers, at 500–700 m above the sea level. During field work carried out in February 2010 we saw bees (*Apis mellifera*) and an unidentified diptera visiting the flowers of *B. nipensis* in the locality of río Piloto.

Phenology — The species has been collected in flower from December to May and in fruit from April to August. When we visited the locality of río Piloto in February 2010 almost all adult plants were in flower.

Conservation status — *Buxus nipensis* has been recently confirmed in three of nine localities in which it has been collected earlier, according to the consulted herbarium specimens. Some historical localities of this species have been affected mostly by the nickel mining industry and by the extraction of timber, and we suppose that some populations of *B. nipensis* could have been affected or may have disappeared. In the last ten years *B. nipensis* has been collected in río Piloto, Loma Mensura and in the ecologic path of Estación de Investigaciones de la Montaña. The populations of Loma Mensura and of the ecologic path of Estación de Investigaciones de la Montaña have six to ten mature individuals, respectively, which are close to one another; in the population of río Piloto we counted at least 50 individuals, both mature and immature, along c. 1000 m. The three populations are protected since Loma Mensura and río Piloto belong to the protected area "Mensura-Piloto" and the third population is protected as well for being included in an ecologic path managed by the Estación de Investigaciones de la Montaña. Based on a suspected population size reduction of ≥50%, a range of less than 500 km² and a decline in the number of locations, the species must be classified as Endangered (EN AB2b) according to IUCN criteria (IUCN 2012).

Discussion of phenotypic characters — Specimens of *Buxus nipensis* had been previously identified as *B. retusa,* but *B. nipensis* can easily be distinguished from that species by the well-developed angular interstylar nectaries and the obliquely upright styles. These develop into upright, apically recurved styles with prominent knoblike, rounded nectaries in the capsule. The internodal morphology of the new species and also the reticulate pattern of protruding anticlinal walls of the adaxial leaf epidermis with sunken anticlinal borders is reminiscent of *B. retusa* but also of *B. braimbridgeorum* Eg. Köhler and *B. triptera* Eg. Köhler. However, the leaf anatomy of *B. nipensis* differs from that of *B. retusa* by the complete absence of secretory cells and by the palisade parenchyma composed of 2 or 3 layers of little-differentiated cells, features that it shares with *B. triptera*. The exine sculpture of the pollen is heterobrochate with murus

segments of different breadth, which are bounding smaller lumina. The muri are broadly crenulate (ribbed), reminiscent of *B. braimbridgeorum*.

Discussion of molecular characters — The first mentioned diagnostic character state for *Buxus nipensis* in the *matK* coding sequence ("A" in position 343) is a synapomorphy for the type and all so-far investigated paratypes with respect to the whole genus *Buxus*. The second diagnostic character state ("G" in position 448) is present in the type and two paratypes but paratype specimen Bx163 exhibits an "A" like all other species of *Buxus*. This case illustrates that there may be infraspecific variation at the molecular level that may affect some of the character states considered as diagnostic. We argue that at least some diagnostic character states are present in all so-far studied individuals of the newly described species, and also, most importantly, that the type specimen exhibits all character states defined as diagnostic. Therefore the species is well defined based on the type. Further research has to address how infraspecific variation can be explained, either through homoplasy in certain individuals as a result of on-site mutation after speciation, through ancient haplotypes still present in individuals of some populations, or even through recent introgression.
The *trnK-matK* region further has two microsatellite regions (poly A/Ts) starting in sequence position 371 of the *trnK* intron 5' part and in position 901 (each referring to the sequence of the type) of the *matK* coding sequence. Both microsatellites are highly variable, including infraspecific variability in *B. nipensis*. A sole individual (Bx163) shows a unique haplotype (13 Ts) in the second microsatellite. The patterns are in line with high mutational rates and high levels of homoplasy in most chloroplast microsatellites (e.g. Tesfaye & al. 2007; Weising & Gardner 1999). Therfore, these characters are unsuitable for use in diagnoses to describe species.

Additional specimens seen (*paratypes*) — CUBA: PROV. HOLGUÍN: Mayarí, Sierra de Nipe, near Woodfred, deciduous woods and thickets, 450–550 m, 20 Dec 1909, *J. Shafer 3219* (NY); near Woodfred, deciduous woods and thickets, 450–550 m, 1 Jan 1910, *J. Shafer 3408* (NY); in charrascales ad Brazo Dolores, c. 800 m, 20 Feb 1918, *E. Ekman 9124* (S); South of lumber camp, crest of Sierra de Nipe, 600–700 m, 16–17 Oct 1941, *C. V. Morton & J. Acuña 3066* (US); Fuente del Arroyo Naranjo, bosque húmedo,

arbusto 1.5–2 m, 750 m, 20 Apr 1960, *Bro. Alain & J. Acuña 7833?* (HAC); arroyo cerca de La Casimba, 19 Apr 1960, *Bro. Alain & J. Acuña 7833* (HAC); Cayo de La Plancha, 7 Apr 1941, *Bro. Leon & al. 20032* (GH, HAC, NY); Sierra de Nipe, Oct 1966, *V. Samek 16193* (HAC); charrascales de la Loma de La Bandera, c. 400 m, Apr 1968, *J. Bisse & E. Köhler HFC 7336* (HAJB, JE); Pinares cerca de la Loma de La Estrella, 800 m, 12 Aug 1970, *J. Bisse & H. Lippold HFC 18102* (HAJB, JE); orillas del arroyo en el camino a Woodfred, 600 m, 2 Nov 1979, *A. Álvarez & al. HFC 36019* (B, HAJB, JE); orillas de las cabezadas del río Piloto, c. 800 m, 30 Oct 1977, *A. Álvarez & al. HFC 35736* (B, HAC, HAJB, JE); arroyo afluente del río Piloto, 10 Aug 1988, *R. Berazaín HFC 66166* (HAJB); orillas de arroyo del Medio cerca de Woodfred, c. 425 m, 7 Mar 1998, *J. Gutiérrez & al. HFC 75431* (BHU, HAJB); arroyo Mensura (río Sabina) alrededores de la Estación de Investigaciones Integrales de la Montaña, 500–600 m, 9 Mar 1998, *J. Gutiérrez & al. HFC 75472* (BHU, HAJB); cabezadas del río Piloto, 689 m, 7 Apr 2003, *J. Gutiérrez & al. HFC 80900* (BHU, HAJB); río Piloto, cascadas altas, 690 m, 7 Apr 2003, *J. Gutiérrez & al. HFC 80905* (BHU, HAJB); cabezadas del río Piloto, 710–724 m, 7 Apr 2004, *J. Gutiérrez & al. HFC 81745* (HAJB); cabezadas del río Piloto, 20 Mar 2005, *J. Gutiérrez & al. HFC 83242* (HAJB); detrás de la Estación de Investigaciones Integrales de la Montaña, c. 670 m, 20 Mar 2005, *J. Gutiérrez & al. HFC 83269* (BHU, HAJB); río Piloto, *T. Borsch & al.* 4164 [= Bx117] (B, HAJB, ULV); Loma Mensura, en el margen de un arroyo con presencia de *Cyrilla* sp., *Tabebuia* sp., *Rondeletia* sp., *Arthrostylidium* sp. y *Leucocroton* sp., 700 msm, 7 Sep 2011, *P. González & al. HFC 87220* [= Bx162] (B, HAJB); sendero ecológico detrás de la Estación de Investigaciones de la Montaña, bosque pluvial con presencia de *Chionanthus domingensis*, *Bactris cubensis*, *Phyllanthus* sp., c. 700 msm, 8 Sep 2011, *P. González & al. HFC 87221* [= Bx163] (B, HAJB).

Buxus cristalensis Eg. Köhler & P. A. González, **sp. nov.** – Fig. 3.8–15.
Holotype: Cuba, province Santiago de Cuba, Segundo Frente, Sierra del Cristal, Arroyo en el camino del Oro a Batista, 20°31' 51"N, 75° 26' 14" W, 700 m, 8 Mar 1998, *J. Gutiérrez, E. Köhler, R. Rankin & I. Silva HFC 75347* (HAJB; isotypes: B, BHU, JE) [= Bx164].

Morphological diagnosis — Leaves elliptic to oblong, apex obtuse, retuse to emarginate and mucronulate. Male tepals broadly ovate, adaxially finely pilose, apex apiculate. Ovary trilobate, dorsal veins deeply sunken; nectaries well developed, angular, wrinkled; styles obliquely spreading, stout. Capsule ellipsoid, dorsal veins scarcely prominent proximally, deeply sunken distally; nectaries prominent, angulose, wrinkled; styles erect then recurved.

Molecular diagnosis — Nucleotide character state "A" in position 1507 of *matK* coding sequence.

Morphological description — *Shrub or tree* to 5 m tall; branchlets angular; internodal folds narrow with slightly prominent ribs, dorsally ± keeled, with 2 lateral keels or variably keeled on each side; internodes 2–5(–8) cm long. *Leaves* dimorphic; *normal-sized leaves* with petiole 5–8 mm long, blade green to yellowish green and slightly shiny adaxially, paler abaxially, elliptic to oblong, 5–7 × 1.5–4 cm, coriaceous, glabrous, base obtuse, apex obtuse, retuse and slightly mucronulate, midvein sunken adaxially, prominent abaxially, secondary veins in 12–15 pairs anastomosing in an intramarginal vein c. 2.5 mm from revolute margin, venation prominent on both surfaces; *smaller decussate leaves* interspersed between normal ones, narrowly lanceolate, c. 7 mm long, apex acute. *Inflorescences* ± sessile, c. 1 cm long, glabrous; bracts triangular, 1.5–2.5 mm long, margin scarcely ciliate, apex acute. *Male flowers* with pedicel 2–3.5 mm long; tepals broadly ovate, c. 2 mm long, adaxially finely pilose, margin narrowly membranous, scarcely ciliate, apex apiculate; stamens 3–4 mm long, filaments slightly flattened, anthers c. 1 mm long with a prominent rounded brownish tip; pistillode rounded-quadrangular, hemispherical, with lateral ellipsoid sinus,

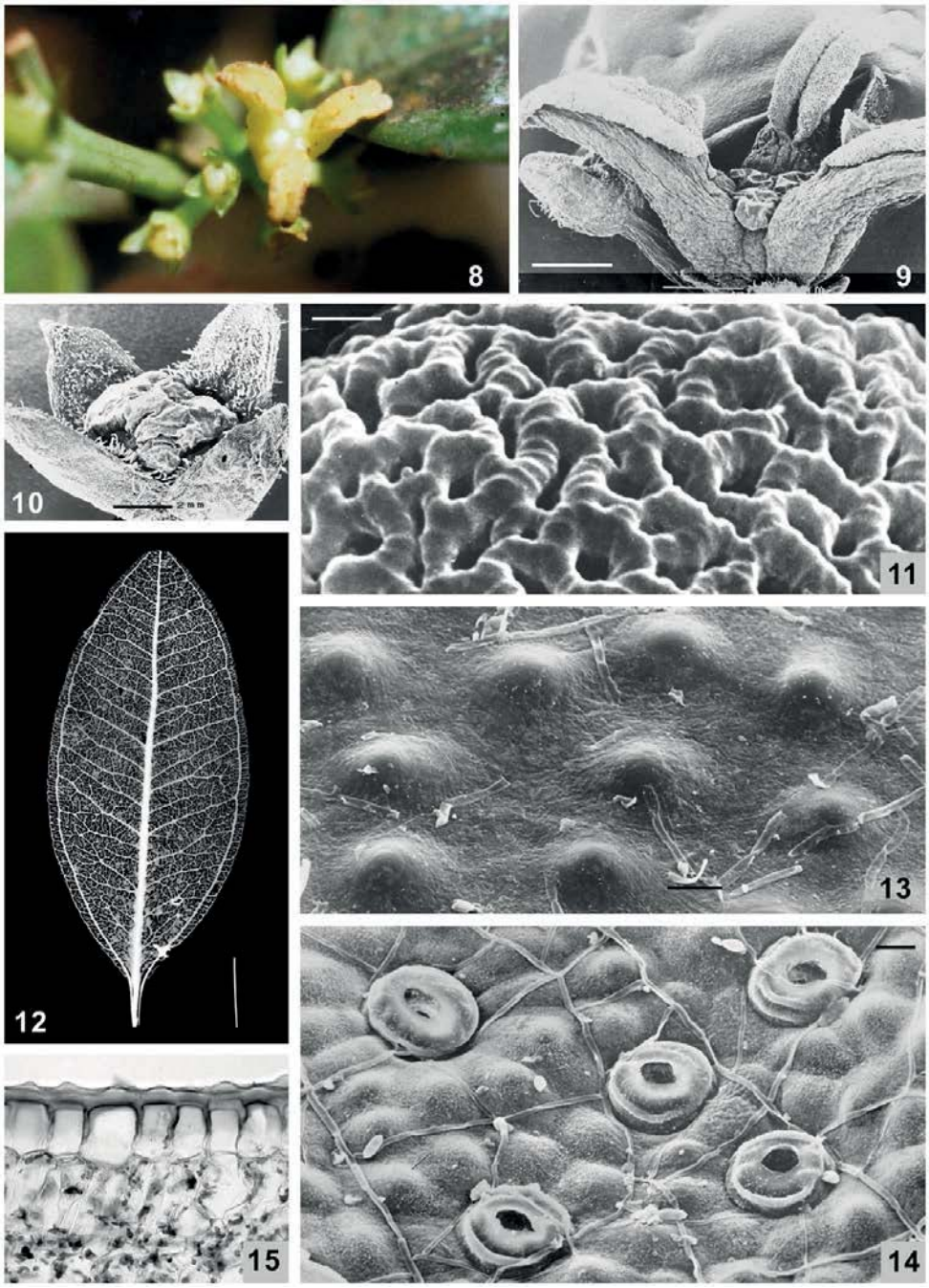


Fig. 3.8-15. *Buxus cristalensis* – 8: inflorescence, female flower, spreading yellowish white thick stylodia, interstylary nectaries; 9: female flower, stout stylodia, nectaries, commissures with collateral furrows, scale bar = 1 mm; 10: male flower, tepals adaxially finely pilose, pistillode, scale bar = 2 mm; 11: reticulate exine, broad, crenulate muri, 10 000×; 12: brochidodromous leaf venation pattern, scale bar = 1 cm; 13: adaxial leaf epidermis, papilla-like (papilloid) protruding periclinal walls, 1000×; 14: abaxial leaf epidermis, ± papilla-like protruding periclinal walls, stomata with peristomal rim 550×; 15: leaf cross-section, adaxial epidermis with protruding periclinal walls, little-differentiated palisade parenchyma, absence of secretory cells. – 8, 12–15 from *HFC 75347* (HAJB, holotype), 9, 10 from specimen *HFC 75349* (B), 12 from specimen *HFC 15938* (HAJB).

wrinkled. *Female flowers* with 5 tepals; tepals triangular with bright tip, 1–1.5 mm long, margin scarcely ciliate; ovary trilobate, 2–2.5 × 2.5 mm; dorsal veins deeply sunken, commissures with lateral furrows apically continued into nectaries; styles white, recurved, thick, glabrous; stigmas broad, deeply folded, 2–3.5 mm long; nectaries angular, wrinkled. *Capsule* green-brownish, ellipsoid, 6–8 × 5–7 mm, glabrous, crowned by 3–4 mm long erect then recurved styles, dorsal veins scarcely prominent proximally, sunken distally, commissures apically slightly protruding, with lateral furrows; nectaries prominent, angular, wrinkled. *Seeds* rounded-trigonous, c. 4 × 2 mm.

Molecular description — Sequences describe the type specimen (code Bx164) and are available in EMBL/GenBank/DDBJ under accession numbers HG004435 (*matK-trnK*) and HG004430 *trnL-trnF*. Further sequences describe a paratype specimen (code Bx026) and are available in EMBL/GenBank/DDBJ under accession numbers HG004434 (*matK-trnK*) and HG004429 *trnL-trnF*.

Pollen morphology — Pollen 3- or 4(or 5)-colporate, colpi 3–5-orate, reticulate, heterobrochate, higher murus segments broader than the lower ones, which are bounding smaller lumina, muri crenulate.

Leaf anatomy — *Buxus cristalensis* is characterized by the absence of secretory cells. The adaxial epidermis consists of high, thin-walled cells, with anticlinal walls only slightly thickened in the apical part, showing a light line. The palisade cells are scarcely differentiated. The periclinal walls of both epidermis are papilla-like and protruding, similar to the species of the *B. gonoclada* Müll. Arg. type (see Köhler & Schirarend 1989). The stomata have a peristomal rim.

Etymology — The specific epithet alludes to Sierra del Cristal, a mountainous region in the northeastern part of Cuba, where this species is endemic.

Distribution — *Buxus cristalensis* is endemic to Sierra del Cristal, municipality of Segundo Frente, in the province of Santiago de Cuba. In Sierra del Cristal the species has been collected near to the rivers Miguel and Levisa (sometimes erroneously written as "Lebisa"), close to the top of Sierra del Cristal, between Los Moreiros and La Zanja,

on the eastern slope of the hill El Gallego, along a brook on the way between El Oro and Batista.

Habitat and ecology — *Buxus cristalensis* grows on serpentines in subspiny xeromorphic thickets and riverine rainforest, at 600–1100 m above the sea level.

Phenology — The species has been collected in flower from December to May and in fruit from April to August.

Conservation status — Although the distribution of *Buxus cristalensis* is restricted to Sierra del Cristal and we have not visited all the recorded populations, it is known that all localities where this species occurs are included in the protected area National Park Pico Cristal managed by the Cuban Enterprise for the protection of the flora and fauna. However, an assessment of the populations in the field is needed before any more reliable conservation status according to IUCN criteria (IUCN 2012) can be determined.

Discussion of phenotypic characters — Herbarium specimens of *Buxus cristalensis* had also been identified as *B. retusa,* but *B. cristalensis* can easily be distinguished from that species by the ovary and capsule with well-developed angular interstylar nectaries. It differs from *B. nipensis* by the obliquely spreading styles rising stoutly out of the carpel, which have a deeply sunken dorsal vein, well pronounced in the upper part of the capsule in contrast to *B. nipensis* and *B. retusa*. In contrast to these species the commissures are narrowly protruding distally, with collateral furrows. The internode morphology of the new species is reminiscent of *B. braimbridgeorum*, *B. nipensis* and *B. retusa*, while the papilla-like protruding periclinal walls of both epidermis layers are different, pointing more to the *B. gonoclada* type (see Köhler & Schirarend 1989). The leaf anatomy of *B. cristalensis* differs from that of *B. retusa* by complete lack of secretory cells and by the palisade parenchyma composed of 1 or 2 layers of little-differentiated cells, features that it shares with *B. nipensis* and ± with *B. triptera*. The anticlinal walls of the adaxial epidermis are only slightly thickened in the apical part, showing a light line, like in *B. nipensis*.

Discussion of molecular characters — The unique molecular diagnostic character states found in *matK* for *Buxus cristalensis* seems synapomorphic for this species. As in *B. nipensis* we did not find any distinctive character state in the *trnL-trnF* sequences of *B. cristalensis*.

Additional specimens seen (paratypes) — CUBA: PROV. SANTIAGO DE CUBA: Segundo Frente, Sierra del Cristal, prope río Lebisa in carrascales, 650–1000 m, 4 Mar 1916, *E. Ekman 6792* (S); at the tributary of río Lebisa, in carrascales, 600–1000 m, 15 Dec 1922, *E. Ekman 15960* (S); charrascos y cumbres del Cristal, rocky places, c. 1000 m, 2–7 Apr 1956, *Bro. Alain & al. 5655* (HAC, HAJB); charrascos y cumbres del Cristal, 2–7 Apr 1956, *Bro. Alain & al. 5697* (HAC, HAJB); Sierra del Cristal, falta sur de la Sierra, cabezadas del río San Miguel, 600–800 m, Apr 1968, *J. Bisse & E. Köhler HFC 8174* (HAJB, JE); camino entre Los Moreiros y La Zanja, Apr 1970, *J. Bisse HFC 15938* (HAJB, JE); Pinares y arroyos en la ladera este de la loma El Gallego, 2 May 1985, *A. Álvarez & al. HFC 57280* (B, BHU, HAJB, JE); charrascos en la subida y firme del Pico Cristal, 800–1100 m, 4 Mar 1998, *J. Gutiérrez & al. HFC 75298* (BHU, HAJB); Sierra del Cristal, arroyo en el camino del Oro a Batista, c. 700 m, 5 Mar 1998, *J. Gutiérrez & al. HFC 75348* (BHU, HAJB); Sierra del Cristal, arroyo en el camino del Oro a Batista, c. 700 m, 5 Mar 1998, *J. Gutiérrez & al. HFC 75349* (BHU, HAJB); Sierra del Cristal, arroyo en el camino entre El Halcón y Batista, 5 Mar 1998, *J. Gutiérrez & al. HFC 75386* [= Bx026] (BHU, HAJB).

Buxus koehleri P. A. González & Borsch, **sp. nov.** – Fig. 3.16–23.
Holotype: Cuba, province Holguín, Mayarí, Sierra de Nipe, Sendero Salto del Guayabo, 20°35' N, 75°45' W, 24 Feb 2010, *T. Borsch, M. Ackermann, C. Panfet, K. Zoglauer, P. González, I. Castañeda & J. Gutiérrez 4091* (HAJB; isotypes: B, ULV) [= Bx055].

Morphological diagnosis — Leaves oblong-lanceolate to narrowly elliptic, apex acute, ± retuse, weakly mucronulate. Male tepals triangular to oblong. Filaments flattened. Ovary rounded, dorsal veins and comissures sunken; nectaries angulose.

Capsule globose, dorsal veins little protruding; nectaries inconspicuous; styles erect then recurved, placed close to each other basally, connate with nectaries.

Molecular diagnosis — Nucleotide character state "A" in positions 612 and 915 of *matK* coding sequence. Nucleotide character state "A" in positions 359 and 392 of *trnL* group I intron and "G" in position 248 of *trnL-F* spacer.

Morphological description — *Tree* to 7 m tall; trunk 20–25 cm in diam.; bark furrowed; branchlets angular; internodal folds narrow with slightly prominent ribs, dorsally variably keeled; internodes 2–6 cm long. *Leaves* dimorphic; *normal-sized leaves* with petiole 4–7 mm long, blade green and shiny adaxially, paler abaxially, oblong-lanceolate to narrowly elliptic, 7–9(–10.5) × 2,5–3.5 cm, coriaceous, glabrous, base acute, apex acute to slightly acuminate, ± retuse and weakly mucronulate, midvein sunken adaxially, prominent abaxially, secondary veins in 15–18 pairs, anastomosing in an intramarginal vein 1.5 mm from margin, venation conspicuous on both surfaces, slightly reticulate; *smaller decussate leaves* interspersed between normal ones, (4–)6–10 mm long. *Inflorescences* with axis 6–7 mm long; bracts triangular, 0.5–1 mm long, apex acute. *Male flowers* with pedicel 4–6 mm long; tepals triangular to oblong, 1–1.5 mm long; stamens 2–4 mm long; filaments white, flattened, anthers c. 1 mm long with a prominent brownish tip; pistillode rounded-quadrangular, with lateral ellipsoid sinus, wrinkled. *Female flowers* with 5 tepals; tepals triangular, c. 1 mm long, with scattered hairs along margin; ovary white to yellowish, rounded, c. 2.5 × 2.5 mm, glabrous, with sunken dorsal veins and commissures; styles erect, recurved, white, thick, c. 3 mm long; stigmas broad, plicate; nectaries prominent, angular. *Capsule* brownish green, globose, 7–10 × 7–8 mm, glabrous, crowned by c. 3.5 mm long erect then recurved styles approaching each other basally, dorsal veins slightly protruding, commissures slightly sunken; nectaries inconspicuous, connate to style bases. *Seeds* rounded-trigonous, c. 6 × 2 mm.

Molecular description — Sequences describe the type specimen (code Bx055) and are available in EMBL/GenBank under accession numbers HG004433 (*matK-trnK*) and HG004428 *trnL-trnF*.

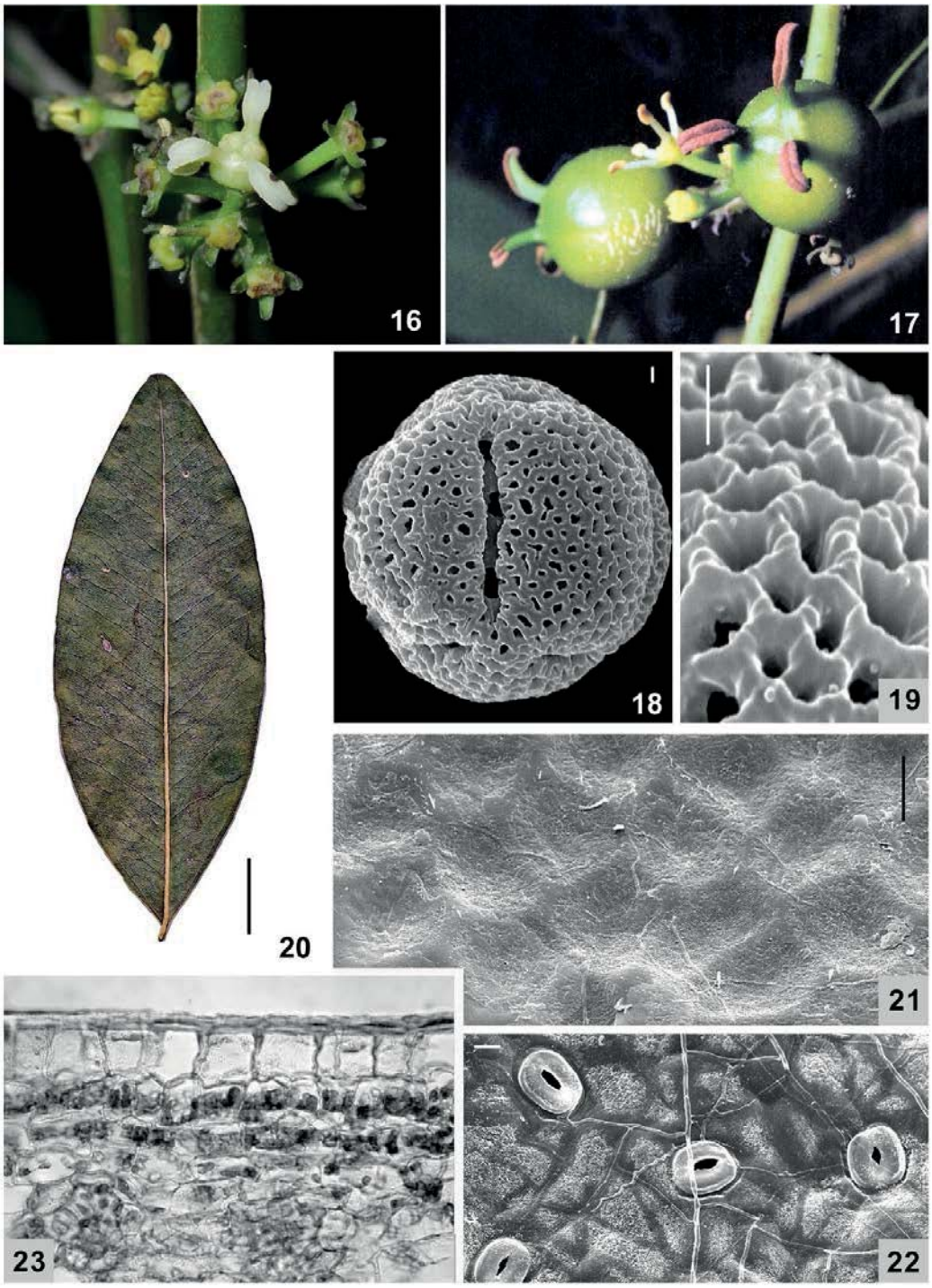


Fig. 3.16-23. *Buxus koehleri* – 16: inflorescence, female flower, male flowers; 17: immature capsule, inconspicuous nectaries; 18: pantocolporate pollen grain, colpus 3-orate, 3500×; 19: reticulate exine, pronounced crenulate muri, 10 000×; 20: brochidodromous leaf venation, scale bar = 1 cm; 21: adaxial leaf epidermis, reticulate pattern of weakly protruding anticlinal walls, 500×; 22: abaxial leaf epidermis, pattern with sunken anticlinal walls, stomata with peristomal rim, 500×; 23: leaf cross-section, adaxial epidermis, little-differentiated palisade cells, absence of secretory cells, 500×. – 16–23 from specimen *Borsch & al. 4091* (HAJB, holotype).

Pollen morphology — Pollen (3–)6–9-pantocolporate, colpi 1–3-orate, reticulate, muri thick, crenulate with protruding ribs.

Leaf anatomy — *Buxus koehleri* is characterized by the absence of secretory cells. The epidermis is formed by isodiametric cells with apically but slightly thickened anticlinal walls. The palisade parenchyma has 2 or 3 layers of little-differentiated cells. The adaxial epidermis has a weakly defined reticulate pattern of protruding anticlinal walls and slightly sunken periclinal walls. The abaxial epidermis has scarcely sunken anticlinal walls. The stomata have a peristomal rim.

Etymology — The name honours Professor Egon Köhler for his significant contributions to the knowledge of *Buxus*.

Distribution — *Buxus koehleri* is a local endemic of Sierra de Nipe, Mayarí, province of Holguín. In Sierra de Nipe it has been collected in Sendero Salto del río Guayabo and in arroyo Woodfred.

Habitat and ecology — In Sendero del Salto del Guayabo *Buxus koehleri* inhabits the understory of rainforest in association with other species such as *Bactris cubensis* Burret, *Calophyllum* sp. and *Dendropanax arboreus* (L.) Decne. & Planch., growing on black and alluvial soils mixed with serpentine, at c. 400 meters above the sea level.

Phenology — *Buxus koehleri* has been collected in flower in February and in fruit in February and September. We have visited the population in Sendero del Salto del Guayabo five times, and saw only a few plants (and always the same plants) with flowers or fruits, which is perhaps due to the low availability of sunlight for most of plants in the understorey of the rainforest.

Conservation status — *Buxus koehleri* has been collected in two localities of Sierra de Nipe. We have visited only the population in Sendero del Salto del Guayabo, where we have estimated the population to consist of 150–200 plants. Most are small trees of 3–7 m in height, but we also found seedlings and juvenile plants. This

population is protected, being located in one area administrated by the Cuban Enterprise for the protection of the flora and fauna. Following IUCN criteria (IUCN 2012) the species must be classified as endangered (EN B2a), mainly because of the small area of occupancy with less than five locatities; the population size appears to be fewer than 250 individuals.

Discussion of phenotypic characters — The most relevant characters in the morphology of *Buxus koehleri* are its habit and the shape of the leaf blade. *B. koehleri* is among the tallest species of Cuban *Buxus* and the tallest growing in Sierra de Nipe. Its apiculate leaf blade also differentiates it from other *Buxus* species that occur in Sierra de Nipe. The leaf form and size are similar to *B. muelleriana* Urb., which possesses, however, broader internodal folds, larger, more petaloid tepals and broader white filaments. The internode morphology and the leaf dimorphy of *B. koehleri* may indicate a relationship to the species of the *B. gonoclada* group that do not have a sharp dorsal keel but are ± variably keeled, like *B. cristalensis, B. excisa* Urb.*, B. nipensis, B. retusa* and *B. triptera*. *B. koehleri* is well-distinguished from these species by its capsule with long erect and only terminally recurved styles that are placed very close to each other and possess only inconspicuous nectaries. In leaf anatomy, the presence of a peristomal rim is indicative of the *B. gonoclada* group, while the absence of secretory cells and the weakly differentiated palisade tissue, which is shared with *B. cristalensis*, *B. nipensis* and *B. triptera*, differentiates it from *B. retusa.* The comparatively coarse reticulum of the pollen exine with thick crenulate muri is reminiscent of *B. triptera* and *B. braimbridgeorum*, the latter of which deviates by the well-developed secretory cells in leaf anatomy.

Discussion of molecular characters — In *Buxus koehleri* the two substitutions in the *trnL* intron and the substitution in the *trnL-trnF* spacer are unique in this species among all taxa in the genus *Buxus* and therefore represent apomorphies. The same applies to the two substitutions in the *matK* coding region. But the "A" in position 612 is only an apomorphy for this species amongst the members of the Caribbean clade. The distant lineage of Eurasian *Buxus* (*B. colchica* Pojark., *B. sempervirens* L., etc.) exhibit the same mutation (González & al. unpubl. data), which must be a convergence.

Considering that the *matK* sequences in *B. koehleri* are typical Caribbean clade sequences with a considerable distance to the Eurasian clade sequences, the Caribbean clade can be unambiguously defined as a reference group for this substitution to be diagnostic. Ongoing sequencing of population samples further indicates that the so-far studied individuals do not show any variation in the diagnostic characters states presented here. *B. koehleri* appears to be the most distinct from all three species newly recognized here when considering plastid genome sequence data.

Additional specimens seen (paratypes) — CUBA: PROV. HOLGUÍN: Mayarí, Sierra de Nipe, Sendero del Salto del Guayabo, al final del sendero, bosque pluvial con presencia de *Calophyllum* sp., *Dendropanax arborea*, *Bactris cubensis*, *Philodendron lacerum*, *Pharus* sp., 7 Sep 2011, *P. González & al. HFC 87164* (B, HAJB, herb. Greuter, JE, ULV); arroyo Woodfred, 2 Apr 1999, *H. Stenzel 742* (BHU).

3.4 Molecular characters supporting the recognition and formal description of new species

The phylogenetic analysis of homologous DNA sequences has not only revolutionized our picture of organismic evolution but sequence data are also increasingly appreciated for identifying species ("DNA barcoding"; e.g., CBOL Plant working group 2009). On the other hand, the taxonomic work process has been traditionally based on morphological characters and the formal description of species relies on characters and their states described in the protologues. Fully integrating the wealth of information that can be obtained from sequence characters into the taxonomic work process, means to also include such data into diagnoses and descriptions of species. Conceptually, a sequence of a genomic region that is obtained from a type specimen describes this particular genomic region. Unlike morphological characters, a species description in a paper will not include the actual sequence in text format but rather the corresponding reference number of a data base such as EMBL or GenBank. Those sequence characters or their states that are found to be diagnostic, should, however, be included in the diagnosis of the taxon to be described. In the case of phenotypically complex species groups this will provide further data that can be

unambiguously attached to the type specimen, and thus allow for a precise positioning of the type specimen amongst other specimens of the study group. The analysis of evolutionarily complex species groups will include the assessment of patterns such as reticulation and incomplete lineage sorting that typically require information from the genome. We therefore argue that diagnoses and descriptions of new species should be complemented by sequence data whenever possible. In our study we have attempted to consider DNA characters in the formal description of three new species of *Buxus* for exactly this reason.

Several issues appear relevant when comparing the use of morphological versus molecular data in the taxonomic workflow. Morphological data are contained in the protologues for all previously described taxa, certainly with varying levels of precision. Along with further data obtained from additional specimens of the study group, and often from re-studying the type specimens, morphological characters can then be comprehensively evaluated during the research process by the specialist researcher who recognizes a taxon as new. Thereby, cladistic or phenetic methods can be applied. The important thing is that all other accepted species in a study group can be considered. Using molecular data this process is more complicated, simply because protologues do not contain such information and because generating new sequence data from historical type specimens is often limited. Using molecular data in the taxonomic work process, therefore, often requires retroactive generation of the sequence data from the previously described species for comparison. What is needed is a comprehensive comparative sequence database, comprising genomic regions that allow the distinction of the respective species. In this context, a phylogenetic tree including putatively new species will help to focus the study of characters supporting the delimitation of a new species on the respective closest relatives. Overview trees that include as many species of a study group (e.g. a genus) as possible with the best possible resolution are needed. This has recently been shown to be feasible by using, e.g., plastid intron sequences, for which large multiple sequence alignments can be constructed (Mansion & al. 2012). In contrast, the typical phylogenetic analysis still contains only between 20 % and 40 % of the species of a study group.

Another challenge in plants is to find molecular markers that provide sufficient information to distinguish closely related species in a taxonomic context. There seems to be increasing awareness that a few standard loci such as partial *matK* and *rbcL* (CBOL Plant Working Group 2009) will not allow to achieve this goal. Recent studies on angiosperm groups such as *Crocus* (Seberg & Petersen 2009) or *Rhipsalideae* of *Cactaceae* (Korotkova & al. 2011) indicate that a combination of various introns and spacer sequences may in fact allow recognition of species-specific character states for most species but this may require combination of some five loci (>3000 nt in total) with also lineage-specific differences as to what are the respective informative genomic regions. In our case, the plastid *trnK* intron including the complete *matK* gene provided diagnostic characters for all three new species of *Buxus*. However, the widely applied *matK* barcoding fragment (CBOL Plant Working Group 2009) contains the two diagnostic characters of *Buxus koehleri* only, while the diagnostic sites for the other two species are located either up- or downstream. In this study we have used comprehensive molecular data sets that are currently being generated and include nearly all species of New World *Buxus* (González & al., in prep.) to find the diagnostic characters. Further genomic regions should definitely be sequenced for the types and other specimens during the further analysis of the evolution of *Buxus* in the Caribbean, including the nuclear genome.

Supporting the formal description of a flowering plant species, Filipowicz & al. (2012) recently recognized a deviant species of *Brunfelsia* L. (*Solanaceae*) from the Andes solely based on a molecular diagnosis. In this case, morphological characters were not apparent to support a morphology-based diagnosis while the newly recognized species and its close morphological allies could be shown to belong to distant subclades of the genus. This indicated that reproductive isolation exists and thus the species circumscription withstands further evolutionary study even in the absence of currently known deviating morphological characters. In other cases both morphology and sequence characters from the *matK* gene diagnosed *Pedersenia volubilis* Borsch & al. as a new species (*Amaranthaceae*, Borsch & al. 2011). However, only one of two diagnostic characters was in the range of the c. 850 nt long barcoding fragment used from *matK*. The available results on molecular diagnostic characters therefore strongly

indicate that additional genomic regions should be sequenced rather than focusing on the markers recommended for barcoding.

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Chapter 4

Cladistic analysis of ITS ribotypes in Caribbean *Buxus* (*Buxaceae*): Different levels of diverging paralogues hint to multiple pseudogenization but also reticulate evolution of some species

Contents

4.1 Introduction

DNA sequences have become the primary data for phylogenetic inference, due to their advantages upon morphological data in phylogenetic reconstruction. The majority of the first sequence-based molecular phylogenies in plants during the 1990ies were exclusively based on plastid genomes. Being aware of the limitations of working only with uniparentally inherited sequences (Doyle 1992), phylogeneticists started to also employ sequences from nuclear genomic regions. Among them the Internal Transcribed Spacer (ITS) region has been the most extensively used (Liston & al. 1996; Álvarez & Wendel 2003; Razafimandimbison & al. 2004; Bayly & Ladiges 2007; Harpke & Peterson 2008; Calonje & al. 2009). The ITS is popular in phylogenetic studies due to its relatively high variability, useful for studies aiming at phylogenetic relationships among species. Moreover, this nuclear region is the easiest to amplify even from herbarium specimens (Baldwin & al. 1995; Álvarez & Wendel 2003) using universal eukaryotic primers (White & al. 1990).

The ITS is part of the nuclear ribosomal array [18S-ITS1-5.8S-ITS2-26S] and have thousands copies in the genome (Baldwin 1992; Baldwin & al. 1995; Álvarez & Wendel 2003; Calonje & al. 2009). The active homogenization of repeat copies within and between loci is known as concerted evolution (Arnheim 1983 reviewed in Álvarez & Wendel 2003; Hillis & al. 1991; Nieto-Feliner & Roselló 2007). When concerted evolution is not fully operational the result is the presence of several different ribosomal sequences or paralogues (Roselló & al. 2007).

Álvarez & Wendel (2003) evaluated the phylogenetic utility of ITS, motivated by the observation that molecular evolutionary patterns of this nuclear marker may not generally be as easy as expected and will eventually confound phylogenetic analyses. This possible confusion in the phylogenetic analyses is because the frequent amplification of paralogues or in some cases contaminants of fungi genomes (Álvarez & Wendel 2003; Nieto-Feliner & Roselló 2007).

A need for phylogeny inference based on nucleic acid sequences is that the genomic regions compared are orthologous as opposed to paralogous (Doyle 1992;

Álvarez & Wendel 2003; Brigandt 2003 and authors therein). While the divergent paralogues of the nuclear ribosomal array are frequently considered to be well homogenized, there are in fact numerous cases of extensive sequence variation, arising from ancient hybridization, lineage sorting, pseudogenes in various states of decay and incomplete array homogenization (Doyle 1992; Razafimandimbison & al. 2004; Bayly & Ladiges 2007; Roselló & al. 2007).

Cloning efforts are necessary to detect and characterize divergent ITS paralogues, but these are rarely conducted in phylogenetic projects and so the levels of intragenomic ribosomal divergence could be underestimated if the ITS genotype is analysed by direct sequencing (e.g. Roselló & al. 2007). However, unreadable pherograms due to widely overlapping sequences or polymorphic sites due to diverging paralogues can be encountered in some or many individuals of a study group. Like in our case, this problem can be overcome by cloning the respective samples.

If cloning is carried out and divergent paralogues are observed, it is also necessary to investigate the putative functionality of such paralogues (Mayol & Roselló 2001; Bayly & Ladiges 2007; Harpke & Peterson 2008). Sequence motifs which can help to identify putative pseudogenes from putative functional ITS ribotypes have been suggested in ITS1 (Liu & Schardl 1994) and 5.8S (Liston & al. 1996; Jobes & Thien 1997; Harpke & Peterson 2008). Particularly important are those located in 5.8S since this gene is assumed to be highly conserved because its secondary structure is required for proper function of the ribosomal complex (Suh & al. 1992; Harpke & Peterson 2008).

The debate about ITS paralogues and pseudogenes and their effects on phylogeny reconstruction has become more intense with a larger number of ITS studies carried out across flowering plants (Mayol & Roselló 2001; Álvarez & Wendel 2003; Bailey & al. 2003; Razafimandimbison & al. 2004; Roselló & al. 2007; Harpke & Peterson 2008). Bailey & al. (2003) consider that a priori exclusion of pseudogenes from gene tree analyses is unjustified and suggest that pseudogene sequences should not be ignored in phylogenetic analyses since they can provide critical information on the adequacy of sampling included in a study, they may also supply important data about DNA sequence diversification and interspecific hybridization. Razafimandimbison & al.

(2004) found that ITS polymorphism may not necessarily mislead phylogenetic inference and that putative pseudogenes can be useful for phylogenetic analyses, especially when no sequences of their functional counterparts are available.

As part of the nuclear genome, the ITS region is inherited biparentally (Álvarez & Wendel 2003; Nieto-Feliner & Roselló 2007). This characteristic makes the detection of reticulation possible when phylogenetic studies include plastid and ITS data sets (Löhne & al. 2008; Fuentes-Bazan & al. 2012). The most common biological phenomena associated to a reticulate pattern are hybridization and incomplete lineage sorting, which are not easy to differentiate from each other (Doyle 1992; Joly & al. 2009). When hybridization or introgression has taken place it is possible to be detected through analysis of ITS sequences if the concerted evolution has not homogenized the copies (Sang & al. 1995; Nieto-Feliner & Roselló 2007).

In Cuba the genus *Buxu*s is represented by 37 species and seven subspecies, most of which inhabit the vegetation on serpentines (Köhler 2014). A comprehensive phylogenetic analysis of a dense sampling of Cuban and other Caribbean species of *Buxus* based on plastid markers has been carried out (Chapter 2). This plastid phylogeny showed the existence of a Caribbean clade, which encloses all Cuban and other Caribbean species of *Buxu*s, except one species (*B. brevipes*), which is enclosed in the Mexican clade (Fig. 2.1 of chapter 2). The chloroplast tree suggests *B. jaucoensi*s to be the sister of the rest of species, which are enclosed in three different subclades: the "Glomerata"-clade, the "Gonoclada"-clade and the "Shaferi"-clade; each of them with particular geographic and ecologic patterns. The chloroplast tree revealed some samples belonging to different populations of the same species as not monophyletic. In addition, some samples belonging to different species share very similar to identical haplotypes. A good example of this is shown in the "Glomerata"-clade, where accessions of *B. glomerata* are placed in two different subclades and two of them (Bx052 and Bx157) are strong related to *B. sclerophylla* (Bx028) [Fig. 2.1 of chapter 2]. This could be associated with incomplete lineage sorting or hybridization and plastid capture (Joly & al. 2009). The hybrid speciation has been suggested as an important mechanism in the evolution of the Cuban flora (Borhidi 1996; López-Almirall 1998). Hybrid taxa have been reported for the flora of Cuba based on morphological studies (León & Alain 1951;

Granda & Fuentes 1985; Caluff & Shelton 2003; Sánchez & Regalado 2003; Sánchez & al. 2006).

Initial efforts to generate an ITS sequence matrix for *Buxus* based on direct sequencing failed in some samples due to unreadable pherograms. Roselló & al. (2007) found a similar difficulty in a study focused on *B. balearica* and after cloning detected divergent ITS paralogues which allowed testing a phylogeographic split in this species and to suggest that the concerted evolution of rDNA has not been fully operational in *Buxus.* Extensive cloning work was therefore conducted in this study with the aim to obtain good quality ITS sequences.

The principal goal of this study is to explore in how far reticulate evolution has contributed to the origin of species diversity of *Buxus* in Cuba and the Caribbean. In particular, the origin and limits of species which were not unambiguously found as monophyletic in the chloroplast tree should be illuminated, such as *B. glomerata*, *B. gonoclada* and *B. shaferi*. Since considerable infragenomic variability with often more than two types of ITS sequences in an individual was observed in several cases, we aimed to classify the sequences into putative functional ribotypes and putative pseudogenic ribotypes and to explore their relationships to better understand comprehensive ITS gene trees.

4.2 Materials and methods

4.2.1 Taxon sampling and plant material

Most of the plant material was collected during field work in Cuba or from the living collection of *Buxus* in National Botanical Garden of Cuba (Rankin-Rodríguez & al. 1999; Köhler 2001). Other samples were obtained from the living collections of Botanical Garden of Berlin, from the Arboretum of the Humboldt University of Berlin, or from herbarium specimens. In the case of the samples collected during field work or in living collections, few young and healthy leaves were collected and dried in silica gel and at the same time a herbarium voucher was made and preserved.

The sampling of this study comprises sequences of 96 samples of 71 taxa of *Buxaceae*. *Buxus* is represented by 55 species and four subspecies, of which 34 species and four subspecies are from Cuba. Of some Cuban species (e.g. *B. foliosa*, *B. glomerata*, *B. gonoclada* ssp. *gonoclada* and *B. shaferi*) samples from different populations were included. Other *Buxus* samples belong to species from Hispaniola (Dominican Republic), Jamaica, Mexico, Panama, Puerto Rico, and regions from Africa and Eurasia. The outgroup comprises sequences of 12 other taxa of *Buxaceae* belonging to the genera *Pachysandra*, *Sarcococca* and *Styloceras*.

Most of sequences were generated during the course of this study and few other were generated in previous studies (Balthazar & al. 2000; Roselló & al. 2007). Information about species, specimens, samples and their codes, localities, collectors and vouchers is shown in appendix 4.1.

4.2.2 DNA extraction, amplification, cloning and sequencing

The Total DNA was isolated from silica-gel-dried leaf tissue or herbarium specimens using a triple CTAB extraction method (Borsch & al. 2003) or the Nucleo Spin Plant II extraction kit (Macherey Nagel, Düren, Germany).

The amplification of ITS was performed in reaction volumes of 50 µL, containing 2 µL of extracted DNA (with a concentration of 10–20 ng/µL), 14.7 µL of H_2O, 5 µL of 10× peqLab Taq. buffer S containing $MgCl_2$, 3 µL of $MgCl_2$ (25 mM), 10 µL of betaine monohydrate (5 M), 1 µL of BSA (10 ug/µl), 2 µL of forward primer (20 pm/µl), 2 µL of reverse primer (20 pm/µl), 10 µL dNTPs (each 0.25 mM) and 0.3 µL Taq polymerase 5 units/µl (PeqLab, Erlangen Germany). The universal primers ITS-4 and ITS-5 of White & al. (1990) were used in the amplification and sequencing. PCR (polymerase chain reaction) conditions were: 35 cycles of denaturation (60 s at 97 ˚C), annealing (60 s at 48 ˚C), extension (45 s at 72 ˚C) and a final extension step (7 min at 72 ˚C).

A group of samples were selected for cloning. The main reasons of this selection were that most of them yielded ITS pherograms with polymorphic sites or totally

illegible. Other samples (e.g. Bx028, Bx052, Bx122, Bx140) were also selected for cloning under the criteria that they could be involved in hybridization events considering the results of the previous phylogenetic reconstruction based on plastid markers. Cloning was conducted using the TOPO TA Cloning kit for Sequencing (Invitrogen, Life technologies, Carlsbad, CA 92008 USA) following the guidelines of the manufacturers. For each cloned sample, 19 colonies were randomly selected for sequencing.

Fragment purification and sequencing were performed by Macrogen Inc., South Korea (http://www.macrogen.com).

4.2.3 Editing of pherograms and alignment of sequences

The pherograms were edited and aligned using PhyDE v.0 995 (Müller & al. 2007). Due to the risk to amplify contaminant DNA of other organisms when using widely universal primers a BLAST search (Altschul & al. 1997) in the GenBank/EMBL nucleotide databases was carried out.

The edited sequences were manually aligned. The regions of uncertain homology were removed from the matrix used for the analysis. The indels were coded using the Simple Index Coding Method (Simons & Ochoterena 2000) with SeqState v. 1.4.1 (Müller 2005a).

The boundaries of ITS1, 5.8S and ITS2 were annotated using as reference the ITS sequence of *B. sempervirens* generated by Rosselló & al. (2007).

4.2.4 Sequences characterization and classification

In order to characterize and classify the sequences of ITS the percentage of Guanine-Cytosine (GC) was calculated and the length of ITS1, 5.8S and ITS2 was measured. This analysis was carried out in line with Roselló & al. (2007), since it is the most extensive publication about *Buxus* on this topic. The analysis of the ITS data set for this purposes was conducted with SeqState v. 1.4.1 (Müller 2005a). Additionally, each sequence was visually explored to search for motifs suggested as conserved in functional sequences of angiosperms. In ITS1 the motif was: GGCRY-(4 to 7n)-GYGYCAAGGAA (Liu & Schardl 1994) and in the 5.8S the motifs analysed were: GATATC (Liston & al. 1996), GAATTGCAGAATCC (Jobes & Thien 1997), TTTGAAYGCA (Harpke & Peterson 2008) [hereafter referred as motif I of Harpke & Peterson (2008)], CGATGAAGAACGYAGC (Harpke & Peterson 2008) [hereafter referred as motif II of Harpke & Peterson (2008)]. Considering the results of the previous analyses the ITS sequences were classified in putative functional ribotypes and putative pseudogenic ribotypes [Appendix 4.2].

4.2.5 Phylogenetic analyses

Two matrices of ITS were aligned. A first matrix of 50 sequences included 37 sequences of Cuban and Caribbean *Buxus* generated from direct PCR sequences without polymorphic sites and other 13 sequences belonging to *Buxus* from other geographic regions, to *Pachysandra* and *Sarcococca*. A plastid matrix including sequences of the same taxa was assembled in order to conduct comparative studies and to explore the possible occurrence of reticulate patterns. The second matrix of ITS included all ITS sequences generated either from direct PCR products or from cloning.

Maximum parsimony (MP) and Bayesian Inference (BI) analyses were carried out. The BI analyses were conducted using MrBayes 3.1 (Huelsenbeck & Ronquist 2001). The optimal nucleotide substitution models were determined following the Akaike Information criterion (AIC) in Modeltest 2.3 (Posada & Crandall 1998); for the three

plastid markers analysed separately the model selected was GTR + G (Chapter 2), and for ITS data sets the selected model was GTR + G as well. A binary (restriction site) model was implemented for the coded indels. All analyses were performed with four independent runs of Markov Chains Monte Carlo (MCMC) each with four parallel chains. Each chain was performed for 1 000 000 generations, saving one random tree every 100th generation. For the BI analyses of ITS the burn in was set to 200 and a majority consensus trees were computed with the remaining trees.

The MP analyses were made through the Parsimony Ratchet (Nixon 1999) using the software PRAP (Müller 2004) in combination with PAUP v. 4.0b10 (Swofford 1998). Ratchet settings were 200 ratchet iterations with 25% of the positions randomly up weighted (weight = 2) during each replicate and 10 random addition cycles. The command files generated with PRAP were then run in PAUP, using the heuristic search with the following parameters: all characters have equal weight, gaps are treated as ‘‘missing’’, TBR branch swapping, initial swapping on 1 tree already in memory, Maxtrees set to 100 (auto increased by 100) and branches collapsed actively if branch length is zero. The Jackknife (JK) support for branches was also performed in PAUP with 10 000 replicates, using a TBR branch swapping algorithm with 36.788% of characters deleted and one tree held during each replicate following Müller (2005b).

In order to explore the congruence among the plastid and nuclear data sets, an Incongruence Length Difference (ILD) test (Farris & al. 1995) was carried out in PAUP v. 4.0b10 (Swofford 1998) as the Partition Homogeneity Test and using the following parameters: 10 000 repetitions, two replications each repetition, holding two trees each step, saving no more than five trees. This test was conducted for the reduced ITS data set and a respective plastid data set.

4.3 Results

4.3.1 Phylogenetic analysis of ITS sequences directly obtained by sequencing PCR products and the corresponding plastid partition

The final plastid matrix of 50 sequences including indels comprised 4473 characters (4402 nt and 71 indels), of which 388 (8.67%) were parsimony informative. The MP search resulted in 61 shortest trees (Length=747, CI=0.890, RI=0.941). The consensus trees from MP and BI analyses have similar topologies. The BI tree with PP and JK support values is shown in Fig. 4.1-A. This phylogenetic tree based on a reduced data set has a similar topology than the tree reconstructed with a denser sampling (Fig. 2.1 of chapter 2). It shows a strongly supported monophyletic *Buxus* (1 PP / 100% JK) and within this clade the species of Eurasia, Africa and America, respectively, are enclosed in three independent subclades (Fig. 4.1-A and Fig. 2.1 of chapter 2). Within the American *Buxus* subclade, the Mexican species and *B. brevipes* from western Cuba are enclosed in an independent clade, although the relationship of these species is poorly supported (Fig. 4.1-A and Fig. 2.1 of chapter 2). Within the Cuban and Caribbean *Buxus*, *B. jaucoensis* is sister of three well supported clades. These clades are hereafter called “Gonoclada”-clade, “Shaferi”-clade and “Glomerata”-clade (Fig. 4.1-A and Fig. 2.1 of chapter 2).

The final ITS matrix of 50 sequences including indels comprised 752 characters (644 nt and 108 indels), of which 297 (39.49%) were parsimony informative. The MP search resulted in 442 shortest trees (Length=884, CI=0.657, RI=0.773). The MP and BI analyses generated trees, which are largely congruent with only some inconsistently inferred nodes. The BI tree with PP and JK support values is shown in the figure 4.1-B. A difference detected among the topologies of both trees is the position of the Mexican species *B. mexicana*. In the MP tree (not shown) *B. mexicana* is related to *B. braimbridgeorum* and *B. nipensis* but this relationship is poorly supported (63% JK), whereas in the BI tree *B. mexicana* is resolved as sister of all other American species of *Buxus* but this relationship has low support (Fig. 4.1-B).

In the tree based on ITS, *Buxus* is monophyletic with maximum support and the species from Africa, Eurasia and America are enclosed in different clades. The Eurasian and African clades are strongly supported (1 PP / 100% JK), but the node linking them is poorly supported (0.64 PP / 73% JK). Like in the plastid tree, the American species are monophyletic with strong support (1 PP / 100% JK). Within the American clade three internal clades are recovered (Fig. 4.1-B). Two of them recovered partially the species composition of the clades defined in the plastid tree, hence they are called " P. Shaferi"-clade. and "P. Glomerata"-clade. In the names of the clades "P" means partial. The "Gonoclada"-clade is totally recovered with one additional species, *B. leivae*. *B. leivae* was enclosed in the "Shaferi"-clade based on the plastid reconstruction with strong support (1 PP / 100% JK). In the ITS tree this species is related to *B. excisa*, *B. triptera*, *B. retusa*, *B. revoluta* and *B. pilosula* ssp. *cacuminis* ("Gonoclada"- clade) also with strong support (1 PP / 99% JK, Figs. 4.1-A and -B).

The "P. Shaferi"-clade recovered in the ITS tree encloses *B. foliosa, B. pseudaneura, B. shaferi* and *B. yunquensis* (except *B. leivae*, see results above). Within this clade *B. glomerata* Bx031 and Bx035 ("Glomerata"- clade based on plastid sequences) are close related to *B. shaferi* and *B. yunquensis* (1 PP / 86% JK) and the sister clade to them encloses *B. glomerata* Bx106 and Bx143 and *B. vahlii* (0.98 PP / 59% JK) all of them within "Glomerata"-clade based on plastid sequences (Figs. 4.1-A and –B).

The "P. Glomerata"-clade recovered in the ITS tree encloses *B. acunae, B. arborea, B. bahamensis, B. ekmanii, B. marginalis, B. olivacea, B. portoricensis* and *B. wrightii*, except three species enclosed in the "Glomerata"-clade of the plastid reconstruction. These are *B. glomerata* and *B. vahlii* (within "P. Shaferi"-clade, see results above) and *B. sclerophylla* related to *B. jaucoensis* (1 PP / 100% JK, Figs. 4.1-A and-B). Ironically all representatives of the species *B. glomerata*, after which this clade was previously named (Chapter 2), are enclosed (accessions Bx031, Bx035, Bx106 and Bx143) and related (accession Bx052) to the "P. Shaferi"-clade (Fig. 4.1-B). Within this clade, *B. brevipes* (Mexican clade based on plastid sequences) is related to *B. marginalis* (0.96 PP / <50 % JK; Fig. 4.1-A and 4.1-B).

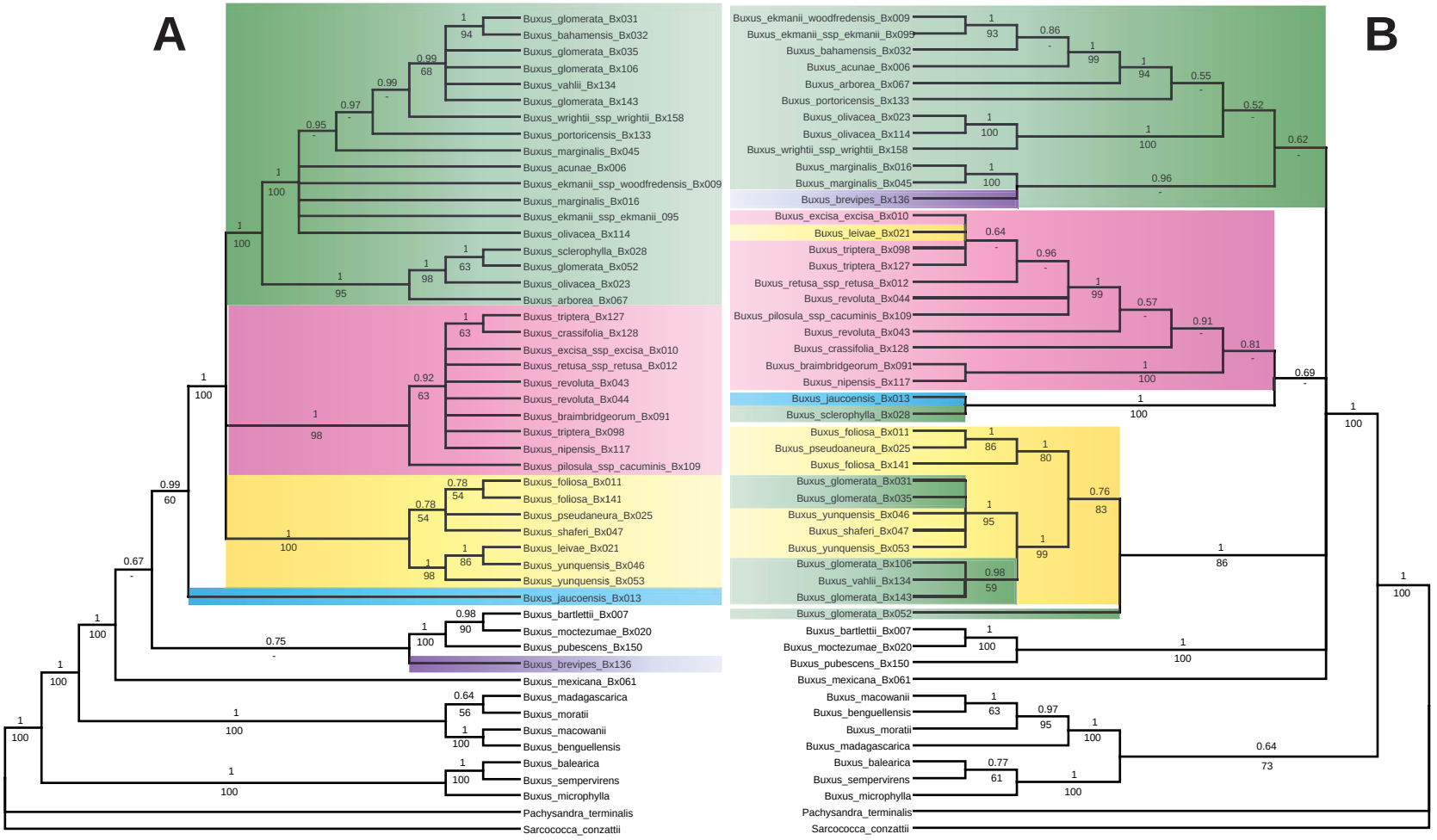


Fig. 4.1.-A- Bayesian majority rule tree based on the 50 accessions dataset of plastid markers *trnL-F+petD+trnK-matK*. Bayesian posterior probabilities (PP) are shown above branches and Jackknife values (JK) are shown below branches. B.- Bayesian majority rule tree based on the 50 accessions dataset of ITS. Bayesian posterior probabilities (PP) are shown above branches and Jackknife values (JK) are shown below branches. The "Glomerata"-clade is represented by green boxes, the " Gonoclada"-clade is represented by pink boxes, the " Shaferi"-clade is represented by yellow boxes, *B. jaucoensis* is represented by blue boxes and *B. brevipes* is represented by violet boxes.

The different positions of the sequences of *B. brevipes*, *B. glomerata*, *B. jaucoensis*, *B. leivae*, *B. vahlii* and *B. sclerophylla* in the plastid tree as compared to the ITS trees indicate a reticulate pattern. These topological deviations are corroborated by results of the incongruence test ILD, which showed that the respective partitions of ITS and the plastid data sets are significantly incongruent at P = 0.0010.

4.3.2 Characteristics of ITS sequences obtained through cloning

Several of the first generated pherograms were unreadable due to polymorphic sites. Only 44 samples (37 of them belonging to Cuban and Caribbean *Buxus*) yielded good quality sequences from direct PCR amplifications, e.g. *B. bahamensis* (Bx032), *B. brevipes* (Bx136) and *B. shaferi* (Bx 047) [Appendix 4.2].

A total of 36 samples were selected for cloning and from 32 (c. 89%) of them 1 to 15 clones were successfully *Buxus* sequences (Appendix 4.2). The samples from which no clone sequences were obtained belong to the species *B. jaucoensis* (Bx013), *B. leivae* (Bx021) and *B. rheedioides* (Bx041, Bx119). About 500 ITS sequences were generated after cloning, although a BLAST search showed that c. 45% matched *Buxus*. In a few samples (e.g. *B. aneura*, Bx001; *B. imbricata*, Bx054) only one of 19 sequences matched *Buxus* while the rest of pherograms represented fungal sequences. Based on the comparison with sequences available in GenBank/EMBL the fungal ribotypes represent genera such as *Cladosporium*, *Hypoxylon*, *Microdochium* and *Rhodotorula*, which are reported for Cuba (Mayra Caminó, specialist on Cuban fungi, personal communication).

B. acuminata (Bx005) and *B. olivacea* (Bx094) are the samples with the highest number of successfully *Buxus* sequences, with 15 ITS sequences each. From the 15 sequences of the sample Bx094 of *B. olivacea* four of them are identical. No identical clones shared by different samples of the same species or by different species were detected (Appendix 4.2).

4.3.3 Length of ITS sequences and GC content

Almost all fragments, consisting of ITS1, 5.8S and ITS2, are in the range of 519 to 677 nt. Only the sequence of *B. olivacea* (Bx094, clone 6) is outstandingly large with 1863 nt and is considered a pseudogene (see discussion below, Appendix 4.2). Within the *Buxaceae* analysed the largest sequences belong to *Pachysandra* (676–677 nt), Asian *Sarcococca* (669-671 nt) and *Styloceras* (647 nt) generated by Balthazar & al. (2000). Within *Buxus* the largest sequence belongs to (*B. balearica* AF245423 of Balthazar & al. (2000). In the Cuban and Caribbean species of *Buxus* the length of ITS varies from 599 nt (*B. koehleri*, Bx055-c58) to 653 nt (*B. glomerata*, Bx031). The African species *B. benguellensis*, *B. hildebrandtii*, *B. macowanii*, *B. madagascarica*, *B. moratii* and the Mexican species *B. bartletii*, *B. moctezumae*, *B. pubescens* have the shortest ITS sequences (Appendix 4.2). The length of the 5.8S is 157–162 nt, although in 92% of sequences the length of 5.8 S is 160 nt. The largest 5.8S copies were found in the sequence of *Styloceras brokawii* (161 nt) generated by Balthazar & al. (2000) and in *B. olivacea* (Bx094-c6, 162 nt) [Appendix 4.2].

The GC content in ITS1 and ITS2 ranges from 48% (*B. olivacea*, Bx094-c6) to 72.6–72.9% (*B. ekmanii* ssp. *ekmanii*, Bx095; *B. acunae*, Bx006). In the sequences of putative functional ribotypes of *Buxus* from Eurasia the range of GC content is c. 66–68 %, in the African species is c. 61–66 % and in the American it is 63–72 % (Appendix 4.2).

4.3.4 The conserved motif in ITS1 indicating functionality

In all species of *Buxus* and other *Buxaceae* included in this study, the conserved motifs of ITS1 has 21 nt (GGCRY-(5 nt)-GYGYCAAGGAA). The internal element which has been reported to be 4–7 nt long in flowering plants (Liu & Schardl 1994), has 5 nt in all sequences of *Buxaceae* analysed. This motif is conserved in 133 sequences. It has one to four mutated positions in 160 sequences. A total of 29 different mutated ITS1 motifs were detected. Some of these mutated motifs are characteristic of a sole or few clones of the same sample; however others are shared by sequences of clones from

different samples, such as: G**A**CGC-(5nt)-GCGTCAAG**A**AA detected in ITS sequences of *B. braimbridgeorum* (Bx091), *B. gonoclada* ssp. *gonoclada* (Bx038, Bx051, Bx079), *B. nipensis* (Bx117) and *B. serpentinicola* (Bx029); G**A**CGT-(5nt)-GCGCCAAGGAA detected in several sequences of *B. glomerata* (Bx052), *B. gonoclada* ssp. *gonoclada* (Bx038, Bx051, Bx079) and *B. sclerophylla* (Bx028) and GGCGC-(5nt)-**A**C**A**CCAAGGAA detected in clones of *B. moana* (Bx018), *B. shaferi* (Bx004, Bx122) and *B. glomerata* (Bx052, Bx155) [Appendices 4.2, 4.3].

4.3.5 The conserved motifs in 5.8S indicating functionality

The conserved motif GATATC in 5.8S characterized by Liston & al. (1996) is not conserved in a total of 38 sequences. Eight different mutated motifs were found. The most common changes in this motif are: i) **A**ATATC detected in 13 clones of *B. citrifolia* (Bx151), *B. crassifolia* (Bx002) and *B. sclerophylla* (Bx028); and ii) GATAT**T** detected in 18 clones of *B. acuminata* (Bx005), *B. glomerata* (Bx052) and *B. sclerophylla* (Bx028) [Appendices 4.2, 4.4].

The motif GAATTGCAGAATCC suggested as a conserved element by Jobes & Thien (1997) is not conserved in 100 sequences. Fourteen different mutated motifs were found. Most of those sequences in which this motif is not conserved, have 1 or 2 mutations. The ITS sequence of *Styloceras brokawii* (AF245431) generated by Balthazar & al. (2000) has four mutations in this motif. Unique mutations in this conserved motif are characteristic for seven clones (see Appendix 4.5). Seven other mutations of them are shared by more than one taxon, sample or clones. The mutated motif GAATTGCA**C**AATCC, is characteristic of several clones or direct amplified PCRs of seven samples of six taxa (Appendices 4.2, 4.5).

The motif I (TTTGAAYGCA) as outlined by Harpke & Peterson (2008) is not conserved in 53 sequences. A total of nine different mutated motifs were detected. Six of them are characteristic of one or several clones of the same sample (e.g. for one clone *B. glomerata* Bx 155-c15 or for several clones *B. citrifolia* Bx151, see Appendix 4.6).

The most common mutations in this motif are TTTGAAC**A**CA and TTTG**G**ACGCA (Appendices 4.2, 4.6).

The motif II (CGATGAAGAACGYAGC) as outlined by Harpke & Peterson (2008) is not conserved in 127 sequences. In these sequences, 24 different mutated motifs were found. Fourteen of these are characteristic of one and eight are shared by sequences generated from more than one clones or species. The most common mutation is CGATGAAGAA**TT**TAGC, shared by clones of seven taxa (Appendices 4.2, 4.7).

4.3.6 Selection of putative functional ribotypes and putative pseudogenic ribotypes

In this study we consider putative pseudogenic ribotypes to: i) sequences anomalously too large; ii) sequences with at least one mutated motif (especially in the 5.8 S partition) and iii) sequences with considerably low (less than c. 60%) percentage of GC %, which does not have mutated motifs. According to these criteria, other ITS sequences obtained, without any of the mentioned characteristics, were classified as putative functional ribotypes (Appendix 4.2).

A total of 84 (29 %) ITS sequences were considered putative functional ribotypes and 210 (71 %) were considered putative pseudogenic ribotypes. From several samples only putative pseudogenic ribotypes were obtained. Examples of this are: Bx005 (*B. acuminata*), Bx008 (*B. bissei*), Bx018 (*B. moana*), Bx038, Bx051 and Bx079 (*B. gonoclada ssp. gonoclada*), Bx094 (*B. olivacea*) and Bx140 (*B. foliosa*). The direct sequencing of PCR products of *B. olivacea* (Bx023), *B. braimbridgeorum* (Bx091) and *B. vahlii* (Bx134) were classified as putative pseudogenic ribotypes, although the pherograms of these no cloned samples did not have polymorphic sites (Appendix 4.2).

4.3.7 Phylogenetic relationships among putative functional ribotypes and putative pseudogenic ribotypes

The final ITS matrix including all generated ITS sequences, from direct PCR and from cloning, comprised 870 characters (670 nt and 200 indels), of which 598 (68.7%) were parsimony informative. The MP search resulted in 1927 shortest trees (Length=3384, CI=0.346, RI=0.857). The MP and BI analyses generated trees with similar topologies. The phylogram obtained from the BI analysis is shown in Fig. 4.2. In this phylogram the sequences considered as putative pseudogenic ribotypes are annotated with specific symbols indicating mutations in the specific ITS1 and 5.8S motifs (Figure 4.2).

The Eurasian *Sarcococca* form a strongly supported clade (0.94 PP / 97% JK), the Mexican *Sarcococca conzattii* is placed alone, between the clade of the Eurasian *Sarcoccoca* and *Pachysandra*, with moderate to strong support (0.81 PP / 100% JK). *Pachysandra* is strongly supported as monophyletic (0.92 PP / 100% JK) [Fig. 4.2].

The clade enclosing all representatives of *Buxus* is well supported (0.93 PP / 96% JK) and within this clade the species of *Buxus* from Africa, Eurasia and America are enclosed in three well defined clades (Fig. 4.2). The relationships of *B. natalensis* (AF245425) and *B. hildebrandtii* (AF245415) with the remaining African representatives are weakly supported (0.56 PP / <50% JK). These two sequences are classified as putative pseudogenic ribotypes as suggested by Roselló & al. (2007). The other African representatives, classified as putative functional ribotypes, are strongly related (0.94 PP / 100% JK; Fig. 4.2)

The clade enclosing all Eurasian representatives of *Buxus* is high supported enclosing also the putative pseudogenic ribotype of *B. henryi* (AF245409) (Roselló & al. 2007; this study Fig. 4.2).

All American species of *Buxus* are enclosed in a clade (0.69 PP / 100% JK), however the relationships within this clade are largely unresolved (Fig. 4.2). The Mexican species *B. bartlettii*, *B. moctezumae* and *B. pubescens*, which are putative

functional ribotypes, are enclosed in a clade (0.94 PP / 100% JK) recovering the Mexican clade (based on the plastid data set, Fig. 4.1-A).

ITS sequences of the Cuban and Caribbean species of *Buxus* appear in 18 different subclades. Eight of them enclose only putative pseudogenic ribotypes (hereafter called PPs1–PPs8), eight clades enclose a mix of sequences of putative functional ribotypes and putative pseudogenic ribotypes (hereafter called Mx1–Mx8) and two clades enclose only sequences of putative functional ribotypes (hereafter called PFR1, PFR2). It is noteworthy that almost all sequences generated from one individual through cloning are polyphyletic in different clades (marked in red in Fig. 4.2).

Clades comprising putative pseudogenic ribotypes (PPs clades)

The PPs1 clade shows putative pseudogenic ribotype with two to five mutated motifs in ITS1 and 5.8S belonging to samples of six taxa (0.71 PP / <50% JK; Fig. 4.2). Some supported relationships are: i) among putative pseudogenic ribotypes of *B. gonoclada* ssp. *gonoclada* (Bx038, Bx051, Bx079), *B. gonoclada* ssp. *orientensis* (Bx146) and *B. pilosula* ssp. *pilosula* (Bx024) [0.97 PP / 91% JK]; ii) among putative pseudogenic ribotypes of *B. crassifolia* (Bx002) [0.97 PP / 100% JK] and iii) among putative pseudogenic ribotypes of *B. moana* (Bx018) and *B. shaferi* (Bx022, Bx122) [0.72 PP / 82% JK; Fig. 4.2].

The PPs2 clade shows putative pseudogenic ribotype with two, three and five mutated motifs in ITS1 and 5.8S belonging to samples of four taxa (0.73 PP / 56% JK). Internally the relationships among putative pseudogenic ribotypes are strongly supported: i) *B. acuminata* (Bx005) to *B. glomerata* (Bx155) [0.97 PP / 100% JK], and ii) related to them *B. rotundifolia* (Bx096) [0.95 PP and 96% JK], finally iii) the putative pseudogenic ribotypes of *B. crassifolia* (Bx002) [0.97 PP / 100% JK; Fig. 4.2].

The PPs3 clade shows putative pseudogenic ribotype with one to three mutated motifs in ITS1 and 5.8S of seven different taxa (0.96 PP / 100% JK). The internal nodes are well supported too (see Fig. 4.2). Within this clade all the putative pseudogenic ribotypes

generated for *B. bissei* (Bx008) are enclosed in one subclade [098 PP / 95% JK; Fig. 4.2].

The clade PPs4 shows putative pseudogenic ribotype with one to three mutated motifs of ITS1 and 5.8S of *B. shaferi* (Bx022), *B. moana* (Bx018) and *B. foliosa* (Bx140) [0.97 PP / 100% JK]. All these taxa included in this clade were enclosed in the "Shaferi"-clade of the plastid tree (Fig. 4.2, 4.1-A and Fig. 2.1 of chapter 2).

The PPs5 clade shows putative pseudogenic ribotype with two to five mutated motifs of ITS1 and 5.8S, which belong to *B. acuminata* (Bx005), *B. glomerata* (Bx052), and *B. sclerophylla* (Bx028) [0.97 PP / 100 % JK; Fig. 4.2]. These three species were enclosed in the "Glomerata"-clade of the plastid tree (Fig. 2.1 of chapter 2).

The PPs6 shows only two putative pseudogenic ribotype of *B. shaferi* (Bx122-c80 and -c85) [0.97 PP / 100% JK; Fig. 4.2]. Similar to this one is the PPs7 clade, but in this case the sequences belong to different accession of *B. shaferi* (Bx122-c81 and Bx004-c49) [0.83 PP / 88 % JK; Fig. 4.2].

The PPs8 clade shows a putative pseudogenic ribotype of *B. moana* (Bx018) with two mutated motifs, in ITS1 and 5.8S, and putative pseudogenic ribotypes of two different samples of *B. glomerata* (Bx052, Bx155) with one mutation in the motif of ITS1 (0.95 PP / 100% JK; Fig. 4.2).

Clades comprising putative functional ribotypes and putative pseudogenic ribotypes (Mx clades)

The Mx1 clade comprise putative pseudogenic ribotype sequences with two or four mutated motifs in ITS1 and 5.8S of the species *B. citrifolia* (Bx151) and *B. olivacea* (Bx094) and also sequences of putative functional ribotypes of *B. marginalis* (Bx016, Bx045) and *B. brevipes* (Bx136) in a polytomy (0.7 PP / <50% JK). Within the Mx1 three subclades are recovered: i) *B. citrifolia* (Bx151) with seven clones of the same accession (0.97 PP / 100% JK); ii) *B. olivacea* (Bx094) with eight clones of the same

accession (0.98 PP / 100 % JK); and iii) two accessions of *B. marginalis* (Bx016 and Bx045; Fig. 4.2).

The Mx2 clade also depicts two sequences of putative functional ribotypes of *B. citrifolia* (Bx151-c85) and *B. portoricensis* (Bx133) and two sequences of putative pseudogenic ribotypes with one or two mutated motifs in ITS1 and 5.8S of *B. citrifolia* (Bx151-c84) and *B. gonoclada* (AF245427). The support of this clade is 0.96 PP but only <50% JK (Fig. 4.2).

The Mx3 clade (0.91 PP / <50% JK) shows two clear subclades: i) sequences of six taxa from Cuba and Jamaica which are putative functional ribotypes (0.77 PP / 92 % JK) and ii) three clones of putative pseudogenic ribotypes with two mutated motifs in 5.8S belonging to *B. sclerophylla* (Bx028) [0.98 PP / 100% JK; Fig. 4.2].

The Mx4 clade (0.97 PP / 86% JK) consists of three subclades: i) sequences of putative functional ribotypes of *B. glomerata* Bx031, Bx035; *B. shaferi* Bx047 and *B. yunquensis* Bx046; Bx053 from Cuba (0.99 PP / 96% JK); ii) sequences of putative functional ribotypes of *B. glomerata* Bx106, Bx143 from Hispaniola and *B. vahlii* (Bx134) from Puerto Rico (1 PP / 84% JK) and iii) three putative pseudogenic ribotypes of *B. glomerata* (Bx157-c21, -c28 and -c38) from Cuba (0.99 PP / 100% JK; Fig. 4.2).

The Mx5 clade shows 14 sequences of the species *B. glomerata* obtained from samples of geographically different localities; Bx052 and Bx157 from Guantánamo in southeastern Cuba, and Bx155 from Cienfuegos in central Cuba (0.97 PP / 100% JK). The putative functional ribotypes form a subclade with 13 clones (0.95 PP / 94 % JK) and related to them is *B. glomerata* Bx052-c34, a putative pseudogene with two mutated motifs (Fig. 4.2).

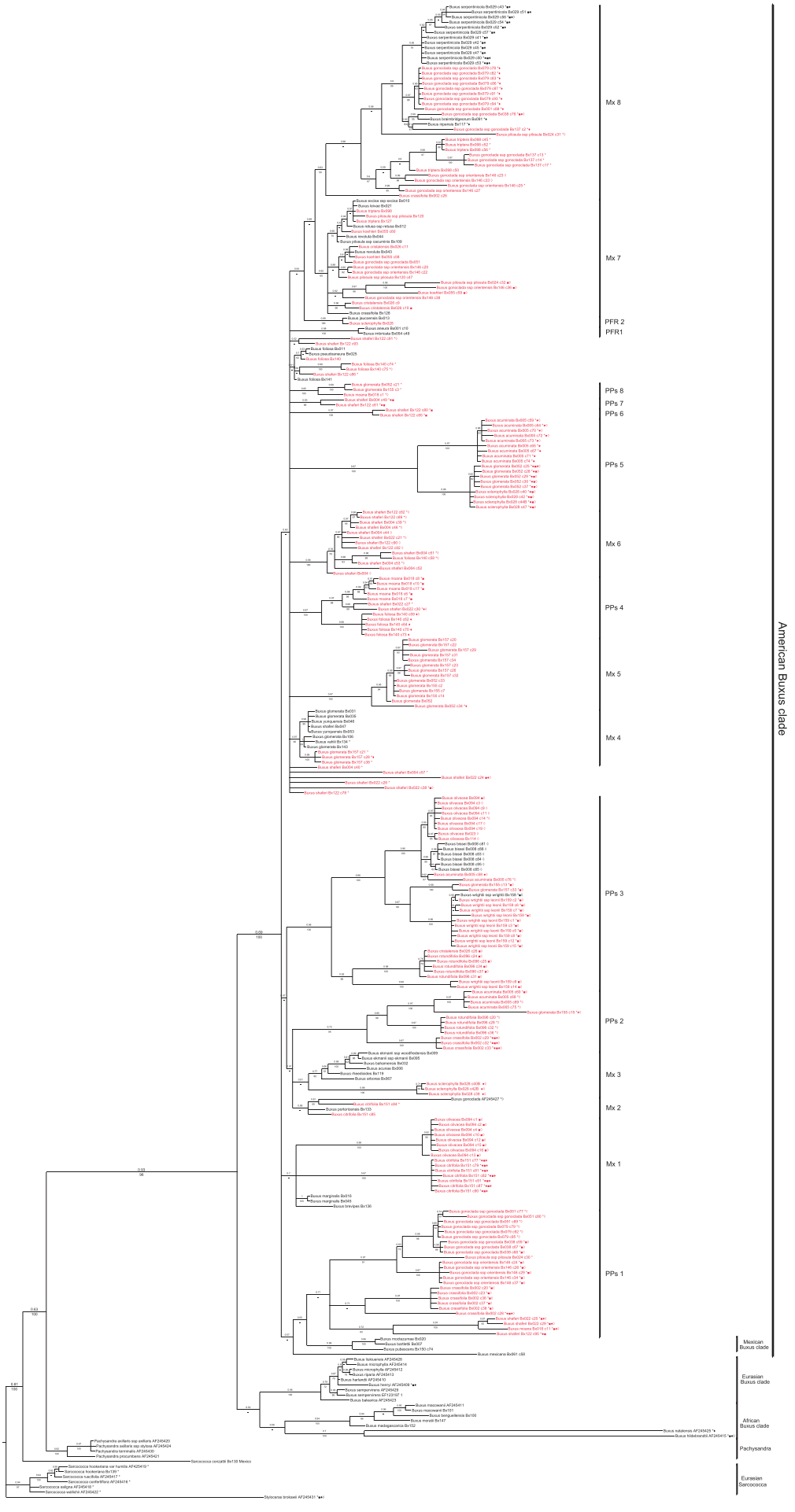
The Mx6 clade (0.96 PP / 100% JK) consists mostly of putative pseudogenic ribotypes with one or two mutated motifs in ITS1 and 5.8 S, of *B. shaferi* (Bx004, Bx022, Bx122) and *B. foliosa* (Bx140-c58), and also a putative functional ribotype of *B. shaferi* (Bx004-c52) [Fig.4.2].

The Mx7 clade (0.92 PP / 60% JK) depicts four subclades: i) a putative pseudogenic ribotype (*B. cristalensis* Bx026-c10) strongly related to a putative

functional ribotype of the *B. cristalensis* (Bx026-c9) [0.96 PP / 96% JK], ii) four putative pseudogenic ribotypes of *B. koehleri* (Bx055-c59), *B. gonoclada* ssp. *orientensis* (Bx146-c36, -c38) and *B. pilosula* ssp. *pilosula* (Bx024-c32) [0.92 PP / <50% JK], iii) putative functional ribotypes of five taxa; *B. cristalensis* (Bx026-c11), *B. revoluta* (Bx043), *B. koehleri* (Bx055-c58), *B. gonoclada* ssp. *gonoclada* (Bx051), *B. gonoclada* ssp. *orientensis* (Bx146-c20, -c22) and *B. pilosula* ssp. *pilosula* (Bx120-c47) [0.89 PP / <50% JK], iv) putative functional ribotypes of eight taxa; *B. excisa* ssp. *excisa* (Bx010), *B. leivae* (Bx021), *B. triptera* (Bx098, Bx127), *B. pilosula* ssp. *pilosula* (Bx120), *B. retusa* ssp. *retusa* (Bx012), *B. koehleri* (Bx055-c60), *B. revoluta* (Bx044) and *B. pilosula* ssp. *cacuminis* (Bx109) [0.95 PP / 75% JK]. Finally, the putative functional ribotype of *B. crassifolia* (Bx128) is also included to this clade but not resolved in any of the subclades (Fig. 4.2).

The Mx8 clade shows mostly putative pseudogenic ribotypes with one to four mutated motifs of ITS1 and in 5.8S, and three sequences of putative functional ribotypes (0.83 PP / 50% JK).The sequence of a putative functional ribotype of *B. crassifolia* (Bx002-c26) is placed an external position sister of the remaining sequences. The second sequence of a putative functional ribotype of *B. gonoclada* ssp. *orientensis* (Bx146-c27) is related to a putative pseudogenic ribotype of the same taxon (*B. gonoclada* ssp. *orientensis* Bx146-c25) [0.99 PP / 95% JK]. The third sequence of a putative functional ribotype, *B. triptera* (Bx098-c50) is related to the putative pseudogenic ribotype of the same taxon *B. triptera* (Bx098-c45, -c52, -c56) and to the putative pseudogenic ribotype of *B. gonoclada* ssp. *gonoclada* (Bx137-c13,-c14, -c17) [0.6 PP / <50% JK; Fig. 4.2]. All clones generated from *B. serpetinicola* (Bx029), which are putative pseudogenic ribotypes, form a monophyletic group (0.95 PP / 78% JK; Fig. 4.2). The remaining putative pseudogenic ribotypes included into this clade are: i) a subclade of *B. gonoclada* ssp. *gonoclada* (nine clones of Bx079, and one of Bx051) [0.97 PP / 98 % JK], ii) a subclade of *B. gonoclada* ssp. *gonoclada* (Bx038-c76, 137-c2), *B. braimbridgeorum* (Bx091) and *B. nipensis* (Bx117) [Fig. 4.2].

Fig. 4.2. Bayesian majority rule tree based on the 293 accessions of ITS data set including coded indels. Bayesian posterior probabilities (PP) are shown above branches and Jackknife values (JK) below branches. Species names in red represent the clones from the same sample which ones are non-monophyletic. Species names tagged with symbols (*●■♦◊) denote sequences of putative pseudogenic ribotypes and each symbol represents that a specific motif is not conserved: *- the motif of Liu & Schardl (1994) in ITS1 is not conserved, ●- the motif of Liston & al. (1996) is not conserved, ■- the motif of Jobes & Thien (1997) is not conserved, ♦- the motif I of Harpke & Peterson (2008) is not conserved, ◊- the motif II of Harpke & Peterson (2008) is not conserved **(next page).**


Mx 8
Mx 7
PFR 2
PFR1
PPs 8
PPs 7
PPs 6
PPs 5
Mx 6
PPs 4
Mx 5
Mx 4
PPs 3
PPs 2
Mx 3
Mx 2
Mx 1
PPs 1
American Buxus clade
Mexican Buxus clade
Eurasian Buxus clade
African Buxus clade
Pachysandra
Eurasian Sarcococca

Clades comprising putative functional ribotypes (PFR clades)

The PFR1 clade shows two sequences of *B. aneura* (Bx001-c10) and *B. imbricata* (Bx054-c49) [0.98 PP / 100% JK; Fig. 4.2].

The PFR2 clade is composed of two sequences of *B. jaucoensis* (Bx013) and *B. sclerophylla* (Bx028) [0.99 PP / 100% JK; Fig. 4.2].

4.4 Discussion

This study complements the previous plastid analysis (Chapter 2) with data from the nuclear genomic partition. Gene trees do not necessarily show the real picture of a species tree (Doyle 1992), but are broadly used in order to detect phylogenetic relationships and also reticulation patters (Löhne & al. 2008; Fuentes-Bazan & al. 2012). In this study the comparison of trees generated from plastid and nuclear markers showed that *Buxus* turns out as a genus with partially reticulate patterns at species level, but also unravels a more complicated picture of ITS evolution due to the detection of putative pseudogenic and putative functional ribotypes.

4.4.1 Degeneration of functional ITS sequences and mutations indicating non-functionality

The relics of former genes that no longer possess biological functions are known as pseudogenes. Such putative nonfunctional copies came usually from duplicate genes generated either by DNA or RNA mediated duplication. The pseudogene birth and loss rates vary significantly across species (Podlaha & Zhang 2010). Moreover, genes that have many copies in the genome tend to generate pseudogenes, as has been well

documented for ITS in the angiosperms (Mayol & Roselló 2001; Bailey & al. 2003; Bayly & Ladiges 2007; Roselló & al. 2007).

Pseudogenization is the process by which a functional gene becomes a pseudogene and usually occurs in the first few million years after duplication if the duplicated gene is not under any selection (Zhang 2003). Pseudogenes are characterized by the gradual accumulation of degenerative mutations, especially in conserved motifs of the gene. They lead to the loss of gene function together with changes in the size of the gene and decrease of the GC content (Mayol & Roselló 2001; Roselló & al. 2007). In the ITS region of plants conserved motifs have been found in the different partitions which are useful to detect putative functional and pseudogenic ribotypes (see material and methods).

The size of normal ITS sequences in angiosperms is usually <700 nt (Baldwin & al. 1992). In the case of *Buxus,* differences in size were used to differentiate functional sequences from putative pseudogenic ribotypes (Roselló & al. 2007). Only one sequence analysed here (*B. olivacea* (Bx094-c6) was excessively large. Considering this and the mutations in the conserved motifs and the low GC content, it was classified as a putative pseudogenic ribotype (Appendix 4.2). The size of all other sequences analysed here are in the normal range of ITS sequences reported for angiosperms (Baldwin & al. 1992).

The 5.8S partition of ITS is 160 nt long in 92% of the analysed sequences, a similar result was found by Roselló & al. (2007) in paralogues of the Eurasian species *B. balearica* and *B. sempervirens* (Appendix 4.2). In this study it is also detected that the cloned copies of *B. bissei* (Bx008) have a shorter 5.8S fragment (157 nt) and all were classified as putative pseudogenic ribotypes because of the presence of two mutations in the motif II of this partition (Harpke & Peterson 2008, Appendix 4.2).

Another result of this study is that conserved motif of ITS1 (Liu & Schardl 1994) has 21 nt because the variable intern fragment has 5 nt instead 4–7 nt. This is consistent with the results of Roselló & al. (2007) and considering the dense sampling of *Buxaceae* analysed in this study it seems to be a stable characteristic of this family.

Regarding the mutations in all five motifs analysed in this study, it is interesting that some specific mutations are shared by sequences generated from different samples and taxa. In the case of mutations shared by species which are distantly related, these are to be considered as convergences, as in the case of species of *Sarcococca* and Cuban *Buxus* which share the same mutation in the motif of Liu & Schardl (1994) in ITS1 (Appendix 4.3) or the case of the Eurasian species *B. henryi* and two Cuban species of *Buxus* (*B. glomerata* and *B. shaferi*) which share the same mutation in the motif I of Harpke & Peterson (2008) in 5.8S (Appendix 4.6).

The GC content reported by Roselló & al. (2007) for ITS sequences of presumed functional ribotypes of Eurasian species of *Buxus* is in the range of 66 to 66.8%. This is the only published information about the GC content in ITS sequences of *Buxus* and thus the only reference for comparative analyses. Of the c. 300 sequences analysed here, only c. 10% have a GC content in the range reported by Roselló & al. (2007). The GC content of the ITS sequences of other Eurasian species such as *B. harlandii*, *B. liukiuensis* and *B. riparia* generated by Bathazar & al. (2000) are in this range or only slightly higher, in line with the results of Roselló & al. (2007) and indicating that a GC content of 66–68% may be the normal range in Eurasian species of *Buxus*. Among the ITS sequences of Eurasian species of *Buxus* only the GC content of the sequence of *B. henryi* is slightly lower (64.9%). This sequence was considered a putative pseudogene by Roselló & al. (2007) and ratified here as such considering other characteristics (Appendix 4.2).

Regarding the range of the GC content, more variability was found among the African and American species. This is evident even if only sequences of putative functional ribotypes are compared. As exposed in the results the range of the GC content in the sequences of the putative functional ribotypes of *Buxus* from Africa is c. 61–66% and 63–72% in the American species (Appendix 4.2). These results show that the GC content in putative functional ribotypes is less variable in the Eurasian species (66–68%); nevertheless the number of species studied from this continent is still insufficient.

Commonly the sequences of clones with the highest number of mutated motifs in ITS1 and 5.8S (4–5 mutated motifs) and with more mutated positions within each motif have the lowest GC content compared with sequences of putative functional ribotypes of

the same sample. This is for example the case among the putative pseudogenic ribotypes of *B. citrifolia* (Bx151-c81, -c82) and its putative functional ribotype (Bx151-c85) [Appendix 4.2]. Thus a good indicator of putative pseudogenic ribotype sequences could be the presence of one or more mutated motifs in ITS1 and 5.8S, associated with lower GC content.

It is noteworthy that 71% of ITS sequences generated in this study are considered putative pseudogenic ribotypes. Moreover, stepwise examples of pseudogenization could be shown by the phylogenetic relationships between putative functional and putative pseudogenic ribotypes from the same sample. In this sense, illustrative examples are: i) *B. cristalensis* (Bx026-c9, -c10) [Fig. 4.2, clade Mx7]; ii) *B. gonoclada* ssp. *orientensis* (Bx146-c25, -c27) [Fig. 4.2, clade Mx8]; iii) *B. triptera* (Bx098-c50, -c45, -c52, -c56) [Fig. 4.2, clade Mx8] and iv) *B. crassifolia* (Bx002) with three (c20, c23, c36, c37 and c38) and five (c28) mutated motifs (Fig. 4.2, clade PPs1). Additionally, it appears that a decreasing size of the 5.8S gene, a decreasing GC content in connection with a higher number of mutated motifs could represent putative pseudogenic ribotypes in different stages of pseudogenization.

The within-individual polymorphism found in several samples of *Buxus* points out that the concerted evolution has acted slowly or has not been fully operational on most of copies of ITS (Bayly & Ladiges 2007). Roselló & al. (2007), after finding within-intraindividual polymorphism in *B. balearica* and *B. sempervirens* stated that there is no reason to suggest that this phenomenon would be restricted to these two species in *Buxus* and with the results achieved in this study such statement is corroborated. These authors also mention that since *B. balearica* and *B. sempervirens* have a relatively long generation time, this could imply a detectable generation-time effect that could slow down rates of concerted evolution. This explanation would also fit to the Cuban and Caribbean species of *Buxus* where deviating paralogues appear to originate from different ancestors [e.g. deviating paralogues of *B. crassifolia* (Bx002) placed in clades PPs1 and PPs2, deviating paralogues of *B. gonoclada* ssp. *gonoclada* (Bx079) and B. *gonoclada* ssp. *orientensis* (Bx146) placed in clades PPs1 and Mx8; Fig. 4.2].

Direct sequencing of some PCR products showed two problems: i) totally illegible pherograms and ii) editable pherograms but with polymorphic sites. Both problems could be caused by the presence of overlapping copies of ITS. The current ITS data set also contains seuquences obtained from direct sequencing of PCR products [e.g. *B. brevipes* (Bx136) and *B. bahamensis* (Bx032)]. The paralogue diversity of the samples included in this study may be still underestimated due to PCR bias.

4.4.2 Phylogenetic relationships among putative functional and putative pseudogenic ribotypes

There are controversial opinions about the inclusion of putative pseudogenic ribotypes in phylogenetic studies. Some authors stated that their phylogenetic signal can distort the reconstruction of the tree (Mayol & Roselló 2001). Others pointed out that divergent putative pseudogenes can be useful in phylogenetic analyses, especially when no sequences of their functional counterparts are available Razafimandimbison & al. (2004).

In this study it has been detected i) putative pseudogenic ribotypes close related to a sister clade of putative functional ribotypes generated from the same sample or from other samples of the same species (Fig. 4.2, clade Mx5) and ii) putative functional ribotypes related to a sister clade of putative pseudogenic ribotypes (see examples of stepwise pseudogenization above). These relationships show pseudogenization events.

On the other hand it was also detected that putative pseudogenic ribotypes generated from the same sample appear inconsistent in different positions that are not immediately resolving clades of functional copies sister to clades of the respective pseudogenic copies. This seems to be the case in *B. shaferi* (Bx004 and Bx122), which sequences are included in the clade Mx6 or in polytomies along the tree (Fig. 4.2). Such inconsistent placements could be caused by saturation at certain sites in the degenerated copies that lead to the loss of hierarchical phylogenetic signal (Moreira & Philippe 2000) or also to long branch attraction effects.

4.4.3 Reticulate patterns in the evolution of the Caribbean species of *Buxus*

The incongruent positions of taxa in the plastid versus ITS phylogenies can be signals of reticulate evolution (ancient hybridization). The analyses performed in this study showed this pattern in several species: *B. glomerata* (Bx31, Bx35, Bx052, Bx106, Bx143), *B. sclerophylla* (Bx028), *B. leivae* (Bx021), *B. gonoclada* ssp. *gonoclada* (Bx051, Bx079), *B. gonoclada* ssp. *orientensis* (Bx146), *B. olivacea* (Bx023, Bx094), *B. vahlii* (Bx134) [Figs. 4.1 and 4.2]. The divergent copies of species belonging to *B. gonoclada* ssp. *gonoclada* (Bx051, Bx079) and to *B. gonoclada* ssp. *orientensis* (Bx146) show this pattern of relationships in the clades PPs1 and Mx8 and in the clades PPs1, Mx7 and Mx8, respectively (Fig. 4.2). Similar relationships are evident in other taxa such as *B. acuminata* (Bx005) in the clades PPs2 and PPs5, *B. olivacea* (Bx094) in the clades Mx1 and PPs3 and *B. crassifolia* (Bx002) in the clades PPs1 and PPs2 (Fig. 4.2). These patterns could suggest that probably the origin of these taxa has been driven by ancestral hybridization. The ILD test also indicated significant incongruence between plastid and ITS data sets.

A noteworthy characteristic of sequences generated through cloning regardless of its putative functional status is that only two samples (*B. bissei*, Bx008; *B. serpentinicola* Bx029) possess ITS ribotypes that exclusively are resolved as monophylum. Thus, reticulate evolution could be the prevalent mechanism in speciation of neotropical *Buxus*. The reticulate evolution could be even a mechanism of speciation in species which were found to be monophyletic such as *B. bissei* and *B. serpentinicola* considering that in these cases ITS copies could be already homogenized (Wendel & al. 1995) or were not picked up by PCR.

Papers focusing on incongruences between plastid and nuclear phylogenies (e.g.: Stefanovic & Costea 2008; Martín-Bravo & al. 2010; Pelser & al. 2010; Fuentes-Bazan & al. 2012), suggest that the different positions of some taxa in the plastid and ITS trees could be caused by phenomena such as incomplete lineage sorting (ILS) or hybridization. Topological incongruence caused by hybridization and ILS can be

difficult to distinguish, because both phenomena may result in similar topological differences (Doyle 1992; Pelser & al. 2010).

The divergence ages of the Cuban and other Caribbean species of *Buxus* have been estimated (Fig. 2.5 and Table 2.2 of chapter 2). The ancestor of the Cuban species of *Buxus* diverged c. 12.3 million years ago (Mya) into two lineages, one of them enclosing a single species, *B. jaucoensis* (Bx013), which is sister of all other Cuban and Caribbean *Buxus* taxa (Fig. 2.1 of chapter 2). All other Cuban and Caribbean taxa are enclosed in three internal clades, the "Glomerata"-clade, "Shaferi"-clade and "Gonoclada"-clade, which started to diverge 5.3 Mya, 4.8 Mya and 3.2 Mya, respectively (Fig. 2.5 and Table 2.2 of chapter 2). Most of the taxa that are involved in the reticulation pattern in the Cuban *Buxus* (Fig. 4.1 and 4.2) are included in these three rather young clades, e.g. *B. glomerata*, *B. leivae*, *B. vahlii* and *B. sclerophylla*.

In this study *B. glomerata*, the most widespread species of *Buxus* in Cuba and the Caribbean, is represented by several accessions which show different reticulations patterns probably caused by hybridizations with other species of *Buxus* occurring along its distribution range. Such an explanation could also be in line with the observation of the non-monophyly of the different *B. glomerata* accessions in a previous phylogeny based on plastid markers. In the plastid trees *B. glomerata* accessions appeared either included in a polytomy, related to *B. bahamensis* (Bx032, Bx033, Bx034) or to *B. sclerophylla* (Bx028)[Fig. 2.1 of chapter 2]. In the ITS tree sequences of *B. glomerata* were found related to other taxa in five clades: i) *B. glomerata* (Bx155-c15) related with *B. acuminata* (Bx005-c60, -c68, -c69, -c75) in the clade PPs2, ii) *B. glomerata* (Bx155-c13, Bx157-c33) related with *B. wrightii* ssp. *wrightii* (Bx158) and *B. wrightii* ssp. *leonii* (Bx159, several clones) in the clade PPs3, iii) *B. glomerata* (Bx031, Bx035) with *B. shaferi* (Bx047) and *B. yunquensis* (Bx046, Bx053) in the clade Mx4, iv) *B. glomerata* (Bx052) with *B. sclerophylla* (Bx028) in the clade PPs5, and v) *B. glomerata* (Bx052-c21, Bx155-c3) with *B. moana* (Bx018-c1) in the clade PPs8 (Fig. 4.2). In the particular case of the hybridization which involves *B. sclerophylla* (Bx028) with its putative parental lines *B. glomerata* (Bx052) and *B. jaucoensis* (Bx013) convincing evidences were found. A first evidence was detected in a previous phylogenetic reconstruction based on plastid markers, where samples of *B. glomerata* (Bx052, Bx157) were strongly

related to *B. sclerophylla* (Bx028) [Fig. 2.1 of chapter 2]. Second evidence is that in the ITS trees (Fig. 4.1-B and 4.2) *B. jaucoensis* (Bx013) and *B. sclerophylla* (Bx028) share identic ITS sequences and are close related with strong support. This relationship is quite noteworthy because in the plastid phylogeny, *B. jaucoensis* is placed alone being sister of other Cuban and Caribbean *Buxus* (Fig. 2.1 of chapter 2). Third evidence is that a strong phylogenetic relationship was detected among sequences of putative pseudogenic ribotypes of *B. glomerata* (Bx052) and *B. sclerophylla* (Bx028) (PPs5 clade; Fig. 4.2). The putative pseudogenic ribotypes of these samples even share identical mutations in four motifs of ITS1 (1) and 5.8S (3) [Appendices 4.3, 4.4, 4.5, 4.7].

Currently the populations represented by these three species are distributed in the southeastern part of Cuba, in the current province of Guantánamo. *B. sclerophylla* is exclusive from an area between Abra de Mariana and Los Ciguatos. In this locality there is also a population of *B. glomerata*, where the sample Bx052 was collected. *B. jaucoensis* is endemic in the locality of Jauco, which is located c. 70 Km east from Abra de Mariana-Los Ciguatos (Fig. 4.3). The distribution areas of these species could have been closer to each other or even overlapped in the past making hybridization possible. Considering the argumentation above, *B. sclerophylla* (Bx028) could be a species of hybrid origin, with the parents being ancestors of *B. glomerata* (Bx052) and *B. jaucoensis* (Bx013). In this case, *B. glomerata* would represent the female parent, due to the affinities of this taxon with *B. sclerophylla* in the plastid tree (Fig. 2.1 of chapter 2), and *B. jaucoensis* the male parent. Pollen could have been transported by the wind (Lázaro & Traveset 2005; Lázaro & Traveset 2006; Rosselló & al. 2007) or insects (Fig. 1.4 of chapter 1). *B. jaucoensis, B. glomerata* and *B. sclerophylla* are morphologically well defined species and so far there has not been any reference about a possible hybridization, based on intermediate morphological characters between these or any other species of Cuban *Buxus* (Köhler 2014).

Other possible causes associated to the reticulation patterns detected in *B. glomerata* is that may be some specimens identified under this name, that in fact belong to other species. These would then be cryptic species, which cannot be differentiated from one another through the usually used morphological characteristics. Ongoing

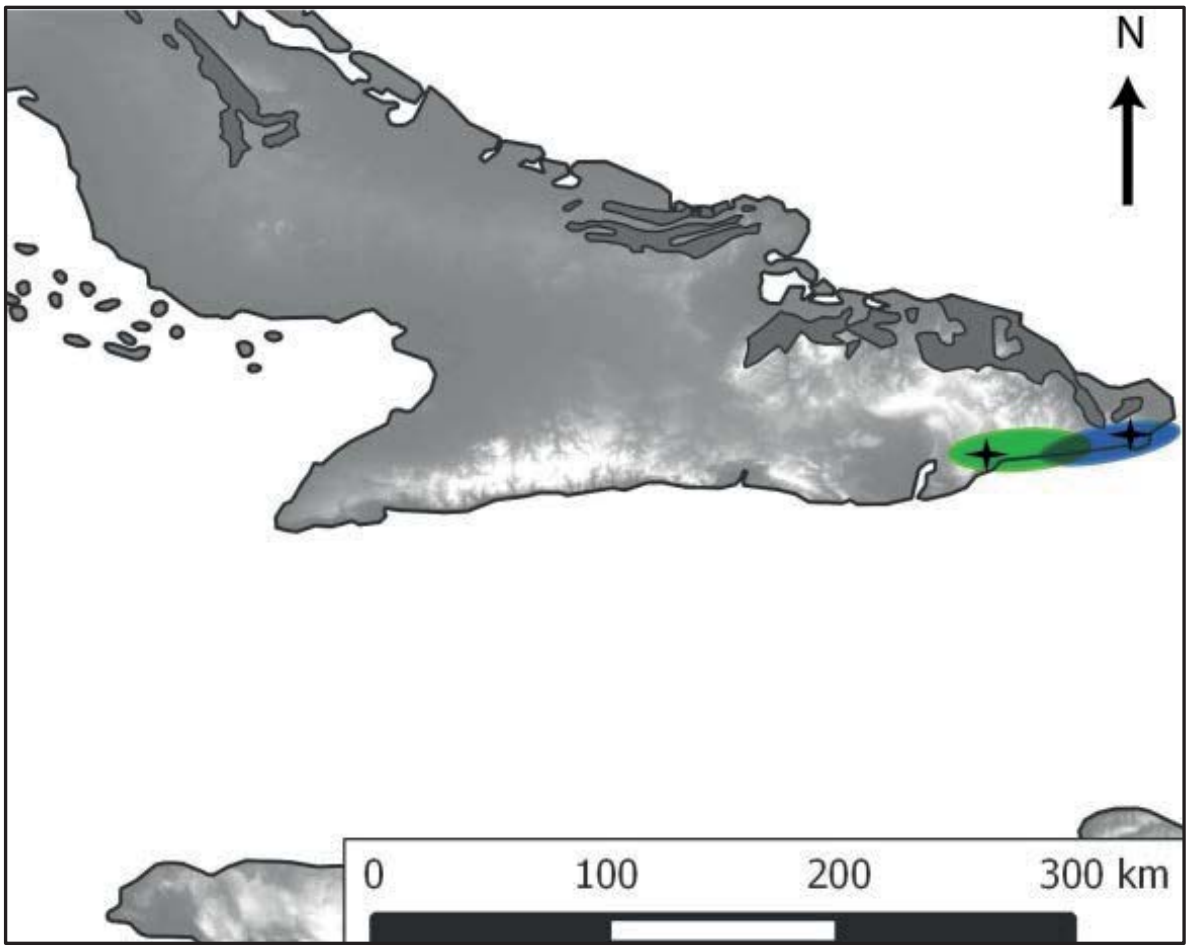


Fig. 4.3 Map of eastern Cuba showing the distribution of *Buxus jaucoensis* in Jauco (cross within the blue oval).and *B. glomerata* and *B. sclerophylla* in Abra de Mariana–Los Ciguatos (cross within the green oval). The ovals represent the hypothetical ancestral distribution of both species where the hybridization could happen.

palynological studies (Egon Köhler, personal communication) showed that some Hispaniolan specimens identified as *B. glomerata* have a pollen morphology distinct from the Cuban specimens of this species.

Other examples of evident incongruences suggesting possible hybridization are: i) *B. leivae* (Bx021), which in the plastid phylogeny is enclosed in the "Shaferi"-clade (Fig. 2.1 of chapter 2), but its ITS sequence is related to species of the "Glomerata"-clade (Fig. 4.1; Fig. 4.2 clade Mx7), and ii) *B. olivacea* (Bx023, Bx094, Bx114), *B. acuminata* (Bx005) and *B. bissei* (Bx008) [Fig. 4.2 clade PPs3]. In this second case the position of *B. olivacea* (Bx023) in the plastid phylogeny probably shows an event of chloroplast capture (Fig. 2.1 of chapter 2).

The hybridization of *Buxus* has also been documented in cultivated plants (Laere & al. 2011) but no reports about the occurrence of natural hybrids are so far published. The evidence of hybridization in Cuban *Buxus* probably is the first case of natural hybridization documented by molecular methods for the flora of Cuba. All hybrids cited so far for Cuba are only based on intermediary morphology between putative parents and often just regard to recent hybridization rather than ancient hybridization that led to the evolution of fertile species. León & Alain (1951) reported a hybrid in the genus *Coccoloba* (*C. uvifera* × *C. diversifolia*) based on leaf morphology. Granda & Fuentes (1985) documented a hybrid for the genus *Rauvolfia* (*Apocynaceae*) based on morphometric studies. The most recent reports of hybrids for Cuba are ferns, which have been identified by detecting of abortive spores (Caluff & Shelton 2003, Sánchez & Regalado 2003, Sánchez & al. 2006).

4.4.4 Overall phylogenetic relationships of *Buxus* – evidence from nuclear and plastid genomic compartments

Through both phylogenetic reconstructions, based on plastid markers and on the nuclear ITS, three major clades of *Buxus* were recovered, African clade, American clade and Eurasian clade. The phylogenetic reconstruction based on plastid markers showed that except *B. brevipes*, all other Cuban and Caribbean species of *Buxus* form a

monophyletic group (Caribbean clade). Within the Caribbean clade it was found that *B. jaucoensis* constitutes a unique linage being sister of the remaining species, which are enclosed in three strong supported intern clades: "Glomerata"-clade, "Gonoclada"-clade and "Shaferi"-clade. In spite of some inconsistences, incongruences and the low support of some clades, the phylogeny based on ITS largely recovered the major clades detected in the plastid phylogeny. In this sense, the clades PPs1, Mx7 and Mx8 partially recover the "Gonoclada"-clade, the clades PPs2, PPs3, PPs5, Mx1 and Mx5 partially recover the "Glomerata"-clade and the clades PPs4, PPs6, PPs7, Mx6 and PFR1 partially recover the "Shaferi"-clade (Fig. 2.1 of chapter 2; Fig. 4.2).

4.4.5 Diversity of co-amplified fungal sequences

A difficulty found during this study was that not all the sequences generated from clones matched with ITS sequences of *Buxus*, but to genera of fungi. These genera of fungi probably grow associated as ectophytes or endophytes to the species of *Buxus* studied here since they have been reported to Cuba (Dr. Mayra Camino Vilaró, specialist in Cuban fungi, personal communication). This difficulty is mostly related to the use of universal primers (Álvarez & Wendel 2003; Razafimandimbison & al. 2004; Nieto-Feliner & Roselló 2007; Jestrow & al. 2010). This fact points how important is the selection and preparation of the samples in the field.

4.5 Conclusions and future work

The reticulate evolution and specifically the hybridization seem to have contributed to the species diversity of *Buxus* in Cuba and the Caribbean. The no monophyly showed by several taxa in the plastid and ITS phylogenies could be mostly caused by reticulate evolution. Nevertheless, other possible causes such as the occurrence of cryptic species should be explore in further work focused on this group of Caribbean plants.

A wider population sampling of the species involved in the detected reticulation patterns (e.g. *B. jaucoensis*, *B. sclerophylla*, *B. leivae*) is necessary in order to support the hypotheses suggesting the occurrence of hybridization.

The diversity of ITS copies of some taxa included in this study has been probably underestimated because i) they were not cloned and ii) due to the use of universal primers and standard PCR conditions. Thus, it could be presumed that other reticulations patterns associated to hybridization were probably not detected in this study. Further ITS studies on Cuban and Caribbean *Buxus* should consider the design of specific ITS primers for *Buxus* and cloning of all samples independently of the quality of the sequences obtained from direct PCR products. This suggestion would allow the PCR to pick up and amplify other copies (divergent paralogues). Additionally will be necessary to consider the use of other nuclear regions such as “EST, LFY” to confirm the pattern obtained with ITS.

Appendix 4.1. Taxa, samples codes, herbarium vouchers, localities, GenBank accessions codes. HFC- series Herbarium Florae Cubensis.

Species name	Project code	Herbarium voucher	Locality	ITS
Buxus acuminata (Griseb.) Müll. Arg.	Bx 005	R. Berazaín & al. HFC 71535 (HAJB)	Cuba: Guantánamo, Baracoa, Yunque de Baracoa.	this study
Buxus acunae Borhidi & O. Muñiz	Bx 006	T. Borsch & al. 4260 (B, HAJB, ULV)	Cuba: Holguín, Moa, Yamanigüey.	this study
Buxus aneura Urb.	Bx 001	J. Gutiérrez & al. HFC 78357 (B, HAJB)	Cuba: Holguín, Mayarí, Rosa Castillo.	this study
Buxus arborea Proctor	Bx 067	E. Köhler 85 (B)	Jamaica: Parish of Trelawny, Island View Hill.	this study
Buxus bahamensis Baker	Bx 032	P. A. González HFC 85861 (B, HAJB)	Cuba: Holguín, Gibara, Los Cañones.	this study
Buxus balearica Lam.		v. Balthazar 98030 (Z)	not available	AF245423
Buxus bartlettii Standl.	Bx 007	E. Köhler 75 (B)	Mexico: Oaxaca, Chiltepec, carretera Chiltepec-Tuxtepec.	this study
Buxus benguellensis Gilg	Bx 100	J. Gutiérrez & Maiato 85996 (HAJB)	Angola, Lubango.	this study
Buxus bissei Eg. Köhler	Bx 008	J. Gutiérrez & al. HFC 77565-A (HAJB)	Cuba: Holguín, Moa, camino entre Alto de La Calinga y Revuelta de Los Chinos.	this study
Buxus braimbridgeorum Eg. Köhler	Bx 091	J. Gutiérrez & al. HFC 83347 (B, HAJB)	Cuba: Holguín, Moa, Ascenso al Toldo.	this study
Buxus brevipes (Müll. Arg.) Urb.	Bx 136	R. Rankin HFC 87054 (B, HAJB)	Cuba: cultivated in Botanical Garden of Havana from P. del Río, río San Juán.	this study
Buxus citrifolia Spreng.	Bx 151	C. Galdames 6848 (B)	Panamá: Distrito de Panamá, Cerro Azul. Alt. 750 m.	this study
Buxus crassifolia (Britton) Urb.	Bx 002	R. Berazaín & al. HFC 74056 (B, HAJB)	Cuba: Holguín, Moa, río Limones.	this study
Buxus crassifolia (Britton) Urb.	Bx 128	M. Ackermann & al. 1001 (B, HAJB, ULV)	Cuba: Guantánamo, Baracoa, Santa María, Alto de Iberia.	this study
Buxus cristalensis Eg. Köhler & P. A. González	Bx 026	J. Gutiérrez & al. HFC 75386 (HAJB)	Cuba: Santiago de Cuba, Segundo Frente, en el camino entre El Halcón y Batista.	this study
Buxus ekmanii Urb. subsp. *ekmanii*	Bx 095	J. Gutiérrez & al. HFC 79695 (HAJB)	Cuba: Guantánamo, Baracoa, Yunque de Baracoa.	this study
Buxus ekmanii subsp. *woodfredensis* Eg. Köhler	Bx 009	J. Gutiérrez & al. HFC 75432 (B, HAJB)	Cuba: Holguín, Mayarí, S. Cristal, Mandinga, Arroyo Claro, al sur de Mina J. Martí.	this study
Buxus excisa Urb. subsp. *excisa*	Bx 010	J. Gutiérrez & al. HFC 77577 (HAJB)	Cuba: Holguín, Moa, Alto de La Calinga.	this study
Buxus foliosa Urb.	Bx 011	R. Berazaín & al. HFC 74072 (HAJB)	Cuba: Holguín, Moa, camino a La Melba, alrededores del antiguo aserrío.	this study
Buxus foliosa Urb.	Bx 140	T. Borsch & al. 4315 (B, HAJB, ULV)	Cuba: Holguín, Moa, Entre los kilometros 22 y 26 del camino a La Melba.	this study
Buxus foliosa Urb.	Bx 141	M. Ackermann & al. 955 (B, HAJB, ULV)	Cuba: Holguín, Moa, Entre el km 26 km y las Comadres, camino a La Melba.	this study
Buxus glomerata (Griseb.) Müll. Arg.	Bx 031	P. A. González HFC 85860 (B, HAJB)	Cuba: Holguín, Gibara, Floro Pérez, cerro de San Marcos.	this study
Buxus glomerata (Griseb.) Müll. Arg.	Bx 035	P. A. González HFC 85864 (B, HAJB)	Cuba: Holguín, Rafael Freire, Bahía de Naranjo, serpentines.	this study
Buxus glomerata (Griseb.) Müll. Arg.	Bx 052	J. Gutiérrez & al. HFC 83461 (B, HAJB)	Cuba: Guantánamo, Los Ciguatos.	this study
Buxus glomerata (Griseb.) Müll. Arg.	Bx 143	R. García & al. 4839 (B)	Dominican Republic: Sierra de Bahoruco, Barahona.	this study
Buxus glomerata (Griseb.) Müll. Arg.	Bx 155	P. A. González 1108-2 HFC 87215 (B, HAJB)	Cienfuegos, Jagua	this study
Buxus glomerata (Griseb.) Müll. Arg.	Bx 157	R. Berazaín & al. HFC 72281 (HAJB)	Cuba: Guantánamo, Los Ciguatos.	this study
Buxus glomerata (Griseb.) Müll. Arg.	Bx 106	A. H. Liogier 16559 (NY)	Dominican Republic: Arroyo Francés, 4 miles W of Puerto Plata, serpentine.	this study
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*		E. Köhler & al. HFC 77434 (B, HAJB)	Mayabeque, Santa Cruz del Norte. Cuabales de Canasí	AF245427
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*	Bx 137	J. Bisse & al. HFC 40211 (HAJB)	Cuba: Mayabeque, Canasí, Lomas de Galindo.	this study
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*	Bx 051	E. Köhler sn (B)	Cuba: Villa Clara, Santa Clara.	this study
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*	Bx 079	J. Matos sn (B)	Cuba: Villa Clara, Santa Clara.	this study
Buxus gonoclada (Griseb.) Müll. Arg. subsp. *gonoclada*	Bx 038	P. A. González HFC 85867 (B, HAJB)	Cuba: Holguín, Rafael Freire, Bahía de Naranjo, serpentines.	this study
Buxus gonoclada ssp. *orientensis* Eg. Köhler	Bx 146	J. Gutiérrez & al. HFC 80656 (B, HAJB)	Cuba: Guantánamo, Yateras, loma del Mulo.	this study
Buxus harlandii Hance		v. Balthazar 98003 (Z)	not available	AF245410
Buxus henryi Mayr		v. Balthazar 98001 (Z)	not available	AF245409
Buxus hildebrandtii Baill.		v. Balthazar 98011 (Z)	not available	AF245415
Buxus imbricata Urb.	Bx 054	J. Gutiérrez & al. HFC 75300 (HAJB)	Cuba: Holguín, Mayarí, Pico Cristal.	this study
Buxus jaucoensis Eg. Köhler	Bx 013	R. Berazaín & al. HFC 72333 (B, HAJB)	Cuba: Guantánamo, Maisí, Paredones del Río Jauco.	this study

Appendix 4.1. Continued

Species name	Project code	Herbarium voucher	Locality	ITS
Buxus koehleri P. A. González & Borsch	Bx 055	T. Borsch & al. 4091 (B, HAJB, ULV)	Cuba: Holguín, Mayarí, Sierra de Nipe, sendero del río Guayabo.	this study
Buxus leivae Eg. Köhler	Bx_021-b	T. Borsch & al. 4317 (B, HAJB, ULV)	Cuba: Holguín, Moa, entre los kilometros 22 y 26 del camino a La Melba	this study
Buxus liukiuensis Makino		Shih 3727 (NSYSU)	not available	AF245428
Buxus macowanii Oliv.	Bx 101	P. A. González sn (B#100507478)	South Africa (cultivated in Baumschulenweg, Berlin).	this study
Buxus macowanii Oliv.		v. Balthazar 98004 (Z)	not available	AF245411
Buxus madagascarica Baill.	Bx 152	G. McPherson & Rabenantoandro 18322 (MO)	Madagascar	this study
Buxus marginalis (Britton) Urb.	Bx 016	R. Berazaín & al. HFC 73923 (HAJB)	Cuba: Holguín, Moa, km 8 del camino a La Melba.	this study
Buxus marginalis (Britton) Urb.	Bx 045	P. A. González HFC 85877 (B, HAJB)	Cuba: Guantánamo, Baracoa, Santa María.	this study
Buxus mexicana Brandegee	Bx 061	Köhler sn (B)	Mexico	this study
Buxus microphylla Siebold & Zucc.		v. Balthazar 98006 (Z)	not available	AF245412
Buxus microphylla Siebold & Zucc.		v. Balthazar 98009 (Z)	not available	AF245414
Buxus moana Alain	Bx 018	R. Berazaín & al. HFC 74025 (B, HAJB)	Cuba: Holguín, Moa, orillas del río Yagrumaje.	this study
Buxus moctezumae Eg. Köhler, R. Fernández & Zamudio	Bx 020	E. Köhler sn (B)	Mexico	this study
Buxus moratii G. E. Schatz & Lowry	Bx 147	Barthelat 474 (B)	Mayotte: Grande Terre, Chiconi.	this study
Buxus natalensis Oliv.		Abbott 7303 (Z)	not available	AF245425
Buxus nipensis Eg. Köhler & P. A. González	Bx 117	T. Borsch & al. 4164 (B, HAJB, ULV)	Cuba: Holguín, Mayarí, Sierra de Nipe, Cabezadas del río Piloto.	this study
Buxus olivacea Urb.	Bx 023	R. Berazaín & al. HFC 72162 (HAJB)	Cuba: Guantánamo, Yateras, entre Diamante y Montecristo.	this study
Buxus olivacea Urb.	Bx 094	J. Gutiérrez & al. HFC 75433 (B, HAJB)	Cuba: Holguín, Mayarí, suroeste de Mandinga, Arroyo Claro, al sur de Mina J. Martí.	this study
Buxus olivacea Urb.	Bx 114	R. Berazaín & al. HFC 72260 (B, HAJB)	Cuba: Guantánamo, Yateras, charrascos entre el Alto de la Clarita y Montecristo.	this study
Buxus pilosula subsp. *cacuminis* Eg. Köhler	Bx 109	J. Gutiérrez & al. HFC 75299 (B, HAJB)	Cuba, Santiago de Cuba, Segundo Frente, subida y firme del Pico Cristal.	this study
Buxus pilosula Urb. subsp. *pilosula*	Bx 024	J. Gutiérrez & al. HFC 78358 (HAJB)	Cuba: Holguín, Mayarí, Pinares de Mayarí, charrascal La Cueva.	this study
Buxus pilosula Urb. subsp. *pilosula*	Bx 120	T. Borsch & al. 4185 (B, HAJB, ULV)	Cuba: Holguín, Mayarí, Pinares de Mayarí, charrascal La Cueva.	this study
Buxus portoricensis Alain	Bx 133	A. Areces 6873 (NY)	Puerto Rico: Bosque Estatal Guajataca, Calizas.	this study
Buxus pseudaneura Eg. Köhler	Bx 025	J. Gutiérrez & al. HFC 78267 (B, HAJB)	Cuba: Guantánamo, Parque Nacional Alejandro de Humboldt, Cayo Fortuna.	this study
Buxus pubescens Greenm.	Bx 150	F. Chiang & Flores 1131 (IEB)	Mexico: Mexico, Nayarit, isla María Madre, antena de telecomunicaciones.	this study
Buxus retusa Müll. Arg. subsp. *retusa*	Bx 012	J. Gutiérrez & al. HFC 77583 (B, HAJB)	Cuba: Holguín, Moa, camino entre el Alto de La Calinga y subida a El Toldo.	this study
Buxus revoluta (Britton) Mathou	Bx 043	P. A. González HFC 85874 (B, HAJB)	Cuba: Holguín, Moa, al Este de Yamanigüey.	this study
Buxus revoluta (Britton) Mathou	Bx 044	P. A. González HFC 85875 (B, HAJB)	Cuba: Holguín, Moa, al Este de Yamanigüey.	this study
Buxus rheedioides Urb.	Bx 119	Borsch & al. 4166 (B, HAJB, ULV)	Cuba: Holguín, Mayarí, río Piloto.	this study
Buxus riparia Makino		v. Balthazar 98007 (Z)	not available	AF245413
Buxus rotundifolia (Britton) Mathou	Bx 096	J. Gutiérrez & al. HFC 83327 (B, HAJB)	Cuba: Holguín, Moa, ascenso al Toldo.	this study
Buxus sclerophylla Eg. Köhler	Bx 028	R. Berazaín & al. HFC 72282 (HAJB)	Cuba: Guantánamo, San Antonio del Sur, Los Ciguatos-Abra de Mariana.	this study
Buxus sempervirens L.		v. Balthazar 99005 (Z)	not available	AF245429
Buxus serpentinicola Eg. Köhler	Bx 029	R. Berazaín & al. HFC 72288 (HAJB)	Cuba, Guantánamo, Maisí, Peladero de Jauco.	this study
Buxus shaferi Urb.	Bx 122	T. Borsch & al. 4319 (B, HAJB, ULV)	Cuba: Holguín, Moa, entre los kilometros 22 y 26 del camino a La Melba	this study
Buxus shaferi Urb.	Bx 004	J. Gutiérrez & al. HFC 75297 (B, HAJB)	Cuba: Santiago de Cuba, Segundo Frente, subida y firme del Pico Cristal.	this study
Buxus shaferi Urb.	Bx 022	J. Gutiérrez & al. HFC 77598 (B, HAJB)	Cuba: Guantánamo, Baracoa, orillas del río Báez, km. 7 del camino a Mina Amores.	this study
Buxus shaferi Urb.	Bx 047	P. A. González HFC 85879 (B, HAJB)	Cuba: Guantánamo, Baracoa, Alto de Iberia.	this study
Buxus triptera Eg. Köhler	Bx 098	J. Gutiérrez & al. HFC 83396 (B, HAJB)	Cuba: Holguín, Moa, km 26 del camino a la Melba.	this study

Appendix 4.1. Continued

Species name	Project code	Herbarium voucher	Locality	ITS
Buxus triptera Eg. Köhler	Bx 127	M. Ackermann & al. 956 (B, HAJB, ULV)	Cuba: Holguín, Moa, Entre el km 26 km y las Comadres, en el camino a La Melba.	this study
Buxus vahlii Baill.	Bx 134	A. Areces 6874 (NY)	Puerto Rico: Mogote del Parque de Las Ciencias, Bayamón, San Juán.	this study
Buxus wrightii subsp. *leonii* (Britton) Eg. Köhler	Bx_159	A. Álvarez de Zayas & al. HFC 43556 (HAJB)	Cuba: Cultivated in Botanic Garden of Havana from Pinar del Río, Las Pozas.	this study
Buxus wrightii Müll. Arg. subsp. *wrightii*	Bx_158	P. A. González & R. Rankin HFC 86142 (HAJB)	Cuba: cultivated in Botanical Garden of Havana from Pinar del Río.	this study
Buxus yunquensis Eg. Köhler	Bx 046	P. A. González HFC 85878 (B, HAJB)	Cuba: Guantánamo, Baracoa, Santa María.	this study
Buxus yunquensis Eg. Köhler	Bx 053	J. Gutiérrez & al. HFC 79696 (B, HAJB)	Cuba: Guantánamo, Baracoa, subida al Yunque de Baracoa.	this study
Pachysandra axillaris Franch. subsp. *axillaris*		v. Balthazar 98024 (Z)	not available	AF245420
Pachysandra axillaris subsp. *stylosa* Dunn		v. Balthazar 98031 (Z)	not available	AF245424
Pachysandra procumbens Michx		v. Balthazar 98025 (Z)	not available	AF245421
Pachysandra terminalis Ziebold & Zucc.		v. Balthazar 99006 (Z)	not available	AF245430
Sarcococca confertiflora Sealy		v. Balthazar 98018 (Z)	not available	AF245416
Sarcococca conzattii (Standl.) I.M.Johnst.	Bx 130	M. Chazaro & R. Sánchez 9759 (B, XAL)	Mexico: Jalisco, Ayutia County, Sierra of Cacoma	this study
Sarcococca hookeriana Baill.	Bx 139	Schwerdtfeger 22670 (B)	Asia (cultivated in Botanical Garden Berlin-Dahlem)	this study
Sarcococca hookeriana var. *humilis* Baill.		v. Balthazar 98023 (Z)	not available	AF425419
Sarcococca ruscifolia Stapf		v. Balthazar 98021 (Z)	not available	AF245417
Sarcococca saligna (D. Donn) Müll. Arg.		v. Balthazar 98022 (Z)	not available	AF245418
Sarcococca wallichii Stapf		v. Balthazar 98027 (Z)	not available	AF245422
Styloceras brokawii A. Gentry & R. Foster		Nee 49511 (NY)	not available	AF245431

Appendix 4.2. Characteristics of ITS sequences of Buxus and other genera of Buxaceae regarding Guanine-Cytosine (G+C) content, analysis of motifs in ITS1 and 5.8S.

Taxa_Genbank code or Bx code_clone number (identic clones)	ITS1+ITS2+5.8S Length (nt)	ITS1 Length (nt)	ITS2 Length (nt)	ITS1+ITS2 Length (nt)	ITS1+ITS2 GC%	5.8S Length (nt)	5.8S GC%	ITS1 motif Liu & Schardl (1994)	5.8S motif Liston & al. (1996)	5.8S motif Jobes & Thien (1997)	5.8S motif I Harpke & Peterson (2008)	5.8S motif II Harpke & Peterson (2008)	Criteria
Buxus_acuminata_Bx005_c58	621	239	225	464	62.5	157	51.592	C	1 m	C	C	2 m	P
Buxus_acuminata_Bx005_c59	638	238	240	478	58.996	160	48.75	2 m	1 m	C	C	1 m	P
Buxus_acuminata_Bx005_c60	616	226	230	456	60.746	160	50.625	1 m	C	1 m	C	1 m	P
Buxus_acuminata_Bx005_c64	639	238	240	478	59.414	161	48.447	2 m	1 m	C	C	1 m	P
Buxus_acuminata_Bx005_c65	638	238	240	478	58.787	160	50	1 m	1 m	C	C	C	P
Buxus_acuminata_Bx005_c67	638	238	240	478	58.996	160	49.375	2 m	1 m	C	C	C	P
Buxus_acuminata_Bx005_c68	616	226	230	456	60.746	160	51.875	1 m	C	C	C	1 m	P
Buxus_acuminata_Bx005_c69	615	225	230	455	60.879	160	51.875	1 m	C	C	C	1 m	P
Buxus_acuminata_Bx005_c70	638	238	240	478	58.787	160	48.75	2 m	1 m	C	C	1 m	P
Buxus_acuminata_Bx005_c71	638	238	240	478	58.996	160	49.375	2 m	1 m	C	C	C	P
Buxus_acuminata_Bx005_c72	638	238	240	478	59.205	160	48.75	2 m	1 m	C	C	2 m	P
Buxus_acuminata_Bx005_c73	637	237	240	477	58.91	160	48.75	2 m	1 m	C	C	1 m	P
Buxus_acuminata_Bx005_c74	638	238	240	478	58.996	160	49.375	2 m	1 m	C	C	C	P
Buxus_acuminata_Bx005_c75	616	226	230	456	60.746	160	51.875	1 m	C	C	C	1 m	P
Buxus_acuminata_Bx005_c76	609	227	225	452	60.619	157	50.955	1 m	C	C	C	2 m	P
Buxus_acunae_Bx006	642	241	241	482	72.925	160	57.812	C	C	C	C	C	F
Buxus_aneura_Bx001_c10	643	243	240	483	66.874	160	53.75	C	C	C	C	C	F
Buxus_arborea_Bx067	624	221	243	464	70.366	160	58.125	C	C	C	C	C	F
Buxus_bahamensis_Bx032	646	245	241	486	72.016	160	58.125	C	C	C	C	C	F
Buxus_balearica_AF245423	659	262	237	499	66.333	160	55.938	C	C	C	C	C	F
Buxus_bartlettii_Bx007	594	194	240	434	72.35	160	58.125	C	C	C	C	C	F
Buxus_benguellensis_Bx100	539	162	217	379	65.963	160	56.25	C	C	C	C	C	F
Buxus_bissei_Bx008_c81	622	239	226	465	61.29	157	50.955	C	C	C	C	2 m	P
Buxus_bissei_Bx008_c83 **(=c84)**	623	239	227	466	61.373	157	51.592	C	C	C	C	2 m	P
Buxus_bissei_Bx008_c84 **(=c83)**	623	239	227	466	61.373	157	51.592	C	C	C	C	2 m	P
Buxus_bissei_Bx008_c85	623	239	227	466	61.373	157	51.592	C	C	C	C	2 m	P
Buxus_bissei_Bx008_c88	623	239	227	466	61.373	157	51.592	C	C	C	C	2 m	P
Buxus_bissei_Bx008_c95	623	239	227	466	61.373	157	51.592	C	C	C	C	2 m	P
Buxus_braimbridgeorum_Bx091	635	237	238	475	62.526	160	53.438	2 m	C	C	1 m	C	P
Buxus_brevipes_Bx136	635	237	238	475	70.105	160	56.25	C	C	C	C	C	F
Buxus_citrifolia_Bx151_c77 **(=c79, c81, c87)**	626	228	238	466	59.657	160	48.75	2 m	1 m	1 m	2 m	C	P
Buxus_citrifolia_Bx151_c79 **(=c77, c81, c87)**	626	228	238	466	59.657	160	48.75	2 m	1 m	1 m	2 m	C	P
Buxus_citrifolia_Bx151_c81 **(=c77, c79, c87)**	626	228	238	466	59.657	160	48.75	2 m	1 m	1 m	2 m	C	P
Buxus_citrifolia_Bx151_c82	626	228	238	466	60.086	160	48.75	3 m	1 m	1 m	2 m	C	P
Buxus_citrifolia_Bx151_c84	630	234	236	470	68.936	160	56.875	1 m	C	C	C	C	P
Buxus_citrifolia_Bx151_c85	623	236	227	463	69.114	160	57.5	C	C	C	C	C	F
Buxus_citrifolia_Bx151_c87 **(=c77, c79, c81)**	626	228	238	466	59.657	160	48.75	2 m	1 m	1 m	2 m	C	P
Buxus_citrifolia_Bx151_c90	626	228	238	466	59.442	160	48.75	2 m	1 m	1 m	2 m	C	P
Buxus_citrifolia_Bx151_c91	626	228	238	466	59.657	160	48.75	2 m	1 m	1 m	2 m	C	P

Appendix 4.2. Continued

Taxa_Genbank code or Bx code_clone number (identic clones)	ITS1+ITS2+5.8S Length (nt)	ITS1 Length (nt)	ITS2 Length (nt)	ITS1+ITS2 Length (nt)	ITS1+ITS2 GC%	5.8S Length (nt)	5.8S GC%	ITS1 motif Liu & Schardl (1994)	5.8S motif Liston & al. (1996)	5.8S motif Jobes & Thien (1997)	5.8S motif I Harpke & Peterson (2008)	5.8S motif II Harpke & Peterson (2008)	Criteria
Buxus_crassifolia_Bx002_c20	629	231	238	469	63.966	160	53.125	2 m	C	1 m	C	1 m	P
Buxus_crassifolia_Bx002_c23	629	231	238	469	63.966	160	53.125	2 m	C	1 m	C	1 m	P
Buxus_crassifolia_Bx002_c26	641	238	243	481	62.786	160	55	C	C	C	C	C	F
Buxus_crassifolia_Bx002_c28	625	227	238	465	63.226	160	50.625	1 m	1 m	1 m	1 m	2 m	P
Buxus_crassifolia_Bx002_c29	618	227	231	458	61.79	160	50.625	1 m	1 m	1 m	1 m	2 m	P
Buxus_crassifolia_Bx002_c32	618	227	231	458	61.572	160	50.625	1 m	1 m	1 m	1 m	2 m	P
Buxus_crassifolia_Bx002_c33	618	227	231	458	61.79	160	51.25	1 m	2 m	1 m	1 m	2 m	P
Buxus_crassifolia_Bx002_c36	629	231	238	469	64.179	160	52.5	2 m	C	1 m	C	1 m	P
Buxus_crassifolia_Bx002_c37	629	231	238	469	63.966	160	53.125	2 m	C	1 m	C	1 m	P
Buxus_crassifolia_Bx002_c38	629	231	238	469	64.392	160	53.125	2 m	C	1 m	C	1 m	P
Buxus_crassifolia_Bx128	624	242	222	464	70.69	160	57.5	C	C	C	C	C	F
Buxus_cristalensis_Bx026_c9	623	241	222	463	70.626	160	57.5	C	C	C	C	C	F
Buxus_cristalensis_Bx026_c10	624	242	222	464	70.474	160	57.5	C	C	1 m	C	C	P
Buxus_cristalensis_Bx026_c11	627	242	225	467	69.165	160	56.875	C	C	C	C	C	F
Buxus_cristalensis_Bx026_c26	638	239	239	478	62.762	160	51.875	C	C	1 m	C	2 m	P
Buxus_ekmanii_ssp_ekmanii_Bx095	642	241	241	482	72.614	160	58.125	C	C	C	C	C	F
Buxus_ekmanii_ssp_woodfredensis_Bx009	630	229	241	470	71.915	160	57.188	C	C	C	C	C	F
Buxus_excisa_ssp_excisa_Bx010	626	244	222	466	70.708	160	56.875	C	C	C	C	C	F
Buxus_foliosa_Bx011	643	244	239	483	70.083	160	57.5	C	C	C	C	C	F
Buxus_foliosa_Bx140 (partial)	435	244	31	275	69.273	160	57.5	C	C	C	C	C	F
Buxus_foliosa_Bx140_c58	623	242	221	463	66.955	160	51.875	2 m	C	C	C	1 m	P
Buxus_foliosa_Bx140_c59	624	238	226	464	61.207	160	53.125	C	C	C	1 m	1 m	P
Buxus_foliosa_Bx140_c62	624	238	226	464	60.776	160	52.5	C	C	C	1 m	C	P
Buxus_foliosa_Bx140_c64	624	238	226	464	60.56	160	52.5	C	C	C	1 m	C	P
Buxus_foliosa_Bx140_c70	623	238	226	464	60.776	159	52.83	C	C	C	1 m	C	P
Buxus_foliosa_Bx140_c73	624	238	226	464	60.776	160	52.5	C	C	C	1 m	C	P
Buxus_foliosa_Bx140_c74	634	235	239	474	66.878	160	56.875	2 m	C	C	C	C	P
Buxus_foliosa_Bx140_c75	634	235	239	474	66.667	160	56.875	1 m	C	C	C	1 m	P
Buxus_foliosa_Bx141	643	244	239	483	70.393	160	57.5	C	C	C	C	C	F
Buxus_glomerata_Bx031	653	252	241	493	70.183	160	58.125	C	C	C	C	C	F
Buxus_glomerata_Bx035	651	250	241	491	70.468	160	58.125	C	C	C	C	C	F
Buxus_glomerata_Bx052	618	220	238	458	65.721	160	53.75	C	C	C	C	C	F
Buxus_glomerata_Bx052_c21	624	238	226	464	59.914	160	51.25	2 m	C	C	C	C	P
Buxus_glomerata_Bx052_c25	637	236	241	477	60.168	160	48.75	1 m	1 m	2 m	1 m	2 m	P
Buxus_glomerata_Bx052_c28	637	236	241	477	60.377	160	48.125	1 m	1 m	2 m	C	2 m	P
Buxus_glomerata_Bx052_c29	637	236	241	477	60.168	160	48.75	1 m	1 m	2 m	C	2 m	P
Buxus_glomerata_Bx052_c30	637	236	241	477	60.587	160	48.75	1 m	1 m	2 m	C	2 m	P
Buxus_glomerata_Bx052_c33	618	220	238	458	65.721	160	53.75	C	C	C	C	C	F
Buxus_glomerata_Bx052_c34	637	239	238	477	63.312	160	54.375	1 m	C	C	1 m	C	P

Appendix 4.2. Continued

Taxa_Genbank code or Bx code_clone number (identic clones)	ITS1+ITS2+5.8S Length (nt)	ITS1 Length (nt)	ITS2 Length (nt)	ITS1+ITS2 Length (nt)	ITS1+ITS2 GC%	5.8S Length (nt)	5.8S GC%	ITS1 motif Liu & Schardl (1994)	5.8S motif Liston & al. (1996)	5.8S motif Jobes & Thien (1997)	5.8S motif I Harpke & Peterson (2008)	5.8S motif II Harpke & Peterson (2008)	Criteria
Buxus_glomerata_Bx052_c37	637	236	241	477	60.168	160	48.75	1 m	1 m	2 m	C	2 m	P
Buxus_glomerata_Bx106	645	244	241	485	70.515	160	57.5	C	C	C	C	C	F
Buxus_glomerata_Bx143	646	245	241	486	70.782	160	58.125	C	C	C	C	C	F
Buxus_glomerata_Bx155_c2 **(=c14)**	643	245	238	483	66.667	160	53.75	C	C	C	C	C	F
Buxus_glomerata_Bx155_c3	624	238	226	464	59.698	160	51.875	2 m	C	C	C	C	P
Buxus_glomerata_Bx155_c7	643	245	238	483	66.46	160	53.75	C	C	C	C	C	F
Buxus_glomerata_Bx155_c13	632	239	233	472	59.746	160	50.625	2 m	C	1 m	C	2 m	P
Buxus_glomerata_Bx155_c14 **(=c2)**	643	245	238	483	66.667	160	53.75	C	C	C	C	C	F
Buxus_glomerata_Bx155_c15	617	226	231	457	58.425	160	50	3 m	C	C	2 m	2 m	P
Buxus_glomerata_Bx157_c20	642	245	237	482	66.183	160	53.75	C	C	C	C	C	F
Buxus_glomerata_Bx157_c21	646	244	242	486	69.136	160	56.875	1 m	C	C	C	C	P
Buxus_glomerata_Bx157_c22	642	245	237	482	65.975	160	54.375	C	C	C	C	C	F
Buxus_glomerata_Bx157_c23	643	245	238	483	66.046	160	53.75	C	C	C	C	C	F
Buxus_glomerata_Bx157_c26	643	245	238	483	66.046	160	53.125	C	C	C	C	C	F
Buxus_glomerata_Bx157_c28	646	244	242	486	69.136	160	57.5	1 m	C	C	1 m	C	P
Buxus_glomerata_Bx157_c29	642	245	237	482	65.145	160	53.75	C	C	C	C	C	F
Buxus_glomerata_Bx157_c31	642	245	237	482	66.183	160	53.75	C	C	C	C	C	F
Buxus_glomerata_Bx157_c33	632	239	233	472	59.322	160	51.25	2 m	C	1 m	C	2 m	P
Buxus_glomerata_Bx157_c34	642	245	237	482	65.768	160	53.75	C	C	C	C	C	F
Buxus_glomerata_Bx157_c38	646	244	242	486	68.93	160	56.875	1 m	C	C	C	C	P
Buxus_gonoclada_AF245427	635	240	235	475	61.474	160	51.25	1 m	C	C	C	2 m	P
Buxus_gonoclada_ssp_gonoclada_Bx038_c59	629	231	238	469	61.194	160	51.25	1 m	C	1 m	C	1 m	P
Buxus_gonoclada_ssp_gonoclada_Bx038_c67 **(=c68)**	629	231	238	469	61.62	160	51.25	1 m	C	1 m	C	1 m	P
Buxus_gonoclada_ssp_gonoclada_Bx038_c68 **(=c67)**	629	231	238	469	61.62	160	51.25	1 m	C	1 m	C	1 m	P
Buxus_gonoclada_ssp_gonoclada_Bx038_c76	636	237	239	476	61.765	160	53.125	2 m	C	1 m	1 m	1 m	P
Buxus_gonoclada_ssp_gonoclada_Bx051	626	241	225	466	69.099	160	56.25	C	C	C	C	C	F
Buxus_gonoclada_ssp_gonoclada_Bx051_c77	629	231	238	469	61.62	160	50.625	1 m	C	C	C	1 m	P
Buxus_gonoclada_ssp_gonoclada_Bx051_c80	629	231	238	469	62.687	160	50.625	1 m	C	C	C	1 m	P
Buxus_gonoclada_ssp_gonoclada_Bx051_c88	635	237	238	475	61.895	160	54.375	2 m	C	C	1 m	C	P
Buxus_gonoclada_ssp_gonoclada_Bx051_c89	629	231	238	469	61.62	160	51.25	1 m	C	C	C	1 m	P
Buxus_gonoclada_ssp_gonoclada_Bx079_c78 **(=c82, c83, c86, c91, c94)**	635	237	238	475	61.895	160	53.75	2 m	C	C	1 m	C	P
Buxus_gonoclada_ssp_gonoclada_Bx079_c79 **(=c95)**	629	231	238	469	61.834	160	51.25	1 m	C	C	C	1 m	P
Buxus_gonoclada_ssp_gonoclada_Bx079_c82 **(=c78, c83, c86, c91, c94)**	635	237	238	475	61.895	160	53.75	2 m	C	C	1 m	C	P
Buxus_gonoclada_ssp_gonoclada_Bx079_c83 **(=c78, c82, c86, c91, c94)**	635	237	238	475	61.895	160	53.75	2 m	C	C	1 m	C	P
Buxus_gonoclada_ssp_gonoclada_Bx079_c86 **(=c78, c82, c83, c91, c94)**	635	237	238	475	61.895	160	53.75	2 m	C	C	1 m	C	P

Appendix 4.2. Continued

Taxa_Genbank code or Bx code_clone number (identic clones)	ITS1+ITS2+5.8S Length (nt)	ITS1 Length (nt)	ITS2 Length (nt)	ITS1+ITS2 Length (nt)	ITS1+ITS2 GC%	5.8S Length (nt)	5.8S GC%	ITS1 motif Liu & Schardl (1994)	5.8S motif Liston & al. (1996)	5.8S motif Jobes & Thien (1997)	5.8S motif I Harpke & Peterson (2008)	5.8S motif II Harpke & Peterson (2008)	Criteria
Buxus_gonoclada_ssp_gonoclada_Bx079_c87	635	237	238	475	61.895	160	54.375	2 m	C	C	1 m	C	P
Buxus_gonoclada_ssp_gonoclada_Bx079_c91 **(=c78, c82, c83, c86, c94)**	635	237	238	475	61.895	160	53.75	2 m	C	C	1 m	C	P
Buxus_gonoclada_ssp_gonoclada_Bx079_c92	629	231	238	469	61.834	160	51.875	1 m	C	C	C	1 m	P
Buxus_gonoclada_ssp_gonoclada_Bx079_c93	635	237	238	475	61.895	160	53.75	2 m	C	C	2 m	C	P
Buxus_gonoclada_ssp_gonoclada_Bx079_c94 **(=c78, c82, c83, c86, c91)**	635	237	238	475	61.895	160	53.75	2 m	C	C	1 m	C	P
Buxus_gonoclada_ssp_gonoclada_Bx079_c95 **(=c79)**	629	231	238	469	61.834	160	51.25	1 m	C	C	C	1 m	P
Buxus_gonoclada_ssp_gonoclada_Bx137_c2	635	237	238	475	59.579	160	53.125	3 m	C	C	1 m	C	P
Buxus_gonoclada_ssp_gonoclada_Bx137_c13	635	237	238	475	60.842	160	52.5	3 m	C	C	C	C	P
Buxus_gonoclada_ssp_gonoclada_Bx137_c14	635	237	238	475	60.842	160	51.875	3 m	C	C	C	C	P
Buxus_gonoclada_ssp_gonoclada_Bx137_c17	635	237	238	475	60.842	160	51.25	3 m	C	C	C	C	P
Buxus_gonoclada_ssp_orientensis_Bx146_c20	627	242	225	467	68.308	160	56.875	C	C	C	C	C	F
Buxus_gonoclada_ssp_orientensis_Bx146_c22	627	242	225	467	68.522	160	56.875	C	C	C	C	C	F
Buxus_gonoclada_ssp_orientensis_Bx146_c23	638	240	238	478	66.318	160	53.75	C	C	C	C	1 m	P
Buxus_gonoclada_ssp_orientensis_Bx146_c24	629	231	238	469	62.9	160	51.25	1 m	C	1 m	C	1 m	P
Buxus_gonoclada_ssp_orientensis_Bx146_c25	642	244	238	482	64.315	160	51.25	1 m	C	C	C	C	P
Buxus_gonoclada_ssp_orientensis_Bx146_c26 **(=c34, c37)**	629	231	238	469	63.113	160	51.25	1 m	C	1 m	C	1 m	P
Buxus_gonoclada_ssp_orientensis_Bx146_c27	640	242	238	480	65.833	160	51.25	C	C	C	C	C	F
Buxus_gonoclada_ssp_orientensis_Bx146_c29	629	231	238	469	63.326	160	51.875	1 m	C	1 m	C	1 m	P
Buxus_gonoclada_ssp_orientensis_Bx146_c33	640	242	238	480	65.833	160	51.25	C	C	C	C	1 m	P
Buxus_gonoclada_ssp_orientensis_Bx146_c34 **(=c26, c37)**	629	231	238	469	63.113	160	51.25	1 m	C	1 m	C	1 m	P
Buxus_gonoclada_ssp_orientensis_Bx146_c36	620	238	222	460	61.739	160	49.375	C	C	1 m	C	1 m	P
Buxus_gonoclada_ssp_orientensis_Bx146_c37 **(=c26, c34)**	629	231	238	469	63.113	160	51.25	1 m	C	1 m	C	1 m	P
Buxus_harlandii_AF245410	651	251	240	491	66.497	160	56.25	C	C	C	C	C	F
Buxus_henryi_AF245409	616	251	205	456	64.912	160	55	1 m	C	1 m	1 m	C	P
Buxus_hildebrandtii_AF245415	598	214	226	440	56.136	158	45.57	3 m	C	2 m	2 m	2 m	P
Buxus_imbricata_Bx054_c49	643	243	240	483	66.667	160	53.75	C	C	C	C	C	F
Buxus_jaucoensis_Bx013	624	229	235	464	71.336	160	57.5	C	C	C	C	C	F
Buxus_koehleri_Bx055_c58	599	214	225	439	69.932	160	56.875	C	C	C	C	C	F
Buxus_koehleri_Bx055_c59	625	244	221	465	65.161	160	51.875	C	C	1 m	C	1 m	P
Buxus_koehleri_Bx055_c60	626	244	222	466	69.957	160	57.5	C	C	C	C	C	F
Buxus_leivae_Bx021	626	244	222	466	70.815	160	56.562	C	C	C	C	C	F
Buxus_liukiuensis_AF245428	637	238	239	477	67.086	160	56.25	C	C	C	C	C	F
Buxus_macowanii_AF245411	542	162	220	382	63.874	160	56.25	C	C	C	C	C	F
Buxus_macowanii_Bx101	531	161	210	371	64.42	160	56.25	C	C	C	C	C	F
Buxus_madagascarica_Bx152	519	164	195	359	61.56	160	56.875	C	C	C	C	C	F
Buxus_marginalis_Bx016	610	210	240	450	69.778	160	57.5	C	C	C	C	C	F

Appendix 4.2. Continued

Taxa_Genbank code or Bx code_clone number (identic clones)	ITS1+ITS2+5.8S Length (nt)	ITS1 Length (nt)	ITS2 Length (nt)	ITS1+ITS2 Length (nt)	ITS1+ITS2 GC%	5.8S Length (nt)	5.8S GC%	ITS1 motif Liu & Schardl (1994)	5.8S motif Liston & al. (1996)	5.8S motif Jobes & Thien (1997)	5.8S motif I Harpke & Peterson (2008)	5.8S motif II Harpke & Peterson (2008)	Criteria
Buxus_marginalis_Bx045	610	210	240	450	69.556	160	57.5	C	C	C	C	C	F
Buxus_mexicana_Bx061_c58	636	239	237	476	57.563	160	46.875	C	C	C	C	C	P
Buxus_microphylla_AF245412	639	239	240	479	67.641	160	56.25	C	C	C	C	C	F
Buxus_microphylla_AF245414	638	239	239	478	68.201	160	56.25	C	C	C	C	C	F
Buxus_moana_Bx018_c1	624	238	226	464	59.052	160	53.125	2 m	C	C	C	1 m	P
Buxus_moana_Bx018_c6	623	237	226	463	59.827	160	52.5	1 m	C	1 m	C	C	P
Buxus_moana_Bx018_c7	624	238	226	464	60.776	160	51.875	2 m	C	1 m	C	C	P
Buxus_moana_Bx018_c8	625	239	226	465	59.785	160	52.5	1 m	C	1 m	C	C	P
Buxus_moana_Bx018_c10	625	239	226	465	60	160	52.5	1 m	C	1 m	C	C	P
Buxus_moana_Bx018_c11	629	231	238	469	60.768	160	48.125	3 m	C	1 m	1 m	1 m	P
Buxus_moana_Bx018_c17	623	237	226	463	59.611	160	52.5	1 m	C	2 m	C	C	P
Buxus_moctezumae_Bx020	594	194	240	434	72.12	160	58.125	C	C	C	C	C	F
Buxus_moratii_Bx147_c	553	162	231	393	63.104	160	55.625	C	C	C	C	C	F
Buxus_natalensis_AF245425	633	237	236	473	62.368	160	50	1 m	C	C	1 m	C	P
Buxus_nipensis_Bx117	635	237	238	475	61.895	160	53.75	2 m	C	C	1 m	C	P
Buxus_olivacea_Bx023	621	239	225	464	61.53	157	50.955	C	C	C	C	3 m	P
Buxus_olivacea_Bx094	621	239	225	464	61.099	157	50.955	C	C	1 m	C	2 m	P
Buxus_olivacea_Bx094_c1 **(=c4, c10, c12)**	626	231	235	466	63.734	160	51.25	C	C	1 m	C	2 m	P
Buxus_olivacea_Bx094_c2	626	231	235	466	63.734	160	50.625	C	C	1 m	C	2 m	P
Buxus_olivacea_Bx094_c3	621	239	225	464	61.422	157	50.955	C	C	C	C	2 m	P
Buxus_olivacea_Bx094_c4 **(=c1, c10, c12)**	626	231	235	466	63.734	160	51.25	C	C	1 m	C	2 m	P
Buxus_olivacea_Bx094_c6	1863	228	1473	1701	48.15	162		C	**C**	1 m	C	2 m	P
Buxus_olivacea_Bx094_c9	621	239	225	464	61.638	157	50.955	C	C	C	C	2 m	P
Buxus_olivacea_Bx094_c10 **(=c1, c4, c12)**	626	231	235	466	63.734	160	51.25	C	C	1 m	C	2 m	P
Buxus_olivacea_Bx094_c11	621	239	225	464	61.207	157	50.955	C	C	C	C	2 m	P
Buxus_olivacea_Bx094_c12 **(=c1, c4, c10)**	626	231	235	466	63.734	160	51.25	C	C	1 m	C	2 m	P
Buxus_olivacea_Bx094_c13	626	231	235	466	63.734	160	51.875	C	C	1 m	C	2 m	P
Buxus_olivacea_Bx094_c14	621	239	225	464	61.207	157	50.955	1 m	C	C	C	2 m	P
Buxus_olivacea_Bx094_c15	627	231	236	467	63.812	160	51.25	C	C	1 m	C	2 m	P
Buxus_olivacea_Bx094_c16	626	231	235	466	63.734	160	51.875	C	C	1 m	C	2 m	P
Buxus_olivacea_Bx094_c17	621	239	225	464	61.422	157	50.955	C	C	C	C	2 m	P
Buxus_olivacea_Bx094_c19	621	239	225	464	61.422	157	50.955	C	C	C	C	2 m	P
Buxus_olivacea_Bx114	621	239	225	464	61.638	157	50.955	C	C	C	C	3 m	P
Buxus_pilosula_ssp_cacuminis_Bx109	626	244	222	466	70.064	160	57.5	C	C	C	C	C	F
Buxus_pilosula_ssp_pilosula_Bx024_c31	617	231	226	457	62.801	160	50	2 m	C	C	C	1 m	P
Buxus_pilosula_ssp_pilosula_Bx024_c32	620	238	222	460	62.609	160	50.625	C	C	1 m	C	1 m	P
Buxus_pilosula_ssp_pilosula_Bx120 (partial)	505	244	101	345	70.58	160	56.875	C	C	C	C	C	F
Buxus_pilosula_ssp_pilosula_Bx120_c47	628	242	226	468	69.872	160	56.875	C	C	C	C	C	F
Buxus_portoricensis_Bx133	622	225	237	462	68.615	160	58.125	C	C	C	C	C	F

Appendix 4.2. Continued

Taxa_Genbank code or Bx code_clone number (identic clones)	ITS1+ITS2+5.8S Length (nt)	ITS1 Length (nt)	ITS2 Length (nt)	ITS1+ITS2 Length (nt)	ITS1+ITS2 GC%	5.8S Length (nt)	5.8S GC%	ITS1 motif Liu & Schardl (1994)	5.8S motif Liston & al. (1996)	5.8S motif Jobes & Thien (1997)	5.8S motif I Harpke & Peterson (2008)	5.8S motif II Harpke & Peterson (2008)	Criteria
Buxus_pseudaneura_Bx025	643	244	239	483	70.393	160	57.5	C	C	C	C	C	F
Buxus_pubescens_Bx150_c74	576	192	224	416	68.269	160	57.5	C	C	C	C	C	F
Buxus_retusa_ssp_retusa_Bx012	626	244	222	466	70.494	160	57.5	C	C	C	C	C	F
Buxus_revoluta_Bx043	626	241	225	466	69.313	160	56.875	C	C	C	C	C	F
Buxus_revoluta_Bx044	626	244	222	466	70.601	160	56.875	C	C	C	C	C	F
Buxus_rheedioides_Bx119	622	240	222	462	71.645	160	57.812	C	C	C	C	C	F
Buxus_riparia_AF245413	639	239	240	479	67.641	160	56.25	C	C	C	C	C	F
Buxus_rotundifolia_Bx096_c20	619	228	231	459	61.874	160	50.625	1 m	C	C	C	2 m	P
Buxus_rotundifolia_Bx096_c24	638	239	239	478	62.762	160	51.875	C	C	1 m	C	2 m	P
Buxus_rotundifolia_Bx096_c25	638	239	239	478	62.971	160	51.875	C	C	1 m	C	2 m	P
Buxus_rotundifolia_Bx096_c28 **(=c32)**	619	228	231	459	61.656	160	50.625	1 m	C	C	C	2 m	P
Buxus_rotundifolia_Bx096_c31	638	239	239	478	62.762	160	51.25	C	C	1 m	C	2 m	P
Buxus_rotundifolia_Bx096_c32 **(=c28)**	619	228	231	459	61.656	160	50.625	1 m	C	C	C	2 m	P
Buxus_rotundifolia_Bx096_c34	638	239	239	478	62.971	160	51.875	C	C	1 m	C	2 m	P
Buxus_rotundifolia_Bx096_c36	619	228	231	459	61.656	160	50.625	1 m	C	C	C	2 m	P
Buxus_rotundifolia_Bx096_c37	638	239	239	478	62.343	160	51.875	C	C	1 m	C	2 m	P
Buxus_sclerophylla_Bx028	624	229	235	464	71.336	160	57.5	C	C	C	C	C	F
Buxus_sclerophylla_Bx028_c39	641	240	241	481	62.994	160	53.75	C	1 m	C	C	3 m	P
Buxus_sclerophylla_Bx028_c40	637	236	241	477	60.377	160	48.75	1 m	1 m	2 m	C	2 m	P
Buxus_sclerophylla_Bx028_c42 **(=c44B)**	637	236	241	477	60.377	160	48.75	1 m	1 m	2 m	C	2 m	P
Buxus_sclerophylla_Bx028_c42B **(=c40B)**	641	240	241	481	62.37	160	53.125	C	1 m	C	C	2 m	P
Buxus_sclerophylla_Bx028_c44B **(=c42)**	637	236	241	477	60.377	160	48.75	1 m	1 m	2 m	C	2 m	P
Buxus_sclerophylla_Bx028_c47	637	236	241	477	60.168	160	48.75	1 m	1 m	2 m	C	2 m	P
Buxus_sempervirens_AF245429	644	262	222	484	66.942	160	56.25	C	C	C	C	C	F
Buxus_sempervirens_EF123197_1	644	262	222	484	67.562	160	56.25	C	C	C	C	C	F
Buxus_serpentinicola_Bx029_c41	629	231	238	469	61.194	160	53.125	2 m	C	1 m	1 m	C	P
Buxus_serpentinicola_Bx029_c42 **(=c45, c47)**	629	231	238	469	61.194	160	52.5	2 m	C	1 m	1 m	C	P
Buxus_serpentinicola_Bx029_c43	635	237	238	475	61.053	160	51.875	2 m	C	1 m	1 m	C	P
Buxus_serpentinicola_Bx029_c45 **(=c42, c47)**	629	231	238	469	61.194	160	52.5	2 m	C	1 m	1 m	C	P
Buxus_serpentinicola_Bx029_c47 **(=c42, c45)**	629	231	238	469	61.194	160	52.5	2 m	C	1 m	1 m	C	P
Buxus_serpentinicola_Bx029_c50	629	231	238	469	61.194	160	53.125	2 m	1 m	1 m	1 m	C	P
Buxus_serpentinicola_Bx029_c51	628	230	238	468	65.385	160	51.875	C	C	1 m	1 m	C	P
Buxus_serpentinicola_Bx029_c52	635	237	238	475	61.263	160	51.875	2 m	C	1 m	1 m	C	P
Buxus_serpentinicola_Bx029_c53	629	231	238	469	61.194	160	53.125	2 m	1 m	1 m	1 m	C	P
Buxus_serpentinicola_Bx029_c54	635	237	238	475	60.421	160	51.875	2 m	C	1 m	1 m	C	P
Buxus_serpentinicola_Bx029_c56	635	237	238	475	61.053	160	51.875	2 m	C	1 m	1 m	1 m	P
Buxus_serpentinicola_Bx029_c57	629	231	238	469	61.407	160	52.5	2 m	C	1 m	1 m	C	P
Buxus_shaferi_Bx004	623	242	221	463	67.387	160	52.812	C	C	C	C	1 m	P
Buxus_shaferi_Bx004_c39	624	243	221	464	66.595	160	52.5	1 m	C	C	C	1 m	P

Appendix 4.2. Continued

Taxa_Genbank code or Bx code_clone number (identic clones)	ITS1+ITS2+5.8S Length (nt)	ITS1 Length (nt)	ITS2 Length (nt)	ITS1+ITS2 Length (nt)	ITS1+ITS2 GC%	5.8S Length (nt)	5.8S GC%	ITS1 motif Liu & Schardl (1994)	5.8S motif Liston & al. (1996)	5.8S motif Jobes & Thien (1997)	5.8S motif I Harpke & Peterson (2008)	5.8S motif II Harpke & Peterson (2008)	Criteria
Buxus_shaferi_Bx004_c40	625	239	226	465	59.785	160	51.25	2 m	C	C	C	C	P
Buxus_shaferi_Bx004_c44	624	243	221	464	66.164	160	53.125	C	C	C	C	1 m	P
Buxus_shaferi_Bx004_c46	624	243	221	464	66.595	160	53.125	1 m	C	C	C	1 m	P
Buxus_shaferi_Bx004_c49	625	239	226	465	58.495	160	52.5	2 m	2 m	1 m	C	C	P
Buxus_shaferi_Bx004_c52	621	242	221	463	67.387	158	52.532	C	C	C	C	C	F
Buxus_shaferi_Bx004_c53	624	243	221	464	66.164	160	53.125	3 m	C	C	C	1 m	P
Buxus_shaferi_Bx004_c57	626	239	227	466	60.73	160	51.25	2 m	C	C	C	C	P
Buxus_shaferi_Bx022_c20	629	231	238	469	61.407	160	47.5	3 m	C	1 m	1 m	1 m	P
Buxus_shaferi_Bx022_c21	625	244	221	465	66.237	160	53.75	1 m	C	C	C	1 m	P
Buxus_shaferi_Bx022_c24	626	229	237	466	64.163	160	46.25	C	C	1 m	1 m	2 m	P
Buxus_shaferi_Bx022_c26	612	239	213	452	64.602	160	57.5	1 m	C	C	C	C	P
Buxus_shaferi_Bx022_c27	625	239	226	465	60	160	51.875	1 m	C	C	C	C	P
Buxus_shaferi_Bx022_c29	629	231	238	469	60.981	160	47.5	3 m	C	1 m	1 m	1 m	P
Buxus_shaferi_Bx022_c30	625	239	226	465	60	160	51.25	1 m	C	C	1 m	1 m	P
Buxus_shaferi_Bx022_c38	642	241	241	482	64.108	160	55	1 m	C	1 m	C	1 m	P
Buxus_shaferi_Bx047	651	250	241	491	70.468	160	58.125	C	C	C	C	C	F
Buxus_shaferi_Bx122_c78	631	232	239	471	68.153	160	57.5	1 m	C	C	C	C	P
Buxus_shaferi_Bx122_c80	619	233	226	459	57.734	160	50	4 m	C	1 m	C	C	P
Buxus_shaferi_Bx122_c81	625	239	226	465	58.925	160	51.875	2 m	2 m	1 m	C	C	P
Buxus_shaferi_Bx122_c82	624	243	221	464	66.164	160	52.5	1 m	C	C	C	1 m	P
Buxus_shaferi_Bx122_c85	617	231	226	457	57.549	160	50	4 m	C	1 m	C	C	P
Buxus_shaferi_Bx122_c86	630	231	239	470	67.872	160	57.5	1 m	C	C	C	C	P
Buxus_shaferi_Bx122_c89	624	243	221	464	66.164	160	53.125	1 m	C	C	C	1 m	P
Buxus_shaferi_Bx122_c90	624	243	221	464	66.81	160	52.5	C	C	C	C	1 m	P
Buxus_shaferi_Bx122_c91	641	243	238	481	65.281	160	56.25	1 m	C	C	C	2 m	P
Buxus_shaferi_Bx122_c92	624	243	221	464	66.595	160	53.125	C	C	C	C	1 m	P
Buxus_shaferi_Bx122_c93	647	248	239	487	68.172	160	56.875	C	C	C	C	C	F
Buxus_shaferi_Bx122_c95	637	239	238	477	59.539	160	48.75	2 m	2 m	1 m	C	C	P
Buxus_triptera_Bx098_c45 **(=c52, c56)**	635	237	238	475	61.263	160	50	3 m	C	C	C	C	P
Buxus_triptera_Bx098_c50	638	240	238	478	65.69	160	50	C	C	C	C	C	F
Buxus_triptera_Bx098_c52 **(=c45, c56)**	635	237	238	475	61.263	160	50	3 m	C	C	C	C	P
Buxus_triptera_Bx098_c56 **(=c52, c52)**	635	237	238	475	61.263	160	50	3 m	C	C	C	C	P
Buxus_triptera_Bx127	626	244	222	466	70.815	160	56.875	C	C	C	C	C	F
Buxus_vahlii_Bx134	645	244	241	485	69.691	160	58.125	1 m	C	C	C	C	P
Buxus_wrightii_ssp_leonii_Bx159	637	239	238	477	59.434	160	49.375	2 m	C	1 m	C	2 m	P
Buxus_wrightii_ssp_leonii_Bx159_c1	638	239	239	478	58.996	160	49.375	2 m	C	1 m	C	2 m	P
Buxus_wrightii_ssp_leonii_Bx159_c2	638	239	239	478	58.996	160	49.375	2 m	C	1 m	C	2 m	P
Buxus_wrightii_ssp_leonii_Bx159_c3	638	239	239	478	58.787	160	49.375	2 m	C	1 m	C	2 m	P
Buxus_wrightii_ssp_leonii_Bx159_c5	638	239	239	478	58.996	160	48.75	2 m	C	1 m	C	2 m	P

Appendix 4.2. Continued

Taxa_Genbank code or Bx code_clone number (identic clones)	ITS1+ITS2+5.8S Length (nt)	ITS1 Length (nt)	ITS2 Length (nt)	ITS1+ITS2 Length (nt)	ITS1+ITS2 GC%	5.8S Length (nt)	5.8S GC%	ITS1 motif Liu & Schardl (1994)	5.8S motif Liston & al. (1996)	5.8S motif Jobes & Thien (1997)	5.8S motif I Harpke & Peterson (2008)	5.8S motif II Harpke & Peterson (2008)	Criteria
Buxus_wrightii_ssp_leonii_Bx159_c6	638	239	239	478	59.205	160	50	2 m	C	1 m	C	2 m	P
Buxus_wrightii_ssp_leonii_Bx159_c7	638	239	239	478	58.996	160	50	2 m	C	1 m	C	2 m	P
Buxus_wrightii_ssp_leonii_Bx159_c8	638	239	239	478	58.996	160	50.625	2 m	C	2 m	C	2 m	P
Buxus_wrightii_ssp_leonii_Bx159_c9 **(=c14)**	638	239	239	478	60.46	160	52.5	C	C	1 m	C	1 m	P
Buxus_wrightii_ssp_leonii_Bx159_c12	638	239	239	478	58.996	160	49.375	2 m	C	1 m	C	2 m	P
Buxus_wrightii_ssp_leonii_Bx159_c14 **(=c9)**	638	239	239	478	60.46	160	52.5	C	C	1 m	C	1 m	P
Buxus_wrightii_ssp_leonii_Bx159_c15	638	239	239	478	58.996	160	50	2 m	C	1 m	C	2 m	P
Buxus_wrightii_ssp_wrightii_Bx158	638	239	239	478	58.787	160	49.375	2 m	C	1 m	C	2 m	P
Buxus_yunquensis_Bx046	651	250	241	491	70.468	160	58.125	C	C	C	C	C	F
Buxus_yunquensis_Bx053	645	244	241	485	70.309	160	58.125	C	C	C	C	C	F
Pachysandra_axillaris_ssp_axillaris_AF245420	676	284	232	516	63.178	160	55	C	C	C	C	C	F
Pachysandra_procumbens_AF245421	676	285	231	516	63.76	160	55	C	C	C	C	C	F
Pachysandra_axillaris_ssp_stylosa_AF245424	677	285	232	517	63.25	160	55	C	C	C	C	C	F
Sarcococca_confertiflora_AF245416	671	280	231	511	65.166	160	55.625	1 m	C	C	C	C	P
Sarcococca_conzattii_Bx130	648	265	223	488	69.877	160	56.875	C	C	C	C	C	F
Sarcococca_hookeriana_Bx139	670	278	232	510	65.098	160	55.625	1 m	C	C	C	C	P
Sarcococca_hookeriana_var_humilis_AF425419	670	278	232	510	65.098	160	55.625	1 m	C	C	C	C	P
Sarcococca_ruscifolia_AF245417	670	278	232	510	65.294	160	55.625	1 m	C	C	C	C	P
Sarcococca_saligna_AF245418	669	279	230	509	64.047	160	55.625	1 m	C	C	C	C	P
Sarcococca_wallichii_AF245422	670	279	231	510	64.314	160	55	1 m	C	C	C	C	P
Styloceras_brokawii_AF245431	662	284	217	501	52.096	161	40.373	2 m	C	4 m	1 m	3 m	P

Note-Sequences with GenBank codes starting with AF were generated by von Balthazar & al. (2000), GenBank codes starting with EF were generated by Roselló & al. (2007). Bx# represents the code and number of each sample included in this study. Motifs: GGCRY-(4 to 7n)-GYGYCAAGGAA (Liu & Schardl 1994), GATATC (Liston & al. 1996), GAATTG-CAGAATCC (Jobes & Thien 1997), TTTGAAYGCA (motif I of Harpke & Peterson 2008), CGATGAAGAACGYAGC (motif II of Harpke & Peterson, 2008). nt: nucleotides, C: Conserved motif, #m: number of mutations detected. Criteria adopted in this study: F- putative functional ribotype, P- putative pseudogenic ribotype. Samples that yielded readable pherograms from direct PCR appear in bold and italic letters and the cloned samples can be distinguished with c# at the end of the name of each sequence.

Appendix 4.3. Mutations found in the ITS1 motif of Liu & Schardl (1994), GGCRY-(4 to 7n)-GYGYCAAGGAA, and information about taxa and sequences that have them. The mutated positions in the motif are shown bold and underlined

Mutations	Taxa	GenBank code/sample code number (clones)
AG**A**GC-(5nt)-G**A**GCCAAGGAA	*B. shaferi*	Bx004 (c51, c53)
AGCAC-(5nt)-GC**A**CC**T**AGGAA	*B. glomerata*	Bx155 (c15)
AGCRY-(5nt)-GYGCCAAGGAA	*B. acuminata*, *B. rotundifolia*	Bx005 (c60, c68, c69, c75, c76); Bx096 (c20, c28, c32, c36)
AGCGC-(5nt)-**A**CGCCAAGGAA	*B. wrightii* ssp. *leonii* , *B. wrightii* ssp. *wrightii*	Bx158, Bx159 (c1, c2, c3, c5, c6, c7, c8, c12, c15)
AGCGY-(5nt)-GC**A**CCAAGGAA	*B. glomerata* , *B. moana*	Bx018 (c7), Bx155 (c13), Bx157 (c33)
AGCGY-(5nt)-GCGCCAAGGAA	*B. gonoclada* ssp. *orientensis* , *B. moana* , *B. shaferi*	Bx018 (c6, c8, c10, c17), Bx022 (c26, c27, c30), Bx146 (c24, c25, c26, c34, c37)
ATCAC-(5nt)-GC**A**CCAAGGAA	*B. moana* , *B. shaferi*	Bx018 (c11), Bx022 (c20, c29)
G**A**CAC-(5nt)-**AAA**CCAAGGAA	*B. shaferi*	Bx122 (c80, c85)
G**A**CGC-(5nt)-GYGCCAAGGAA	*B. citrifolia* , *B. foliosa*	Bx140 (c75), Bx151 (c84)
G**A**CGC-(5nt)-GTGCCAAGG**G**A	*B. foliosa*	Bx140 (c74)
G**A**CGC-(5nt)-GCGTCAAG**A**AA	*B. braimbridgeorum* , *B. gonoclada* ssp. *gonoclada* , *B. nipensis* , *B. serpentinicola*	Bx029 (c41, c42, c43, c45, c47, c50, c52, c53, c54, c56, c57), Bx038 (c76), Bx051 (c88), Bx079 (c78, c82, c83, c86, c87, c91, c93, c94), Bx091, Bx117
G**A**CGT-(5nt)-**A**CGCCAAGGAA	*B. pilosula* ssp. *pilosula*	Bx024 (c30, c31)
G**A**CGT-(5nt)-GC**A**CCAAGGAA	*B. acuminata*	Bx005 (c59, c64, c65, c67, c70, c71, c72, c73, c74)
G**A**CGT-(5nt)-GCGCCAAGGAA	*B. glomerata* , *B. gonoclada* ssp. *gonoclada* , *B. sclerophylla*	Bx028 (c40, c42, c44B, c47), Bx038 (c59, c67, c68), Bx051 (c77, c80, c89), Bx052 (c25, c28, c29, c30, c34, c37), Bx079 (c79, c92, c95)
G**A**CGT-(5nt)-GTGCCAAGG**G**A	*Styloceras brokawii*	AF245431
G**AT**GC-(5nt)-GCGTCAAG**A**AA	*B. gonoclada* ssp. *gonoclada* , *B. triptera*	Bx098 (c45, c52, c56), Bx137 (c13, c14, c17)
GG**A**GC-(5nt)-G**A**GCCAAGGAA	*B. foliosa*	Bx140 (c58)
GGCG**A**-(5nt)-GCGCCAAGGAA	*B. shaferi*	Bx022 (c21)
GGCGC-(5nt)-**A**CGCCAAGGAA	*B. glomerata* , *B. shaferi*	Bx022 (c38), Bx157 (c21, 38)
GGCGC-(5nt)-**A**C**A**CCAAGGAA	*B. glomerata* , *B. moana* , *B. shaferi*	Bx004 (c40, c49, Bx018 (c1), c57), Bx052 (c21), Bx122 (c81, c95), Bx155 (c3)
GGCGC-(5nt)-G**A**GCCAAGGAA	*B. shaferi*	Bx122 (c78, 86)
GGCGC-(5nt)-GC**A**YCAAGGAA	*B. crassifolia* , *B. henryi* , *B. olivacea* , *B. vahlii*	AF245409, Bx002 (c28, c29, c32, c33), Bx094 (c14), Bx134
GGCGY-(5nt)-**A**CGCCAAGGAA	*B. glomerata* , *B. gonoclada* , *Sarcococca confertiflora* , *S. hookeriana* , *S. hookeriana* var. *humilis* , *S. ruscifolia* , *S. saligna* , *S. wallichii*	AF245427, AF245416, AF245417, AF245418, AF425419, AF245422, Bx139, Bx157 (c28)
GG**T**GC-(5nt)-GC**A**CCAAGGA**T**	*B. hildebrandtii*	AF245415
GG**T**GC-(5nt)-GC**A**CCAAGGAA	*B. citrifolia* , *B. crassifolia*	Bx002 (c20, 23, c36, c37, c38), Bx151 (c77, c79, c81, c87, c90, c91)
GG**T**GC-(5nt)-GC**A**CCAAGGA**G**	*B. citrifolia*	Bx151 (c82)
G**T**CGC-(5nt)-GCGCCAAGGAA	*B. shaferi*	Bx004 (c39, c46), Bx122 (c82, c89, c91)
TGCAC-(5nt)-GCGCCAAGGAA	*B. natalensis*	AF245425

Appendix 4.4. Mutations found in the 5.8S motif of Liston & al. (1996), GATATC, and information about taxa and sequences that have them. The mutated positions in the motif are shown bold and underlined

Mutation	Taxa	GenBank code or sample number (clones)
AATATC	*B . citrifolia* , *B. crassifolia* , *B . sclerophylla*	Bx002 (c28, c29, c32), Bx028 (c39, c40B, c42B), Bx151 (c77, c79, c81, c82, c87, c90, c91)
AA**C**ATC	*B. crassifolia*	Bx002 (c33)
TA**A**ATC	*B. shaferi*	Bx004 (c49)
GATA**C**C	*B . acuminata*	Bx005 (c58)
GATAT**T**	*B . acuminata* , *B . glomerata* , *B . sclerophylla*	Bx005 (59, c64, c65, c67, c70, c71, c72, c73, c74), Bx028, c40, c42, c44B, c47), Bx052 (c25, c28, c29, c30, c37)
G**G**TATC	*B . serpentinicola*	Bx029 (c50)
GA**C**ATC	*B . serpentinicola*	Bx029 (c53)
TA**A**ATC	*B . shaferi*	Bx122 (c81, c95)

Appendix 4.5. Mutations found in the 5.8S motif of Jobes & Thien (1997), GAATTGCAGAATCC, and information about taxa and sequences that have them. The mutated positions in the motif are shown bold and underlined

Mutation	Taxa	GenBank code or sample code number (clones)
AAATTGCAGAATCC	*Buxus henryi*	AF245409
GAAT**C**GCA**C**AATCC	*B. wrightii* ssp. *leonii*	Bx159 (c8)
GAATT**A**CAGAATCC	*B . koehleri* , *B. serpentinicola*	Bx029 (c41, c42, c43, c45, c47, c50, c51, c52, c53, c54, c56, c57), Bx055 (c59)
GAATT**A**CAGAATC**T**	*B. glomerata* , *B. sclerophylla*	Bx028 (c40, c42, c44B, c47), Bx052 (c25, c28, c29, c30, c37)
GAATTGCA**A**AATCC	*B. acuminata* , *B. olivacea*, *B. shaferi*	Bx005 (c60), Bx094 (c1, c2, c4, c10,c12, c13, c15, c16), Bx122 (c80,c85).
GAATTGCA**A**AATC**T**	*B . hildebrandtii*	AF245415
GAATTGCA**C**AATCC	*B. olivacea* , *B. rotundifolia* , *B. wrightii* ssp *leonii* , *B. wrightii* ssp *wrightii*	Bx026 (c26), Bx094, Bx096 (c24, c25, c31, c34, c37), Bx155 (c13), Bx157 (c33), Bx158, Bx159 (c1, c2, c3, c5, c6, c7, c9, c12, c14, c15)
GAATTGCAGAATC**T**	*B . citrifolia* , *B . gonoclada* ssp. *gonoclada* , *B . gonoclada* ssp *orientensis*	Bx038 (c76), Bx146 (c24, c26, c29, c34, c37), Bx151 (c77, c79, c81, c82, c87, c90, c91)
GAATTGCAGA**G**TCC	*B. cristalensis*	Bx026 (c10)
GAATTG**T**AGAATCC	*B. crassifolia* , *B. gonoclada* ssp *orientensis* , *B . moana* , *B . pilosula* ssp. *pilosula* , *B . shaferi*	Bx002 (c20, c23, c36, c37, c38), Bx004 (c49), Bx018 (c6, c7, c8, c10, c11), Bx022 (c20, c24, c29), Bx024 (c32), Bx146 (c36), Bx122 (c81, c95)
GAATT**T**CAGAATCC	*B. gonoclada* ssp. *gonoclada* , *B. shaferi*	Bx022 (c38), Bx038 (c59, c67, c68)
GAATT**T**C**TA**AATC**T**	*Styloceras brokawii*	AF245431
GA**G**TTGCAGAATCC	*B. crassifolia*	Bx002 (c28, c29, c32, c33)
G**T**ATTG**T**AGAATCC	*B. moana*	Bx018 (c17)

Appendix 4.6. Mutations found in the 5.8S motif I of Harpke & Peterson (2008), TTTGAAYGCA, and information about taxa and sequences that have them. The mutated positions in the motif are shown bold and underlined

Mutation	Taxa	GenBank code or project code number (clone number)
CTTGAACGCA	*Buxus glomerata* , *B. henryi* , *B* . *shaferi*	AF245409, Bx022 (c30), Bx157 (c28)
TTTGAAC**A**CA	*B* . *crassifolia* , *B. foliosa* , *B. glomerata* , *B. moana* , *B. shaferi* , *Styloceras brokawii*	AF245431, Bx002 (c28, c29, c32, c33), Bx018 (c11), Bx022 (c20, 24, 29), Bx052 (c25), Bx140 (c59, c62, c64, c70, c73)
TTTGAAT**AA**A	*B. citrifolia*	Bx151 (c77, c79, c81, c82, c87, c90, c91)
TTTGAAT**TA**A	*B. hildebrandtii*	AF245415
TTTGA**CA**GCA	*B. glomerata*	Bx155 (c15)
TTTGA**G**CGCA	*B. natalensis*	AF245425
TTTG**G**ACGCA	*B* . *braimbridgeorum* , *B* . *gonoclada* ssp. *gonoclada, B. nipensis, B. serpentinicola*	Bx029 (c41, c42, c43, c45, c47, c50, c51, c53, c54, c56, c57), Bx038 (c76), Bx051 (c88), Bx079 (c78, 82, c83, c86, c87, c91, c9), Bx091, Bx117
TTTG**G**ACGC**T**	*B* . *gonoclada* ssp. *gonoclada*	Bx079 (c93)
TTTG**G**ATGCA	*B* . *gonoclada* ssp. *gonoclada*	Bx137 (c2)

Appendix 4.7. Mutations found in the 5.8S motif II of Harpke & Peterson (2008), GATGAAGAACGYAGC, and information about taxa and sequences that have them. The mutated positions in the motif are shown bold and underlined

Mutation	Taxa	GenBank code or project code (clone number)
CAATGAAGAACATAGC	*B. gonoclada*	AF245427
CAATGAAGAACGAAGC	*B. acuminata*	Bx005 (c72)
C**A**ATGAAGAACGTAGC	*B. acuminata* , *B. gonoclada* ssp. *gonoclada*	Bx005 (c59, c64, c70, c73), Bx038 (c59, c67, c68), Bx051 (c77, c80, c89), Bx079 (c79, c92, c95)
C**A**ATGAAGAACGTAG**T**	*B. olivacea*	Bx094 (c1, c2, c4, c10, c12, c13, c15, c16)
CGA**A**GAAGAACGTAGC	*B. serpentinicola*	Bx029 (c56)
CGATGAAGAAC**A**TAGC	*B. crassifolia* , *B. gonoclada* ssp. *orientensis* , *B. pilosula* ssp. *pilosula*	Bx002 (c20, c23, c36, c37, c38), Bx146 (c36), Bx024 (c32)
CGATGAAGAACGTA**A**C	*B. gonoclada* ssp. *orientensis*	Bx146 (c33)
CGATGAAGAACGTAG**T**	*B. gonoclada* ssp. *orientensis* , *B. koehleri* , *B. shaferi*	Bx146 (c24, c26, c29, c34, c37), Bx055 (c59), Bx022 (c38)
CGATGAAGAAC**T**TAGC	*B. moana*	Bx018 (c1)
CGATGAAGAATGTACC	*B. hildebrandtii*	AF245415
CGATGAAGAA**T**GTAGC	*B. foliosa* , *B. moana* , *B. shaferi*	Bx140 (c58), Bx018 (c11), Bx004 (c39, c44, c46, c51, c53) Bx022 (c20, c21m c29, c30), Bx122 (c82, c89, c90, c92)
CGATGAAGAA**T**GTAG**T**	*B. sclerophylla*	Bx028 (c40B, c42B)
CGATGAAGAA**T**GTA**T**C	*B. glomerata* , *B. sclerophylla*	Bx052 (c25, c28, c29, c30, c37), Bx028 (c40, c42, c44B, c47)
CGATGAAGAA**TT**TAGC	*B. acuminata* , *B. bissei* , *B. glomerata* , *B. olivacea* , *B. shaferi* , *B. wrightii* ssp. *leonii* , *B. wrightii* ssp. *wrightii*	Bx005 (c58, c76), (c81, c83, c84, c85, c88, c95), Bx155 (c13), Bx157 (c33), Bx094 (c3, c9, c11, c14, c17 , c19), Bx022 (c24), Bx159 (c1, c2, c3, c5, c6, c7, c8, c12, c15), Bx158
CGATGAATAACGAAGC	*B. shaferi*	Bx122 (c91)
CGATGA**G**GAACGTAGC	*B. foliosa* , *B. gonoclada* ssp. *gonoclada*	Bx140 (c59, c75), Bx038 (c76)
CGATGA**G**GAA**T**GTAG**T**	*B. sclerophylla*	Bx028 (c39)
CGATGA**T**GAACGTAGC	*B. pilosula* ssp. *pilosula*	Bx024 (c31)
CG**G**TGAAGAACGTAGC	*B. gonoclada* ssp. *orientensis*	Bx146 (c23)
TGATGAAGAAC**A**TAGC	*B. crassifolia* , *B. glomerata*, *B. rotundifolia*	Bx002 (c28, c29, c32, c33), Bx155 (c15), Bx096 (c20, c28, c32, c36)
TGATGAAGAACGTA**AT**	*Styloceras brokawii*	AF245431
TGATGAAGAACGTAGC	*B. acuminata*	Bx005 (c60, c68, c69, c75)
TGATGAAGAA**T**GTAGC	*B. cristalensis* , *B. rotundifolia*	Bx026 (c26), Bx096 (c24, c25, c31, c34, c37)
TGATGAAGAA**TT**TAGC	*B. olivacea*	Bx023, Bx114

Chapter 5

Haplotype analysis of two species of *Buxus* of the "Shaferi"-clade

Contents

5.1 Introduction

The first molecular studies on plants based on plastid or nuclear markers aimed at answering phylogenetic questions at intrafamilial and infrageneric levels. Lately, the use of plastid or nuclear sequences in studies at the intraspecific and populations level increased (Roselló & al. 2007; Cavers & al. 2013; Rymer & al. 2013; Longo & al. 2014).

Comparative studies of haplotypes have proved to be useful in phylogeographic studies (Roselló & al. 2007; Longo & al. 2014), for testing biogeographic hypothesis and the existence of cryptic species (Cavers & al. 2013), to examine levels of genetic diversity and to evaluate the success of forestry and regeneration projects (Rymer & al. 2013), to reconsider the taxonomic status of relict species (Gugger & Cavender-Bares 2013) and to detect the occurrence of hybridization and introgression (Palme & al. 2004).

So far only few published investigations based on haplotypes have included samples of species distributed in Cuba. Gugger & Cavender-Bares (2013) carried out a comparative investigation based on haplotypes and morphological data and proved that the Cuban oak, *Quercus sagraeana*, should be considered an endemic species, which originated from migrations of *Quercus virginiana* from Florida to Cuba. Cavers & al. (2013) found that the Cuban *Cedrela odorata* is more closely related to northern Central American representatives of this species.

The only comparative study focused on geographically isolated populations of *Buxus* was carried out by Roselló & al (2007). These authors conducted a study on two species of European *Buxus* based functional ribotypes of ITS, and they found the existence of a phylogeographic split between western and eastern Mediterranean populations of *B. balearica*.

The highest species diversity of *Buxus* in Cuba occurs in the mountains of the northeastern region of the island. In localities like La Melba and Mina Iberia up to ten species have been reported (Köhler 2014). Several Cuban species of *Buxus* are only

known through one population of few individuals (e.g. *B. acunae*, *B. jaucoensis*, *B. serpentinicola*) and the most widespread taxa (e.g. *B. gonoclada* ssp. *gonoclada*, *B. glomerata*) are represented by populations that are isolated from one another, some of them located 100 to c. 350 km apart (see distribution maps in Köhler 2014). *B. shaferi* and *B. foliosa*, the species on which this study is focused, are endemic in the northeastern region of Cuba. *B. shaferi* is widespread in several localities of northeastern Cuba from mountains of Sierra del Cristal (west) to the mountains of Moa and Baracoa (east). This species inhabits xeromorphic thickets on serpentines, pine forests rainforests and riverine vegetation, from 600 to 1100 meters above sea level (masl). The distribution of *B. foliosa* is restricted to three nearby localities of the mountains of Moa and Baracoa. This species inhabits in riverine thickets on serpentines in the courses of rivers and brooks, from 300 to 900 masl (Figure 1.3 B of chapter 1; Köhler 2014).

A previous phylogenetic study based on the plastid markers *petD*, *trnL-F* and *trnK-matK* (Chapter 2), in which some species were represented by two or more samples from different localities, showed that some of them are not monophyletic. One of the most interesting examples of this is the case of *B. shaferi*, which is enclosed in the "Shaferi"-clade of the phylogenetic reconstruction based on plastid markers (Figure 2.1 of chapter 2). None of the four accessions included in the above mentioned phylogeny are placed together and on the contrary are closer related to other different species within the "Shaferi"-clade. Moreover, it is interesting that one accession of this species (Bx122) shares an identical plastid haplotype with ascensions of *B. foliosa* (Bx140, Bx141) and such haplotype is not shared by any other sample enclosed in the "Shaferi"-clade. All non-monophyletic ascensions of *B. shaferi* were collected in locations which are isolated from one another whereas the samples of *B. shaferi* (Bx122) and *B. foliosa* (Bx 140, Bx 141) were collected in the same locality, along the road to La Melba in the municipality of Moa, province of Holguín.

Another phylogenetic study based on the nuclear marker ITS in comparison with plastid based phylogenies (Chapter 4) showed incongruent topologies suggesting that the evolution of *Buxus* in Cuba could be driven by reticulation. In this previous study some evidences showed that a plausible explanation for at least a case of reticulation could be the occurrence of hybridization.

Borhidi (1996) mentioned the hybridogenic speciation among the factors with more influence in the process of speciation of the Cuban flora and López-Almirall (1998) suggested that in the flora of Cuba, species which are genetically very close related and spread on near distribution areas, could have been formed may be without efficient anti-crossing barriers, and so evolved complexes of species.

In this study populations or isolated plants of *B. shaferi* and *B. foliosa* from five localities of northeastern Cuba were included, in order to explore their diversity, their relationships and to discuss if any of the resulting patterns could be associated to the hypotheses about hybridization and evolution of species complexes (Borhidi 1996; López-Almirall 1998).

5.2 Materials and methods

The study is based on the plastid marker *trnK-matK* of 49 samples of the species *B. foliosa* and *B. shaferi*. The sampling relied on four populations, two of each species, and seven other samples belonging to sole plants of these two species from other localities were also included. The number of samples collected in each population varied from eight to 13. In the case of the populations corresponding with the codes Bx122, Bx141 and Bx169 all plants found in the populations were sampled, whereas in the case of Bx140, the number of plants sampled was set to 12 considering the number of plants sampled in the other three populations. The localities included in the sampling are shown in the Fig. 5.1 Data about taxa, number of samples per population, localities, herbaria and sequences are available in the appendix 5.1.

5.2.1 DNA isolation and sequencing

Total DNA was isolated from silica-gel-dried leaf tissue using the Nucleo Spin Plant II extraction kit (Macherey Nagel, Düren, Germany). The *trnK-matK* region was amplified and sequenced as explained in González-Gutiérrez & al. (2013) [Chapter 3].

5.2.2 Editing of sequences, alignment and analysis

The sequences were edited and manually aligned using PhyDE v.0 995 (Müller & al. 2007). All sequences were aligned without any difficulties and no regions were excluded from the final matrix. The haplotypes analysis was conducted with the software TCS v. 1.21 using statistical parsimony (Clement & al. 2000). This program has been conceived in order to estimate genealogical relationships among sequences. The input of the analysis was the final matrix in nexus (Maddison & al. 1997).

5.3 Results

A total of 43 samples yielded complete or almost complete *trnK-matK* sequences, but from other five only the first fragment of this region was generated resulting only partial sequences. The length of most of the complete sequences was in the range of 2425 to 2441 bases pairs (bp), without any relevant difference regarding length among species or samples of the same species. All 49 sequences, including the five partial, were included in the total alignment. The resulting aligned matrix had 2442 bp.

5.3.1 Statistical Parsimony Network

The software TCS calculated a 95% parsimony connection limit of 21 steps and generated a haplotype network of eight different haplotypes (Fig. 5.1). Other 18 intermediate haplotypes were inferred by the analysis. Five haplotypes are represented by one (H1, H4, H7, H8) or two samples (H6), whereas the other three haplotypes (H2, H3, H5) are characteristic of ten or more samples (Table 5.1).

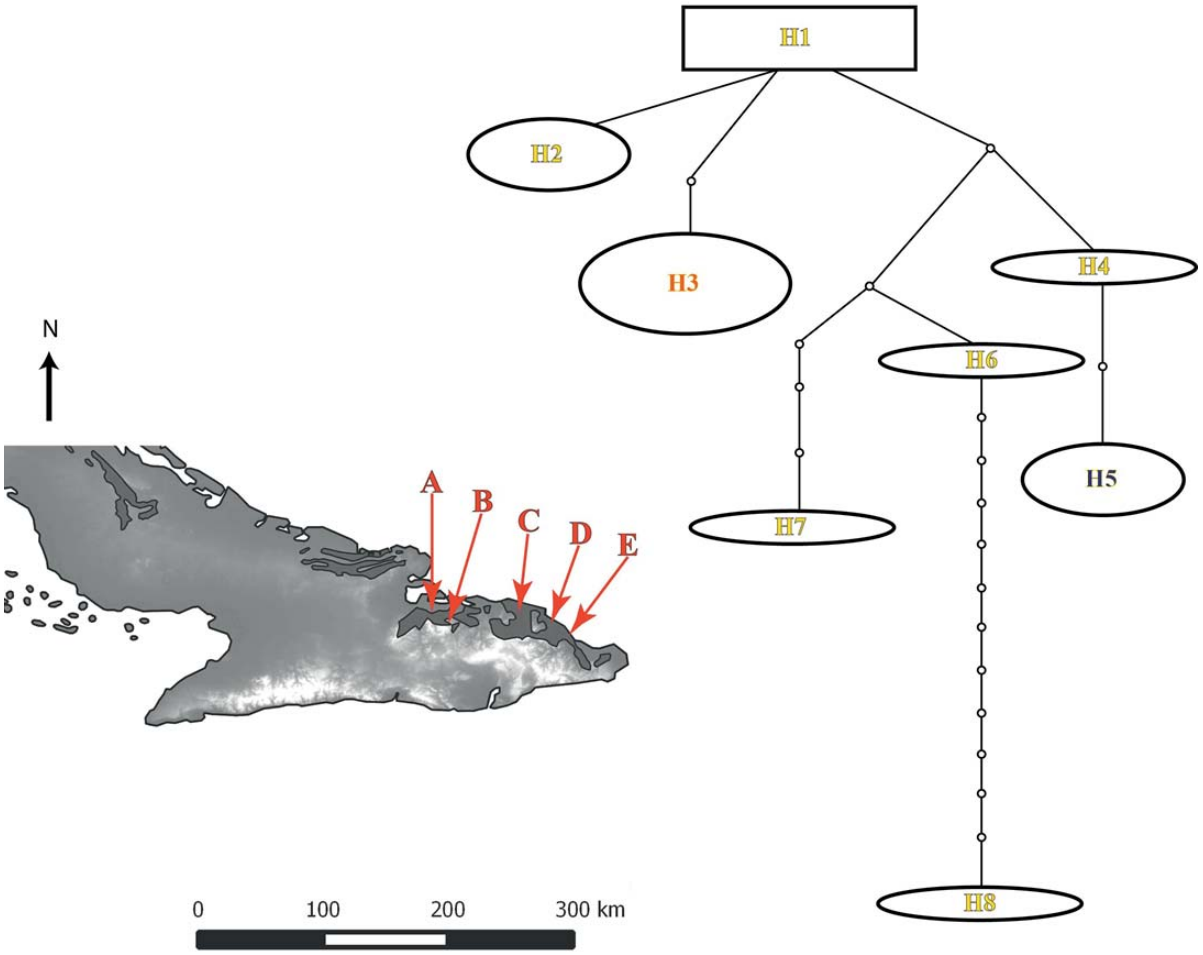


Fig. 5.1. Left: map of east Cuba with the localities sampled in this study. A-Pico Cristal northern slope, haplotypes H1 and H2; B- Pico Cristal, southern slope, haplotype H8; C- Moa, La Melba, haplotypes H3, H5 and H6; D- Baracoa, Alto de Iberia, haplotype H7; E- Baracoa, río Báez, haplotype H4. Right: TCS network of the eight *trnK-matK* haplotypes (H1-H8) found in the 49 samples of *B. foliosa* and *B. shaferi*. Haplotypes in in yellow belong to *B. shaferi*, in dark green to *B. foliosa* and in orange to both species.

Between the haplotype H8, only found in the sample Bx004 of *B. shaferi*, and its closest related haplotype (H6), 12 intermediate haplotypes were inferred by TCS, pointing that H8 is the most divergent haplotype found, followed by H7 with four intermediate inferred haplotypes between this and its closest related haplotype (H6) [Table 5.1, Fig. 5.1]. These two divergent haplotypes were found in plants from the southern slope of National Park Pico Cristal (Bx004) and Alto de Iberia (Bx047) [Fig. 5.1].

The remaining six haplotypes (H1, H2, H3, H4, H5, H6) are closest related to one another (Fig. 5.1) and are spread in the locality of La Zoilita in the northern slope of the National Park Pico Cristal (H1, H2), and in the localities of La Melba, Alto de Iberia and Río Báez (Fig. 5.1, Table 5.1). Among the eight different haplotypes found, six (H1, H2, H4, H6, H7, H8) are exclusive of the widespread species *B. shaferi*, one (H5) is exclusive of *B. foliosa* and one (H3) is shared by both species (Table 5.1, Fig. 5.1). The H3 shared by these two species has been detected only in the locality along the way to La Melba, between the kilometers 22 and 26, where the sampled populations (Bx122, Bx140, Bx141) of these two species do not overlap but are distant only by c. 500 m to c. 2 km.

Table. 5.1. Haplotypes found in the plastid marker *trnK-matK* of 49 samples of *B. foliosa* and *B. shaferi* collected in five localities of northeastern Cuba.

Haplotype	**Species (sample code)**	**Locality**
H1	*B. shaferi* (Bx170)	National Park Pico Cristal (**northern slope**).
H2	*B. shaferi* (Bx168; Bx169 a, b, c, d, e, f, g, h, i; Bx171)	National Park Pico Cristal (**northern slope**).
H3	*B. foliosa* (Bx140 a, b, d, e, f, g, h, j; Bx141 a, c, d), *B. shaferi* (Bx122 a, b, c, d, e, f, g, h, i, l, m).	Moa, La Melba, between kilometers 22, 26 and Las Comadres.
H4	*B. shaferi* (Bx022)	Baracoa, near river Báez.
H5	*B. foliosa* (Bx011; Bx140 c, i, k, l; Bx141 b, e, f, g, h)	Moa, La Melba, between kilometers 22, 26 and Las Comadres.
H6	*B. shaferi* (Bx122 j, k)	Moa, La Melba, between kilometers 22 and 26.
H7	*B. shaferi* (Bx047)	Baracoa: Alto de Iberia.
H8	*B. shaferi* (Bx004)	National Park Pico Cristal (**southern slope**).

5.4 Discussion

This is the first time that a haplotypes study is conducted on endemic Cuban species of the genus *Buxus*. The pattern of the haplotype network shows partial geographic affinities. Contradictorily the haplotypes of *B. shaferi* from the northern slope of National Park Cristal (H1, H2) are more related to the haplotypes of the same species and of *B. foliosa* from the easternmost sampled localities than to H8, characteristic of a *B. shaferi* sample collected in the same mountain massif, but from the southern slope. In spite of the poorly sampled southern slope of National Park Cristal it could be hypothesize that may be this pattern has been modeled by the factors that have been proposed as dispersers of plant groups in Cuba, such as extreme meteorological events like hurricanes (Borhidi 1996) and the intensive rains and overflows of rivers. Most of hurricanes in the Caribbean move from east to west and those facing a mountain massif in their trajectories loose strength (Anonymous 2010-2013). The massif of Sierra del Cristal with most of its areas belonging to the National Park Pico Cristal has the highest mountains in northeastern Cuba, the Pico Cristal has up to 1200 meters above the sea level (Anonymous 1989). Taking into account the ideas exposed above in the case of *B. shaferi* most likely the dispersal has happened from east to west and west to east than between the southern and northern slopes of high massifs like the case of Sierra del Cristal. This hypothesis is only supported by a unique sample from the southern slope of National Park Pico Cristal and further sampling is necessary in order to corroborate this pattern or to explore other of the inferred haplotypes between H8 and the rest of haplotypes.

The haplotypes H1 and H2 are characteristic of *B. shaferi* from the northern slope of National Park Cristal, of plants from a population (Bx169) or growing isolated from one another (Bx168, Bx170 and Bx171). This pattern could indicate that the dispersion of *B. shaferi* at least in this locality has been successful. During field work in localities of northern slope of National Park Pico Cristal was observed that populations and isolated plants of *B. shaferi* grow near to courses of water and small brooks which could contribute to the successful dispersal of this species.

An interesting fact found in this study is that the H3 is shared by 22 samples of three different populations of *B. foliosa* (Bx140, Bx141) and *B. shaferi* (Bx122), which grow relatively near to one another. A similar pattern of samples belonging to different species sharing identical haplotypes has found in an extensive study conducted in the genus *Hordeum* (Jakob & Blattner 2006). In this case, the authors concluded that it is due to persisting polymorphisms together with incomplete lineage sorting, although they state that cannot completely exclude that hybridization is contributing to the observed haplotype pattern in *Hordeum*. Other studies focused on haplotypes have found similar patterns, which have been associated with incomplete lineage sorting or hybridization (Scotti-Saintagne & al. 2013). In a study involving species of *Quercus* from Florida, Central America and Cuba, Gugger & Cavender-Bares (2013), state that shared chloroplast variation among species have been associated to chloroplast capture, but shared ancestral variation owing to incomplete lineage sorting cannot be ruled out.

Incomplete lineage sorting can be defined as the persistence of ancestral polymorphisms through speciation events (Jakob & Blattner 2006). If the coexistence of the same haplotype in populations of different species, in the case studied here, would have been only caused by this phenomenon it could be expectable the existence of the same haplotype (H3) in other samples of *B. shaferi* from other localities. However because the H3 is shared by the largest number of samples of *B. shaferi* and *B. foliosa* collected along the way to La Melba, and at the same time only two plants of *B. shaferi* (Bx122) have a different haplotype (H6) and ten plants of *B. foliosa* (Bx11, Bx140, Bx141) have also an exclusive haplotype (H5), may be the most plausible explanation for the existence of plants of these two different species with the same haplotype (H3) would be the occurrence of ancient hybridization with the corresponding chloroplast capture or introgression. Palme & al. (2004) found identical plastid haplotypes in different species of *Betula* from same geographic areas. According to these authors the causes could be convergence, ancestral polymorphism or hybridization/introgression. They concluded that in their study the most likely cause is the local hybridization because none of the haplotypes that are shared among species are found in different geographical regions in different species.

In these two species Cuban *Buxus*, if the hypothesis of ancient hybridization and chloroplast capture is correct it is not possible to identify the donor of the plastid DNA since plants from populations of both species have haplotypes (H5, H6) that are exclusive of each species. If hybridization happened between these two species, it should have happened in the last c. 4.8 [3.1–6.8] My, which is the time of divergence estimated for the "Shaferi"-clade (Fig. 2.5 of chapter 2). Within the hypothesis of the likely hybridization the idea of ancient instead recent hybridization makes more sense, since nowadays *B. shaferi* and *B. foliosa* are two well defined species, morphologically and ecologically, and no putative hybrids among them could have been detected during the preparation of the taxonomic treatment of *Buxus* for Cuba (Köhler 2014), neither during the recent field work. *B. shaferi* is a shrub that can reach c. 3 meters high, has ovate to oval leaves (Fig. 5.2 A) and grow thickets and forests on serpentines. *B. foliosa* plants are commonly not higher than 1 meter, they typically have linear-spatulate leaves (Fig. 5.2 B) and grow along rivers (Köhler, 2014). The pollination in *Buxus* is ambophilous, by wind or insects (Lázaro & Traveset 2005), or basically entomogamous (Köhler 2014). E. Köhler (personal communication) suggests that the pollinators of the Cuban species of *Buxus* are probably small insects with limited movement or displacement. During the field work insects such as *Apis mellifera*, ants and flies have been seen in the flowers of Cuban *Buxus* (Fig. 1.4 of chapter 1); nevertheless no insects have been observed in the flowers of *B. foliosa* and *B. shaferi*. If crossing pollination between *B. foliosa* and *B. shaferi* took place it could have been favored by insects or by wind, however the knowledge of the reproduction biology of Cuban *Buxus* is still insufficient.

The phylogenetic pattern observed in the "Shaferi"-clade (Fig. 2.1 of chapter 2) and the results obtained here could be indicators that the species enclosed in this clade could have hybridized. However it would be necessary a more extensive sampling including populations of *B. foliosa* and *B. shaferi* from other localities, and populations of other species of this clade, focusing in those taxa with sympatric populations, in order to find out if the existence of similar haplotypes in different species could be also a pattern present in other species of the "Shaferi"-clade.

Figure 5.2 A- leaves and fruits of *B. shaferi* (National Park Pico Cristal, Holguín, Cuba), B- leaves and flowers of *B. foliosa* (cultivated in National Botanical Garden, La Habana, Cuba). Scale bars = 1 cm. Photographs by P. A. González-Gutiérrez (A) and Rosa Rankin-Rodríguez (B).

Appendix 5.1 Taxa, samples codes, herbarium vouchers, localities and sequences data

Species name	Sample code	Number of samples	Herbarium voucher	Locality	*trnK-matK*
B. foliosa	Bx 011	1	R. Berazaín & al. HFC 74072 (HAJB)	Province Holguín, municipality Moa: camino a La Melba, alrededores del antiguo aserrío.	this study
B. foliosa	Bx 140	12	T. Borsch & al. 4315 (B, HAJB, ULV)	Province Holguín, municipality Moa: Entre los kilometros 22 y 26 del camino a La Melba.	this study
B. foliosa	Bx 141	8	M. Ackermann & al. 955 (B, HAJB, ULV)	Province Holguín, municipality Moa: entre el km 26 km y las Comadres, en el camino a La Melba.	this study
B. shaferi	Bx 004	1	J. Gutiérrez & al. HFC 75297 (HAJB)	Province Santiago de Cuba, municipality Segundo Frente: subida y firme del Pico Cristal (southern slope).	this study
B. shaferi	Bx 022	1	J. Gutiérrez & al. HFC 77598 (HAJB)	Province Guantánamo, municipality Baracoa: orillas del río Báez, km. 7 del camino a Mina Amores, entre Camarones y Los Naranjos.	this study
B. shaferi	Bx 047	1	P. A. González HFC 85879 (HAJB)	Province Guantánamo, municipality Baracoa: Alto de Iberia.	this study
B. shaferi	Bx 122	13	T. Borsch & al. 4319 (HAJB, B)	Province Holguín, municipality Moa: entre los kilometros 22 y 26 del camino a La Melba.	this study
B. shaferi	Bx 168	1	S. Fuentes & al. 474 (B, HAJB, PAL-Gr)	Province Holguín, municipality Mayarí: entre La Zoilita y Vega Fresca, Parque Nacional Pico Cristal (northern slope).	this study
B. shaferi	Bx 169	9	S. Fuentes & al. 587 (B, HAJB, PAL-Gr)	Province Holguín, municipality Mayarí: entre La Zoilita y Vega Fresca, Parque Nacional Pico Cristal (northern slope).	this study
B. shaferi	Bx 170	1	S. Fuentes & al. 606 (B, HAJB, PAL-Gr)	Province Holguín, municipality Mayarí: entre La Zoilita y Vega Fresca, Parque Nacional Pico Cristal (northern slope).	this study
B. shaferi	Bx 171	1	S. Fuentes & al. 613 (B, HAJB, PAL-Gr)	Province Holguín, municipality Mayarí: entre La Zoilita y Vega Fresca, Parque Nacional Pico Cristal (northern slope).	this study

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