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Comparative analysis of adipokinetic hormones and their receptors in Blattodea reveals novel patterns of gene evolution

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Abstract

Adipokinetic hormone (AKH) is a neuropeptide produced in the insect *corpora cardiaca* that plays an essential role in mobilising carbohydrates and lipids from the fat body to the haemolymph. AKH acts by binding to a rhodopsin-like G protein-coupled receptor (GPCR), the adipokinetic hormone receptor (AKHR). In this study, we tackle AKH ligand and receptor gene evolution as well as the evolutionary origins of AKH gene paralogues from the order Blattodea (termites and cockroaches). Phylogenetic analyses of AKH precursor sequences point to an ancient AKH gene duplication event in the common ancestor of Blaberoidea, yielding a new group of putative decapeptides. In total, 16 different AKH peptides from 90 species were obtained. Two octapeptides and seven putatively novel decapeptides are predicted for the first time. AKH receptor sequences from 18 species, spanning solitary cockroaches and subsocial wood roaches as well as lower and higher termites, were subsequently acquired using classical molecular methods and *in silico* approaches employing transcriptomic data. Aligned AKHR open reading frames revealed 7 highly conserved transmembrane regions, a typical arrangement for GPCRs. Phylogenetic analyses based on AKHR sequences support accepted relationships among termite, subsocial (*Cryptocercus* spp.) and solitary cockroach lineages to a large extent, while putative post-translational modification sites do not greatly differ between solitary and subsocial roaches and social termites. Our study provides important information not only for AKH and AKHR functional research but also for further analyses interested in their development as potential candidates for biorational pest control agents against invasive termites and cockroaches.

KEYWORDS
adipokinetic hormone, adipokinetic hormone receptor, 'green' pesticide, neuropeptide, termite

INTRODUCTION

The adipokinetic hormones (AKHs) of insects are well-investigated neuropeptides, synthesised and released from the *corpora cardiaca* (CC). The biologically active peptides belong to the well known AKH/RPCH (red pigment-concentrating hormone) family of peptides, of which more than 90 different mature AKHs have been identified by primary sequence or predicted from arthropods (Gäde et al., 1997; Gäde, 1997; Gäde, 2004; Gäde & Marco, 2009; Gäde et al., 2013; Gäde and Marco, 2022). In the biosynthesis and processing pathways in the CC, each AKH is derived from an individual preprohormone that is composed of a signal peptide and two potential peptides: the precursor

of the bioactive AKH and a structurally-unrelated peptide known as the AKH precursor-associated peptide, to which no biological activity has been assigned to date. Following cleavage from the preprohormone and post-translational modification, the mature AKH and the unrelated peptide are ready for release from storage/secretory granules (Van der Horst et al., 2001). Peptides of the AKH/RPCH family share typical features: at least 8 but no more than 10 amino acids in length, a blocked pyroGlu (pQ) N-terminus and a C-terminus blocked by amidation. The aromatic residues phenylalanine (Phe) or tyrosine (Tyr) occur at position 4 and tryptophan (Trp) at position 8 (Gäde, 1997; Gäde, 2004; Li et al., 2016). Investigated insect species may synthesise only one AKH peptide, whereas others produce from two to as many as five different AKH peptides in their CC (Gäde, 2009; Gäde et al., 2013; Marciniak et al., 2022; Marco & Gäde, 2019; Veenstra, 2014). However, the evolutionary origins and relationships between AKH gene paralogues in insects are poorly characterised.

It is a well-established fact that in insects, AKHs play a crucial role in the control of energy mobilisation in various physiological processes, such as in locomotory activity, starvation and stress response (Auerswald et al., 2005; Chino et al., 1989; Gäde, 2004; Gäde & Beenakkers, 1977; Gäde & Marco, 2013; Goldsworthy et al., 1975; Holwerda et al., 1977; Isabel et al., 2005; Kodrík, 2008; Kodrík et al., 2000; Marco & Gäde, 2017; Robinson & Goldsworthy, 1977; Sajwan et al., 2015; Stone et al., 1976; Tang et al., 2020). These physiological functions are mediated by adipokinetic hormone receptors (AKHRs), specifically, GPCRs related to vertebrate gonadotropin-releasing hormone (GnRH) receptors. During energy-demanding circumstances, AKHs are released from the CC into the haemolymph to reach the AKHRs expressed on fat body cells. AKHR binding and activation initiates a signal transduction cascade that causes the activation of either glycogen phosphorylase or triacylglycerol lipase, which in turn activates specific cellular pathways to release trehalose or diacylglycerol and free fatty acids from the fat body (Gäde & Auerswald, 2003; Gáliková et al., 2015). In 1998, an insect GPCR was cloned from *D. melanogaster*, and 4 years later, it was confirmed to be an AKH receptor (Hauser et al., 1998; Park et al., 2002; Staubli et al., 2002). The AKHR from the silkworm *Bombyx mori* was also identified and described in 2002 (Staubli et al., 2002). Since then, AKHRs have been described and some have been functionally studied from a wide variety of insect species and orders, such as *Anopheles gambiae*, *Sarcophaga crassipalpis*, *Glossina morsitans morsitans* and *Bactrocera dorsalis* in Diptera (Belmont et al., 2006; Bil et al., 2016; Caers et al., 2016; Hou et al., 2017; Kaufmann & Brown, 2006), *Manduca sexta* (Lepidoptera) (Ziegler et al., 2011), *Tribolium castaneum* (Coleoptera) (Li et al., 2008), *Periplaneta americana* and *Blattella germanica* (Blattodea) (Hansen et al., 2006; Huang et al., 2012; Wicher et al., 2006), *Schistocerca gregaria* and *Gryllus bimaculatus* (Orthoptera) (Jackson et al., 2019; Konuma et al., 2012), *Acyrthosiphon pisum* and *Rhodnius prolixus* (Hemiptera) (Alves-Bezerra et al., 2016; Jedlička et al., 2012; Zandawala et al., 2015) and *Carausius morosus* in Phasmatodea (Birgul Iyison et al., 2020).

Termites are a relatively small group of social insects consisting of more than 3000 species (Krishna et al., 2013), which were traditionally classified as the order Isoptera. More recent research demonstrates that termites appear as the sister clade to the *Cryptocercus* lineage of wood roaches, nesting within cockroaches and, thus, belong to the order Blattodea (Bourguignon et al., 2014; Evangelista et al., 2019; Inward et al., 2007; Klass et al., 2008; Krishna et al., 2013). Blattodea together with Mantodea (mantids) comprise the superorder Dictyoptera, which share a common recent ancestor (Ware et al., 2008).

The most recent study on the structural diversity of AKHs in 13 termite species (Jedličková et al., 2016), used molecular methods (seven species) and *in silico* bioinformatic searches (six species) from five families of Isoptera for phylogenetic analyses between termites and their closest cockroach relatives. These analyses were, however, carried out without sequence data of representatives from all termite families, and in the absence of data from wood roaches (*Cryptocercus*) since the AKH preprohormone sequence was not known at the time. In fact, at that time, only the mature AKH sequences were known from three termite species (Liebrich et al., 1995) and over 30 cockroach species (Gäde, 2009; Roth et al., 2009). Despite the short peptide sequences of AKHs, these have been useful in some insect orders, including cockroaches, to investigate phylogenetic trends and evolutionary relationships between insect lineages (Gäde, 1989; Gäde & Marco, 2005; Marco et al., 2020; Roth et al., 2009). However, the occurrence of multiple AKH genes, including within Blattodea, can hamper the interpretation of phylogenetic approaches (Bläser et al., 2020; Bläser & Predel, 2020), particularly when dealing with incomplete or missing data and insufficient taxon sampling. For example, while termites possess just one AKH gene, several cockroach species have 2 AKH peptides (Gäde, 2009; Gäde & Rinehart, 1990; Jedličková et al., 2016).

In contrast to the peptide ligands, only a few AKH receptor sequences are known from Blattodea to date. The AKHR of *P. americana* was the first to be identified from a hemimetabolous insect (Hansen et al., 2006). The AKHR of *Blattella germanica* was cloned in 2012 (Huang et al., 2012), while there are two putative AKHR sequences from termites available on NCBI: *Zootermopsis nevadensis* (XM_022082426.1) and *Cryptotermes secundus* (XM_033755069.1). There is, thus, a paucity of information regarding AKHR sequences of Blattodea. Receptor sequences are fundamentally important for further molecular and pharmacological characterisation of receptor-ligand systems, especially in the identification of potential methods to combat pest insect species.

In addition to representing a number of global pest species, cockroaches and termites play a crucial role in ecosystem services, including in the decomposition of deadwood (Bell et al., 2007; Bignell & Eggleton, 2000). Currently, researchers are considering the use of peptide mimetics (as so-called 'green' insecticides), based on the interaction of neuropeptide ligands with receptors, to negatively influence the physiology and/or behaviour of specific pest insects without harming beneficial insects (Altstein & Nässel, 2010; Gäde et al., 2017; Marco et al., 2018). RNAi represents an alternative potential strategy for pest control, enabling downregulation of specific gene targets via the host's RNAi pathway, whereby dsRNA is converted to small interfering RNA (siRNA), in turn, binding to the RNA-induced silencing complex (RISC), which then efficiently locates and eliminates target mRNA and reducing its translation into protein (Arakane et al., 2008;

TABLE 1 AKH peptides of termites and cockroaches. The taxonomic classification is per Evangelista et al. (2019) and Hellemans et al. (2022). Adipokinetic hormone nomenclature: Bladi-HrTH, *Blaberus discoidalis* hypertrehalosemic hormone; Emppe-AKH; *Empusa pennata* AKH; Manto-CC, Mantophasmatodea CC; Micvi-CC, *Microhodotermes viator* CC; Peram-CAH, *Periplaneta americana* cardioacceleratory hormone; Polae-HrTH, *Polyphaga aegyptiaca* hypertrehalosemic hormone; Pyrap-AKH, *Pyrrhocoris apterus* AKH; Tenmo-HrTH, *Tenebrio molitor* hypertreahlosemic hormone. For peptide source details; see Table S1.

Taxonomy sub−/family	Species	AKH name	AKH sequence	References
Blattellinae	*Lobopterella dimidiatipes*	Bladi-HrTH	pQVNFSPGWGTa	This study (source: 1KITE)
	Loboptera decipiens			Roth et al. (2009)
	Symploce sp., *Asiablatta kyotensis, Ischnoptera deropeltiformis, Anallacta methanoides*	Bladi-HrTH putative novel 1	pQVNFSPGWGTa pQLNFSPGWGVa	This study (source: 1KITE)
	Loboptera decipiens	Bladi-HrTH	pQVNFSPGWGTa	This study (source: 1KITE)
	Blattella germanica	Bladi-HrTH putative novel 2	pQVNFSPGWGTa pQLNFSPGWGPa	Gäde and Rinehart (1990); Veenstra and Camps (1990); This study (source: Sanger sequencing)
Nyctiborinae	*Nyctibora* sp.	Bladi-HrTH putative novel 3	pQVNFSPGWGTa pQVNFSPGWGVa	This study (source: 1KITE)
Blaberidae	*Aptera fusca, Archimandrita tessellate, Bantua robusta, Blaptica dubia, Blepharodera discoidalis, Diploptera punctata, Elliptorhina* sp., *Eublaberus distanti, Gyna caffrorum, Luchihormetica grossei, Panaesthia* sp., *Panchlora viridis, Princisia vanwaerebeki*			Roth et al. (2009)
	Blaberus discoidalis	Bladi-HrTH	pQVNFSPGWGTa	Hayes et al. (1986)
	Gromphadorhina portentosa, Leucophaea maderae			Gäde and Rinehart (1990)
	Nauphoeta cinerea			Gäde and Rinehart (1986)
	Panchlora nivea	Bladi-HrTH putative novel 4	pQVNFSPGWGTa pQLNFSPGWGTa	This study (source: 1KITE)
	Blaberus atropos	Bladi-HrTH putative novel 5	pQVNFSPGWGTa pQLNFSPGWGFa	This study (source: 1KITE)
	Princisia vanwaerebeki, Diploptera punctata	Bladi-HrTH	pQVNFSPGWGTa	This study (source: 1KITE)
Pseudophyllodromiinae	*Balta vilis, Ellipsidion* sp., *Supella longipalpa, Euthlastoblatta diaphana*	Bladi-HrTH putative novel 1	pQVNFSPGWGTa pQLNFSPGWGVa	This study (source: 1KITE)
	Sundablatta sexpunctata	Bladi-HrTH putative novel 6	pQVNFSPGWGTa pQINFSPGWGVa	This study (source: 1KITE)
	Cariblatta sp.	putative novel 7	pQINFSPGWGVa pQVNFSPGWGAa	This study (source: 1KITE)
Ectobiinae	*Ectobius sylvestris*	Bladi-HrTH putative novel 1	pQVNFSPGWGTa pQLNFSPGWGVa	This study (source: 1KITE)

Blaberoidea

(Continues)

TABLE 1 (Continued)

Taxonomy sub-/family			Species	AKH name	AKH sequence	References
Solumblattodea	Isoptera	Termitidae	Odontotermes formosanus, Macrotermes natalensis, Nasutitermes takasagoensis, Termes hospes			Jedličková et al. (2016)
			Trinervitermes trinervoides			Liebrich et al. (1995)
			Bulbitermes sp., Dicuspiditermes sp., Globitermes sp., Indotermes sp., Macrotermes subhyalinus, Pericapritermes sp., Promirotermes sp.			This study (source: He et al., 2021)
		Rhinotermitidae	Coptotermes formosanus, Coptotermes gestroi, Prorhinotermes simplex, Reticulitermes flavipes, Reticulitermes speratus	Peram-CAH-I	pQVNFSPNWa	Jedličková et al. (2016)
			Reticulitermes grassei, Prorhinotermes inopinatus			This study (source: He et al., 2021)
		Kalotermitidae	Neotermes castaneus			Jedlickova et al. (2016)
			Cryptotermes sp., Cryptotermes domesticus, Incisitermes marginipennis			This study (source: I. marginipennis: 1KITE; C. domesticus: He et al., 2021; Cryptotermes sp. Sanger sequencing)
			Kalotermes flavicollis	Manto-CC	pQVNFSPGWa	This study (source: Sanger sequencing)
		Archotermopsidae	Hodotermopsis sjöstedti, Zootermopsis nevadensis	Emppe-AKH	pQVNFTPNWa	Jedličková et al. (2016)
		Hodotermitidae	Microhodotermes viator	Micvi-CC	pQINFTPNWa	Liebrich et al. (1995)
		Mastotermitidae	Mastotermes darwiniensis	Peram-CAH-I	pQVNFSPNWa	Liebrich et al. (1995); Jedličková et al. (2016)
	Cryptocercidae		Cryptocercus darwini			Roth et al. (2009)
			Cryptocercus punctulatus			Gäde et al. (1997)
			Cryptocercus pudacoensis, Cryptocercus meridianus	Tenmo-HrTH	pQLNFSPNWa	This study (source: He et al., 2021)
	Lamproblattidae		Lamproblatta albipalpus	Peram-CAH-I	pQVNFSPNWa	This study (source: 1KITE)
	Blattidae		Blatta orientalis			Gäde and Rinehart (1990)
			Brinckia hanstroemi, Celatoblatta sp., Deropeltis erythrocephala, Eurycotis floridana, Neostylopyga rhombifolia, Pseudoderopeltis foveolate, Shelfordella lateralis			Predel and Gäde (2005)
			Periplaneta americana	Peram-CAH-I Peram-CAH-II	pQVNFSPNWa pQLTFTPNWa	Scarborough et al. (1984); Witten et al. (1984); Zeng et al. (2020)
			Shelfordella lateralis, Eurycotis floridana, Methana parva			This study (source: 1KITE)

TABLE 1 (Continued)

Taxonomy sub−/family	Species	AKH name	AKH sequence	References
Tryonicidae	*Tryonicus parvus*	Emppe-AKH Polae-HrTH	pQINFTPNWa pQITFTPNWa	This study (source: 1KITE)
Corydiidae	*Polyphaga aegyptiaca*	Tenmo-HrTH Polae-HrTH	pQLNFSPNWa pQITFTPNWa	Gäde and Kellner (1992)
	Ergaula capucina			König et al. (2005)
	Therea petiveriana	Tenmo-HrTH	pQLNFSPNWa	Gäde et al. (1997)
	Therea bernhardti			This study (source: 1KITE)
	Eucorydia yasumatsui	Pyrap-AKH	pQLNFTPNWa	This study (source: 1KITE)
	Tivia sp.	Peram-CAH-I	pQVNFSPNWa	This study (source: 1KITE)
Nocticolidae	*Nocticola* sp.	Peram-CAH-I	pQVNFSPNWa	This study (source: 1KITE)

Burand & Hunter, 2013; Kapan et al., 2012; Lee et al., 2011; Park et al., 2014; Rewitz et al., 2009; Yu et al., 2023). For such applications, it is imperative to know peptide and receptor sequences and structures in as many pest species as possible, as well as in non-pest species to evaluate the potential collateral damage or success of sequence-based green insecticides.

With the current study, we employ bioinformatic and molecular approaches to characterise AKH precursor sequences and cognate AKHR sequences from a wide diversity of termite and cockroach species, including wood roaches. We combine recently sequenced transcriptomic data with publicly available Sequence Read Archive (SRA) data and classical molecular approaches to conduct a comprehensive sequence analysis of AKH and AKHR in termites and their nearest cockroach relatives. Our aims are to investigate the evolution of AKH peptides and their receptors in Blattodea, as well as resolve the evolutionary origins and relationships of AKH gene duplications in this group. Additionally, we enhance termite AKH and AKHR datasets for comparative analysis, carry out an assessment of patterns of post-translational modification during the evolution of termite sociality and establish a data framework for research aiming to exploit AKH and AKHRs as potential targets for 'green' pesticide development against invasive termites and cockroaches, or for possible RNAi interventions.

RESULTS

AKHs in termites and cockroaches

From the complete list of AKH mature peptides derived from 90 species (Table 1), we were able to obtain sequence information for 85 partial or complete AKH precursors from a total of 62 species spanning diverse families across the order Blattodea. The information was collated from pre-existing (published) data as well as from transcriptome and SRA data and bioinformatic searches of databases, as well as via Sanger sequencing in selected cases to confirm the *in silico* findings. The aligned AKH preprohormone sequences of these species demonstrate that both signal peptides and AKH-associated peptides are conserved at the amino acid level (Figure 1). Precursor sequences in cockroaches, especially for those species in the same family (Table 1), show high levels of sequence conservation, such as only two substitutions between AKH1 of *Shelfordella lateralis* and AKH1 of *P. americana*, or five substitutions between AKH2 of *A. kyotensis* and AKH2 of *S. sexpunctata*.

The mature peptide for each species was classified based on amino acid sequence identity to known AKHs and categorised based on taxonomy. Our new sequence data are compared alongside previously published findings in Table 1. A total of 16 AKH primary sequences from 90 species were identified. Table 1 shows that all predicted bioactive mature peptides consist of 8 or 10 amino acids. In Blaberoidea, only decapeptides were found, with Bladi-HrTH being the most frequently detected peptide (Table 1, Figure 2), which was

found in all species except *Cariblatta* sp. (where two putative novel decapeptides are identified). Further to this, we identified seven putative novel decapeptides in Blaberoidea via *in silico* characterisation.

The presence of the putative novel 2 type in *Blattella germanica* was confirmed via Sanger sequencing. By contrast, species in Solumblattodea (Blattoidea + Corydioidea) contained only octapeptides. The
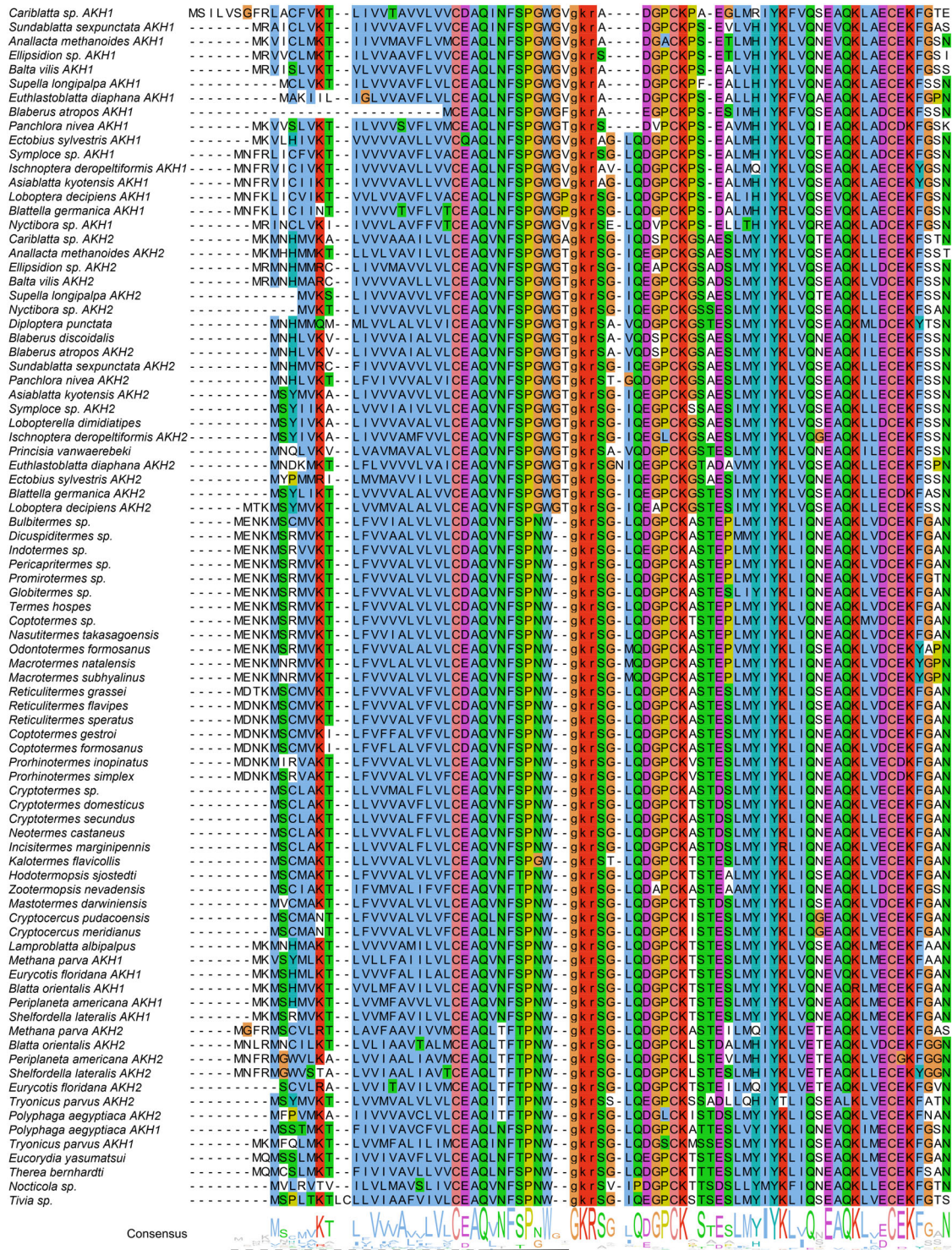

FIGURE 1 Multiple sequence alignment of 85 termite and cockroach Adipokinetic hormone (AKH) preprohormones from 62 species. The sequence logo beneath depicts the consensus sequence and the solid line under the sequence logo identifies the AKH bioactive peptide. The sequence before the bioactive peptide is referred to as the signal peptide (dashed line), while the sequence following is the AKH-associated peptide. Putative amidation and dibasic cleavage sites are indicated in lowercase based on known homologous precursors in other species. Available sequence accession codes are listed in Data S1.

most frequently detected AKH in Solumblattodea was Peram-CAH-I (Figure 2), which occurs not only in most termite families such as Termitidae, Rhinotermitidae, Mastotermitidae and Kalotermitidae but also in the cockroach family Lamproblattidae, Blattidae, Corydiidae and Nocticolidae. Two AKH octapeptides were recorded in some cockroaches: Peram-CAH-I and Peram-CAH-II in Blattidae, Tenmo-HrTH and Polae-HrTH in Corydiidae (*Polyphaga aegyptiaca*) and Emppe-AKH + Polae-HrTH in Tryonicidae (*Tryonicus parvus*). Micvi-CC is exclusively found in the Hodotermitidae (Table 1). The sister group to the termites, the wood roaches (genus *Cryptocercus*) possess a Tenmo-HrTH peptide, which differs only in the occurrence of leucine at position 2 in comparison to valine in Peram-CAH-I. Interestingly, a unique AKH peptide, Manto-CC, in *K. flavicollis* (Kalotermitidae) was identified using Sanger sequencing in this study and further confirmed and validated by mass spectrometry (Marco et al., 2022) for the first time in cockroaches and termites, with a structure that differs from Peram-CAH-I at position 7 by a glycine/asparagine substitution. The other unique octapeptide, Pyrap-AKH in *Eucorydia yasumatsui* (Corydiidae), was found for the first time in Blattodea, with a structure that differs from Tenmo-HrTH at position 5 by a serine/threonine substitution (Table 1).

Phylogenetic analysis of AKH in termites and cockroaches

Phylogenetic trees obtained from two different methods, RAxML and MrBayes, rooted between the major Blattodea clades: Blaberoidea and Solumblattodea (Blattoidea + Corydioidea) (Figure 3, Figure S1) support an AKH duplication event in the ancestor of Blaberoidea; leading to a

Bladi-HrTH decapeptide clade (except *Cariblatta* sp., which possesses a novel decapeptide, although this still groups with high support in the Bladi-HrTH clade) and a clade of novel putative decapeptides. Node support for this hypothetical duplication event is high when using nucleotide data and slightly less so when employing amino acid sequences. Termite AKHs nested paraphyletically within Solumblattodea sequences, with limited bootstrap support at deeper nodes in this clade for both nucleotide and amino acid trees. In terms of sequence evolution, termitid plus *Reticulitermes* and *Coptotermes* AKHs appear to diverge from the remaining lower termite and cockroach sequences.

The relationships between Corydiidae + Nocticolidea (Corydioidea), Blattidae and other lineages within Solumblattodea are generally equivocal. The nucleotide phylogeny suggests that a parallel AKH duplication is likely to have taken place in Blattidae, although this receives limited statistical support in both ML and BI approaches and so should be interpreted with a degree of caution.

AKHRs in termites and cockroaches

A total of 18 new AKH receptor sequences were retrieved bioinformatically and/or sequenced using a classical molecular approach. We aligned the sequences for these 18 species and for the other 4 available species to obtain a better understanding of amino acid variation among blattid and termite AKHR sequences (Figure 4). The ORF (open reading frame) of all termite and cockroach AKHRs contains sequences of between 405 and 467 amino acid residues in length. Except for *Cryptotermes* sp. and *C. secundus*, the shortest amino acid sequences belong to the Blattellinae subfamily: *B. germanica*, *Symploce* sp., *I. deropeltiformis*, *A. kyotensis* and
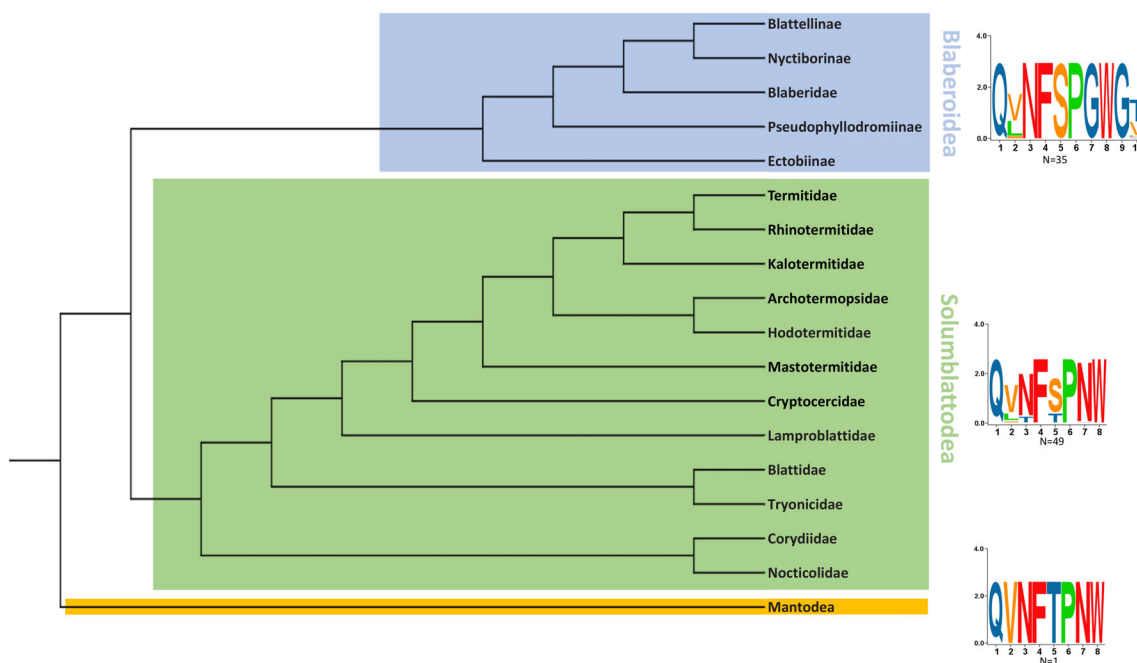

FIGURE 2 Simplified phylogenetic scheme of Blattodea modified from Evangelista et al. (2019) and Hellemans et al. (2022). The sequence logo represents the degree of conservation of amino acids in Adipokinetic hormone (AKH) neuropeptides in Blaberoidea and Solumblattodea. N: number of AKH neuropeptide sequences. Mantodea AKH (Emppe-AKH; Gäde and Marco (2017)) is used as an outgroup.
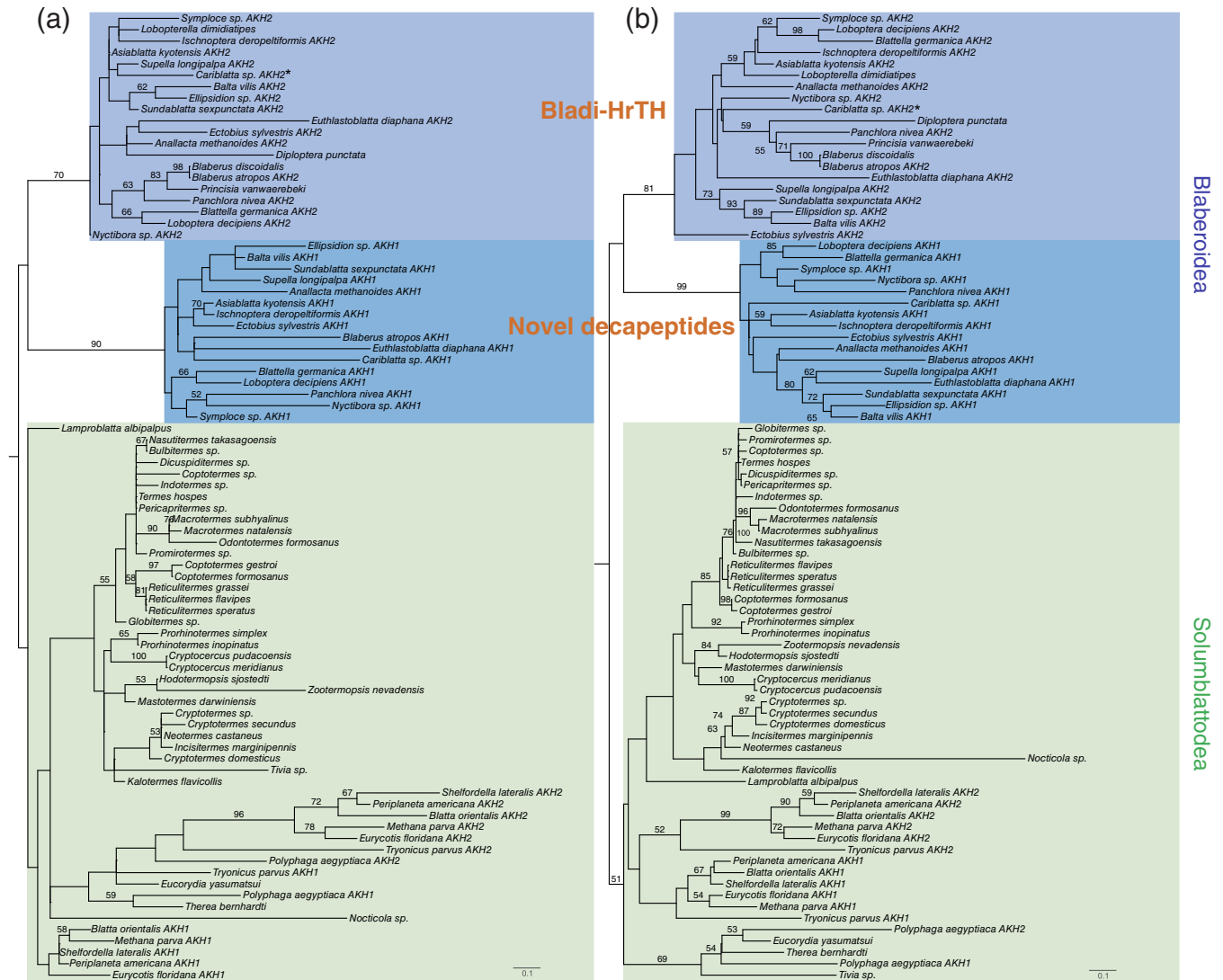
FIGURE 3 ML phylogenetic trees constructed from AKH preprohormones from termites and cockroaches. Trees were reconstructed from alignments of the whole ORF sequence, using amino acids (a) or nucleotides (b). Numbers at each node represent bootstrap support values (in percent), with only values above 50 being shown. The putative novel 7 decapeptide of *Cariblatta* sp. AKH2 (*) grouped with Bladi-HrTH. For comparison, equivalent BI trees are shown in Figure S1.

P. couloniana. The results derived from the aligned sequences demonstrate that termite and cockroach AKH receptors share highly conserved sequence elements, particularly in the seven transmembrane domains (TMs), which corresponds to the typical arrangement found in G-protein-coupled receptors (TM1-TM7 highlighted in Figure 4). Sequence conservation is especially high in TM2, TM6 and TM7, although moderate levels of sequence conservation could be detected across most of the ORF, except close to the N- and particularly the C-termini.

We also compared pairwise identity and similarity at the amino acid level of the AKHRs identified to date of the full 22 Blattodea species (Figure 5). Identities and similarities range from 64.0% to 99.6% and from 68.4% to 99.6%, respectively. Notable levels of identity and similarity include comparisons between solitary cockroaches (Blattidae + Blattellinae) and wood-feeding cockroaches (*Cryptocercus*): 71.6%–81.5%, whereas between solitary

cockroaches and higher termites (Termitidae), identity and similarity values range from 64.0% to 76.0%. Termitidae and *Cryptocercus* are separated by intermediate levels (71.3%–79.0%) of amino acid identity and similarity. AKHR identity and similarity within kalotermitids and rhinotermitids range from 93.6% to 99.3% and 92.2% to 99.6%, respectively, whereas between these families, values range from 81.1% to 88.2%. All other pairwise comparisons are shown in Figure 5.

Phylogenetic analysis of AKHRs in termites and cockroaches

Phylogenetic reconstructions of 22 AKHR sequences are depicted in Figure 6. The evolution of AKHR largely reflects the accepted view of termites and cockroach lineage diversification (Figure 6).

FIGURE 4 Comparative amino acid sequence alignment of termite and cockroach AKHR sequences. Intracellular (ICL) and extracellular (ECL) loops, N-terminus and C-terminus are labelled above the alignment. Transmembrane domains 1–7 are highlighted in grey. Blue and red lines under the alignment show putatively conserved myristoylation and glycosylation sites, respectively.

The topologies of the ML and BI phylogenetic trees are identical. Five AKHRs from Blaberidae cluster together as a monophyletic group. *B. orientalis* and *P. americana* in the Blattidae family are closely clustered with high support and *Cryptocercus* occurs as the nearest lineage to the termites. Within the termites, *Mastotermes*, *Zootermopsis* and Kalotermitidae occur in expected positions in the phylogenies, as do the AKHR sequences belonging to the Neoisoptera (Rhinotermitidae + Termitidae). The rhinotermitids *Reticulitermes* and *Coptotermes* are paraphyletic with respect to Termitidae (instead of appearing as a monophyletic clade). But overall the evolution of AKHR in termites and cockroaches mostly reflects the accepted view of termite lineage diversification and the majority of ancestral nodes receive unequivocal support.

Predicted AKHR post-translational modifications

Post-translational modifications (PTMs) and their potential interactions contribute significantly to the biological functions of proteins, and GPCRs are an important class of proteins that are regulated by PTMs. To investigate the putative PTMs of AKHRs in termites, three modifications, glycosylation, myristoylation and phosphorylation were explored. The results reveal that all 22 species contain putative glycosylation sites in at least two domains (in the ECL1 and C-terminus, Figure 7). Among them, cockroaches in the Blattidae family (*B. orientalis* and *P. americana*) have the most widely distributed glycosylation sites, being found in the following domains: N-terminus, TM1, ECL1, ECL2, ICL3 and the C-terminus.

Two subsocial cockroaches in the genus *Cryptocercus* (*C. pudacoensis* and *C. meridianus*) and in Archotermopsidae (*Z. nevadensis*) have five glycosylation domains in the TM1, ECL1, ECL2, ICL3 and C-terminus. By contrast, *C. formosanus* contains two (ECL1 and C-terminus) and *Indotermes* sp. only three (ECL1, ECL2 and C- terminus). All 22 species contain one glycosylation site in the ECL1. In addition, all cockroach species, except *Symploce* sp. and *Z. nevadensis* contain one glycosylation site in ICL3 (Figure 7a).

In terms of myristoylation sites, all AKHR sequences have at least two putative domains in TM3 and the C-terminus. In addition, *Crypto-termes* sp. and *C. secundus* (Kalotermitidae) contain an other site localised in the N-terminus, while *C. pudacoensis*, *C. meridianus* and *Z. nevadensis* (*Cryptocercus* + *Archotermopsidae*) contain a further myristoylation site in the TM5 (Figure 7b).

Three putative phosphorylation sites (serine, threonine and Tyr residues) were quantified in the intracellular domains. In ICL1, two sites in *B. orientalis* and *P. americana* (Blattidae), *N. castaneus*,
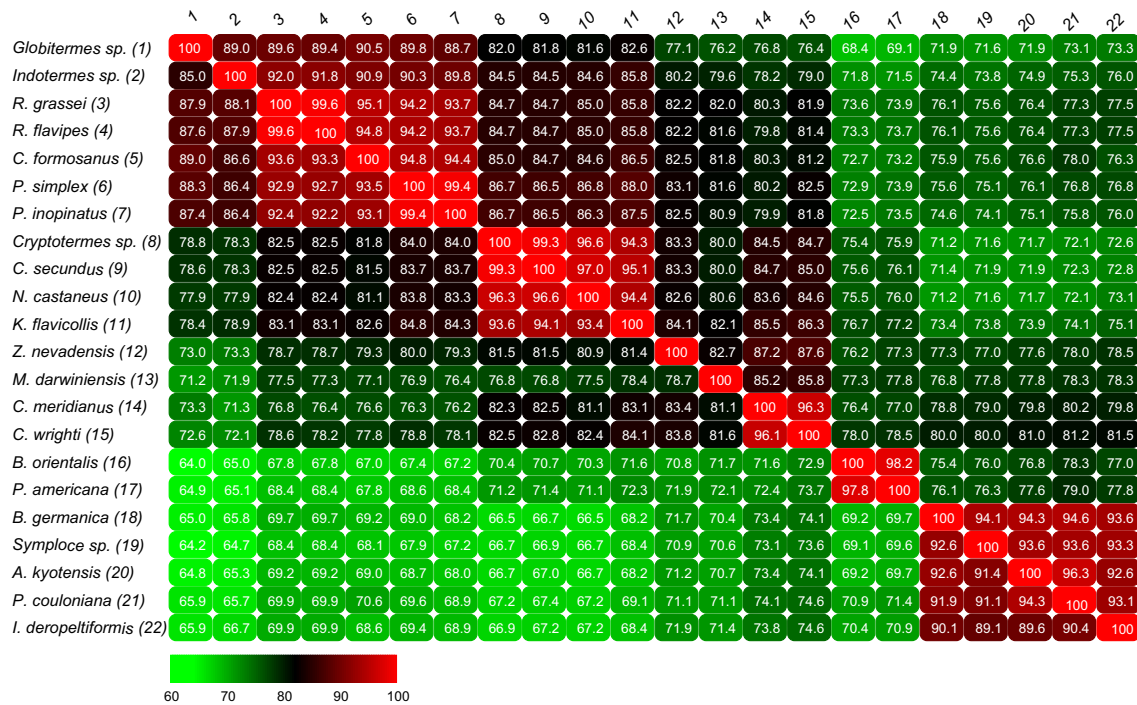

FIGURE 5 Pairwise comparison (%) of amino acid identity (lower triangular) and similarity (upper triangular) from 22 AKHRs of termites and cockroaches.
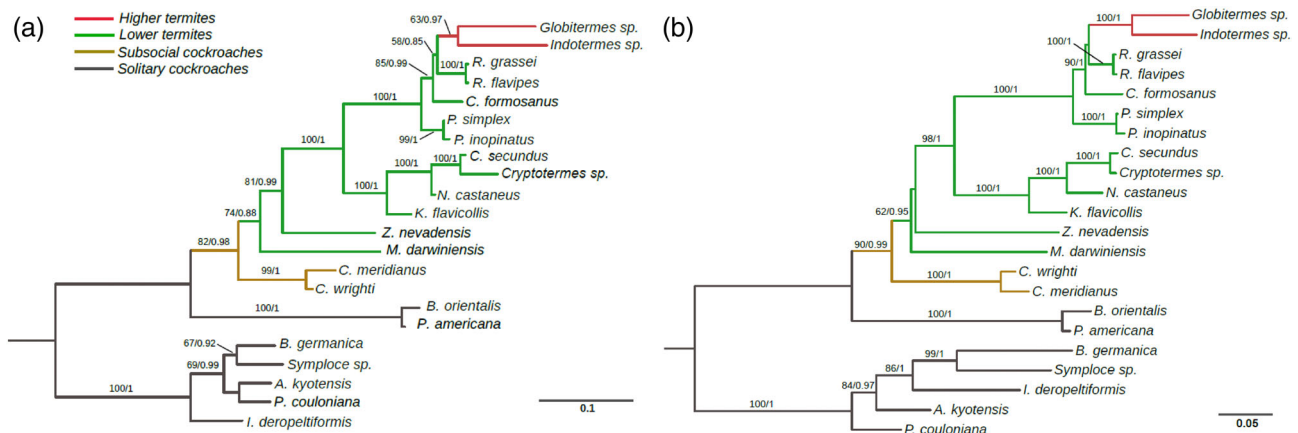

FIGURE 6 Phylogenetic reconstruction of adipokinetic hormone receptors (AKHRs) in termites and cockroaches using amino acids (a) or nucleotides (b). Numbers at each node represent bootstrap support values/posterior probabilities (ML/BI respectively). Different colours indicate traditional classifications of termites and cockroaches (Evangelista et al., 2019). Bootstrap support/posterior probability values above 50/0.8 are displayed.
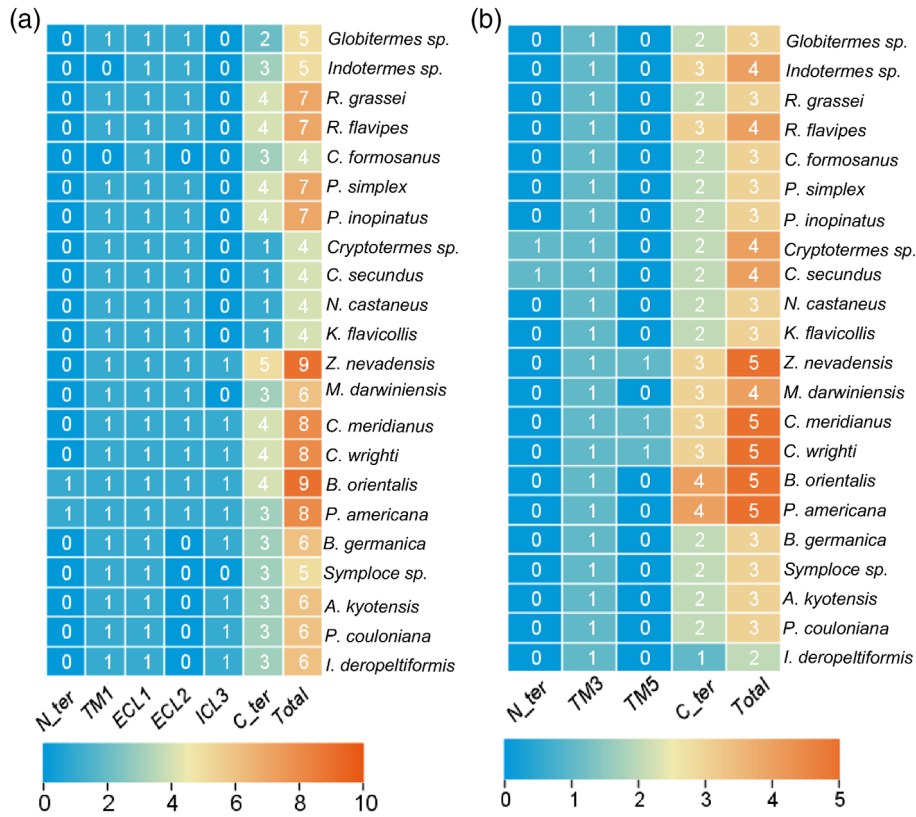
FIGURE 7 Distribution of putative glycosylation (a) and myristoylation sites (b) from AKHRs in 22 blattodean species (13 termite and 9 cockroach species). C_ter, C-terminus; ECL, Extracellular loop; ICL, Intracellular loop; N_ter, N-terminus.
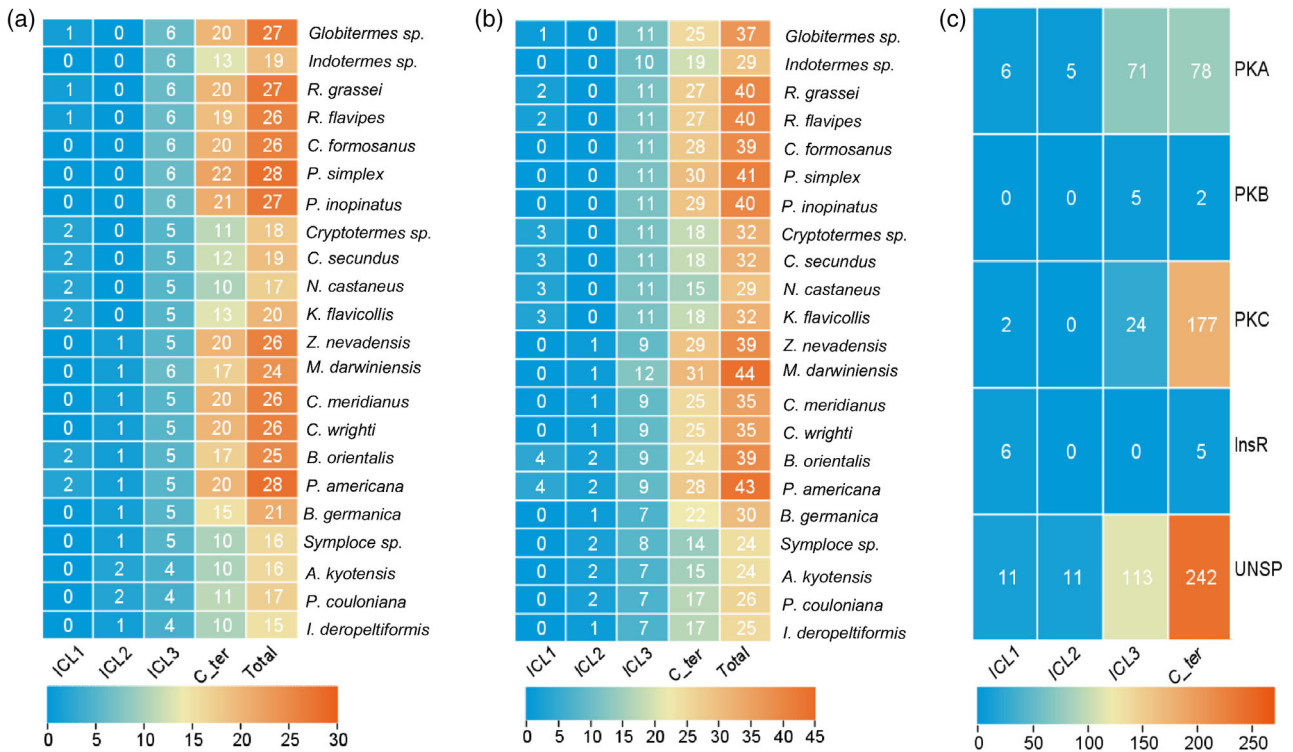

FIGURE 8 Phosphorylation features of cockroach and termite AKHR intracellular domains. (a) distribution of phosphorylation sites (Ser, Thr and Tyr); (b) varieties of putative protein kinase phosphorylation sites and (c) their distribution. C_ter, C-terminus; ICL, Intracellular loop; InsR, Insulin receptor; PKA, Protein kinase A; PKB, Protein kinase B; PKC, Protein kinase C; UNSP, Unspecified kinase.

C. secundus, Cryptotermes sp. and *K. flavicollis* (Kalotermitidae) were detected. One site was found in *R. grassei* and *R. flavipes* (Rhinotermitidae) and *Globitermes* sp. (Termitidae). In ICL2, two sites were found for *A. kyotensis* and *P. couloniana*, and one site was detected in *B. orientalis, P. americana, B. germanica, I. deropeltiformis, C. pudacoensis, C. meridianus, M. darwiniensis* and *Z. nevadensis*. In ICL3, four or five sites in all roach species were recovered, as well as in *Z. nevadensis* and all kalotermitid species, while other termites possess six sites. Regarding the C-terminus, *P. simplex* contains the most sites, followed by *P. inopinatus*. With respect to putative kinase phosphorylation sites, *M. darwiniensis* has the highest number in the ICL and in the C-terminus (Figure 8a, b).

Further analysis of specific kinases indicates that putative PKA and UNSP sites are distributed among three ICL domains and the C-terminus. Most protein kinase phosphorylation sites were found in the C-terminus, while PKB, PKC and InsR were restricted to two or three ICL domains. Termite species possess up to 4 additional protein kinase phosphorylation sites (except *Z. nevadensis*) in the ICL3 compared with wood and solitary cockroaches (Figure 8c, Table S3), while they have lost 1 such site in the ICL 2 (except *M. darwiniensis* and *Z. nevadensis*) compared to wood and solitary cockroaches. Otherwise, PKA, PKC and UNSP sites were regularly predicted to occur in both the ICL3 and the C-terminus of all 22 species, indicating their potentially conserved role in intracellular signalling among blattodean AKHRs.

DISCUSSION

We present 16 structurally different mature AKH peptides from 90 species distributed across a wide range of cockroach and termite families. Evolutionary trees based on phylogenetic analyses using AKH precursor sequences reveal a new family of putative decapeptides in Blaberoidea. Phylogenetic trees indicate that an ancient duplication of AKH genes occurred in the ancestor of this group, yielding the previously described Bladi-HrTH on the one hand, and a group of novel decapeptides on the other. The decapeptide AKH, Bladi-HrTH, was until now the only decapeptide to have been reported from Blattodea (Gäde & Rinehart Jr, 1986; Hayes et al., 1986). Our study identifies seven putative novel decapeptides, significantly increasing the known diversity of decapeptides in Blattodea. The remaining AKHs of Solumblattodea are found to be solely octapeptides in nature, with Peram-CAH-I (pQVNFSPNWamide) accounting for the largest proportion in this clade. Further species with two AKH peptides were also identified in Blattidae, Tryonicidae and Corydiidae. Phylogenies suggest that at least one parallel duplication event also took place at some point during the evolution of the Solumblattodea, although statistical support is low and inconsistently retrieved between nucleotide and amino acid-based analyses.

The presence of more than one mature AKH in a single species may indicate that each AKH plays a role in different regulatory processes (Bártů et al., 2010; Goldsworthy et al., 1997; Kaufmann & Brown, 2008). Indeed, the retention of both AKH genes in many extant species found across Blaberoidea suggests that both peptides serve an important adaptive purpose, potentially serving different physiological functions in these insects. While the functional significance of AKH peptide duplicates in Blaberoidea, and potentially also Solumblattodea, remain under investigation, we hypothesise that these may be related to the capacity of cockroaches to live in diverse and challenging niches, where adaptive responses to diverse pathogens, toxins or fluctuating environments may have necessitated important alterations to AKH-mediated energy, stress or starvation responses.

Multiple AKHs have been reported in other insects, such as one decapeptide and two octapeptides in the migratory locust, *Locusta migratoria* (Oudejans et al., 1991; Siegert et al., 1985; Stone et al., 1976). One octapeptide and two decapeptides are also described from the African froghopper, *Ptyelus flavescens* (Gäde et al., 2017), while certain grasshoppers have three octapeptides (Gäde, 2006) and even five mature peptides (octa-, nona- and decapeptides) have been isolated and sequenced from two species of the lepidopteran genus *Hippotion* (Gäde et al., 2013). We also confirm the presence of Manto-CC in termites, being previously only recorded from the order Mantophasmatodea (Gäde et al., 2005) and also recently verified by mass spectrometry in the termite *K. flavicollis* (Marco et al., 2022). Another surprising discovery from the current work is that Pyrap-AKH is also present in cockroaches, where previously it had been found in Hemiptera (Kodrík et al., 2000; Gäde and Marco, 2022), Orthoptera (Caelifera) (Gäde, 2006) and in some beetle species (Gäde et al., 2019).

A study by Evangelista et al. (2019) provides a recent phylogenomic analysis of Blattodea, while a further study analysing 17 neuropeptide precursor sequences (AKH precursor sequences were not included) revealed consistent topologies (Bläser et al., 2020). Our evolutionary trees based on AKHR genes were broadly in agreement with accepted topologies. An interesting question to ponder here is how to interpret divergent versus conserved patterns of ligand and receptor gene evolution, respectively. Multiple ligand binding may be permitted by the availability of large AKHR binding pockets which could facilitate promiscuous receptor-ligand interactions (Marchal et al., 2018; Marco & Gäde, 2019; Rios et al., 2001; Schwartz, 1994; Stank et al., 2016; Venkatakrishnan et al., 2013; Zhu et al., 2009). A recent study by Jackson et al. (2019) determined the structure of three AKHs in the desert locust, *Schistocerca gregaria* using NMR (Nuclear magnetic resonance) techniques, finding that they interact with the same receptor residues despite having varying chain lengths and sequences. Such flexibility could depend on the receptor's ligand affinity properties, the intrinsic stability of receptor states and long-range allosteric coupling dynamics between the binding pocket and receptor regions residing on the cytoplasmic side (Chen et al., 2020; Cong et al., 2022; Möller et al., 2001; Xia et al., 2022).

We next chose to examine predicted post-translational modification signatures in AKHR. We explored this by characterising putative glycosylation, myristoylation and phosphorylation sites in corresponding TM regions, as well as intra- and extracellular domains of the AKHR amino acid sequence. A principal aim of this analysis was to explore whether patterns of post-translational modification differed between

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social termites and their solitary cockroach relatives. There are some patterns of note, such as 1 fewer and up to 4 additional protein kinase phosphorylation sites in the ICL2 and ICL3 of termites, compared with cockroaches. Neoisopteran sequences also appear to possess an additional predicted PKA site and an additional UNSP site in ICL3 compared with other termites and cockroaches. Kalotermitids (dry wood termites) appear to have fewer putative sites of modifications in general, which may reflect a truncated C-terminus in these species. A study by Yang et al. (2018) implied potential differences in post-translational modification of AKHRs between solitary and social bees. Aside from the minor changes outlined above, our study did not detect obvious phosphorylation site pattern differences between social termites and subsocial or solitary cockroaches. This is perhaps surprising, given the important role of post-translational modification in diverse functions including protein interaction and stability, signalling, β-arrestin recruitment and receptor trafficking (Chou, 2020; Withers & Dong, 2017).

Bioactive neuropeptides and their GPCRs in insects are under investigation for their potential use as a more environmentally friendly alternative to conventional pesticides. This concept of a 'green insecticide' is based on the potentially disruptive consequences of interference at the level of neuropeptide-cognate receptor binding, as discussed elsewhere (Gäde et al., 2017; Gäde & Goldsworthy, 2010; Whetstone & Hammock, 2007). One of the first steps towards identifying such a lead for the chemical development of an AKH peptide mimetic with which to disrupt normal endocrine signalling in crucial metabolic pathways in so-called pest insects is to ascertain the complement of AKHs in insects and to establish whether cross-activity of a ligand is possible also in other insects.

In the case of Blattodea, cockroaches and termites are ecologically important detritivores, especially in the subtropical and tropical regions where they recycle wood and plant matter and represent a major driver of carbon cycles (Bignell, 2019; Ulyshen, 2016). Nevertheless, approximately 10% of termite species are considered severe pest insects where they infect and feed on dry timber in man-made structures and are very costly to combat worldwide (Khan & Ahmad, 2018; Su & Scheffrahn, 2000). Furthermore, less than 1% of cockroach species are recognised as omnivorous pest insects, when they come into close contact with human habitation (Cochran, 1999; presenting potentially serious health threats (Rosenstreich et al., 1997). Table 1 reveals that the AKH octapeptide, Peram-CAH-I, is not pest-insect specific, being shared by 36 cockroach and termite species. In fact, Peram-CAH-I is prevalent also in other insect orders, such as Hemiptera (Gäde and Marco, 2022), Coleoptera (Gäde et al., 2019) and Archaeognatha (Marco et al., 2014) and is, thus, not a suitable lead compound as cross-activity is likely to occur across a wide spectrum of insects. The remaining 6 octapeptide AKHs so far detected in the Blattodea are also not unique (Gäde, 2009), except for Polae-HrTH, which is synthesised in the CC of only 2 cockroach species (Table 1). However, our current study has uncovered a potential wealth in decapeptide AKHs, characterised by both *in silico* approaches as well as Sanger sequencing in the case of *B. germanica*: Table 1 lists a total of 8 decapeptides, 7 of which are novel structures, and all are present only in the Blaberoidea. These AKHs may be sufficiently different to warrant

such a decapeptide as a lead peptide. The strategy is clear and partially shown for the locust pair of AKHs and the cognate AKH receptor where a non-peptide (mimetic) has already been found to act as a competitive substance on the receptor (Jackson et al., 2022), and a case study in which a mimetic could bind the locust AKH receptor but not that of the honeybee (Abdulganiyyu et al., 2020).

Co-application of AKH with pathogens could yet further enhance these effects by interfering with the immune response in some insect species (Adamo et al., 2008; Gautam et al., 2020; Ibrahim et al., 2017; Ibrahim et al., 2018), potentially making them more susceptible to pathogen-mediated control, which in termites has proven difficult to implement, in part due to their social immune defence traits, which prevent the spread of infectious disease (He et al., 2018; Davis et al., 2018; Liu et al., 2019; Hassan et al., 2021).

In conclusion, our study provides valuable comparative data, not only for further research exploring the interactions between AKH and AKHR in termites and cockroaches but also in providing necessary sequence information for functional research in an economically and scientifically important group of non-model insects, with potential applications in the development of mimetic or RNAi-based approaches to pest control. The data also constitute an important molecular framework for future studies seeking to exploit neuropeptides as a sustainable means of controlling globally important termite and cockroach pest organisms.

EXPERIMENTAL PROCEDURES

Insect samples

The termite species *Coptotermes formosanus*, *Kalotermes flavicollis*, *Mastotermes darwiniensis*, *Cryptotermes* sp., *Prorhinotermes inopinatus* and *Reticulitermes flavipes*, as well as cockroach species, *Blattella germanica* were bred from laboratory colonies of the Federal Institute of Materials Research and Testing (BAM), Berlin, Germany. One species of subsocial wood roaches, *Cryptocercus meridianus* was collected in Yunshanping (27°14′N, 100°23′E), Yulongxueshan, Lijiang, Yunnan, China, and one higher social termite, *Indotermes* sp., was collected in Mengla County, Xishuangbanna Dai Autonomous Prefecture, Yunnan, China (21°62′N, 101°58′E).

Sequence retrieval

AKH preprohormone sequences were retrieved from the published literature (Jedličková et al., 2016) and accessible SRA datasets for *Cryptotermes domesticus* (SRR2039534) and *Reticulitermes grassei* (SRR13251 [02–10]). The following AKHR nucleotide sequences were retrieved from SRA datasets and the nucleotide collection from GenBank: *Periplaneta americana* (DQ217786.1), *Blattella germanica* (GU591493.1), *Zootermopsis nevadensis* (XM_022082426.1) and *Cryptotermes secundus* (XM_033755069.1).

In addition, AKHs and AKHRs were searched from assemblies of 18 cockroach and termite transcriptomes (He et al., 2021) and

transcriptome sequence assemblies (TSA) from 'The 1KITE project: evolution of insects' (BioProject: PRJNA183205). We adopted a hidden Markov model (HMM) approach, as described by He et al. (2020) with slight modifications (Data S2). Briefly, candidate AKH and AKHR sequences were searched in the transcriptome assemblies by querying against a reference dataset of diverse AKH precursor and AKHR sequences compiled from NCBI (Table S1). The online tool SignalP 5.0 Server (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) was then used to predict the signal peptide cleavage site from each candidate AKH precursor sequence (Almagro Armenteros et al., 2019).

Sequencing the AKH precursor gene and AKH receptor gene

For some species, the complete open reading frame (ORF) of AKHR could be obtained from assembled transcriptome data alone, including *C. wrighti*, *Globitermes* sp., *B. orientalis*, *P. simplex*, *N. castaneus*, *R. grassei*, *Symploce* sp., *Asiablatta kyotensis*, *Paratemnopteryx couloniana* and *Ischnoptera deropeltiformis*. However, none or only partial AKHR fragment(s) were retrieved from *Indotermes* sp., *C. formosanus*, *C. meridianus*, *K. flavicollis*, *M. darwiniensis*, *P. inopinatus*, *Indotermes* sp. and *R. flavipes*. To obtain these, and to verify the presence of the novel AKH peptide type in *Blattella germanica*, we employed classical molecular methods to attain the full-length AKH/AKHR sequences of these remaining species. ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to determine ORFs in the sequences.

Apart from *C. meridianus*, whose abdomens were cut with sterile scissors, the whole body of other termite species was used for total RNA isolation. Pre-cooled Trizol reagent (Thermo Fisher Scientific) was used to preserve insect tissues and then homogenised with a FastPrep®-24 homogeniser (MP Biomedicals). Recovery of RNA was followed according to the manufacturer's recommendation for Trizol (Thermo Fisher Scientific), with chloroform extraction and isopropanol precipitation, followed by re-dissolving RNA in Nuclease-free water, and subsequent incubation with TurboDNase for 30 min at 37°C to remove remaining DNA (TURBO DNA-free Kit, Ambion). cDNA synthesis was performed with oligo-dT primers using the M-MLV Reverse Transcriptase (Promega). PCR was performed using Taq DNA Polymerase (Red Load Taq Master (5x), NEB) with specific or degenerate forward and reverse primers (Table S2).

Primers were manually selected based on conserved homologous nucleotide sequences or designed online: https://www.ncbi.nlm.nih.gov/tools/primer-blast/ (Ye et al., 2012) using the assembled transcripts and available full-length gene sequences from the species *B. germanica*, *P. americana*, *C. wrighti* and *C. secundus* to guide primer sequence design. PCR products were either cleaned directly with a Monarch® PCR & DNA Cleanup Kit (NEB) (for a specific PCR product) or separated on a 1.5% agarose gel (Agarose NEEO ultra-quality Roth®, Karlsruhe, Germany) and the expected size band excised and extracted using the QIAquick Gel Extraction Kit (Qiagen). Cleaned-up products were then sent for Sanger sequencing (Eurofins Genomic).

Sequence alignment and phylogenetic analysis

The multiple sequence alignment programme MAFFT (Katoh & Standley, 2013) with the E-INS-I algorithm was used to perform multiple sequence alignments for AKH precursor sequences and AKHRs. Alignments were visualised using Jalview (Waterhouse et al., 2009), and the transmembrane domains of the AKHR receptors were predicted with the TMHMM server (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). Pairwise sequence comparisons for AKHR amino acid identity and similarity were computed using the online tool: SIAS (http://imed.med.ucm.es/Tools/sias.html) using the 'length of smallest sequence' option.

To explore the evolutionary relationships of AKH precursor sequences and AKHR sequences, we carried out phylogenetic reconstruction on amino acid or nucleotide-based alignments. Aligned sequences were trimmed using trimAl v1.2 (Capella-Gutiérrez et al., 2009) with the function -gappyout prior to use in phylogenetic tree reconstruction. We employed a maximum likelihood (ML) approach in RAxML v8.2.12 (Stamatakis, 2014) and a Bayesian inference (BI) approach in MrBayes v3.2.7a (Huelsenbeck & Ronquist, 2001) to reconstruct phylogenies.

For ML, we used 1000 rapid bootstrap (RB) replicates with the PROTGAMMAAUTO model (amino acid alignment) or the GTRGAMMAI model (nucleotide alignment). For BI, we employed a model-jumping approach with the command 'prset aamodelpr = mixed' (amino acid alignment), or 'lset nst =mixed' (nucleotide alignment). Four chains of the Markov Chain Monte Carlo (MCMC) with two independent runs of 15 million generations were conducted, sampling every 500 generations. The average standard deviation of split frequencies was inspected to ensure these were less than 0.01. Posterior probabilities (PPs) were calculated from the posterior distribution of trees after discarding 25% as burn-in.

Prediction of AKHR post-translational modifications (PTMs)

Putative glycosylation and myristoylation sites of AKHRs were detected using MotifScan (https://myhits.sib.swiss/cgi-bin/motif_scan) using default settings (Sigrist et al., 2010) and phosphorylation sites of the intracellular domains of AKHRs were predicted using the NetPhos server (https://services.healthtech.dtu.dk/service.php?NetPhos-3.1), displaying only scores higher than a threshold of 0.5 (Blom et al., 1999; Blom et al., 2004).

AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1. Supporting Information.