

Structural data on hypertrehalosaemic neuropeptides from *Cryptocercus punctulatus* and *Therea petiveriana*: how do they fit into the phylogeny of cockroaches?

GERD GÄDE¹*, PHILIPPE GRANDCOLAS² AND ROLAND KELLNER³

¹Zoology Department, University of Cape Town, Rondebosch 7701, South Africa

²E.P. 90 CNRS, Laboratoire d'Entomologie, Muséum National d'Histoire Naturelle, 45 rue Buffon, F-75005 Paris, France

³Institute for Physiological Chemistry and Pathobiochemistry, D-55099 Mainz, Germany

SUMMARY

Hypertrehalosaemic neuropeptides from the corpora cardiaca of the cockroaches *Cryptocercus punctulatus* and *Therea petiveriana* were structurally analysed to gather phylogenetic information independent from that provided by morphoanatomical data. Isolation of the peptides by liquid chromatography and structural elucidation by Edman degradation and mass spectrometry revealed an identical octapeptide for both species: pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp-NH₂. This peptide, denoted Tem-HrTH, was previously found in tenebrionid beetles and in the cockroach *Polyphaga aegyptiaca*. Using this information for phylogenetic analysis yielded a peptide tree that supports the previous morphoanatomical data and thus places the woodroach *Cryptocercus* inside the cockroach subfamily Polyphaginae.

1. INTRODUCTION

Cryptocercus spp. woodroaches are generally regarded as interesting models to understand the earliest stages of the evolution of sociality in termites (Nalepa 1984; Myles 1988; Roisin 1994). They are subsocial and xylophagous, and they harbour flagellates as intestinal symbionts. All of these characters were assumed to be ancestral to termites, and the genus *Cryptocercus* was thus supposed to be representative of the ancestral blattarian-isopteran stock (Cleveland *et al.* 1934; McKittrick 1964, 1965). It was emphasized, however, that the validity of such an assumption depends on the independent establishment of the phylogenetic position of *Cryptocercus* (Grandcolas & Deleporte 1992). If *Cryptocercus* is closely related to termites, its life habits and its intestinal symbiosis could actually be ancestral. If, however, the genus *Cryptocercus* belongs to a cockroach subfamily, its life habits and its symbiosis could be more plausibly explained as being convergent and secondarily acquired.

Unfortunately, the phylogenetic position of *Cryptocercus* was until recently unclear. It was based on unreliable characters and thus could not bring support to any of these different evolutionary scenarios, and the alternative inheritance or convergence remained unresolved (Thorne 1990, 1991; Nalepa 1991). According to the classifications of McKittrick (1964) and Princis (1960), the genus *Cryptocercus* was included in the monogeneric cockroach family Cryptocercidae. Subsequent phylogenetic studies (Deleporte 1988;

Thorne & Carpenter 1992) could not refute this classification because of the lack of synapomorphies that could relate it to other taxa.

Eventually, Grandcolas & Deleporte (1992) and Grandcolas (1994, 1996) placed the genus in the subfamily Polyphaginae of the family Polyphagidae according to additional studies and to the reappraisal of previous studies. Thirty-one morphoanatomical apomorphies unambiguously support the inclusion of *Cryptocercus* in the Polyphaginae and in particular its relatedness to several South Asian genera. This phylogenetic position provided support for the hypothesis that the life history characteristics of *Cryptocercus* are a convergent development (Grandcolas & Deleporte 1992, 1996).

Additional studies are now needed to provide independent data to test these morphoanatomical results. Towards this end, analyses using molecular biological techniques have been carried out. For various reasons, however, these studies cannot be used to substantiate or refute the above arguments. Vawter (1991) and Kambhampati (1995) did not include key taxa from the Polyphagidae, except *Cryptocercus*; Kambhampati (1996) included several Polyphagidae but used a very small portion of the DNA sequence of the mitochondrial 12S rRNA gene. The latter study provided poorly consistent results, which did not substantiate the relationship of *Cryptocercus* with another cockroach (very low bootstrap values and consistency index). Moreover, all three studies cited above yielded trees that were very different from each other.

To gather additional information of a different nature we report in the present study on the structural data of hypertrehalosaemic peptides of *Cryptocercus*

* Author for correspondence: (ggade@botzoo.uct.ac.za)

Table 1. Sequences of peptides used in this study.

genus	peptide name	sequence
<i>Blatta; Periplaneta</i>	Pea-CAH-I	pQVNFSPNWamide
	Pea-CAH-II	pQLTFTPNWamide
<i>Polyphaga</i>	Poa-HrTH	pQITFTPNWamide
	Tem-HrTH	pQLNFSPNWamide
<i>Cryptocercus; Therea</i>	Tem-HrTH	pQLNFSPNWamide
<i>Blaberus, Blatella, Diploptera</i>	Bld-HrTH	pQVNFSPGWTamide
<i>Gromphadorhina, Leucophaea, Nauphoeta, Supella</i>		
<i>Mastotermes, Trinervitermes</i>	Pea-CAH-I	pQVNFSPNWamide
<i>Microhodotermes, Hodotermes</i>	Miv-CC	pQINFTPNWamide
<i>Empusa, Sphodromantis</i>	Emp-AKH	pQVNFTPNWamide

Table 2. Effect of a crude methanolic CC extract from *Cryptocercus punctulatus* and *Therea petiveriana* on haemolymph carbohydrates in the American cockroach

treatment	n	haemolymph carbohydrates (mg ml ⁻¹)*			p**
		0 min	120 min	difference	
control; water	8	14.8 ± 1.7	14.2 ± 2.5	-0.6 ± 2.2	not significant
<i>Cryptocercus</i> (0.2 gland equivalents)	8	14.3 ± 1.4	32.5 ± 8.2	18.2 ± 8.6	< 0.001
<i>Therea</i> (0.2 gland equivalents)	6	13.5 ± 2.4	23.6 ± 5.2	10.1 ± 4.4	< 0.005
<i>Periplaneta</i> (0.1 gland equivalent)	8	14.2 ± 3.8	36.4 ± 8.4	22.2 ± 7.5	< 0.001

* Values are means ± standard deviation.

** Significance of difference of pre- versus post-injection values was calculated using Student's paired *t*-test.

punctulatus and of *Therea petiveriana*, a well-known member of the Polyphaginae. Hypertrehalosemic peptides are neuropeptides synthesized in the corpora cardiaca and are responsible for the control of carbohydrate homeostasis. These peptides belong to a large family of peptides, the adipokinetic/red pigment-concentrating family, members of which have been sequenced in each major class of insects (Gäde 1990, 1996; Gäde *et al.* 1994). Such studies included peptides from cockroaches, mantids and termites (Gäde 1989, 1991; Gäde & Kellner 1992; Liebrich *et al.* 1995; for structures, see table 1), but not previously from *Cryptocercus*. The structural information is then used for phylogenetic analysis.

2. MATERIALS AND METHODS

Adult specimens of *Cryptocercus punctulatus* of both sexes were a gift of Dr C. Nalepa (Entomology Department, North Carolina State University, USA) and adult males of *Therea petiveriana* were obtained from a colony in Paimpont, France kept at 25 °C and fed with dog food. This culture was established using females collected near Pondicherry, India (a gift from Dr D. De Francesci).

Corpora cardiaca were dissected and methanolic extracts were prepared as outlined previously (Gäde *et al.* 1984). These extracts were taken up in water for the hypertrehalosaemic bioassay in American cockroaches (Gäde 1980) or in 15% acetonitrile for chromatography. The material was applied to a C₁₈ column for reversed-phase high performance liquid chromatography (RP-HPLC) using equipment and methods described previously (Gäde 1985;

see also legend to figure 1). The biologically active material of this step was subjected to digestion with pyroglutamate aminopeptidase according to Gäde *et al.* (1988). The blocked (intact) and deblocked peptides were separated by RP-HPLC using the same column as above, but a gradient from 33 to 53% B in 40 min was applied. The deblocked peptides were subjected to automated Edman degradation using a Model 477A sequencer connected to an online Model 120 phenylthiohydantoin amino acid analyser (both from Applied Biosystems, Foster City, CA, USA). The intact peptides were used for electrospray mass spectrometry (VG Quattro ES-MS, VG Organic, Altrincham, England). The peptide, denoted Tem-HrTH, was then synthesized by standard solid-phase chemistry.

Phylogenetic analysis was carried out following the classical procedure without weighting the data obtained from peptide analysis. Taxa, or specifically, their peptides are clustered in monophyletic groups on the basis of apomorphic amino acids. The same weight is applied to any transformation between different amino acids. Further details about this standard procedure may be found in Swofford & Olsen (1990). Twelve cockroach genera were taken as the ingroup, whereas mantid and termite genera (respectively *Empusa*, *Sphodromantis* and *Mastotermes*, *Trinervitermes*, *Microhodotermes*, *Hodotermes*) were selected as outgroup taxa. To simplify the analysis, if several genera had identical peptides, they were considered as one unique terminal taxon. Using this principle, there were four cockroach terminal taxa: [*Blatta*, *Periplaneta*] (family Blattidae), [*Polyphaga*] (family Polyphagidae), [*Therea*, *Cryptocercus*] (family Polyphagidae), [*Supella*, *Blatella*, *Blaberus*, *Nauphoeta*, *Leucophaea*, *Gromphadorhina*, *Diploptera*] (families Blaberidae, Pseudophyllodromiidae and Blattellidae). Characters were coded as multistate and non-additive, each state being represented by

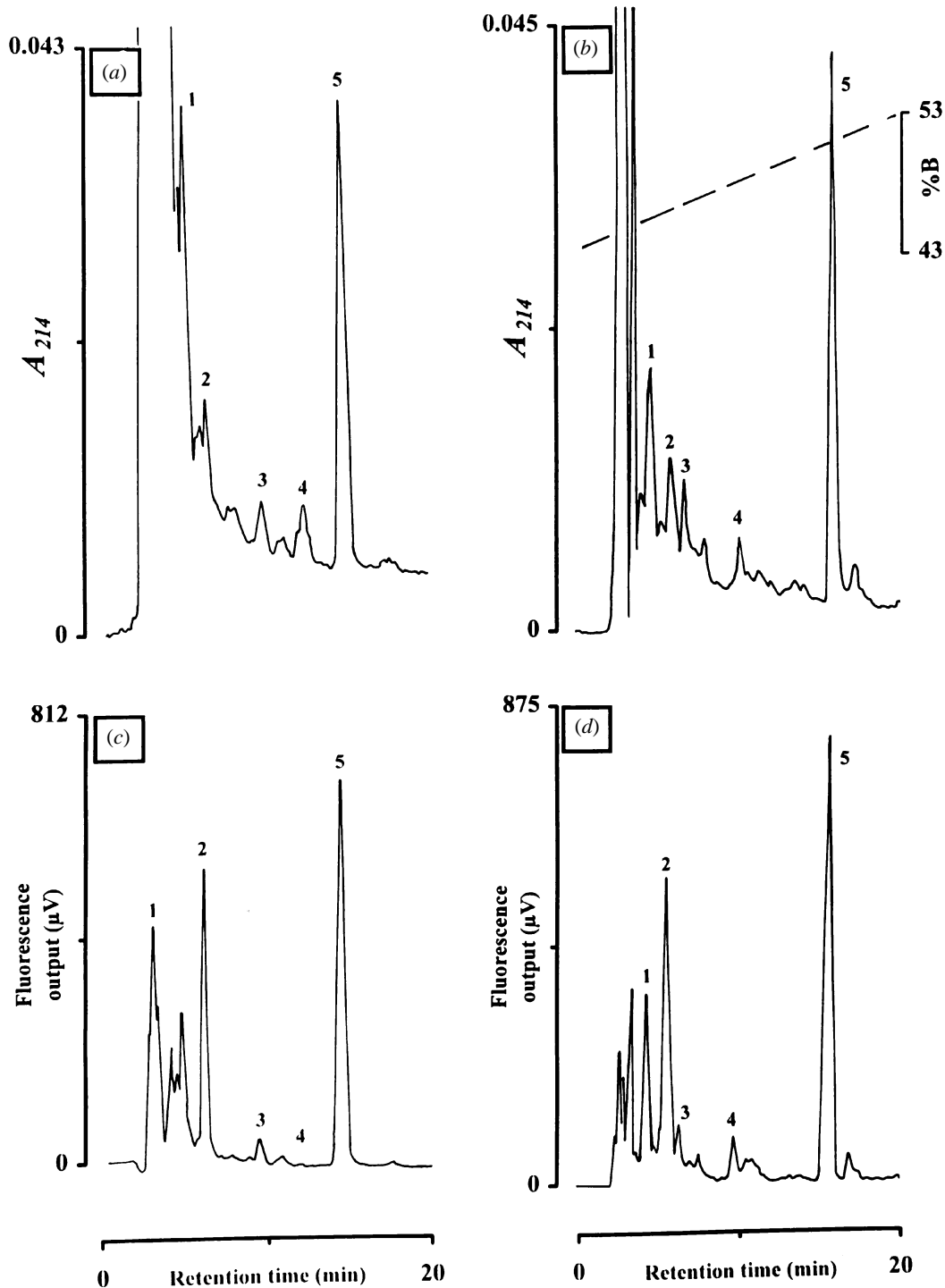


Figure 1. Purification of the corpus cardiacum peptides of *Cryptocercus* (*a, c*) and *Therea* (*b, d*) by HPLC. A methanolic extract of 7 (*a, c*) and 20 (*b, d*) glands each was applied to a Nucleosil C-18 column (5 μm particle size; dimensions: 4.6 \times 250 mm) equilibrated with 43% solvent B (solvent A: 0.11% trifluoroacetic acid; solvent B: 0.1% trifluoroacetic acid in 60% acetonitrile). After injection of samples, a linear gradient of 43–53% B within 20 min was started at a flow rate of 1 ml min^{-1} . The elution was monitored either with a uv detector at 214 nm (*a, b*) or with a fluorimeter at 276 nm (excitation)/350 nm (emission) (*c, d*). The peak fraction at 15.74 min had hypertrehalosaemic activity.

a different amino acid. Only the peptide occurring in Blaberidae, Pseudophyllodromiidae and Blattellidae contains ten amino acids instead of the usual eight in cockroaches; in this case, amino acids in positions 9 and 10 were coded as inapplicable (question mark). In two taxa (*[Blatta, Periplaneta]* and *Polyphaga*) two peptides exist. The possibility of their origin via gene duplication has been considered in the following way. First, a peptide tree was built using each

peptide as terminal taxa (taxa with two peptides were thus included twice in the analysis). The more parsimonious tree(s) was(were) searched for using the program Hennig86 and its most exhaustive search algorithm, command implicit enumeration (see Farris 1988). Second, the taxon tree was inferred by making a consensus tree between the two monophyletic parts of the peptide tree that comprise the same set of taxa. Both strict consensus and Nelson consensus

trees were built. Strict consensus retains only the dichotomies that are resolved in all the trees, and Nelson consensus retains the dichotomies that are resolved in at least one of the trees.

3. RESULTS

Crude extracts of both cockroach species under investigation effectively increased the level of carbohydrates in the haemolymph of American cockroaches (table 2). Thus, both species apparently contained hypertrehalosaemic peptides in their corpora cardiaca. RP-HPLC elution profiles of both extracts showed multiple distinct UV-absorbance peaks (numbered 1 to 5) for each species (figure 1*a* and 1*b*). One peak, at a retention time of 15.74 min (numbered 5) in each species was exceptionally high and also gave a clear fluorescence signal indicating the presence of Trp (figure 1*c* and 1*d*). This peak only had biological activity when an aliquot (representing 0.2 gland equivalents) was injected into American cockroaches (results not shown). Material corresponding to this peak from both species was separately digested with pyroglutamate aminopeptidase and, after rechromatography, the deblocked material of each species yielded the following sequence (concentration in pmol in brackets): *Cryptocercus*: Leu(17)-Asn(10)-Phe(14)-Ser(6)-Pro(5)-Asn(6)-Trp(2); *Therea*: Leu(16)-Asn(8)-Phe(10)-Ser(5)-Pro(4)-Asn(4)-Trp(2). The native peptides of each species, when subjected to mass spectrometry, yielded peaks at $m/z = 1009$, which were attributed to the $[M + Na]^+$ forms of the peptides. Thus, both peptides have a mass of 986. The combined dataset assigns the complete structure (N-terminus as pGlu by enzymatic digestion; C-terminus as amidated by extra mass unit) as pGlu-Leu-Asn-Phe-Ser-Pro-Asn-TrpNH₂, which corresponds to a peptide previously denoted Tem-HrTH found in beetles of the family Tenebrionidae (Gäde & Rosinski 1990; Gäde 1994). Coelution of native and synthetic peptides on different columns and in various solvents confirmed the peptide sequence (data not shown).

A single consistent peptide tree was obtained (CI = 0.87 and RI = 0.90) from phylogenetic analysis. This

tree placed the peptides of termites as well as the peptides of Blattidae and Polyphagidae cockroaches into two different groups. This leads to the hypothesis of a single origin of hypertrehalosaemic peptides, followed by a duplication in an ancestor of [termites + cockroaches], and two convergent losses of the duplicated peptide in [*Cryptocercus*, *Therea*] and in [*Supella*, *Blattella*, *Blaberus*, *Nauphoeta*, *Leucophaea*, *Gromphadorhina*, *Diploptera*].

The strict consensus of the two parts of the peptide tree is very poorly resolved and shows only one dichotomous node, which clusters *Polyphaga* and [*Therea*, *Cryptocercus*] (figure 2*a*). The Nelson consensus is almost fully resolved (figure 2*b*): mantids are the outgroup of termites and cockroaches; two groups of termites are the sister group of cockroaches that are monophyletic. In cockroaches, *Cryptocercus*, *Polyphaga* and *Therea* are closely related.

4. DISCUSSION

We have successfully identified the primary structure of an identical hypertrehalosaemic peptide from the corpora cardiaca of the two cockroach species *Cryptocercus* and *Therea*. The octapeptide, denoted Tem-HrTH, was previously sequenced from corpora cardiaca of tenebrionid beetles (Gäde & Rosinski 1990; Gäde 1994), but was also identified as one of two peptides in the meloid beetle, *Decapotoma lunata* (Gäde 1995), and the cockroach, *Polyphaga aegyptiaca* (Gäde & Kellner 1992). The second peptide in *Polyphaga*, denoted Poa-HrTH, is unique to this species.

When this information is combined with previous analyses of peptides (Gäde 1989, 1991) for carrying out a phylogenetic analysis, the peptide Tem-HrTH appears apomorphic in its structure in (*Polyphaga* + [*Therea*, *Cryptocercus*]), and the lack of Poa-HrTH in *Therea* and *Cryptocercus* resulted from a secondary disappearance after an ancestral duplication. The presence of Tem-HrTH in some beetles may only be interpreted as a total convergence; several sites may have changed to account for convergent states (amino acids) with those of the hyper-

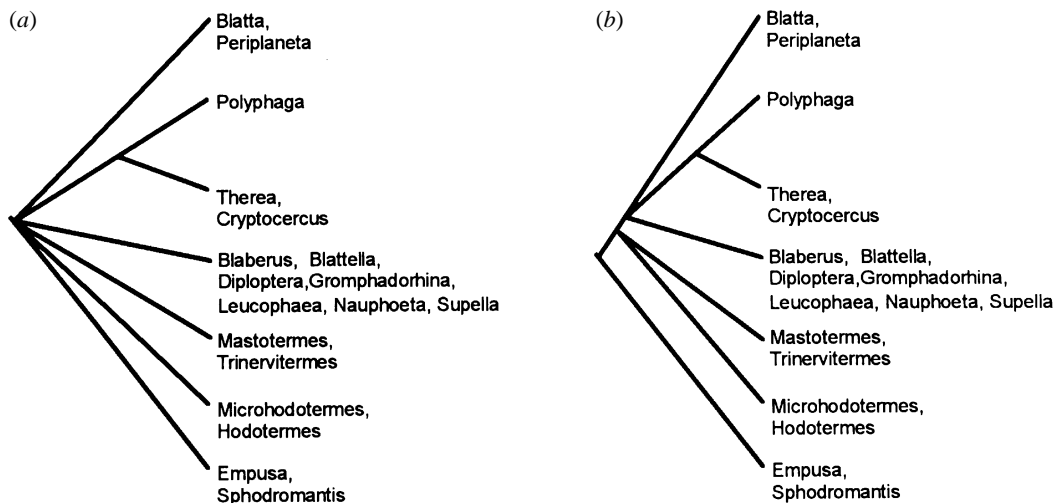


Figure 2. Phylogenetic trees obtained from consensus of two parts of the peptide tree. (a) strict consensus; (b) Nelson consensus. Terminal taxa are genera with identical peptides.

trehalosaemic peptide in *Therea* and *Cryptocercus*. Other cockroaches and also all related groups, such as mantids, termites and orthopteran groups, do not contain Tem-HrTH (Gäde 1989, 1991, 1995, 1996; Gäde & Kellner 1992; Liebrich *et al.* 1995). Therefore, the peptide cannot be considered a primitive one that has remained unchanged only in Polyphagidae (*Polyphaga*, *Therea* and *Cryptocercus*), but must be considered a derived one that has appeared in the ancestor of Polyphagidae cockroaches. Analyses may be carried out using differential weighting according to the genetic code: if one change of amino acid is coded by one change of base instead of three changes of bases for another amino acid (according to the respective codon composition), the first change may be weighted less than the second one. Using this analytical technique (all possible codon choices) gives an identical result of interpretation as with amino acids. We conclude that the Tem-HrTH structure characterizes the subfamily Polyphaginae. This supports the phylogenetic position of *Cryptocercus* in this subfamily as previously assessed on the basis of morphoanatomical studies (Grandcolas & Deleporte 1992; Grandcolas 1994, 1996). This, in turn, substantiates the theory of convergent development as outlined in the Introduction. *Cryptocercus* is clearly a true cockroach, bearing mainly derived and modern traits in its social behaviour and intestinal symbiosis (Grandcolas & Deleporte 1992, 1996).

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