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Peptidomics-Based Phylogeny and Biogeography of Mantophasmatodea (Hexapoda)

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Abstract.—The insect order Mantophasmatodea was described in 2002. Prior to that time, several generations of entomologists had assumed that all major insect taxa were known; thus, its description was a sensation for zoologists. Since then, a surprising abundance and species diversity of this taxon have been found, particularly in the winter rainfall region of South Africa. To learn more about the evolutionary lineages, speciation, and biogeography of Mantophasmatodea, we applied an unusual peptidomics approach. We collected specimens of almost all known and novel taxa of these insects, developed methods for immediate sample preparation in the field, introduced peptide mass fingerprints for the unambiguous identification of taxa, and subsequently analyzed the most extensive data set on peptide hormones ever compiled for insect taxa. To account for intraspecific variation, we analyzed several individuals per putative species. Increased taxon sampling was preferred over a further increase in the number of characters to optimize the accuracy of phylogenetic analyses. The large data set made it possible to test the validity of using neuropeptide sequences, which coevolve with their respective receptors, to analyze phylogenetic relationships among closely related taxa. Altogether, the data from 71 populations of Mantophasmatodea were sufficient to clearly separate the major clades of Mantophasmatodea, including previously undescribed taxa such as *Pachyphasma*, *Striatophasma*, and Austrophasmatidae gen. et sp. nov. "RV." The data confirm the monophyly of Austrophasmatidae and show a relatively recent and extensive radiation in the winter rainfall region of South Africa but also suggest that the species-level diversification of Namibian *Mantophasma* is less marked than previously thought. We discuss the biogeographical and ecological factors that may have resulted in different regional patterns of endemism and species diversity in Mantophasmatodea. The unique development of the neuroendocrine *capa*-neurons in the ventral nervous system is described as synapomorphy of Mantophasmatodea + Grylloblattodea and is a further argument for a close relationship between these insect taxa. [Biogeography; insects; Mantophasmatodea; neuropeptides; nonadaptive radiation; peptidomics; phylogeny.]

The taxon Mantophasmatodea has been described as a new insect order because its representatives lack characteristics that would support their assignment to one of the already existing insect orders (Klass et al. 2002). The scientific name alludes to a superficial resemblance to praying mantises (Mantodea) and stick insects (Phasmatodea). Indeed, sequencing of the entire mitochondrial genome suggests that Mantophasmatodea and Phasmatodea are sister groups (Cameron et al. 2006). On the basis of other DNA sequence data and morphological characters, however, a sister group relationship with Grylloblattodea has been postulated (Terry and Whiting 2005; Beutel and Gorb 2006; Kjer et al. 2006; Ishiwata et al. 2010; Wipfler et al. 2011). Damgaard et al. (2008) have provided a comprehensive review of the current literature on Mantophasmatodea phylogeny.

Following the spectacular discovery of the first Mantophasmatodea species (Klass et al. 2002), a surprising abundance and species diversity of this taxon have been described, particularly in the winter rainfall region of South Africa (Picker et al. 2002; Klass et al. 2003). However, species delimitation, particularly the assignment of specimens from new localities to already described species based on morphological characters, is often difficult. This uncertainty may be due the fact that most species have been defined based on rather small groups of individuals; within-species variation is poorly studied in Mantophasmatodea.

In 2005, easily obtainable mass fingerprint data based on peptide hormones (neuropeptides) from several South African Mantophasmatodea populations were published (Predel et al. 2005). These data, obtained by screening of neuroendocrine tissues from single specimens, facilitated the clear separation of morphologically similar individuals into different populations and/or species. A similar approach was later successfully used to distinguish subspecies of grasshoppers (Roth et al. 2007). In both cases, the peptide hormone mass fingerprints of different specimens from selected populations have been shown to be identical, regardless of the larval stage or sex of the specimen (Predel et al. 2005). Indeed, peptide (not neuropeptide) or protein mass fingerprints have been employed to support species recognition in many organisms, particularly in groups (such as microorganisms) that do not exhibit clear morphological differences (Claydon et al. 1996; Bizzini and Greub, 2010; Ferreira et al. 2010; Murray 2010; Spanu et al. 2011). Recently, Feltens et al. (2010) have introduced protein profiling of single insect specimens as a method to discriminate closely related *Drosophila* species.

The aim of the current study is to obtain, by means of a peptidomics approach, detailed data on lineage

evolution within Mantophasmatodea. Two novel genera (*Striatophasma* and *Pachyphasma*) identified during this study have been described in a separate publication (Wipfler et al. 2012). The first part of this project was to acquire detailed neuropeptide mass fingerprint data for the Namibian species of Mantophasmatodea and to complement this data set with records from additional South African species. This approach included all currently known lineages of Mantophasmatodea except *Tanzaniophasma* from southern Tanzania, which is known only from a single museum specimen (Klass et al. 2002). Our results permit the unambiguous characterization of presently known Mantophasmatodea populations, and the method of peptide mass fingerprinting will be important not only for future studies dealing with the taxonomy and phylogeography of Mantophasmatodea but also for the study of other arthropods.

A second aim of the current study was to identify the neuropeptide sequences that contributed to the mass fingerprints. Neuropeptides are the structurally most diverse messenger molecules in animals and influence, via specific transmembrane receptors, a wide range of physiological processes. The biologically active neuropeptides are posttranslationally processed from larger precursor sequences which often contain multiple sequence-related peptide copies. For this reason, the expression of a single neuropeptide gene may result in the processing of numerous peptides, including the neuropeptides which coevolve with their respective receptors and spacer sequences. Such neuropeptide copies within a precursor sequence can be assigned as paracopies; neuropeptide copies at the same position in homologous precursor sequences of other species can be designated as orthocopies (see Wegener and Gorbashov 2008). In *Drosophila*, the sequences of single copy peptides are more conserved than the sequences of multiple copy peptides and the sequences of orthocopies are generally more conserved than the sequences of ortholog spacer peptides (Wegener and Gorbashov 2008). Thus, different peptides that originate from neuropeptide genes are not subject to the same kind of stabilizing selection and, likely, this also affects the usefulness of the different peptide species for phylogenetic studies. In our study, not less than 25 homologous neuropeptides from specimens representing 71 populations were analyzed, respectively. In sheer number (approximately 2000 neuropeptides), this surpasses all previous analyses using peptidomics approaches. The considerable data accumulated in this study made it possible to test the usefulness of neuropeptide sequences in analyzing phylogenetic relationships among closely related taxa.

Previously published phylogenetic studies on Mantophasmatodea (Klass et al. 2003; Damgaard et al. 2008) based on morphological characters and DNA sequence data have revealed a sister group relationship between Mantophasmatidae ([*Praedatophasma* + *Tyrannophasma*] + [*Mantophasma* + *Sclerophasma*]) and South African Austrophasmatidae. The relationships among the many taxa of Austrophasmatidae have not been resolved, and it is assumed that the Namibian *Mantophasma* clade in particular contains numerous species with limited distributions (Zompro and Adis 2006). The neuropeptide sequence data obtained in this study complement the morphological and DNA sequence data obtained previously. In contrast to a recent case study on neuropeptides in Blattodea (Roth et al. 2009), which included selected species representing distantly related taxa, we considered many more neuropeptides from single specimens and analyzed all available populations of the well-defined taxon Mantophasmatodea.

In theory, analyzing the genes that encode neuropeptides would seem to be a more convenient approach than analyzing the neuropeptides themselves. In most cases, however, only small portions of neuropeptide genes are highly conserved. This conservation applies specifically to the gene regions that encode the mature peptides. Thus, primers that can be successfully used to identify neuropeptide genes in a given insect species may fail to recognize the orthologous genes in a related species (Derst C., Roth S., Predel R., unpublished data). However, recent improvements in mass spectrometric techniques (see Aebersold and Mann 2003) have made it possible to rapidly identify mature neuropeptides from single insect specimens (e.g., Predel et al. 2010), thereby circumventing the genomic approach. The rapid development of instrumentation for high-throughput mass spectrometry is likely to greatly accelerate the use of such peptidomics approaches.

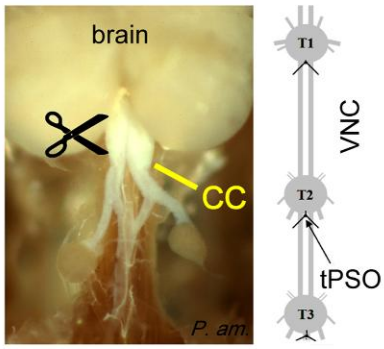
The data presented here demonstrate the extent to which neuropeptide data can be used for phylogenetic purposes. The resulting phylograms clearly separate the major clades of Mantophasmatodea, including hitherto unknown taxa, such as *Pachyphasma*, *Striatophasma*, and Austrophasmatidae gen. et sp. nov. "RV." Furthermore, our extensive sampling and subsequent peptidomic analyses provide comprehensive insights regarding the biogeography and species richness of Mantophasmatodea, including the recognition of presumed phylogenetic relict and endemic taxa and of recently successful and widespread taxa. The data show that the distribution of Mantophasmatodea in southern Africa coincides with areas of exceptionally high biodiversity.

Materials and Methods

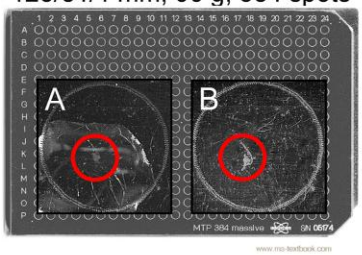
An overview of the effort that is necessary to obtain peptidomics data, including all experimental steps involved in the complete analysis of a single specimen, are illustrated in Figure 1.

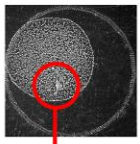
Insects

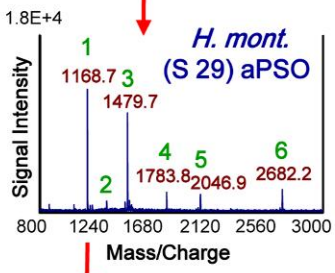
Living specimens were collected during field trips, placed in separate plastic containers, and fed with insects. In South Africa, up to 10 specimens per location were collected and transported to the laboratory, where nymphs were usually reared to adulthood. In Namibia, most specimens were dissected in the field using a stereomicroscope, and neuroendocrine tissues

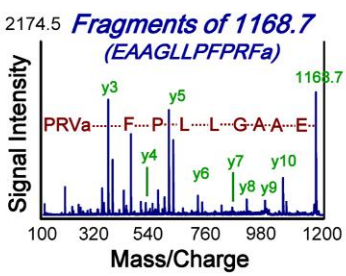


123/81/1 mm; 90 g; 384 spots









steps | **time** | **specific costs**

1. Collecting insects
note: research/collecting permits required
unpredictable | travel costs

2. Dissection of neurohemal organs from single specimens
CC - corpora cardiaca, tPSO/aPSO - thoracic/abdominal perisympathetic organs, VNC - ventral nerve cord (abdominal ganglia not shown; see Predel et al. 2005 for complete information)
30 min per specimen | no

3. Transfer of isolated organs onto a sample plate for MALDI
note: transfer with insect pins or glass capillaries into a drop of water (inset A) to reduce salt contamination; removal of water with capillary. Samples (inset B) usable for months if not exposed to light.
20 sec-1 min per organ | no

4. Deposition of matrix salt solution (CHCA) on the sample spots
note: For Mantophasmatodean samples, we applied approx. 0.2 µl with an Eppendorf pipette; Samples are now ready for analysis and usable for months if not exposed to light.
±10 sec per spot | no

5. MALDI-TOF mass fingerprint
note: Selected substances within the chosen mass range will be, subsequently, fragmented from the same spot. Here, the neuropeptide at $[M+H]^+$ at 1168.7 has been selected.
±5 sec per spot | In-house analysis: no costs or Service (e.g. Chem. Dept.)

6. Fragmentation of the selected peptides
C-terminal fragments are marked (y-series)
±10 sec per peptide | In-house analysis: no costs or Service (e.g. Chem. Dept.)

7. Fragment analysis
1-30 min per peptide | no

8. Alignment of homologous neuropeptides and phylogenetic analysis of the concatenated sequences

FIGURE 1. Overview of the peptidomics approach used to analyze the phylogeny of Mantophasmatodea. The different steps can be performed by the same person; the estimated time and noticeable costs required for an experienced scientist to perform each step are shown. If the mass spectrometric analysis is run by a service unit of the university, extra costs may arise.

(neurohemal organs) were placed on sample plates for mass spectrometry. This approach resulted in the complete sample preparation of up to 10 specimens per day. After dissection of the neurohemal organs, the remains of the insects and intact specimens from the same localities were placed in 70% ethanol and kept for species identification. All species of Mantophasmatodea are wingless in both sexes; thus, these insects have restricted vagility. In most cases, the distances between sampling localities were at least 20 km, and we assumed no genetic contact among specimens from different sampling sites. The term "population" is used

TABLE 1. Collection localities for Mantophasmatodea, including coordinates and preliminary species assignments

Locality	Coordinates in decimal degrees	Code[1]	Preliminary assignment
Omuhonga mountains	−17.35, 13.12	N01	*Mantophasma* sp.
Opuwo	−18.20, 13.82	N02	*Mantophasma* sp.
Grootberg Pass	−19.85, 14.10	N03	*Mantophasma* sp.
50 km South of Kamanjab	−19.88, 14.91	N04	*Mantophasma* sp.
Zuwas mountains	−20.45, 14.56	N05	*Mantophasma* sp.
Zawixams mountains	−20.48, 15.29	N06	*Mantophasma* sp.
Ugab Terrace	−20.35, 15.45	N07	*Mantophasma* sp.
Langeberg mountains	−19.60, 16.85	N08a,b	*Mantophasma* sp.
Otavi	−19.65, 17.41	N09	*Mantophasma* sp.
Tsumeb	−19.29, 17.68	N10	*Mantophasma* sp.
West of Grootfontein	−19.68, 17.98	N11	*Mantophasma* sp.
East of Grootfontein	−19.37, 18.32	N12	*Mantophasma* sp.
Outjo	−20.11, 16.13	N13a,b	*Mantophasma* sp.
Paresis mountains	−20.40, 16.31	N14a,b	*Sclerophasma paresisense*
Waterberg	−20.52, 17.23	N15a,b	*Mantophasma* sp.
Etjo mountains	−20.94, 16.28	N16	*Mantophasma* sp.
Elefantenberg	−21.32, 15.65	N17	*Mantophasma* sp.
Elefantenberg 2	−21.32, 15.65	N18	*Mantophasma* sp.
Omatoko	−21.26, 16.82	N19	*Mantophasma omatokoense*
Spitzkoppe	−21.83, 15.19	N20	*Mantophasma* sp.
Erongo mountains	−21.75, 15.80	N21	*Mantophasma kudubergense*
Ojakajongo	−21.70, 16.17	N22	*Mantophasma* sp.
Okahandja	−21.95, 16.77	N23	*Mantophasma* sp.
Otjipatera mountains	−22.27, 15.85	N24a,b	*Mantophasma* sp.
Tsaobis	−22.48, 15.86	N25a,b	*Mantophasma* sp.
Witwater mountains	−22.71, 15.86	N26a,b	*Mantophasma* sp.
Onanis	−22.86, 15.73	N27a,b	*Mantophasma* sp.
Khomas mountains	−22.62, 16.72	N28a,b	*Mantophasma* sp.
Windhoek	−22.58, 17.17	N29	*Mantophasma* sp.
Kleeberge	−22.58, 17.74	N30	*Striatophasma* sp.
Gamsberg Pass	−23.27, 16.32	N31a,b	*Mantophasma* (*gamsbergense*)[a]
Gamsberg Pass	−23.27, 16.32	N32	*Striatophasma* sp.
Nauchas	−23.61, 16.37	N33	*Striatophasma* sp.
Remhoogte Pass	−23.96, 16.30	N34a,b	*Striatophasma* sp.
Naukluft mountains	−24.15, 16.32	N35	*Striatophasma naukluftense*
Tsaris Pass	−24.93, 16.42	N36	*Striatophasma* sp.
Richtersveld	−28.08, 16.90	N37	*Praedatophasma maraisi*
Brandberg	−21.16, 14.58	N38	*Tyrannophasma gladiator*
Brandberg	−21.16, 14.58	N39	*Pachyphasma brandbergense*
Richtersveld	−28.17, 17.03	S01	Austrophasmatidae gen. et sp. nov. "RV"[b]
Richtersveld	−28.29, 16.98	S02	*Namaquaphasma ookiepense*
West of Steinkopf	−29.26, 17.48	S03	*Namaquaphasma ookiepense*
Steinkopf	−29.27, 17.71	S04	*Namaquaphasma ookiepense*
Springbok	−29.67, 17.90	S05	*Namaquaphasma ookiepense*
Karootjie	−29.81, 17.36	S06	*Namaquaphasma ookiepense*
North of Kamieskroon	−30.17, 17.93	S07	*Namaquaphasma ookiepense*
Kamieskroon mountains	−30.13, 18.10	S08a,b	*Namaquaphasma ookiepense*
Leliefontein	−30.33, 18.11	S09	*Namaquaphasma ookiepense*
West of Platbakkies	−30.34, 18.32	S10	*Namaquaphasma ookiepense*
West of Spoegrivier	−30.26, 17.65	S11	*Namaquaphasma ookiepense*
Garies	−30.55, 17.97	S12	*Namaquaphasma ookiepense*
Bitterfontein	−31.11, 18.35	S13	*Namaquaphasma ookiepense*
Strandfontein	−31.77, 18.23	S14	*Namaquaphasma ookiepense*
Loeriesfontein	−31.04, 19.31	S15a,b	*Karoophasma botterkloofense*
Botterkloof Pass	−31.80, 19.27	S16a,b	*Karoophasma botterkloofense*
Driefontein Farm	−32.02, 19.22	S17a,b	*Karoophasma biedouwense*
Vanrhynsdorp	−31.67, 18.89	S18	Austrophasmatidae sp. nov. "VR"[c]
Redelinghuys	−32.46, 18.55	S19a,b	*Lobatophasma redelinghuysense*
Clanwilliam	−32.18, 18.87	S20a,b	*Karoophasma cf. botterkloofense*
Clanwilliam Dam	−32.20, 18.89	S21a,b	*Viridiphasma clanwilliamense*

Continued

TABLE 1. *Continued*

Locality	Coordinates in decimal degrees	Code[1]	Preliminary assignment
Algeria	−32.35, 19.01	S22	*Lobatophasma cf. redelinghuysense*
St. Helena Bay	−32.77, 18.04	S23	*Austrophasma c.f. rawsonvillense*
Worcester	−33.61, 19.45	S24	*Austrophasma rawsonvillense*
Ashton	−33.83, 20.08	S25	*Austrophasma rawsonvillense*
Montagu	−33.76, 20.12	S26	*Hemilobophasma montaguense*
Garcia Pass	−33.80, 21.13	S27	*Hemilobophasma montaguense*
VanWyksvlei	−33.73, 21.60	S28	*Hemilobophasma montaguense*
Calitzdorp	−33.52, 21.84	S29	*Hemilobophasma montaguense*
Oudtshoorn	−33.59, 22.18	S30	*Hemilobophasma montaguense*
De Rust	−33.51, 22.50	S31	*Hemilobophasma sp.n.*[d]
Gansbaai	−34.54, 19.40	S32	*Austrophasma gansbaaiense*

Notes: [1]Several localities contained individuals that were distinguishable by at least one amino acid substitution. For these localities, the respective specimens are designated as a- or b-type (e.g., N13a and N13b) (hatched circles in Fig. 2).
[a]Zompro and Adis (2006) described *Mantophasma gamsbergense* from female specimens. This description was not sufficient for us to clearly assign one of the 2 sympatric species from the Gamsberg to *M. gamsbergense.*
[b]Nymphs.
[c]*Austrophasmatidae sp.* n. 2 in Damgaard et al. (2008).
[d]*Hemilobophasma sp.* n. De in Damgaard et al. (2008).

to describe multiple specimens from such localities. Table 1 lists the collection sites of the populations that were successfully analyzed. Preliminary assignment of specimens to previously described taxa was based primarily on sampling from the same localities (Damgaard et al. 2008). Several Austrophasmatidae specimens (taxa for which mass fingerprints were published in Predel et al. 2005) were determined by K.-D. Klass. *Galloisiana* sp. (Grylloblattodea), which was used as an outgroup species, was collected in the Ussuri River Basin (Russia) by Frank Walther (Jena, Germany).

Dissection of Neurohemal Organs and Sample Preparation for Mass Spectrometry

The different neurohemal organs that accumulate peptide hormones were dissected as described for insects in Predel (2001). Briefly, these organs (corpora cardiaca and thoracic and abdominal perisympathetic organs) were dissected using fine scissors and transferred by means of an insect pin to a sample plate for matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass spectrometry. A single sample plate can take up to 384 tissue samples that are placed on consecutively numbered spots. On the sample plate, each dried organ was rinsed with water. Subsequently, about 0.2 µl of matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in methanol/water) was injected onto the dried sample with an Eppendorf pipette. More detailed protocols for the use of MALDI–TOF mass spectrometry to directly analyze the peptidome of neuroendocrine tissues of insects are listed in Wegener et al. (2010). The neurohemal organs of a single specimen were usually sufficient to obtain the different peptide hormone sequences. In most cases, however, several specimens from the same locality were analyzed to verify that the sequences were identical within populations. At the beginning of our study, we additionally used electrospray ionization quadrupole time-of-flight (ESI-Q–TOF) mass spectrometry as an alternative approach for the sequence elucidation. For that, 5 neurohemal organs were dissected into 5 µl 0.1 trifluoroacetic acid, the extract was then briefly sonicated, and centrifuged. To concentrate the neuropeptides, the supernatant was loaded onto a homemade C-18 micro column (purification capillary for electrospray mass spectrometry).

Mass Spectrometry

MALDI–TOF mass spectrometry.—Mass spectra from the collected tissue samples were obtained using a Voyager DE biospectrometry workstation and a 4700 Proteomics Analyzer or a 4800 Proteomics Analyzer with TOF/TOF optics (Applied Biosystems, Framingham, MA). Prominent neuropeptide signals detected in the mass fingerprints (mass range 800–2500 Da) were selected and fragmented by tandem mass spectrometry using the same sample. Resulting fragments were subsequently analyzed to determine the respective sequences; deduced sequences were counterchecked using the ProteinProspector (http://prospector.ucsf.edu/prospector). Due to the mass ambiguities of the isomeric Leu/Ile and similar mass Gln/Lys amino acid pairs, the complete data set was prepared with Leu (in the case of Leu/Ile ambiguities) and Gln (in the case of Gln/Lys ambiguities) since the software used for phylogenetic analysis (see below) does not accept specific symbols for ambiguities (e.g., J for Leu/Ile).

ESI-Q–TOF mass spectrometry.—In a few cases, the data from MALDI–TOF/TOF fragmentation experiments did not contain sufficient information to reveal the complete sequences of the peptides of interest. To fill the respective sequence gaps, nanoelectrospray mass spectra were acquired in the positive-ion mode using an

API QStar Pulsar (Applied Biosystems) fitted with a Protana (Odense, Denmark) nanoelectrospray source. Typically, 950–1000 V was applied as ionspray voltage. Before, the samples were purified using a homemade spin column. Approximately 1–2 mm of Poros R2 designed for reversed-phase chromatography (PerSeptive Biosystems, Foster City, CA) was loaded into a 2 cm capillary column with a needle tip. Liquids are passed through the column by securing the capillary column to a purification needle holder (Proxeon Biosystems A/S, Odense, Denmark) and centrifugation. After the column was equilibrated in 5% formic acid, the samples were loaded and rinsed with 5% formic acid. Peptides were eluted from the column with 30% methanol (5% formic acid) and collected into a metal-coated nanoelectrospray capillary. The purified samples were then loaded onto the source and analyzed. After determining the mass-to-charge ratio (m/z) of the peptides in mass spectrometry mode, the collision energy (10–40 V) was applied. Fragmentation data of the different peptides were acquired over 3–10 min each and manually analyzed.

Immunohistochemistry

Sample preparation and immunostaining were performed as described in Neupert et al. (2009). As the primary antiserum, we used a polyclonal anti-CAPA-PVK serum raised against *Periplaneta americana* periviscerokinin-2 in rabbit at a concentration of 1:4000 (kindly provided by Dr M. Eckert, Jena). After immunofluorescent labeling, samples were examined with a confocal laser scanning microscope (Zeiss LSM 510 Meta system; Jena, Germany) equipped with a Helium-Neon1 laser (wavelength 543 nm). Images were exported and processed with Adobe Photoshop 7.0 software.

Taxon Sampling, Sequence Alignment, and Phylogenetic Analysis

Sequences of peptide hormones from the corpora cardiaca (e.g., adipokinetic hormone, corazonin, myosuppressin, pyrokinins) and from thoracic (extended FMRFamides) and abdominal perisympathetic organs (CAPA-peptides) were identified for 71 populations of Mantophasmatodea and the outgroup taxon *Galloisiana* sp. (Grylloblattodea). The sequences of the peptide hormones of the other outgroup taxa, *Periplaneta americana* and *Locusta migratoria*, had been sequenced in earlier studies (see Predel 2006; Clynen and Schoofs 2009). Matrices consisting of homologous peptides were aligned using the ClustalX program package (parameter settings: gap penalty = 1; protein weight matrix = BLOSUM). Assignment of single neuropeptides to homologous gene products was facilitated due to their location in specific neurohemal organs (see above) and their characteristic C-terminal sequences. So, peptides were first assigned to products of a specific neuropeptide gene. After aligning the products of the different genes separately, all individual alignments were subsequently put together to one data set. Phylogenetic analyses employed Bayesian analysis (BA) in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Gaps and missing characters were treated as missing data. Three different analyses of the peptide data set were performed by treating it: (i) as a concatenated data set including 3 outgroup taxa, (ii) as a concatenated data set without outgroup taxa, and (iii) as a data set with partitions. The 5 partitions comprised products of the *fmrf*-gene (11 peptides), *pyrokinin*-gene (4 peptides), *capa*-gene (5 peptides), and 2 sets of peptides from the corpora cardiaca, namely adipokinetic hormone + myosuppressin + corazonin (single copy peptides from 3 different neuropeptide genes) and corpora cardiaca peptides 8 and 9 (not assigned to a specific neuropeptide gene; see Table 2). In all analyses, the protein substitution model was selected using the mixed analysis that can average over 10 available models (see Huelsenbeck et al. 2008). In addition, we compared these results with selected models by using ProtTest 3 (Abascal et al. 2005), again for the partitioned data sets and the whole data set. If not stated otherwise, the JTT amino acid substitution model (Jones et al. 1992) was implemented as a fixed rate model. This is in agreement to another study on protein evolution models in insect neuropeptides (Wegener and Gorbashov 2008). For all analyses, Markov Chain Monte Carlo (MCMC) sampling was run for 3,000,000 generations. Trees were saved every 100 generations. Likelihood values were observed with Tracer v1.4 (Rambaut and Drummond 2005), and all trees sampled before the likelihood values stabilized were discarded as burn-in. Stationarity was reassessed using the convergence diagnostics in MrBayes (i.e., the average standard deviation of split frequencies [values <0.05] and the potential scale reduction factor [values ± 1.00]). A burn-in of 10% of all sampled trees was sufficient to ensure that suboptimal trees were excluded. The remaining trees were used to construct a 50% majority rule consensus tree. Posterior probabilities (biPP) with values $\geqslant 90\%$ are presented. Bayesian trees were formatted for presentation using TreeView (Win32) 1.6.6 and FigTree 1.3.1. In addition, a simple parsimony analysis was performed by using the default settings of PAUP* ver. 4.0b10 (Swofford 2002) to characterize our data set in terms of parsimony and character fit information. All phylogenetic trees presented in this study were deposited in TreeBASE, accession number TB2:S11103. Peptide sequences are deposited at UniProtKB, accession numbers P86995-9, and regex B[0,3,O][M,U,A][0,2,3,7,8][5-9,A-L,T,U,X][0-9].

RESULTS

Living specimens of Mantophasmatodea were collected from numerous locations in South Africa and Namibia (Fig. 2). All South African species and *Praedatophasma maraisi*, which was found on the southern and northern banks of the Orange River, were collected during winter (July to September). In contrast,

TABLE 2. List of neuropeptides identified from 3 distantly related species of Mantophasmatodea

	Namaquaphasma ookiepense (S11)	*Striatophasma naukluftense* (N35)	*Mantophasma kudubergense* (N21)
aPSO			
1	ESAGLLPFPRV-NH_2	EAAGLLSFPRV-NH_2	AEAAGLLPFPRV-NH_2
2	EDRGDGASAAPRS-OH	EDREDGASAAPRS-OH	EDRGDSASVA*GDVPE*-OH
3	SGGGEGSGMWFGPRL-NH_2	SGGGEGSGMWFGPRL-NH_2	SGGGEGSGMWFGPRL-NH_2
4	EAAPPHEPMLYATEVE-OH	EAAPPHEPMLYATEVE-OH	EAAPPHEPLLYAAEVA-OH
5	SGLQFAVLDGQGFLPFPRV-NH_2	SGLQFAVLDGQGFLPFPRV-NH_2	SGLQFAVLDGQGFLPFSRV-NH_2
tPSO			
1	AQSFLRL-NH_2	AQSFLRL-NH_2	AQSFLRL-NH_2
2	SDYLQLAR-NH_2	ADYLQLAR-NH_2	ADYLQLARA-OH
3	GPDSAFLRL-NH_2	GPESAFLRL-NH_2	GPESAFLRL-NH_2
4	GVDSSFLRL-NH_2	GVDSSFLRL-NH_2	GVDSSFLRL-NH_2
5	TDRNFLRL-NH_2	TDRNFLRL-NH_2	TDRNFLRL-NH_2
6[a]	GRAENFLRL-NH_2	GRADNFLRL-NH_2	—
7	ARSDNFVRL-NH_2	ARSDNFVRL-NH_2	ARSDNFVRL-NH_2
8	ARTDNFVRL-NH_2	ARSDNFVRL-NH_2	ARSDNFVRL-NH_2
9	GRGGASNYVRL-NH_2	GRGGASNYVRL-NH_2	GRGGSSNYVRL-NH_2
10	PAPESNFVRDP-OH	PVPDSSFLRDP-OH	PAASESGFRRDP-OH
11	NANNADSAVGFGRGQL-OH	NANNADAAVGFGRGQL-OH	NANSADAAVGFGRGQL-OH
12	SPSLDDERNDNFVRL-OH	SPALDDEHNDNFLRL-NH_2	SPGALEDEHNDNFLRF-NH_2
CC			
1	DGYTPRL-NH_2	DGYTPRL-NH_2	DGYTPRL-NH_2
2	SPPFAPRL-NH_2	SPPFAPRL-NH_2	SPPFAPRL-NH_2
3	*DP*PFSPRL-NH_2	*DP*PFSPRL-NH_2	DPPFAPRM-NH_2
4	—	*AE*LPQGLWVQPRL-NH_2	*AE*LPQGLWVRPRL-NH_2
5	pQVNFTPGW-NH_2	pQVNFTPSW-NH_2	pQVNFSPGW-NH_2
6	pQDVDHVFLRF-NH_2	pQDVDHVFLRF-NH_2	pQDVDHVFLRF-NH_2
7	pQTFHYSQGWTN-NH_2	pQTFQYSRGWTN-NH_2	pQTFQYSRGWTN-NH_2
8	SVGVPTAFRENPPF-OH	SVGVPTAFRENPR-OH	*DV*AAPTPAFRENPR-OH
9	PSSYLRTYPVLTTD-OH	PSSYLRAYPVLTTD-OH	PSSYLRTYPVLTTD-OH

Notes: The numbering is in accordance with the fingerprint data (Supplemental Material 1; see also Supplemental Material 2 for the complete data set). Note that the complete data set for phylogenetic analyses was prepared with Leu (in the case of Leu/Ile ambiguities) and Gln (in the case of Gln/Lys ambiguities). aPSO = abdominal perisympathetic organ (CAPA-peptides); tPSO = thoracic perisympathetic organ (extended FMRFamides); CC = corpora cardiaca (1–4: pyrokinins, 5: adipokinetic hormone, 6: myosuppressin, 7: corazonin, and 8 and 9: not assignable).
[a]Not included in phylogenetic analyses.

the Namibian species were exclusively collected following heavy rains during the summer season (February to May). In a rainy season with sufficient precipitation, the density of Mantophasmatodea in Namibia can be exceptionally high, up to 10 individuals in a bush about 1 m tall. This density made it possible to thoroughly analyze specimens from neighboring populations. Generally speaking, the distribution of Mantophasmatodea in Namibia and South Africa seems to be largely confined to the westernmost upfoldings of the Great Escarpment. In the Southern Cape (South Africa), a few species also inhabit the coastal region, including the Strandveld Succulent Karoo, and Dune Thicket (Fynbos); no specimens have been found on the Cape Peninsula. The distribution map suggests that a dispersal barrier may currently exist between the species found in the winter rainfall area of South Africa and those living in the summer rainfall region of Namibia. In the North, the genus *Mantophasma* is likely to cross the Kunene River into southwestern Angola, a region that was not explored in our study.

The Neuroendocrine System of Mantophasmatodea

In analogy to the hypothalamic–pituitary axis of vertebrates, peptide hormones produced in the central nervous system of insects are stored in neurohemal organs, mainly the corpora cardiaca and the thoracic and abdominal perisympathetic organs. The neurohemal organs from different tagmata (head, thorax, and abdomen) accumulate tagma-specific peptide hormones, thus increasing the diversity of the neuroendocrine system (Predel 2001). For example, the insect perisympathetic organs store extended FMRFamides (thoracic perisympathetic organs) and CAPA-peptides (abdominal perisympathetic organs), which are produced in the respective ganglia of the ventral nerve cord (see Predel et al. 2004). In addition, the corpora cardiaca accumulate gene products from neuroendocrine cell clusters of the brain (e.g., corazonin, myosuppressin, pyrokinins) and also contain endocrine cells which produce adipokinetic hormone. The peptide hormones processed from homologous precursors in different species often contain substitutions that are of potential diagnostic and taxonomic value.

In Mantophasmatodea, the ventral nerve cord consists of 3 thoracic ganglia, 6 unfused abdominal ganglia, and the terminal ganglion. The thoracic ganglia and the unfused abdominal ganglia each possess a single perisympathetic organ; 2 perisympathetic organs arise from the terminal ganglion. The cellular origin of the CAPA-peptides is particularly noteworthy. As shown in Figure 3a, 2 ventrolateral *capa*-cells of each abdominal ganglion are accompanied by 3 unpaired median

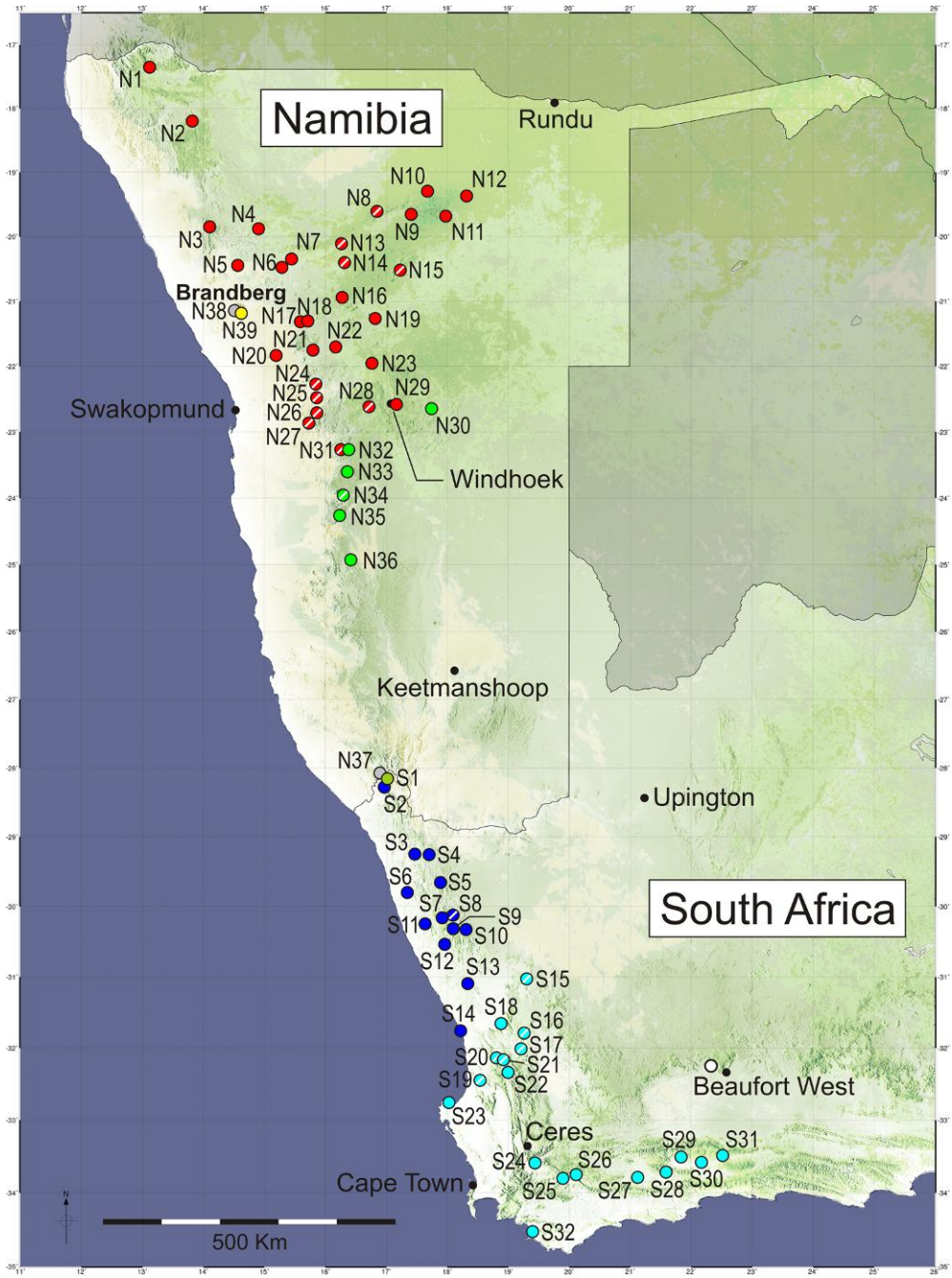


FIGURE 2. Collection localities of Mantophasmatodea that were analyzed in this study. Identical colors represent related taxa as revealed by analysis of neuropeptide sequences (blue, Austrophasmatidae [dark blue: *Namaquaphasma ookiepense*]; gray, *Tyrannophasma* and *Praedatophasma*; green, *Striatophasma*; light green, Austrophasmatidae gen. et sp. nov. "RV"; and yellow, *Pachyphasma*; red, *Mantophasma*). The distribution of Mantophasmatodea in Namibia and South Africa seems to be confined mainly to the westernmost upfoldings of the Great Escarpment. The map suggests that a putative dispersal barrier may exist between the species found in the winter rainfall area of South Africa and those living in the summer rainfall region of Namibia. The South African Austrophasmatidae probably do not enter the summer rainfall region of South Africa; a single species has been found in the transition zone at Beaufort West (white circle; see Damgaard et al. 2008). See Table 1 for locality codes.

capa-neurons. These neurons have projections that extend into the abdominal perisympathetic organs. An identical cellular pattern has been found in Grylloblattodea (outgroup species *Galloisiana* sp.; Fig. 3b) but not in other Neoptera (not shown).

Differentiation of Populations/Species by Peptide Hormone Mass Fingerprints

To account for intraspecific variation, usually 3–5 specimens were prepared from each of the collecting sites; at least 3 abdominal perisympathetic organs, 2

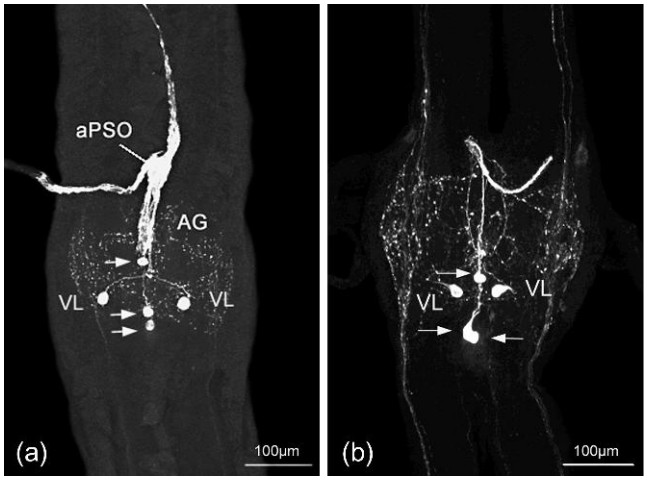


FIGURE 3. Neuropeptide (CAPA-periviscerokinin-like) immunofluorescence staining in whole-mount preparations of an unfused abdominal ganglion (AG) from (a) *Mantophasma kudubergense* and (b) *Galloisiana* sp. (Grylloblattodea). The 2 ventrolateral *capa*-cells (VL) are generally typical of insects. The expression of CAPA-peptides in 3 unpaired median neurons (arrows), however, has been found only in Mantophasmatodea and Grylloblattodea. The perisympathetic organ (PSO) of *Galloisiana* sp. was removed for peptide identification prior to immunostaining.

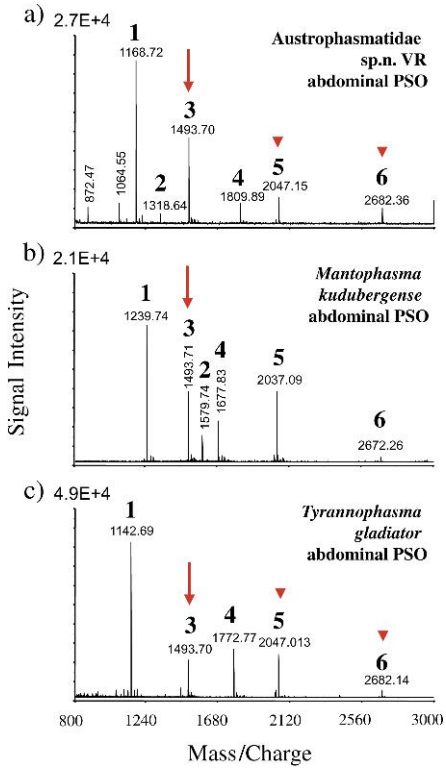


FIGURE 4. MALDI–TOF mass spectra (mass fingerprints) obtained by direct profiling of abdominal perisympathetic organs from 3 distantly related species of Mantophasmatodea. The prominent ion signals are labeled. The numbers mark orthocopies; homology was confirmed by subsequent fragmentation analysis. Note that the monoisotopic masses of most neuropeptides differed between the 3 preparations; only 1 orthocopy (arrow) was identical in all species and 2 orthocopies (arrowheads) were found to be identical in Austrophasmatidae sp. nov. "VR" and *Tyrannophasma gladiator*. Assignment of the CAPA peptides: 1: CAPA-periviscerokinin-1; 5: CAPA-periviscerokinin-2; 6: extended CAPA-periviscerokinin-2; 3: CAPA-pyrokinin; 2 + 4: putative spacer peptides of the *capa*-gene.

thoracic perisympathetic organs, and 2 pieces of the paired corpora cardiaca were analyzed per individual. The resulting MALDI–TOF mass spectra yielded highly reproducible mass fingerprints (Figs. 4–6; Supplemental Material 1, available at http://datadryad.org, doi:10.5061/dryad.r6ng71d4.). In 53 of the 71 locations, all specimens had identical mass fingerprints. The remaining 18 localities contained individuals in which at least one ion signal was replaced by another ion signal (putative amino acid substitution). Specimens from these localities are specifically indicated by their respective locality codes (e.g., S18a and S18b). Some ion signals were useful to assign specimens at the family or genus level, whereas others appeared to be species- or population-specific, with a few cases of variability at even lower levels (populations and phylogeographical units). Although mass fingerprints should not be used to define species, they contain sufficient information to unambiguously assign specimens to recognized species and, in the case of Mantophasmatodea, to different populations. This feature makes peptide mass fingerprints an ideal tool to follow the migration of populations and to detect hybridization between neighboring populations or tentatively described species. Indeed, we found a few heterozygous specimens from localities with otherwise homozygous individuals that were distinguishable by at least one ion signal (see above). In these specimens, signals that differed among individuals from their respective localities were combined in a single spectrum (Fig. 7). An assignment of these hybrid signals to homologous neuropeptides was subsequently performed by sequence analysis (see below).

Because the mass spectra from the corpora cardiaca preparations were more variable than those from other organs with respect to the relative intensity of their neuropeptide signals, only corpora cardiaca peptides that were structurally elucidated in subsequent experiments (see below) were included in the fingerprint data set. Altogether, 25 of the most prominent neuropeptide signals were collected from each of the 71 populations of Mantophasmatodea. These fingerprint data already allowed the grouping of populations or related species. For example, a prominent peptide signal at $[M + H]^+$ 1350.8 was typical of the corpora cardiaca preparations from all South African Austrophasmatidae (see Supplemental Material 1). Because this distinct neuropeptide signal did not occur in any other known Mantophasmatodean species and was also not found in Grylloblattodea (see below), it represents an apomorphic character of the family Austrophasmatidae sensu Klass et al. (2003). The fingerprint data set also indicates the close relationship of the majority of "*Mantophasma*-like" specimens, which show many easily detectable character transitions. All

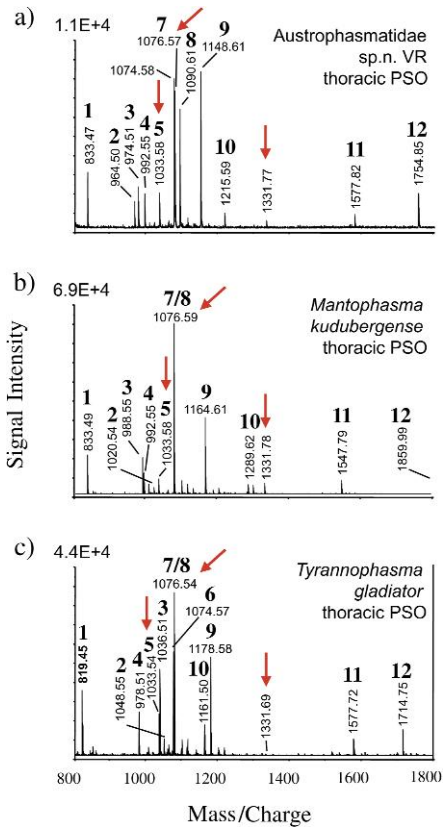


FIGURE 5. Mass fingerprints obtained by direct profiling of thoracic perisympathetic organs from the 3 species shown in Figure 4. Due to the large number of peptide signals, the shown mass range was reduced to 800–1800 Da. The numbers mark orthocopies; homology was confirmed by subsequent fragmentation analysis. The monoisotopic masses of most neuropeptides differed between the 3 preparations; only 3 orthocopies (arrows) were identical in all species. The peptide signals likely represent products of the *extended fmrf*-gene of Mantophasmatodea.

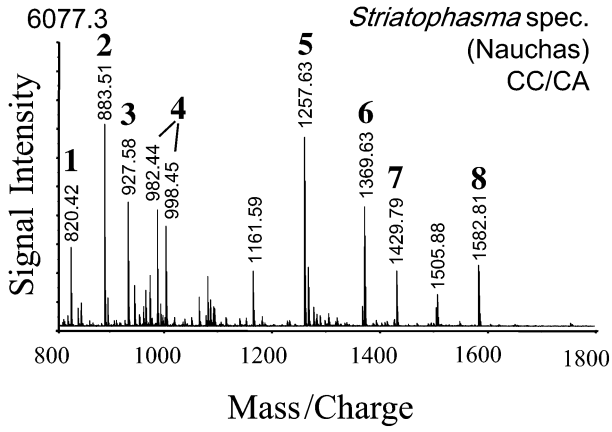


FIGURE 6. Mass fingerprint obtained by direct profiling of corpora cardiaca of a member of the novel genus *Striatophasma* collected at Nauchas (Namibia). The numbering corresponds to corpora cardiaca peptides whose sequences were revealed by fragment analysis and were included in the phylogenetic analyses. Assignment of the neuropeptides: 1: pyrokinin (PK)-1; 2: PK-2; 3: PK-3; 4: adipokinetic hormone ($[M+Na]^+/[M+K]^+$ adduct ions); 5: myosuppressin; and 6: corazonin. Peptides 7 and 8 could not be assigned to a known neuropeptide gene.

peptides that contributed to the mass fingerprint data set were subsequently sequenced (see below). Therefore, a thorough taxonomic interpretation of the fingerprint data was unnecessary. However, these data can be used in future investigations to assign unknown Mantophasmatodean specimens from newly explored populations to previously studied taxa in a kind of peptide barcoding.

Sequence Analysis of Peptide Hormones

All neuropeptides that contributed to the mass fingerprint data set were fragmented using mass spectrometric techniques. First, prepurified extracts of neurohemal organs (corpora cardiaca, abdominal perisympathetic organs, and thoracic perisympathetic organs) from 3 to 5 adult specimens of *Austrophasma gansbaaiense*, *Mantophasma kudubergense*, and *Tyrannophasma gladiator* were each analyzed by nanospray ESI-Q–TOF mass spectrometry. Manual fragment analyses of the collision-induced dissociation spectra revealed the neuropeptidomes of these species. Subsequently, the homologous neuropeptides of the remaining species were elucidated by direct profiling of tissue samples using MALDI–TOF/TOF mass spectrometry (see Medzihradszky et al. 2000). The same samples that were used to obtain the mass fingerprints were usually sufficient to determine sequence substitutions (see Fig. 1). Identical mass ion signals were not fragmented in all species but were fragmented in at least the more distantly related taxa. In a few cases, fragment analyses resulted in different but identical mass neuropeptides (Fig. 8; Supplemental Material 1). Most of the novel neuropeptides are products of known neuropeptide genes, such as the *capa*-gene (abdominal perisympathetic organ peptides 1, 3, and 5; paracopies), *extended fmrf*-gene (thoracic perisympathetic organ peptides 1–12; paracopies), *myosuppressin*-gene (corpora cardiaca peptide 6; single copy peptide), *adipokinetic hormone*-gene (corpora cardiaca peptide 5; single copy peptide), *pyrokinin*-gene (corpora cardiaca peptides 1–4; paracopies), and *corazonin*-gene (corpora cardiaca peptide 7; single copy peptide, see Table 2). Some nonamidated peptides that could not be assigned to biologically active peptides of other insects represent putative spacer peptides of the CAPA-precursor (abdominal perisympathetic organ peptides 2 and 4; ortholog spacer peptides) or could not be assigned to a specific insect neuropeptide gene (corpora cardiaca peptides 8 and 9). Altogether, the different neuropeptides represent products of 7 loci. In a parallel analysis, the neuropeptides of *Galloisiana* sp. (Grylloblattodea), which was included as an outgroup species in the subsequent phylogenetic analysis, were identified from a single specimen.

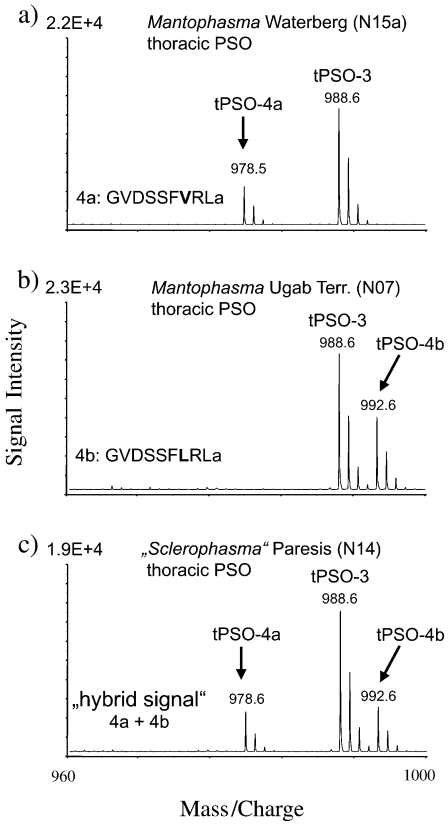


FIGURE 7. Mass fingerprints of thoracic perisympathetic organ preparations from 3 *Mantophasma*/"*Sclerophasma*" specimens (selected mass range of 960–1000). Specimens from Waterberg (a; collection site N15) and Ugab-Terrace (b; collection site N07) can be distinguished by different tPSO-4 orthocopies (arrow; single amino acid substitution). The specimen from the Paresis mountains (c; collection site N14) expressed both orthocopies. This hybrid pattern indicates that the respective parental neuropeptide genes encoded for different tPSO-4 orthocopies (heterozygous). We also analyzed homozygous specimens from the same area. The Paresis mountains are located between Waterberg (100 km east of the Paresis mountains) and the Ugab Terrace (100 km west of the Paresis mountains).

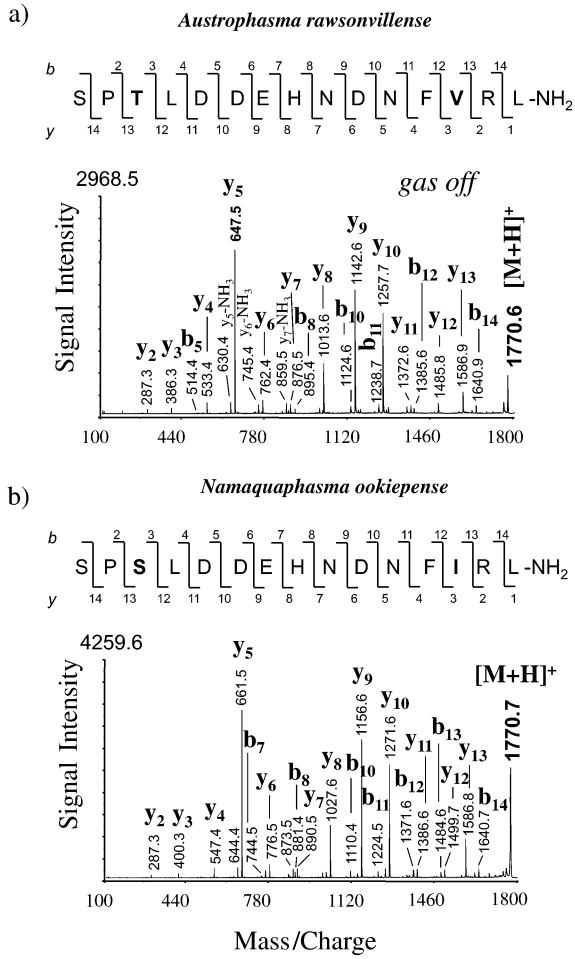


FIGURE 8. Examples of MALDI–TOF/TOF fragment spectra of thoracic perisympathetic organ orthocopies #12 from (a) *Austrophasma rawsonvillense* (S07) and (b) *Namaquaphasma ookiepense* (S24). Fragments were analyzed manually. Assigned fragments are labeled, and the resulting sequences are indicated. Note that these peptides represent a rare case of identical mass orthocopies with different sequences.

Alignments of Homologous Neuropeptides and Phylogenetic Analyses

Sequences of the identified products of 7 neuropeptide genes (25 peptides per population) were first aligned separately and subsequently combined. The length of the combined sequences ranged from 275 to 305 amino acids (for individual peptide sequences see Table 2 and Supplemental Material 2) and, including gaps, the alignment contained 320 characters. Variations in length of the single neuropeptides as well as species with missing data are shown in Table 3. Within Mantophasmatodea, all peptides could be unambiguously aligned (Supplemental Material 2). This ease of alignment was particularly surprising for the extended FMRFamides, which are highly variable in sequence and in the number of paracopies as shown for example in Diptera (see Wegener and Gorbashov 2008; Rahman et al. 2009). However, the assignment of extended FMRFamides from Mantophasmatodea to those of the outgroup species (*L. migratoria*, *P. americana*, and *Galloisiana* sp.) was complicate due to difficulties in the assignment of orthocopies.

Statistics from parsimony and BAs are presented in Table 4. Analysis of the complete data set, utilizing the option to average inference over a set of fixed amino acid models in MrBayes, returned JTT (Jones et al. 1992) as the best amino acid substitution model (see Table 4). This model was also obtained using ProtTest 3 (Abascal et al. 2005). The model averaging approach with MrBayes was also performed on each of the partitioned data sets (CAPA-peptides, extended FMRFamides, pyrokinins, and 2 × corpora cardiaca peptides) independently. Since mitochondrial or chloroplast models were selected for some of the peptide partitions (data not shown), this procedure was found inappropriate (see Huelsenbeck et al. 2008), probably due to the limited length of each partition. When excluding such nonappropriate models a priori in ProtTest 3, the

TABLE 3. Sequence length of homologous peptides from different species or populations of Mantophasmatodea, list of missing data (for species codes see Table 1), and number of amino acid substitutions within the different homologous peptides

	Sequence length (minimum–maximum)	Species with missing data	Number of different sequences
aPSO			
1[a]	11–12		8
2[b]	10–13	N38	15
3[a]	15–18		2
4[b]	16		12
5[a]	17–20		6
tPSO			
1[a]	7		4
2[a]	8		9
3[a]	9		7
4[a]	9		3
5[a]	8		2
7[a]	9		1
8[a]	9		4
9[a]	11		8
10[a]	11–12	N37, N39	18
11[a]	16	N37, S05	9
12[a]	15–16	N37	14
CC			
1[a]	7		1
2[a]	8		1
3[a]	8		2
4[a]	13	S01–S32	2
5[c]	8		3
6[c]	10		1
7[c]	11		2
8	11–15	N37, N38	7
9	14		8

[a]Multiple copy peptide.
[b]Spacer peptide.
[c]Single copy peptide.

testing of individual partitions returned several models (e.g., WAG, JTT, or VT) with similar or almost identical Akaike weights (AIC about 0.24) that differed only in third decimal place (data not shown). Therefore, the model selected for the whole data set (JTT) could also apply for the BA of unlinked partitions (see Table 4).

Figure 9 shows a Bayesian consensus tree inferred from the neuropeptide sequences of the 71 Mantophasmatodea populations and 3 outgroup taxa, which are Grylloblattodea (*Galloisiana*), Blattodea (*Periplaneta*), and Orthoptera (*Locusta*). Because slightly different peptidomes (designated as a- and b-type) were identified in specimens from 18 locations, the total number of Mantophasmatodean terminal taxa was 89. The phylogenetic tree revealed long branches for the outgroup taxa, indicating evolutionary divergence of neuropeptide sequences between the outgroup taxa and Mantophasmatodea. The Mantophasmatodea samples form 2 well-supported subclades. Subclade 1 includes the known members of Austrophasmatidae and several undescribed but closely related taxa, as well as a distinct new taxon of Austrophasmatidae from the Richtersveld area (Austrophasmatidae gen. et sp. nov. "RV") and the novel southern Namibian genus *Striatophasma*. Subclade 2 (= Mantophasmatidae) contains the endemic taxa from the Brandberg Massif (*Tyrannophasma* and the novel genus *Pachyphasma*), representatives of "*Mantophasma*" from northern and central Namibia and *Praedatophasma maraisi* from the Orange River (Richtersveld).

JTT was selected as the best-fit amino acid substitution model (post probability >0.95) in this analysis (see Table 4). In an alternative analysis, we tested the most simple model (Poisson distribution) which assumes equal stationary state frequencies and equal substitution rates for all amino acids as fixed rate model resulting in an identical topology of the tree but partly increased Bayesian inference posterior probability (biPP) values (Fig. 9).

Since ambiguities in the assignment of orthocopies from the outgroup taxa (extended FMRFamides; see above) might result in homoplasies and thereby affect our main goal, the study the phylogenetic relationships within Mantophasmatodea, we repeated the BAs without outgroups. For this, we performed first a BA using concatenated data sets, that is, treating the sequences of the identified products of 7 genes as a single linked data set. In a second approach, we ran an analysis with the peptides grouped into 5 unlinked data sets, allowing for estimation of substitution rates in the different peptides (see Table 4). We used the JTT model for all analyses. Both approaches revealed identical tree

TABLE 4. Statistics for the major analyses under parsimony (PA) and BA

	PA characters				PA statistics					BA variables		
Analysis	Total	Constant	Not informative	Informative	CI	HI	RI	RC	Tree length	Mean log likelihood	Protein Model	Substitution rates ± SE
Mantophasmatodea + outgroup, analysis 1a	320	174	70	76	0.763	0.236	0.94	0.732	279	−2838.389	JTT (model average)	2.21 ± 0.85
Mantophasmatodea + outgroup, analysis 1b										−3116.27	Poisson = setting	$1.98 \pm 3.53 \times 10^{-3}$
Mantophasmatodea without outgroup	320	227	28	65	0.665	0.335	0.948	0.631	188	−2329.56	JTT (model average)	$1.27 \pm 2.89 \times 10^{-3}$
Mantophasmatodea without outgroup Partitioned data set												
1. Total analysis										−2305.43	JTT = setting	$1.23 \pm 2.22 \times 10^{-3}$
2. Data set 1	125	79	17	29	0.664	0.372	0.925	0.630	102		JTT = setting	$1.33 \pm 1.50 \times 10^{-3}$
3. Data set 2	31	27	0	4	1.000	0.000	1.000	1.000	4		JTT = setting	$0.23 \pm 1.20 \times 10^{-3}$
4. Data set 3	47	44	0	3	1.000	0.000	1.000	1.000	3		JTT = setting	$0.28 \pm 1.98 \times 10^{-3}$
5. Data set 4	87	59	7	21	0.795	0.204	0.971	0.773	49		JTT = setting	$1.11 \pm 1.26 \times 10^{-3}$
6. Data set 5	30	18	4	8	0.882	0.117	0.968	0.870	17		JTT = setting	$1.17 \pm 4.00 \times 10^{-3}$

Notes: PA was performed by using the default settings of PAUP* (heuristic search, TBR branch-swapping algorithm, maximum number of trees = 100). Variables of BA consider results of 2 runs. Partitioned data sets: Set 1—extended FMRFamides (multiple copy peptides); Set 2—adipokinetic hormone, myosuppressin, and corazonin (single copy peptides of 3 different genes); Set 3—pyrokinins (multiple copy peptides); Set 4—CAPA-peptides (multiple copy peptides); and Set 5—CC-peptides 8 and 9 (see also Table 2).

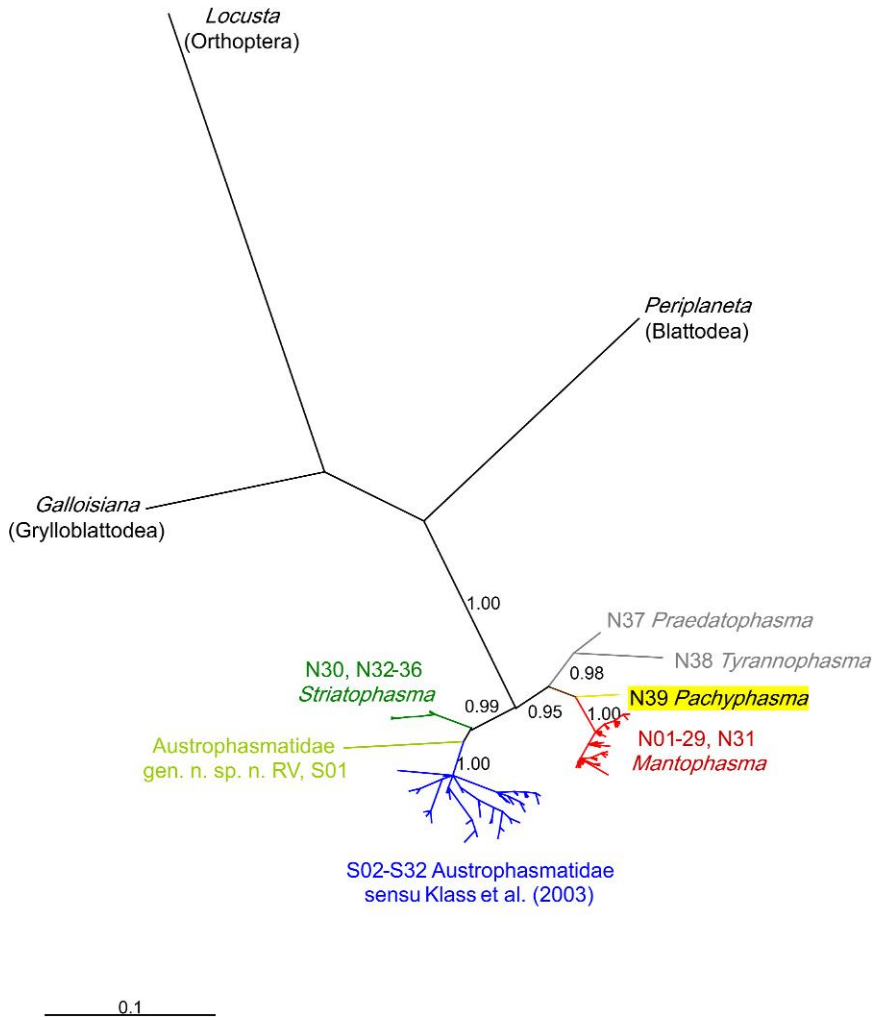


FIGURE 9. Radial consensus tree from Bayesian phylogenetic analysis of the concatenated data set of 25 neuropeptides from 89 Mantophasmatodea samples and 3 outgroup taxa. Posterior probabilities >0.90 are shown.

topologies and almost identical biPP values (for likelihood values see Table 4), and therefore, only the tree from the concatenated data set is presented (Fig. 10). The backbone topology of the tree with the outgroup species (Fig. 9) was confirmed, and some intraordinal clades became better supported.

The Namibian populations of the genus *Striatophasma* and all South African Austrophasmatidae were monophyletic (biPP 1). Within the South African Austrophasmatidae, the position of the undescribed taxon Austrophasmatidae gen. et sp. nov. "RV," which shows an exceptionally large number of autapomorphies, exhibits the most conflict (biPP = 0.76). All other South African Mantophasmatodea taxa were forming one well-supported clade, within which the relationships are uncertain (polytomy). Nevertheless, most of the described genera (except *Austrophasma*) and species receive some support.

Namaquaphasma ookiepense has the most widespread distribution of the sampled species of Austrophasmatidae. The 14 sampled populations of this species occupy a large area ranging from Strandfontein in the south (Strandveld Succulent Karoo, altitude of 10 m; S14) to the Richtersveld Park in the northern Cape Province (Little Succulent Karoo, 500 m; S02), and the Kamieskroon Mountains in the east (Northwestern Mountain Renosterveld, up to 1500 m; S08) (see Fig. 2). The phylogenetic analysis also supports the monophyly of *Hemilobophasma* (different populations of *H. montaguense*), *Karoophasma* (*K. botterkloofense* and *K. biedouwense*), and *Lobatophasma* (*L. redelinghuysense*). *Karoophasma botterkloofense and K. biedouwense* form a well-supported sister group to an undescribed species of Mantophasmatodea (Austrophasmatidae gen. et sp. nov. "VR," S18; biPP 1); *L. redelinghuysense* is the poorly supported sister group of *Viridiphasma clanwilliamense* (Eberhard et al. 2011; S21a,b; biPP 0.86). The 2 species of *Austrophasma* that were included in this study (*A. gansbaaiense* and *A. rawsonvillense*) do not appear to form a monophyletic group. However, the phylograms support a close relationship between *A. rawsonvillense* from Worcester/Ashton (Fig. 2, S24/S25) and a Mantophas-

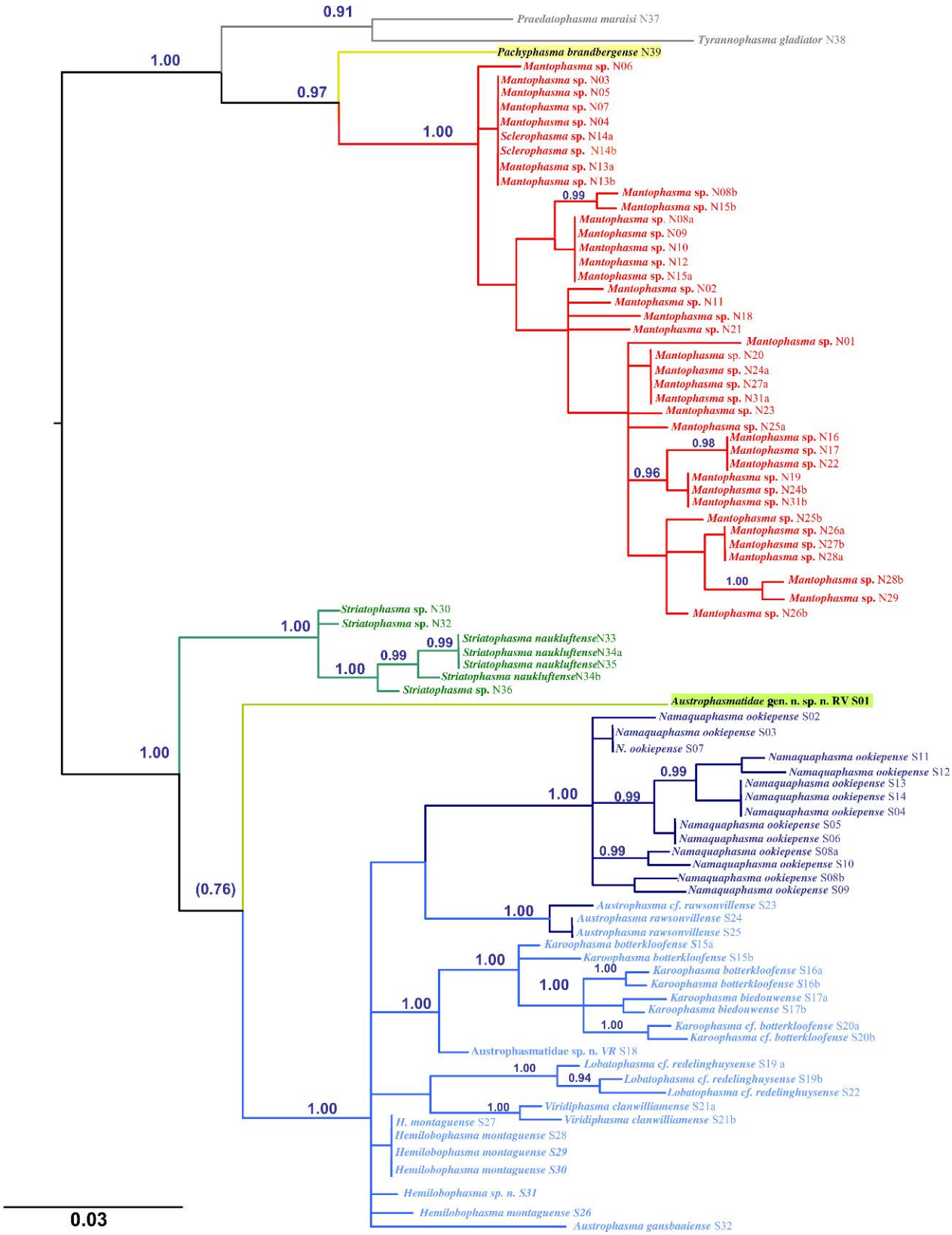


FIGURE 10. Consensus tree (midpoint rooted) from Bayesian phylogenetic analysis of the concatenated data set of 25 neuropeptides from 89 Mantophasmatodea samples. The JTT amino acid substitution model was implemented as a fixed rate model in MrBayes. Posterior probabilities >0.90 are shown. Colors are in accordance with colors in Figure 2.

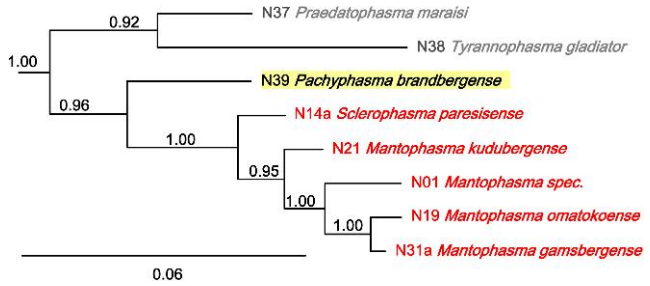


FIGURE 11. Subtree of the consensus tree from Bayesian phylogenetic analysis of the concatenated data set (as in Fig. 9) of 44 Mantophasmatodea samples and 3 outgroup taxa. For the Namibian *Mantophasma* clade (see Fig. 9), a reduced data set consisting of 5 already described taxa (see Damgaard et al. 2008) plus *Mantophasma* sp. from Omohunga was analyzed. Posterior probabilities >0.90 are shown.

matodea population from Helena Bay on the west coast (Fig. 2; S23).

Within the Namibian Mantophasmatidae, the endemic species *Tyrannophasma gladiator* (Brandberg Mountains) and *Praedatophasma maraisi* (restricted distribution along the Orange River) constitute a single clade that is clearly separated from *Pachyphasma* + *Mantophasma* (Fig. 10). All our analyses recovered a well-supported sister group relationship between *Pachyphasma brandbergense* and *Mantophasma* (Fig. 10). The relationships of the numerous *Mantophasma* populations from many locations in central and northern Namibia (mopane, thornbush, and highland savannas) are poorly resolved in the phylogenetic analyses. For this taxon, therefore, we use the currently accepted generic name in combination with the collection localities to designate different populations throughout the manuscript. In a separate analysis, we tested only specimens from locations with already described *Mantophasma* species (*M. kudubergense*, *M. omatokoense*, *M. gamsbergense*, and *Sclerophasma paresisense*) and the northernmost location (Omuhonga, N01). This analysis retrieved strong branch support for *M. kudubergense*, *M. gamsbergense*, *M. omatokoense*, and *Sclerophasma paresisense* and for *Mantophasma* from Omuhonga (Fig. 11). With the inclusion of more *Mantophasma* populations (see Fig. 10), however, polyphyletic branching and a gradual decrease in posterior probability as a measure of branch support occurred, indicating that *Mantophasma* consists of many informal phylogeographic groups rather than numerous distinct species. The detection of heterozygous individuals (see Fig. 7) supports the same interpretation, confirming that *Mantophasma* populations are closely related and that the genus may include described taxa with uncertain species status.

DISCUSSION

The use of neuropeptide sequence analysis to determine phylogenetic relationships is still uncommon. Due to their role as ligands that must bind to their respective receptors, these messenger molecules are under considerable evolutionary constraint. Consequently, the regions of neuropeptide genes that encode mature peptides may be highly conserved, making them especially suitable for the reconstruction of phylogenetic relationships within higher taxa. This topic has been comprehensively discussed in a recent paper (Roth et al. 2009). In the present study, we used mass spectrometry to analyze a large number of homologous neuropeptides from closely related insects, all of which belong to the recently discovered group Mantophasmatodea. Except for *Tanzaniophasma subsolanum*, which is not found in southern Africa, and *Austrophasma caledonense*, this study includes all species and most of the known populations of these distinctive insects. As we have shown, neuropeptide sequences contain useful phylogenetic signals and are sufficient to provide an overview of sister group relationships within Mantophasmatodea.

Our peptidomic approach yields peptide mass fingerprints and sequences for use in phylogenetic analyses from the same sample and by using the same equipment. Mass fingerprints, which are essential by-products of sequencing attempts, contain information about the neuropeptides to be sequenced. We do not claim the taxonomic assignment should mainly based on mass fingerprinting data but we consider this method as a helpful tool to sort taxa of Mantophasmatodea. Moreover, it easily reveals heterozygous individuals as it was shown for the species complex of *Mantophasma*.

Within Mantophasmatodea, the sequenced peptides could be unambiguously aligned across all species, even when putative spacer peptides that lack ligand–receptor coevolution (e.g., several peptides from the abdominal perisympathetic organs) were included. In a few cases, the search for orthocopies failed, and the missing data were coded with question marks in the alignments. The observed similarities in the numbers and sequences of paracopies, particularly those of the extended FMRFamides, which are highly variable in insects, indicate a close relationship among all studied Mantophasmatodea. The outgroup species, however, exhibited a widely divergent peptidome, creating alignment problems for the extended FMRFamides. As a result, the existing data are not yet sufficient to determine the relationship of Mantophasmatodea to other insect taxa; although the neuropeptides of Grylloblattodea and Blattodea share certain similarities with those of Mantophasmatodea. An extension of the existing data set with further multiple copy peptide families (e.g., tachykinin-related peptides and allatostatins) may provide greater insight regarding this topic.

Morphological characters of the neurohemal organ system, rather than peptide sequences, provide hints for interordinal relationships between Mantophasmatodea and other Neoptera. Our analysis of the architecture of the abdominal ventral nerve cord and the respective neurosecretory *capa*-neurons favors a close relationship between Mantophasmatodea and Grylloblattodea. Among hemimetabolous insects, the presence of

6 unfused abdominal ganglia (abdominal neuromere 1 fused with the metathoracic ganglion, neuromeres 2–7 represented by unfused ganglia, and the remaining neuromeres fused to the terminal ganglion) occurs only in a few basal Neoptera, namely Phasmatodea, Embioptera, Grylloblattodea (see Niven et al. 2008), and Mantophasmatodea. These insect taxa also possess 8 abdominal perisympathetic organs; the first neuromere does not develop an abdominal perisympathetic organ, and the terminal ganglion has 2 perisympathetic organs. These characters separate Mantophasmatodea from insect groups such as Blattodea and Dermaptera (5 unfused abdominal ganglia and 8 abdominal perisympathetic organs), Mantodea (3 unfused abdominal ganglia and 8 abdominal perisympathetic organs), and Orthoptera (most of which have 4 unfused abdominal ganglia and 7 abdominal perisympathetic organs) (Predel R. and co-workers; unpublished). With respect to the number of *capa*-neurons in the unfused abdominal ganglia, Phasmatodea and Embioptera can easily be distinguished from Mantophasmatodea and Grylloblattodea. Embiopterans possess only 2 ventrolateral *capa*-neurons (plesiomorphic character; Predel R. and co-workers; unpublished), and Phasmatodeans have 3 additional median *capa*-cell clusters with numerous neurons in each one (see Predel 2006). The latter character state is also typical of other Neoptera, such as Blattodea (Eckert et al. 2002), Mantodea (Köhler and Predel 2010), and Orthoptera (Swales and Evans 1995). Only in Mantophasmatodea and Grylloblattodea are the 2 ventral *capa*-neurons accompanied by 3 separated median unpaired neurons. This feature is unique in Neoptera and presents an obvious and remarkable apomorphy of Mantophasmatodea + Grylloblattodea.

Phylogenetics of Mantophasmatodea

Using peptide sequences from single species of Grylloblattodea, Orthoptera, and Dictyoptera as outgroups, Mantophasmatodea was monophyletic, but these data would need additional sequences (e.g., from Phasmatodea) to test the monophyly of the order. Regarding the intraordinal relationships, our phylogenetic analyses of neuropeptide sequences support the monophyly of 2 subclades with Mantophasmatidae and Austrophasmatidae. To avoid describing additional families, we preliminarily place the novel taxa *Striatophasma* and Austrophasmatidae gen. et sp. nov. "RV" within Austrophasmatidae and retain *Praedatophasma* and *Tyrannophasma* within Mantophasmatidae. The 2 major clades of Mantophasmatodea are discussed below. *Tanzaniophasma* may represent a third clade; specimens of this taxon were not available for this study.

Mantophasmatidae.—Within Mantophasmatidae, our analyses support a sister group relationship between *Tyrannophasma* + *Praedatophasma* and *Mantophasma* + *Pachyphasma*. The proposed close relationship between *Tyrannophasma* and *Praedatophasma* (see Damgaard et al. 2008), which is corroborated by their similar appearance, may result from the complete absence of related species within this clade. Peptide sequences suggest that these species are actually not very closely related to each other. Nevertheless, the *Praedatophasma* + *Tyrannophasma* clade exhibits distinct peptide sequences that place these species at a separate position in our phylogenetic tree. This finding supports previous suggestions that these taxa should receive a higher taxonomic rank (e.g., family status; see Klass et al. 2003; Damgaard et al. 2008). Data from the genus *Mantophasma* do not reveal conclusive lineage sorting within this taxon, although numerous populations were studied. On the contrary, even the relationships of previously described and available species of this genus (*M. gamsbergense*, *M. omatokoense*, and M. *kudubergense*; Zompro and Adis 2006) and *Sclerophasma paresisense* (Klass et al. 2003) are poorly resolved.

Austrophasmatidae.—The family Austrophasmatidae, which was originally erected to encompass related taxa from the Western and Northern Cape Provinces of South Africa, contains numerous species and has been comprehensively investigated using morphological (Klass et al. 2003) and molecular characters (Damgaard et al. 2008). The data obtained in the present study confirm the monophyly of Austrophasmatidae sensu Klass et al. (2003) and thus corroborate the findings of Damgaard et al. (2008).

In their peptide sequences, the members of Austrophasmatidae sensu Klass et al. (2003) show distinct synapomorphies. Substitutions in the sequences of pyrokinin-3, adipokinetic hormone, and corazonin are particularly important; the sequences of the latter 2 peptides are usually conserved in closely related insects. It is highly unlikely that such substitutions have occurred twice during the evolution of Mantophasmatodea. These peptides separate Austrophasmatidae sensu Klass et al. (2003) from the remaining Mantophasmatodea, whose, for example, corazonin sequences are identical to those of related insects, such as Grylloblattodea (this study) and Blattodea (Predel et al. 2007).

The backbone relationships of the genera of Austrophasmatidae sensu Klass et al. (2003) (*Namaquaphasma*, *Karoophasma*, *Lobatophasma*, *Hemilobophasma*, *Viridiphasma*, and *Austrophasma*) remain unresolved (polytomy). This ambiguity has been found previously in an analysis of mitochondrial DNA sequences (Damgaard et al. 2008). These taxa may have evolved within a rapidly speciating lineage, resulting in a monophyletic group of allopatric species (see below). Currently, many of these taxa are defined as monotypic genera (Klass et al. 2003; Eberhard et al. 2011). The Austrophasmatid genus with the most widespread distribution is *Namaquaphasma*, which may be represented by a single species (*N. ookiepense*). Our analyses neither support the proposed sister group relationship of *Namaquaphasma* to the other previously defined clades of Austrophasmatidae nor the existence of northern and

southern groups within *Namaquaphasma* (Damgaard et al. 2008). Extensive collecting yielded many populations with some variability but without polarity. Regarding the other genera of Austrophasmatidae sensu Klass et al. (2003), only *Austrophasma* (*A. gansbaaiense* and *A. rawsonvillense*) was not supported as monophyletic by our data. In the Richtersveld area, we found specimens of a new taxon (Austrophasmatidae gen. et sp. nov. "RV") with neuropeptide sequences that point to an intermediate position between Mantophasmatidae and Austrophasmatidae sensu Klass et al. (2003). Whereas the corazonin sequence of Austrophasmatidae gen. et sp. nov. "RV" is of the basal (Mantophasmatidae) type, its adipokinetic hormone and pyrokinin-3 sequences are typical of Austrophasmatidae sensu Klass et al. (2003). However, a sister group relationship between this taxon and Austrophasmatidae sensu Klass et al. (2003) is not strongly supported. Notably, the novel taxon (Austrophasmatidae gen. et sp. nov. "RV") shows an exceptionally large number of autapomorphies. Analysis of a reduced set of taxa reveals an alternative scenario in which *Striatophasma* + Austrophasmatidae gen. et sp. nov. "RV" forms the sister-group of previously known South African Austrophasmatidae, but this relationship is also not strongly supported (not shown).

As its designation implies, we classify this new taxon in Austrophasmatidae. The family Austrophasmatidae is also extended by a strongly supported novel sister group (BiPP: 1.0) of previously known Austrophasmatidae + Austrophasmatidae gen. et sp. nov. "RV," the genus *Striatophasma*, which occurs in Namibia, south of the region inhabited by *Mantophasma*. *Striatophasma* is characterized by the presence of several neuropeptides that are found only in this genus, most notably by the expression of a particular adipokinetic hormone. A total of 3 different adipokinetic hormones have been found in Mantophasmatodea. The first form is typical of all Namibian taxa except *Striatophasma* (Gäde et al. 2005; this study); a second adipokinetic hormone is found in all members of South African Austrophasmatidae (Predel et al. 2005); and the third adipokinetic hormone is found in *Striatophasma*. The fact that *Striatophasma* expresses the conserved corazonin typical of Mantophasmatidae and Austrophasmatidae gen. et sp. nov. "RV" but shares its pyrokinin-3 sequence with all South African Austrophasmatidae also implies that this taxon occupies an intermediate position between the 2 families.

Biogeography and Speciation of Mantophasmatodea

All records of extant Mantophasmatodea are from Africa south of the equator. Fossil records from the Middle Jurassic in China (Huang et al. 2008) and from Eocene Baltic amber (Zompro 2001), however, confirm the worldwide distribution and long evolutionary history of this taxon. If the suspected sister group relationship with Grylloblattodea can be conclusively demonstrated in future analyses, a Laurasian–Gondwanan split similar to the separation at the subordinal level within Plecoptera might be postulated (Grimaldi and Engel 2005). Its recent distribution, with numerous taxa in southern Africa and a single species known from the poorly surveyed region of eastern Africa, might suggest colonization in either direction through the Pleistocene "arid corridor" (Balinsky 1962; Verdcourt 1969; Poynton 1995). This corridor probably consisted of arid savanna and not true desert (Irish 1990), thereby fitting the ecological adaptation of the extant species.

Within the extensive southern African range, many species of Mantophasmatodea are rather common and widespread insects. In fact, Mantophasmatodea successfully compete with Mantodea (praying mandids), which inhabit the same habitats, and are also sit-and-wait predators with a similar prey spectrum (Roth S., Predel R., unpublished data). In contrast to the praying mantises, however, the diversity of species in a given geographical area is low. As a rule, a single species inhabits a given biotope; with very few exceptions (see below), we found no sympatric occurrences of different taxa. Conceivably, successfully expanding taxa (e.g., *Mantophasma*) may have displaced other species of Mantophasmatodea within their ranges. As a result, endemic species such as *Tyrannophasma gladiator* and *Pachyphasma brandbergense* from the Brandberg may have survived only in isolated biotopes outside the currently accessible ranges of expanding taxa and may thus represent phylogenetic relicts that have been isolated for millennia following climate change (Simmons et al. 1998). The Brandberg Massif is isolated from the longitudinal escarpment of Namibia; this isolation has generally ensured its significance as a natural habitat for numerous endemic organisms (see Simmons et al. 1998; Kirk-Spriggs and Marais 2000). Our peptidomics data also indicate obvious variation in peptide sequences, particularly within widespread taxa, such as *Mantophasma*, *Striatophasma*, and *Karoophasma*. This phenomenon is much less distinct in other insects such as Mantodea and Blattodea that have been collected in the same region (Predel R. and co-workers; unpublished). Possibly, apterism and a biological lifestyle featuring low mobility and restricted passive dispersal have resulted in extremely limited gene flow across the continuous ranges of Mantophasmatodean species. Hence, nondistinctive local patterns of their characters (as shown here for neuropeptides) may be insufficient to explain species integrity (see Endler 1977) in Mantophasmatodea.

Mantophasma is the most widely distributed genus and provides a good case study to examine speciation processes in Mantophasmatodea. Populations of this taxon occur from the well-vegetated mountains of central Namibia to Grootfontein in the northeast but also inhabit the mopane savanna (Kaokoveld) in the northwest. Therefore, southern Angola is probably also populated by *Mantophasma*. The Namib forms the western boundary of the distribution, whereas the Kalahari functions as a natural barrier in the east. Within the currently known range of *Mantophasma*, no other

Mantophasmatodean taxon has been observed. In most of their range, these insects are common in grasses, shrubs, and even trees if sufficient rainfall occurs. This abundance may have led to distribution patterns lacking isolated patches, thus preventing extensive speciation within *Mantophasma*, although Zompro and Adis (2006) have suggested that "apparently every mountain range support(s) endemic congeners". In fact, extensive sampling has yielded numerous transitional forms between the slightly variable *Mantophasma* populations, including *Sclerophasma paresisense*; putative hybridization of specimens with distinguishable peptidomes has even been observed in several cases. Such heterozygous individuals carry 2 different alleles of the same neuropeptide gene (see Fig. 7). These hybrid signals confirm gene flow and counteract speciation, suggesting that the observed variations are still essentially random and that genetic recombination breaks down diversifying allele combinations in these species. In a strict interpretation of the currently available data, the *Mantophasma* lineage (including *Sclerophasma paresisense*; Klass et al. 2003) is thus consisting of a single species with several distinct units on the population level. Alternatively, relatively small groups of individuals might be treated as separate species, leading to substantial taxonomic inflation.

The southernmost known distributional limit of *Mantophasma* is equivalent to the northern limit of a distinct lineage within Mantophasmatodea, the novel genus *Striatophasma* (Wipfler et al. 2012). In the Gamsberg area, members of both taxa sometimes occur in the same bushes, but the general distribution of these taxa (see Fig. 2) suggests that *Striatophasma* is better adapted to conditions of lower rainfall and scattered vegetation typical of the Great Escarpment in Namibia south of the Tropic of Capricorn. Whether *Striatophasma* consists of several closely related species or constitutes a single variable species (see the Discussion section on *Mantophasma*) with different morphotypes has yet to be resolved.

The currently known distribution of Mantophasmatodea shows a gap between 25° and 28° south latitude. Our phylogenetic analyses and the recent distribution suggest that *Striatophasma*-like species crossed this region in the past from the North and established a founder population of what is today the Austrophasmatidae of South Africa. Unlike *Striatophasma*, all the South African Austrophasmatidae are adapted to conditions of winter rain. The adaptation of these univoltine insects likely occurred in a transition zone between the winter and summer rainfall regions, such as the Richtersveld just south of the Orange River. Indeed, a putative sister taxon of the hitherto described South African Austrophasmatidae (Austrophasmatidae gen. et sp. nov. "'RV") is found in the Richtersveld. In that region, a rapid west–east transition from the Succulent Karoo to the Nama-Karoo is associated with a change in rainfall season. The Richtersveld, a part of the Great Escarpment, is an arid biodiversity hotspot and a particular center of endemism within the Succulent Karoo (Hilton-Taylor 1996), which itself is the world's only recognized arid biodiversity hotspot (Myers et al. 2000).

Another important finding of this study is the occurrence of 3 relatively distantly related Mantophasmatodean taxa in this area, namely Austrophasmatidae gen. et sp. nov. "RV," *Praedatophasma maraisi* (Mantophasmatidae), and *Namaquaphasma ookiepense* (Austrophasmatidae). Our phylogenetic analysis indicates that the population of the latter species does not represent a link to the remaining South African Austrophasmatidae but is rather part of the *Namaquaphasma* clade, which may have reached the Richtersveld from the south.

The Austrophasmatidae of South Africa are the most derived Mantophasmatodea. Our phylogenetic analyses, as well as characteristic substitutions found only in the neuropeptides of Austrophasmatidae, confirm the monophyly of this taxon. Modification of the life cycle to live under conditions of winter rain was probably the key adaptation of a *Striatophasma*-like ancestor. Population(s) of this taxon may have given rise to a new lineage that entered the Namaqualand and the southwestern Cape, where conditions of winter rainfall developed about 4 million years ago. A scenario that may have led to the current diversity of Austrophasmatidae in South Africa is a rapid and even colonization of the winter rainfall region due to the availability of unoccupied niches. The even distribution of this taxon may have been interrupted by Pleistocene climate changes. In the Succulent Karoo, glacial wetting was always followed by interglacial drying, and it is assumed, at least for many plants, that regions such as the Richtersveld retained an appreciable core range under peak glacial conditions (Midgley et al. 2005). Isolated populations of Austrophasmatidae may have developed into a multitude of geographically separated species and recolonized the current biotopes after such primarily nonadaptive allopatric speciation. Such nonadaptive radiation (see, e.g., Wright 1931; Gittenberger 1991; Rundall and Price 2009) could result in reticulate phylogenetic relationships such as those obtained in our phylogenetic analyses of South African Austrophasmatidae (but see Whitfield and Kjer 2008). Alternatively, the polyphyly of the currently accepted genera of Austrophasmatidae could result from incomplete lineage sorting following recent speciation (Funk and Omland 2003); in this case, the genera would be better downgraded to species. All major taxa of South African Austrophasmatidae have populations in mountainous regions, an observation that favors the first scenario. Our collection data also suggest that neither vegetation type nor altitude is a key factor for the distribution of these species. The different taxa of Austrophasmatidae more or less continuously occupy the Succulent Karoo and southwestern Cape without clear geographical separation but also without distinct sympatry. If this pattern of distribution can be confirmed in future analyses, it might mean either that expanding taxa just meet each other or, more likely, that the most competitive taxa slowly replace other taxa (see Discussion section on *Mantophasma*). Consequently, a single species will always dominate

in a given region. The general distribution of Austrophasmatidae suggests that these insects have not succeeded in readapting to conditions of summer rainfall. Of 13 (putative) Austrophasmatid species, only one has been collected in the eastern transition zone between winter and summer rainfall at Beaufort West (see Damgaard et al. 2008).

Conclusions

We used a peptidomics approach to study the phylogenetic relationships and biogeography of Mantophasmatodea. We collected and analyzed known and previously undescribed taxa of this recently described insect order, developed methods for immediate sample preparation in the field, introduced mass fingerprints for the unambiguous identification of taxa, and analyzed the most extensive peptide hormone data set that has been compiled to date for related taxa. These data were sufficient to separate the major clades of Mantophasmatodea (including hitherto unknown higher taxa). Diversification at the species level seems to be less marked than expected, particularly in *Mantophasma*. Our extensive sampling provides a more complete knowledge of the distribution and biogeography of Mantophasmatodea, and we expect that the major southern African clades have now been detected. The taxonomic status of Austrophasmatidae gen. et sp. nov. "RV" is a subject of our ongoing research. Considering that these insects remained undetected until recently, it is surprising to note the wide distribution and abundance of Mantophasmatodea in southern Africa.

Supplementary Material

Supplementary material, including data files and/or online-only appendices, can be found at http://datadryad.org and in the Dryad data repository (DOI:10.5061/dryad.r6ng71d4).

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