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Higher phylogeny of zygaenid moths (Insecta: Lepidoptera) inferred from nuclear and mitochondrial sequence data and the evolution of larval cuticular cavities for chemical defence

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Abstract

Zygaenid moths are capable of releasing hydrogen cyanide in their defense by enzymatic break-down of cyanoglucosides, but only larvae of chalcosiine and zygaenine moths store cyanogenic compounds in cuticular cavities and thus are able to discharge defense droplets, which effectively deter potential predators. A previously proposed phylogeny of Zygaenidae hypothesized a sister group relationship of chalcosiine and zygaenine moths because of their similar larval defense system. Not all chalcosiine taxa possess cuticular cavities, however, and a comparable defense mechanism has been reported in larvae of the zygaenoid family Heterogynidae. Considering sequence data of seven molecular loci, the present study estimates the posterior probability of phylogenetic hypotheses explaining the occurrence of larval cuticular cavities. The molecular data confirm the previous exclusion of Himantopteridae from Zygaenidae and suggest their close affinity to Somabrachyidae. The sequence data also corroborate the recently proposed exclusion of the Phaudinae from the Zygaenidae, because this subfamily is recovered in a reasonably well supported species cluster consisting of members of the families Lacturidae, Limacodidae, Himantopteridae, and Somabrachyidae. We consequently agree to raise Phaudinae to family rank. Within Zygaenidae, the subfamilies Callizygaeninae, Chalcosiinae, and Procridinae most likely constitute a monophyletic group, which is sister to the Zygaeninae. Our results imply that cuticular cavities were probably present in the larvae of the most recent common ancestor of Zygaenidae. Heterogynidae cannot be confirmed as sister taxon to this family, but appear at the very first split of the Zygaenoidea, although with poor support. The specific pattern of taxa in the molecular phylogeny showing larval cuticular cavities opens the possibility that these structures could have been already present in the most recent common ancestor of the Zygaenoidea.
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1. Introduction

Zygaenidae are a species rich family of predominantly diurnal moths with a world-wide distribution, being most diverse in tropical and subtropical Asia and the Palaearctic region (Epstein et al., 1999). The group has been of great interest to lepidopterists and evolutionary biologists owing to the extensive individual and geographic variation of its brightly colored species and due to an intriguing chemical defense system (Naumann et al., 1999); all Zygaenidae studied so far are capable of releasing highly toxic hydrogen cyanide (HCN) upon disturbance or tissue disruption (Jones et al., 1962) by enzymatic break-down of the two cyanoglucosides linamarin and lotaustralin (Davis and Nahrstedt, 1979; Witthohn and Naumann, 1987). In addition, the larvae of two major zygaenid lineages

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(Chalcosiinae, Zygaeninae) store considerable amounts of cyanic defense fluid in cuticular cavities (Naumann and Feist, 1987; Witthohn and Naumann, 1984), which are connected through specialized opening mechanisms to the exterior (Naumann, 1990; Naumann and Povolný, 1987). This chemical defense has enabled some species to participate in complex mimicry systems (Epstein et al., 1999; Naumann et al., 1999; Sbordoni et al., 1979; Yen et al., 2005a). In this sense, zygaenid moths have many aspects in common with the New World butterflies of the genus *Heliconius* (Epstein et al., 1999; Naumann et al., 1999), a taxon extensively used to investigate the evolution of mimicry, co-evolution of plants and insects, and processes of speciation (Brower, 1996a, 1997; Mallet et al., 1998; Sheppard et al., 1985). However, while much effort has been spent on elucidating the relationships of passion-vine butterflies (Heliconiini) by examining morphological, ecological, and molecular data (e.g., Beltrán et al., 2002; Brower, 1994, 1996a,b; Brower and DeSalle, 1998; Brower and Egan, 1997; Brown, 1981; Penz, 1999), the affinities of Zygaenidae and allied groups received little attention and remained elusive (Epstein et al., 1999; Fänger and Naumann, 2001; Fänger et al., 1998).

The systematics of zygaenid moths has a chaotic history involving many groups of the superfamily Zygaenoidea and encompassing even non-zygaenoid taxa (Fänger and Naumann, 2001; Yen et al., 2005b). Alberti (1954, 1981), the first author who treated the systematics of zygaenid moths in a comprehensive manner, recognized seven subfamilies: Anomoeotinae, Chalcosiinae, Charideinae, Himantopterinae, Phaudinae, Procridinae, and Zygaeninae. His demarcation of the Zygaenidae goes back to Jordan (1907–1909) and was found to be based essentially on plesiomorphic characters (Naumann, 1985). To satisfy the demands of a natural system, the Himantopterinae and Anomoeotinae have been excluded subsequently from the Zygaenidae and raised to family rank by Fletcher and Nye (1982); and Minet (1991) transferred the Charideinae to the Thyrididae, a non-zygaenoid family. On the other hand, a number of genera associated with the genus *Lactura* and formally included among the Yponomeutidae (superfamily Yponomeutoidea) and Immidae (superfamily Immoidea) had been temporarily assigned to the Zygaenidae (Common, 1990), until Heppner (1995) excluded them again by establishing the new zygaenoid family Lacturidae. At about the same time, Tarmann (1994) expelled the genus *Callizygaena* and its relatives from the Procridinae and established the new subfamily Callizygaeninae. Recent morphological investigations on last instar larvae finally suggested excluding the Phaudinae from the Zygaenidae (Fänger et al., 1998), and Fänger (1999) and Yen et al. (2005b) already treat them as separate family. As a consequence of the systematic changes, the Zygaenidae currently consist of the following four subfamilies (Epstein et al., 1999): Callizygaeninae, Chalcosiinae, Procridinae, and Zygaeninae.

The retrospective view of the zygaenid moth systematics in the previous paragraph illustrates that understanding the evolutionary history of this family is closely linked with the similarly confusing systematics of the whole Zygaenoidea (Epstein et al., 1999; Yen et al., 2005b). For a long time this large superfamily has been considered as 'waste-paper-basket taxon' (Epstein et al., 1999) consisting of more or less well defined family groups that seemed to have little more in common than plesiomorphic characters (Epstein et al., 1999; Naumann et al., 1999). It is therefore not surprising that the number of families included in the Zygaenoidea has been varied over the years by transferring species groups from this superfamily to others and vice versa (Brock, 1971; Epstein et al., 1999; Heppner, 1984) and by creating new families for isolated lineages with uncertain phylogenetic affinities (Fletcher and Nye, 1982; Heppner, 1995; Minet, 1991). Nonetheless, recent efforts to reconstruct a natural system of the Zygaenoidea based on morphological characters of preimaginal stages revealed at least seven potential synapomorphies, which allow characterizing the Zygaenoidea and suggest its monophyly (Epstein et al., 1999; Fänger et al., 1998, 2002). According to these investigations, the following families are recognized within Zygaenoidea (Epstein et al., 1999): Aididae, Anomoeotidae, Cyclotornidae, Dalceridae, Epipyropidae, Heterogynidae, Himantopteridae, Lacturidae, Limacodidae, Megalopygidae, Somabrachyidae, and Zygaenidae.

The formally erroneous characterization of the Zygaenidae has been changed in the course of time by a thorough examination of their morphology and chemical ecology (Naumann et al., 1999 and references therein) and by more strictly applying phylogenetic principles (Naumann, 1985). Naumann et al. (1999) enumerate three characters to define the Zygaenidae: firstly, the capacity to biosynthesize the cyanoglucosides linamarin and lotaustralin from the amino acids valine and isoleucine; secondly, the presence of two crossed muscles around the mid gut of the larvae; and thirdly, the occurrence of paired, highly specialized secondary accessory glands in the internal female genitalia (Bode and Naumann, 1988; Naumann, 1988). These characters have not been studied in the family groups that were recently established, however (e.g., Callizygaeninae). Furthermore, the interpretation of at least one character depends on a particular assumption; within Zygaenoidea, the capability to biosynthesize the cyanoglucosides linamarin and lotaustralin is possibly not restricted to zygaenid moths as it has also been reported in Anomoeotinae, Heterogynidae, Limacodidae, and Megalopygidae (Witthohn and Naumann, 1987). Yen (unpublished) casts doubts on these reports, however, and regards cyanogenesis only in the family Heterogynidae as confirmed. Treating cyanogenesis as an apomorphy to justify the monophyly of Zygaenidae implies that at least Heterogynidae evolved a similar chemical ecology, but our knowledge of zygaenoid relationships is still fragmentary and thus it cannot be ruled out that cyanogenesis in the Zygaenidae represents a plesiomorphic trait.

In order to understand the complex distribution of traits connected with the chemical defense system in zygaenid moths, we decided to perform a phylogenetic reconstruction of major zygaenoid lineages with emphasis on taxa of the family Zygaenidae by simultaneously analyzing mitochondrial and nuclear markers. Applying a Bayesian approach, our intention is to evaluate phylogenetic hypotheses, which have a major impact on the interpretation of the evolutionary history of chemical defense in Zygaenidae. In particular: (i) are the Zygaenidae monophyletic? Until recently, the Phaudidae have been placed in this family although characters supporting this relationship were absent or had not been studied (Epstein et al., 1999; Fänger et al., 1998). (ii) Are Heterogynidae and Zygaenidae sister taxa? This hypothesis has been corroborated by the fact that the larvae of Heterogynidae have cuticular cavities with openings comparable to those found in chalcosiine and zygaenine moths (Epstein et al., 1999) and in which cyanoglucosides may occur as well (Naumann, 1985; Witthohn and Naumann, 1987). However, a recent investigation (Vegliante and Zilli, 2004) on the genus *Heterogynis* revealed traits shared exclusively between Heterogynidae and Phaudidae and between Heterogynidae and taxa of the limacodid family group (i.e., Limacodidae, Megalopygidae, Somabrachyidae, Aididae, and Dalceridae; Epstein et al., 1999). (iii) Do the two different types of cuticular cavities found in most larvae of chalcosiines and in all zygaenines represent a synapomorphy of these two subfamilies as implicitly hypothesized by Witthohn and Naumann (1987) and Naumann et al. (1999)? Although there is no evidence for the presence of cuticular cavities in procridines and phaudines, cuticular cavities are also absent in the western Palaearctic chalcosiine *Aglaope*. Fänger and Naumann (2001) therefore discussed alternative phylogenetic scenarios, including one in which the genus *Aglaope* forms the adelphotaxon to Chalcosiinae (without *Aglaope*) and Zygaeninae. (iv) Is it true that *Callizygaena* and the remaining Procridinae do not form a monophylum, thus justifying the raise of the subfamily Callizygaeninae? *Callizygaena* has been excluded from the Procridinae due to its highly differing genital structure (Tarmann, 1994), but the affinities of the newly established subfamily remained unclear. We finally address phylogenetic relationships within the subfamily Zygaeninae in some depth to provide a systematic framework for a species phylogeny of the genus *Zygaena*, which is currently in preparation.

Having a robust phylogenetic hypothesis of the major zygaenoid lineages and zygaenid subfamilies at hand, the occurrence of characters related to the larval chemical defense system found in zygaenoid moths will hopefully become more plausible and lead to a better understanding of the evolution of this trait. This may stimulate future investigations dealing with the relation of larval cyanogenesis and aposematism.

2. Materials and methods

2.1. Taxon sampling

We sampled 40 ingroup species taxa representing the zygaenoid moth families Heterogynidae, Himantopteridae, Lacturidae, Limacodidae, Phaudidae, Somabrachyidae, and Zygaenidae. Most of the type genera of the ingroup families were included to represent an adequate taxon sampling. We added further taxa to characterize important lineages other than that of the type genus of each family. Within Zygaenidae, all currently recognized subfamilies (i.e. Callizygaeninae, Chalcosiinae, Procridinae, and Zygaeninae) are considered. For Chalcosiinae, we used *Neochalcosia*, closely related to the nominotypical genus *Chalcosia* (Yen et al., 2005b), and two additional genera to represent the major lineages of this subfamily. Taxon sampling in the Procridinae and Zygaeninae cover all major Palaearctic genera. The Ethiopian, Nearctic, and Australian procridines were not considered since appropriately preserved material was not available and their association with the Palaearctic members of this subfamily was corroborated by the phylogenetic analysis of Yen et al. (2005b). Material of Lacturidae for molecular applications is extremely difficult to obtain. We used *Gymnogramma* to represent this family since this genus is morphologically not substantially distinct from the Oriental *Anticrates* and Indo-Australian *Lactura* (Yen, pers. observ.). In contrast to the highly diverse larval morphology, the adult features of Limacodidae are fairly conservative among groups and no infrasubfamilial classification was ever suggested; we therefore only included *Apoda* to represent this family. For outgroup comparison, a sesioid and a tortricoid species were investigated. Species names and the current systematic classification are summarized in Table 1. Voucher specimens are stored in the Alexander Koenig Research Institute and Museum of Zoology (ZFMK) in Bonn, Germany.

2.2. Molecular procedures

The present phylogenetic analysis is based on complete sequences of the mitochondrial (mt) genes NADH dehydrogenase subunit 1 (ND1), tRNA-leucine (tRNA-Leu), large subunit ribosomal RNA (16S rRNA), tRNA-valine (tRNA-Val) as well as of a major fragment of the mt small subunit ribosomal RNA (12S rRNA). In addition to this approximately 3100 base pair long mitochondrial sequence section, we compiled 2.3 kb (kilobases) of nuclear (nc) DNA comprising the (almost) complete small subunit ribosomal RNA (18S rRNA) and the 5′-end of the large subunit ribosomal RNA (28S rRNA). DNA extraction and PCR amplification followed molecular protocols described by Niehuis et al. (2006).

The compilation of the mt sequence data will subsequently focus only on the genes ND1 and 16S rRNA (including flanking tRNAs) since appropriate 12S rRNA

Table 1
Species names and EMBL accession numbers to sequences of taxa analyzed in the present study

Taxa	Sampling locations	Accession numbers				
		ND1	16S rRNA[a]	12S rRNA	18S rRNA	28S rRNA
Sesioidea						
Sesia bembeciformis (Hübner, [1806])	Germany	AJ844306	AJ831588	AJ785615	AJ830746	AJ844024
Tortricoidea						
Cacoecimorpha pronubana (Hübner, 1799)	Germany	AJ844307	AJ831589	AJ785616	AJ830747	AJ844025
Zygaenoidea						
Heterogynidae						
Heterogynis penella (Hübner, 1819)	Italy	AJ844308	AJ831590	AJ785617	AJ830748	AJ844026
Himantopteridae						
Himantopterus dohertyi (Elwes, 1890)	Vietnam	AJ844309	AJ831591	AJ785618	AJ830749	AJ844027
Lacturidae						
Gymnogramma flavivitella (Walsingham, 1881)	South Africa	AJ844310	AJ831592	AJ785619	AJ830750	AJ844028
Limacodidae						
Apoda limacodes Hufnagel, 1766	Germany	AJ844311	AJ831593	AJ785620	AJ830751	AJ844029
Phaudidae						
Phauda mimica Strand, 1915	Taiwan	AJ844318	AJ831600	AJ785627	AJ830758	AJ844036
Somabrachyidae						
Somabrachys aegrota (Klug, 1830)	Tunisia	AJ844312	AJ831594	AJ785621	AJ830752	AJ844030
Zygaenidae						
Callizygaeninae						
Callizygaena splendens Candeze, 1927	unknown	AJ844313	AJ831595	AJ785622	AJ830753	AJ844031
Chalcosiinae						
Aglaope infausta (Linnaeus, 1767)	Germany	AJ844314	AJ831596	AJ785623	AJ830754	AJ844032
Aglaope labasi labasi Oberthür, 1922	Morocco	AJ844315	AJ831597	AJ785624	AJ830755	AJ844033
Neochalcosia remota (Walker, 1854)	Japan	AJ844316	AJ831598	AJ785625	AJ830756	AJ844034
Pidorus atratus Butler, 1877	Japan	AJ844317	AJ831599	AJ785626	AJ830757	AJ844035
Procridinae						
Adscita geryon (Hübner, [1813])	Germany	AJ844319	AJ831601	AJ785628	AJ830759	AJ844037
Adscita mannii (Lederer, 1853)	Greece	AJ844320	AJ831602	AJ785629	AJ830760	AJ844038
Adscita mauretanica (Naufock, 1932)	Morocco	AJ844321	AJ831603	AJ785630	AJ830761	AJ844039
Jordanita hector (Jordan, 1907)	Turkey	AJ844322	AJ831604	AJ785631	AJ830762	AJ844040
Rhagades brandti (Alberti, 1938)	Iran	AJ844323	AJ831605	AJ785632	AJ830763	AJ844041
Rhagades pruni ([Denis & Schiffermüller], 1775)	Germany	AJ844324	AJ831606	AJ785633	AJ830764	AJ844042
Theresimima ampellophaga (Bayle-Barelle, 1808)	Turkey	AJ844325	AJ831607–8	AJ785634	AJ830765	AJ844043
Thyrassia penangae (Moore, 1859)	China	AJ844326	AJ831609	AJ785635	AJ830766	AJ844044
Zygaenoprocris persepolis (Alberti, 1938)	Iran	AJ844327	AJ831610	AJ785636	AJ830767	AJ844045
Zygaeninae						
Pryeria sinica Moore, 1877	Japan	AJ844328	AJ831611	AJ785637	AJ830768	AJ844046
Epizygaenella c. caschmirensis (Kollar, 1844)	Pakistan	AJ844329	AJ831612	AJ785638	AJ830769	AJ844047
Neurosymploca caffra (Linnaeus, 1764)	South Africa	AJ844330	AJ831613–4	AJ785639	AJ830770	AJ844048
Neurosymploca concinna (Dalman, 1823)	South Africa	AJ844331	AJ831615–6	AJ785640	AJ830771	AJ844049
Neurosymploca sp. 1 (morphotype '*atomarina*')	South Africa	AJ844332	AJ831617	AJ785641	AJ830772	AJ844050
Neurosymploca sp. 2 (morphotype '*geertsemai*')	South Africa	AJ844333	AJ831618	AJ785642	AJ830773	AJ844051
Neurosymploca sp. 3 (morphotype '*magnifica*')	South Africa	AJ844334	AJ831619	AJ785643	AJ830774	AJ844052
Orna nebulosa (Guérin-Méneville, 1832)	South Africa	AJ844335	AJ831620	AJ785644	AJ830775	AJ844053
Praezygaena agria (Distant, 1892)	South Africa	AJ844336	AJ831621–2	AJ785645	AJ830776	AJ844054
Praezygaena ochroptera (Felder, 1874)	South Africa	AJ844337	AJ831623	AJ785646	AJ830777	AJ844055
Reissita simonyi yemenicola Tremewan, 1959	Yemen	AJ844338	AJ831624	AJ785647	AJ830778	AJ844056
Zygaena carniolica virginea Müller, 1766	Italy	AJ844344	AJ831630	AJ785653	AJ830784	AJ844062
Zygaena exulans exulans (Hohenwarth, 1792)	Italy	AJ844428	AJ831723	AJ785737	AJ830868	AJ844146
Zygaena filipendulae gemina Burgeff, 1914[b]	Spain	AJ844429	AJ831724	AJ785738	AJ830869	AJ844147
Zygaena loti macedonica Burgeff, 1926	Greece	AJ844434	AJ831729	AJ785743	AJ830874	AJ844152
Zygaena manlia manlia Lederer, 1870	Iran	AJ844401	AJ831690–1	AJ785710	AJ830841	AJ844119
Zygaena sarpedon lusitanica Reiss, 1936	Spain	AJ844418	AJ831713	AJ785727	AJ830858	AJ844136
Zygaena sogdiana sogdiana Erschoff, 1874	Uzbekistan	AJ844370	AJ831657	AJ785679	AJ830810	AJ844088
Zygaena viciae confusa Staudinger, 1881	Kazakhstan	AJ844445	AJ831740	AJ785754	AJ830885	AJ844163
Zygaena youngi youngi Rothschild, 1926	Morocco	AJ844376	AJ831663	AJ785685	AJ830816	AJ844094

[a] Including tRNA-Leu and tRNA-Val.
[b] Type-species of the family Zygaenidae.

sequences are provided by Niehuis et al. (2006) (accession numbers given in Table 1). Binding positions and directions of PCR primers are illustrated in Fig. 1. Oligonucleotide sequences are listed in Table 2.

ND1 gene sequences were in most samples derived by means of two PCRs. Amplification of the first fragment with the 3′-end of the gene was accomplished applying primers ND1f2 (rarely ND1f1) and ND1r4. Primer mismatch or poor genomic DNA required amplifying smaller fragments in some cases using combinations of the following oligonucleotides: ND1f3, ND1f4, ND1f5, ND1r1, ND1r2, and ND1r3. The second part of the gene was obtained with the aid of the primers ND1f6 and ND1r7. This PCR also provided us with sequences of domain VI of the 16S rRNA and the tRNA-Leu gene. As in the first fragment, primer mismatch problems, poor genomic DNA or impeding AT-repeats at the 3′-end of the 16S rRNA gene required invoking additional primers in order to achieve complete sequence data for all taxa: ND1f7, ND1f8, ND1f9, 16Sf1, ND1r5, ND1r6.

Sequence data of the gene cluster tRNA-Leu, 16S rRNA, and tRNA-Val were completed with three additional PCRs. We applied the primer set 16Sf2 and 16Sr1 to amplify the domains V and IV, and obtained domain II using the oligonucleotides 16Sf4a (rarely 16Sf4b) and 16Sr2a. In some cases, we received better results using the primers 16Sf3 and/or 16Sr2b/c instead of 16Sf4a and 16Sr2a. The final fragment, comprising domain I of the large subunit rRNA and the tRNA-Val gene, was amplified using primer set 16Sf5a and 16Sr5a. If no product was visible, we used the oligonucleotides 16Sf5b or/and 16Sr5b instead.

18S rRNA sequences of all studied species are provided by Niehuis et al. (in press) (accession numbers are listed in Table 1). For each taxon, we added about 550 bp of nuclear sequence data by investigating the 5′-end of the 28S rRNA gene. Using the primer sets 28Sf1/28Sr1 and 28Sf2/28Sr2b, we obtained two overlapping fragments comprising almost the entire domain I of the large subunit rRNA. Primer sequences are listed in Table 2.

All PCR products were sequenced in both directions following the protocol described by Niehuis et al. (2006). In addition to the PCR oligonucleotides, which were also used in cycle sequencing reactions, we applied internal primers (16Sr3, 16Sr4, 28Sf3, and 28Sr2) for sequencing the 5′-end of the 16S rRNA gene and the 3′-end near part of the second 28S rRNA gene fragment (Table 2). All new sequences have been submitted to the EMBL Data Library (accession numbers given in Table 1).

2.3. Sequence alignment and secondary structure analysis

DNA sequences of the ND1 gene were manually aligned using BioEdit 7.0.0 (Hall, 1999). The alignment was subsequently checked by translating the DNA data into corresponding amino acid sequences using GeneDoc 2.6.02 (Nicholas et al., 1997) and applying the invertebrate mitochondrial gene code. 12S and 18S rDNA sequence alignments were adopted unchanged from Niehuis et al. (2006, in press), in which we had inferred secondary structure models of the small ribosomal RNAs in zygaenoid moths. The remaining genes (tRNA-Leu, tRNA-Val, 16S rRNA, and 28S rRNA) were aligned using Clustal X (Thompson et al., 1997) with the default settings. These alignments were next improved by considering secondary structure models of reference organisms (Cannone et al., 2002; Wuyts et al., 2004) in more conserved sections of the molecules. We finally performed comparative sequence analyses taking data of additional 98 taxa of *Zygaena* (published separately) into account and derived taxon specific secondary structure models for all genes as described by Niehuis et al. (2006). Structure masks incorporating the derived structural information were added to the alignments to allow for the application of specific RNA substitution models in the phylogenetic analysis.

2.4. Phylogenetic analysis

Prior to the phylogenetic analysis, we tested for homogeneity of base frequencies among taxa using the program PAUP* 4.0b10 (Swofford, 2003) and considering various reasonable character sets (e.g., all sites, just variable sites, first and second codon positions only) for different data partitions (given below).

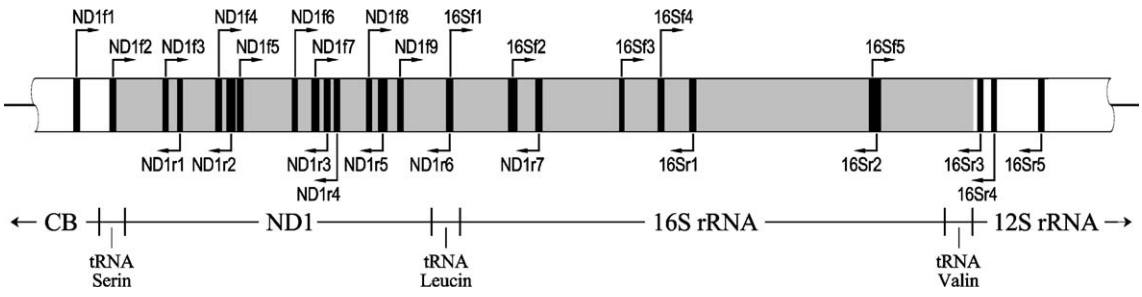

Fig. 1. Binding positions and directions of oligonucleotide primers used to amplify and sequence the mitochondrial gene cluster ND1, tRNA-Leu, 16S rRNA, and tRNA-Val in moths.

Table 2
Primers used to amplify and sequence the mitochondrial gene cluster ND1, tRNA-Leu, 16S rRNA, tRNA-Val and the 5′-end of the nuclear gene 28S rRNA in moths

Name	Direction	Sequence (5′ → 3′)	Source
ND1f1	Forward	*ACATGAATTGGAGCYCGACCWGT*	Simon et al. (1994) (mod. CTBA)
ND1f2	Forward	*CTTGTCTATAAGCATTTGTTTTG*	Niehuis, present study
ND1f3	Forward	*TTAAAGAAACAGGTAAATAAC*	Niehuis, present study
ND1f4a	Forward	*TTAAAGAAACAGGTAAATAAC*	Niehuis, present study
ND1f4b	Forward	*TAAAATTCTAGAGTATTCAG*	Niehuis, present study
ND1f4c	Forward	*TAAAATTCTAGAGTATTCAGCC*	Niehuis, present study
ND1f4d	Forward	*TAAAATTCTTGAATACTCTGCT*	Niehuis, present study
ND1f5a	Forward	*AAACTAATTCTCTTTCACCCTC*	Niehuis, present study
ND1f5b	Forward	*AAACTAATTCTCTTTCACCTTC*	Niehuis, present study
ND1f6	Forward	*AAGCTATTCTTACTTCATAAG*	Simon et al. (1994) (mod. FawND1B)
ND1f7a	Forward	*TAGAATTAGAAGATCAACCAGC*	Simon et al. (1994) (alias FawND1A)
ND1f7b	Forward	*TTAGAAGATCAACCAGCAACT*	Niehuis, present study
ND1f8a	Forward	*AATATAGGATAAGTTTGTTC*	Niehuis, present study
ND1f8b	Forward	*AATATAGGATAAGTTTGCTC*	Niehuis, present study
ND1f9	Forward	*ATAACCTAATAATTTTCGCTC*	Niehuis, present study
ND1r1	Reverse	*TTTACCTCGTTATCGTTATG*	Niehuis, present study
ND1r2a	Reverse	*GAATATAGAAGAGGAGGATTTG*	Niehuis, present study
ND1r2b	Reverse	*GAATATAGAAGAGGAGGGTTTG*	Niehuis, present study
ND1r2c	Reverse	*ATGTAGAATATAGAAGAGG*	Niehuis, present study
ND1r2d	Reverse	*TTTAATGTTGAATATAGAAGAGG*	Niehuis, present study
ND1r2e	Reverse	*TTTAATGTTGAATATAGAAGTGG*	Niehuis, present study
ND1r3a	Reverse	*TTTTTTGTTGTACAAGATTAGG*	Niehuis, present study
ND1r3b	Reverse	*TTTTTTGTTGTACAAGATTGGG*	Niehuis, present study
ND1r3c	Reverse	*TTTTTTGTTGTACAAGAGTAGG*	Niehuis, present study
ND1r3d	Reverse	*TTTTTAGTTGTTTAAGGTTAGG*	Niehuis, present study
ND1r4	Reverse	*ATATAATTAGATTTAATTTAGG*	Niehuis, present study
ND1r5a	Reverse	*TATTTTACAGCCATTTTCTGATG*	Niehuis, present study
ND1r5b	Reverse	*TATTTTACAGCCTTTTTCTGATG*	Niehuis, present study
ND1r5c	Reverse	*TATTTTACAGCCTTTTTCAGATG*	Niehuis, present study
ND1r5d	Reverse	*ATTAATAGGTATTTTACAGCC*	Niehuis, present study
ND1r6a	Reverse	*GACAGATAATTGTGATGATTTTAG*	Niehuis, present study
ND1r6b	Reverse	*GACAGATAATTGTGATGGTTTTAG*	Niehuis, present study
ND1r7a	Reverse	*GCGACCTCGATGTTGGATTAA*	Simon et al. (1994) (alias Faw16S2)
ND1r7b	Reverse	*GTTTGAGACCTCGATGTTGGA*	Niehuis, present study
16Sf1a	Forward	*AATCATCACATTTATCTGTCA*	Niehuis, present study
16Sf1b	Forward	*AATCATCACATTTATCTGCCA*	Niehuis, present study
16Sf2	Forward	*GCTCCGGTTTGAACTCAGAT*	Misof et al. (2001) (alias LR-J-New)
16Sf3	Forward	*AACTCTATGGGGTCTTC*	Niehuis, present study
16Sf4a	Forward	*AATGATTATGCTACCTTTGT*	Niehuis, present study
16Sf4b	Forward	*CTAATGATTATGCTACCTTTG*	Niehuis, present study
16Sf5a	Forward	*ATTAATAAACTCTGATACAC*	Niehuis, present study
16Sf5b	Forward	*AAACTCTGATACACAAGATAC*	Niehuis, present study
16Sr1	Reverse	*CGGCCGCCTGTTTATCAAAAACAT*	Xiong and Kocher (1991) (mod. primer A)
16Sr2a	Reverse	*ATTTTAATTTATTGTATCTTG*	Niehuis, present study
16Sr2b	Reverse	*GTTTTAAATTAATGTATCTTG*	Niehuis, present study
16Sr2c	Reverse	*ATTTTAGTTTAATGTATCTTG*	Niehuis, present study
16Sr3	Reverse	*TGAAATAAGTCGTAACAAAGT*	Niehuis, present study
16Sr4	Reverse	*TGTACATATTGCCCGTCGCT*	Niehuis, present study
16Sr5a	Reverse	*AAAATTAAATCAGATCAAGATG*	Niehuis, present study
16Sr5b	Reverse	*AAATTAAATCAGATCAAGATGC*	Niehuis, present study
28Sf1	Forward	*CCCSSGTAATTTAAGCATATTA*	Whiting (2002) (alias 28S rD1.2a)
28Sf2	Forward	*TAAATATTACCGCGAGACCGATAG*	Niehuis, present study
28Sf3a	Forward	*CGGCCGCGCGCCTCGATG*	Niehuis, present study
28Sf3b	Forward	*GGGGGCGCGCCTCGATGTCGTC*	Niehuis, present study
28Sf3c	Forward	*GGCCGCTACTCTCGATGTCGTC*	Niehuis, present study
28Sr1	Forward	*TGAACGGTTTCACGTACTMTTGA*	Whiting (2002) (alias 28S rD3.2b)
28Sr2a	Forward	*ATTACTAAGAGAGAAGTG*	Niehuis, present study
28Sr2b	Forward	*GCGATGCATTACTAAGAGAGAAG*	Niehuis, present study

We applied a Bayesian approach for phylogenetic inference[3] using Markov Chain Monte Carlo (MCMC) estimation of the posterior probability distribution as implemented in the software package PHASE 2.0-alpha (Hudelot et al., 2003; Jow et al., 2002) and analyzed the molecular datasets simultaneously selecting different substitution models for RNA stems and loops, and the protein coding region. An introduction to the method is provided by Huelsenbeck et al. (2001, 2002) and Nylander et al. (2004), which we refer the more interested reader to. Since we had no strong assumption about any particular prior distribution of trees and model parameters, we applied uniform priors. Specifically, we assumed all topologies to be equally likely and used uniform positive priors with a cut-off at some upper limit for substitution rate parameters, gamma rate variation, and invariable sites (Hudelot et al., 2003; Jow et al., 2002). A Dirichlet distribution prior was chosen for nucleotide and base pair frequency parameters, respectively (i.e., all sets of nucleotide or base pair frequencies summing to 1.0 were equally likely). An exponential (10) prior was selected for branch length. This put 50% prior probability on branch lengths less than 0.1, which fits better than a uniform prior with our *a priori* expectations.

Analyzing heterogeneous molecular datasets by specifying partition-specific substitution models is a particular challenge for model selection. Fitting substitution models for each partition independently using one of several proposed criteria (e.g., hierarchical likelihood-ratio test, Akaike information criterion; Posada and Crandall, 2001) ignores information in other partitions. However, since all partitions in Bayesian analyses of heterogeneous data are typically linked by a common phylogeny, the optimal combination of models can be different from that derived from each partition separately, because different partitions may favor different topologies thus influencing model ranking. This issue is addressed in more detail by Nylander et al. (2004). Unfortunately, testing all possible model combinations is in most cases impractical. We therefore applied a heuristic approach for finding a proper composite model. For each partition, we first selected the best fitting substitution models by means of Bayes model comparison based on Bayes factors (Nylander et al., 2004), and subsequently tested the most reasonable combinations. The best model combination was again selected based on Bayes factors, but the behavior of individual model parameters was taken into account as well to meet problems related with over-parameterization. As an estimator of the model likelihood, a quantity necessary for applying Bayes factors, we calculated the harmonic mean of the likelihood values sampled during the last 1,000,000 cycles of the stationary phases of the MCMC runs (Newton and Raftery, 1994).

The molecular dataset was split into five partitions: mt RNA loop regions plus non-protein coding mt DNA, mt RNA stem regions, a (mt) protein coding section, nc RNA loop regions, and nc RNA stem regions. For the RNA loop regions and the protein coding section, we applied standard DNA substitution models: Jukes-Cantor (JC69; Jukes and Cantor, 1969), Kimura (K80; Kimura, 1980), Hasegawa-Kishino-Yano (HKY85; Hasegawa et al., 1985), Tamura-Nei (TN93; Tamura and Nei, 1993), and General Time Reversible (REV; Yang, 1994). To account for correlations in paired sites of RNA stem regions, we used RNA substitution models: RNA6A (Savill et al., 2001), RNA6B (Tillier, 1994), RNA7A (Savill et al., 2001), and RNA7D (Tillier and Collins, 1998). Rate heterogeneity among sites was considered applying the discrete gamma-model of Yang (1994) with four categories; an extra parameter governed the proportion of invariable sites.

During model selection, we ran MCMC with 1,700,000 cycles and sampled trees after initial 200,000 iterations every 100 cycles. After selecting a composite model, we ran six independent MCMC with 6,200,000 cycles each and random start parameters applying either nuclear or mitochondrial sequence data only or both data sets combined. The initial burn-in period was in every run 3,200,000 cycles. Trees were sampled during the subsequent 3,000,000 iterations every 100 cycles. This resulted in 30,000 trees for each run and 180,000 trees in total for each data set. To examine whether or not the mitochondrial and nuclear data supported incompatible species clusters, we analyzed mitochondrial and nuclear data first separately and determined whether incompatible clades with posterior probability values (pP) higher than 95% exist. We did not test for incongruence among genes within the mitochondrial and nuclear data because we assumed a more or less tight linkage of the analyzed markers. For all runs, we plotted likelihood values of sampled trees and model parameters in Microsoft Excel to assess convergence and mixing of chains. Since the fraction a tree appears in the sample is an approximation of its posterior probability (given a model and prior probabilities for branch lengths, model parameters, and topologies; Hudelot et al., 2003), we eventually summarized results considering all 180,000 trees. A consensus topology was inferred using the majority-rule consensus method as implemented in the software package PHYLIP (Felsenstein, 1989), and maximum likelihood estimates of branch-length were calculated for this topology using the program *optimizer*, which is part of the PHASE software package, starting 10 independent runs with random start parameters.

[3] Parametric methods of tree reconstruction can become statistically inconsistent when rates at which sequence sites evolve change non-identically over time (Kolaczkowski and Thornton, 2004). Maximum parsimony has been shown to outperform maximum likelihood and Bayesian phylogenetics under specific situations (Kolaczkowski and Thornton, 2004), although Steel (2005) and Gaucher and Miyamoto (2005) put these findings into perspective. We are aware of the limitations accompanied by relying on only a single optimality criterion in the present investigation. However, to our knowledge current implementations of the maximum parsimony method do not allow considering co-variation of paired nucleotide sites in aligned RNA sequence data, rendering a meaningful comparison with a phylogenetic tree inferred under this optimality criterion inappropriate.

To estimate the probability that a certain character was present in the most recent common ancestor of Zygaenoidea, we applied the parsimony method to reconstruct ancestral states in sampled trees utilizing the 'Ancestral State Reconstruction Packages' available for the software MESQUITE 1.05 (Maddison and Maddison, 2001). The posterior probability of the hypothesis was approximated by counting the number of trees that were consistent with it. To weigh up the prior probability for the hypothesis due to taxon sampling, we generated random trees consistent with a monophyletic ingroup of the same size using PAUP*. Bayes factors were finally calculated to measure the favor of the hypothesis when considering the molecular data (Huelsenbeck and Imennov, 2002).

3. Results

3.1. Sequence alignment and structure mask

The data set initially consisted of 5,912 sites. Characterizing the secondary structure of the 16S and 28S rRNA as well as of the two small tRNAs was in most species possible (structure models for *Zygaena sarpedon* provided in the appendices A and B), but not in the taxon *Heterogynis penella*. We were not able to reliably identify the mt tRNA valine gene in this species and therefore assigned ambiguity symbols at the corresponding site in the alignment. Minor modifications of the applied models were necessary when implementing a structure mask, since we previously had found evidence for taxon specific structure deviations among moths in certain parts of the molecules (12S rRNA: helix 31 and 47 (Niehuis et al., 2006); 16S rRNA: helix C1, D7, and D17). We eventually removed 1,044 positions from the data set, which could not be reliably aligned. The sequence alignment with structural co-notation is available upon request.

3.2. Homogeneity of base frequencies

RNA stem and loop regions did not show any considerable deviation from homogeneity of base frequencies among taxa independent of whether or not constant sites had been considered. Exclusion of constant sites in the protein coding partition had a tremendous effect indicating a highly significant departure from the expected distribution. A more thorough examination of the ND1 sequence data pointed to a significant inhomogeneity of base frequencies in the third codon position. We therefore decided to exclude the third codon position (i.e. 306 sites) from the data set, since all substitution models applied in the present investigation explicitly assume homogeneity of base frequencies among taxa. Test results are summarized in Table 3.

3.3. Model selection

The estimated model likelihood of DNA and RNA substitution models applied on different data partitions are shown in Table 4 and 5. The protein coding region fitted best with the general time reversible substitution model allowing for rate heterogeneity and invariable sites. Excluding model parameters almost always had a strong negative effect on the estimated model likelihood when 'strong' was defined as being expressed by a Bayes factor $\geqslant 20$. Ignoring the invariable site parameter changed the estimated model likelihood only slightly ($B_{10} = 13.5$). The mt DNA was best explained by the REV+Γ+inv model, but Bayes factors indicated that the number of rate parameters seems to have just a minor influence on the model likelihood ($B_{10} \approx 17$ for both, TN93 and HKY85). The less variable nc DNA partition finally called for a REV+inv model. Taking just three or two rate parameters into account changed the estimated model likelihood again only slightly ($B_{10} < 1.7$).

None of the DNA substitution models showed signs of over-parameterization. This concern proved to be justified when applying parameter rich RNA models. Although the best estimated model likelihood for the mt RNA partition was gained with the RNA6A+Γ+inv model, rate parameters were highly fluctuating, showed extreme values, and reached the fixed upper bound in PHASE (examples shown in Fig. 2). The model with the highest estimated model likelihood still properly converging in all parameters was RNA6B+Γ. The less variable nc RNA partition allowed only for the application of the RNA6B model.

Table 3
Tests for homogeneity of base frequencies among taxa

Partition	# sites	χ^2		p
mtND1/all sites	918	92.007	(df = 123)	0.9833
mtND1/constant sites excluded	483	177.591	(df = 123)	**0.0009**
mtND1/1st, 2nd codon positions and constant sites excluded	267	250.956	(df = 123)	**0.0000**
mtND1/3rd codon positions and constant sites excluded	216	67.326	(df = 123)	1.0000
mtDNA/all sites	723	18.116	(df = 123)	1.0000
mtDNA/constant sites excluded	243	60.370	(df = 123)	1.0000
mtRNA/all sites	848	26.984	(df = 123)	1.0000
mtRNA/constant sites excluded	299	64.012	(df = 123)	1.0000
ncDNA/all sites	1,169	4.115	(df = 123)	1.0000
ncDNA/constant sites excluded	114	33.400	(df = 123)	1.0000
ncRNA/all sites	1,210	5.773	(df = 123)	1.0000
ncRNA/constant sites excluded	111	73.882	(df = 123)	1.0000

Table 4
Estimated model likelihood of DNA substitution models separately applied on different data partitions

Model	Γ	Inv	# parameters	$\log_e \hat{f}(X\|M_i)$		
				ND1[a]	mtDNA	ncDNA
JC69			0	−5478.5	−6058.16	−3201.75
JC69	+		1	−4917.0	−5483.62	−3095.16
JC69		+	1	−5017.8	−5557.84	−3071.58
JC69	+	+	2	−4901.8	−5469.78	−3129.12
K80			1	−5479.1	−6059.54	−3190.05
K80	+		2	−4915.8	−5483.00	−3084.83
K80		+	2	−5016.9	−5555.01	−3059.60
K80	+	+	3	−4899.4	−5469.67	−3115.65
HKY85			4	−5255.0	−5595.21	−3145.94
HKY85	+		5	−4652.0	−4942.81	−3041.14
HKY85		+	5	−4787.9	−5040.94	**−3017.36**
HKY85	+	+	6	−4641.5	**−4919.95**	−3074.78
TN93			5	−5224.1	−5595.99	−3140.93
TN93	+		6	−4646.2	−4941.16	−3037.29
TN93		+	6	−4764.0	−5039.08	**−3017.06**
TN93	+	+	7	−4633.9	**−4920.04**	−3074.34
REV			8	−5127.1	−5541.35	−3140.73
REV	+		9	**−4608.9**	−4931.12	−3040.38
REV		+	9	−4699.0	−5006.90	**−3015.71**
REV	+	+	10	**−4595.4**	**−4902.95**	−3074.38

[a] 3rd condon position excluded.

Table 5
Estimated model likelihood of RNA substitution models separately applied on different data partitions

Model	Γ	Inv	# parameters	$\log_e \hat{f}(X\|M_i)$	
				mtRNA	ncRNA
RNA6B			7	**−3319.69**	**−2000.38**
RNA6B	+		8	**−3057.95**[b]	−1929.17[b]
RNA6B		+	8	**−3115.79**	**−1918.26**[b]
RNA6B	+	+	9	**−3056.84**[b]	−1936.18[b]
RNA6A			19	−3258.13	−2010.26[b]
RNA6A	+		20	−3044.63[b]	−1981.55[b]
RNA6A		+	20	−3132.79[b]	−1919.43[b]
RNA6A	+	+	21	−3039.03[b]	−1937.87[b]
RNA7D			9	−4507.92	−2519.61[a]
RNA7D	+		10	−4123.69	−2377.12[b]
RNA7D		+	10	−4222.37	−2370.66[b]
RNA7D	+	+	11	−4127.53	−2388.70[b]
RNA7A			26	−4513.91[b]	−2530.44[b]
RNA7A		+	27	−4236.11[b]	−2368.42[b]
RNA7A	+		27	−4113.54[b]	−2379.35[b]
RNA7A	+	+	28	−4108.04[b]	−2390.71[b]

[a] Likely to be overparameterized.
[b] Clearly overparameterized.

Given the previous results, we chose 13 reasonable combinations of partition specific substitution models shown in Table 6. These included again some parameter rich RNA models since we expected a positive influence on parameter convergence by the combined analysis of data. The composite model with the highest estimated model likelihood had a total of 49 free parameters and consisted of the sub-models REV+Γ+inv (ND1), REV+Γ+inv (mt DNA), RNA6B+Γ (mt RNA), REV+inv (nc DNA), and RNA6B (nc RNA). However, reducing the number of rate parameters from six to two in the model applied on the nc DNA partition and omitting the invariable site parameter when modeling the mt RNA section had a minor effect on the estimated model likelihood ($B_{10} = 15$), but limited the number of free parameters to just 44. Therefore, we selected this composite model for the subsequent phylogenetic inference: REV+Γ+inv (ND1), REV+Γ +inv (mt DNA), RNA6B+Γ (mt RNA), HKY85+inv (nc DNA), RNA6B (nc RNA partition).

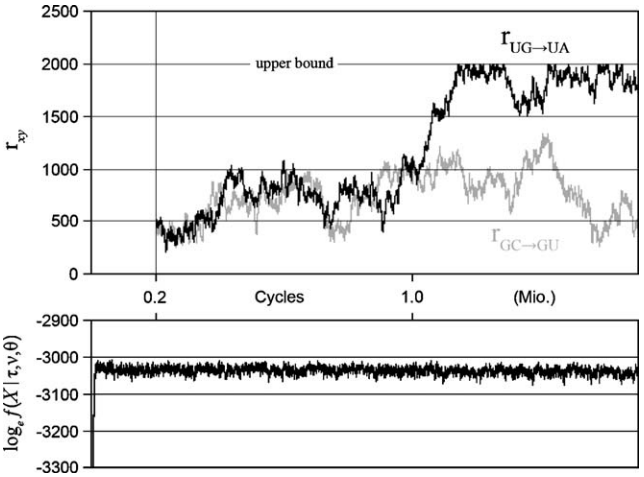

Fig. 2. Example of an MCMC analysis with an over-parameterized model (RNA6A+Γ+inv applied on the mt RNA partition). Despite stationary of the overall model likelihood after initial 200,000 cycles, individual rate parameters show high fluctuation and reach the pre-defined upper bound [2000].

3.4. Phylogenetic analyses

Separate analyses of nuclear and mitochondrial genome data applying the substitution models given above provided us with two compatible consensus topologies when nodes with posterior probability values higher than 95% were regarded as indicative only. We therefore did not reject our *a priori* expectation that both genomes share the same phylogenetic history and pooled the sequence data to take advantage of their capability to resolve relationships at different phylogenetic levels.

The phylogeny of zygaenoid moths inferred from the Bayesian analyses of the combined molecular data is shown in Fig. 3. More than 98% of the sampled trees were congruent with the hypothesis of a monophyletic origin of Zygaenoidea (node A). *Heterogynis* was shown as most basal taxon of this superfamily, but with weak support (node B). Zygaenidae appeared as natural group (node C). The Phaudidae do not seem closely related to any of the other zygaenid taxa. In fact, the posterior probability for a taxon Zygaenidae including the Phaudidae was zero (Table 7). The same was true for Himantopteridae, formally included in the Zygaenidae, too; the molecular data suggested a close relationship to Somabrachyidae (node D). Within Zygaenidae, all subfamilies being represented by more than

Table 6
Estimated model likelihood of composite substitution models applied on partitioned sequence data

Model					# parameters	$\log_e \hat{f}(X\|M_i)$
ND1[a]	mtDNA	mtRNA	ncDNA	ncRNA		
REV+Γ+inv	REV+Γ+inv	RNA6A	REV+inv	RNA6B	59	−18,027.3
REV+Γ+inv	REV+Γ+inv	RNA6B+Γ+inv	REV+inv	RNA6B	49	**−17,755.7**
REV+Γ+inv	REV+Γ+inv	RNA6B+inv	REV+inv	RNA6B	48	−17,831.0
REV+Γ+inv	REV+Γ+inv	RNA6B+Γ	REV+inv	RNA6B	48	**−17,762.7**
REV+Γ+inv	REV+Γ+inv	RNA6B+Γ	TN93+inv	RNA6B	45	**−17,763.8**
REV+Γ+inv	REV+Γ+inv	RNA6B+Γ	HKY85+inv	RNA6B	44	**−17,765.6**
REV+Γ+inv	REV+Γ+inv	RNA6B	REV+inv	RNA6B+inv[b]	48	−17,936.3
REV+Γ+inv	REV+Γ+inv	RNA6B	REV+inv	RNA6B	47	−18,058.7
REV+Γ+inv	REV+Γ+inv	RNA6B	HKY85+inv	RNA6B	44	−18,061.9
REV+Γ+inv	HKY85+Γ+inv	RNA6B+Γ	REV+inv	RNA6B	44	−17,781.2
REV+Γ+inv	HKY85+Γ+inv	RNA6B+Γ	HKY85+inv	RNA6B	44	−17,786.2
REV+Γ	REV+Γ+inv	RNA6B+Γ	REV+inv	RNA6B	47	−17,783.5
REV+Γ	REV+Γ+inv	RNA6B+Γ	HKY85+inv	RNA6B	44	−17,786.5

All parameters were allowed to be partition specific.
[a] 3rd condon position excluded.
[b] Likely to be overparameterized.

one species were shown as monophyletic (nodes E_{1-3}). No tree was recovered confirming Chalcosiinae as sister taxon to Zygaeninae, however. The posterior probability of this hypothesis proved to be zero (Table 7). Rather the subfamily Zygaeninae seemed sister to a group consisting of Callizygaeninae, Chalcosiinae, and Procridinae (node F). Given the fact that Callizygaeninae appeared more closely related to Chalcosiinae than to Procridinae, we also evaluated a slightly modified hypothesis in which the Callizygaeninae were included in the Chalcosiinae. Yet, the posterior probability for this scenario was zero (Table 7).

Among Zygaeninae, the genus *Pryeria* was shown as the most basal taxon of this subfamily whereas all other investigated genera (i.e., *Neurosymploca*, *Orna*, *Praezygaena*, *Reissita*, *Epizygaenella*, and *Zygaena*) emerged as a derived, subordinated taxon (node G). Interestingly, the genus *Orna*, formally considered as most primitive afrotropical representative of Zygaeninae appeared as sister taxon to *Praezygaena* in the crown of the tree (node H). Whether this group or another taxon of the crown or even all of them are sister to the large monophyletic genus *Zygaena* (node I) cannot unequivocally be assessed with the present molecular data, however.

Since the occurrence of cuticular cavities in Zygaenidae and Heterogynidae has given rise to the idea that both taxa together are a monophyletic group, we finally evaluated this hypothesis with the molecular data; independent of whether Heterogynidae is adelphotaxon to Zygaenidae or Heterogynidae is a taxon within Zygaenidae, the posterior probability proved to be very low ($p \leqslant 0.25\%$)

3.5. Ancestral state reconstruction

The molecular data contradicted a sister group relationship of Zygaenidae and Heterogynidae and implied that the latter could be the most basal representative of zygaenoid moths. There was uncertainty in the exact position of the Heterogynidae in the inferred phylogeny, however. The coincidence in the differentiation of cuticular cavities and their opening mechanisms between chalcosiine and zygaenine moths, on the other hand, strongly suggests that these structures are likely homologous within Zygaenidae (see discussion) and that therefore cuticular cavities were probably present in the ancestor of this family. Hence, we were interested in evaluating the hypothesis that cuticular cavities were already present in the most recent common ancestor of the superfamily Zygaenoidea taking the uncertainty of the phylogenetic reconstruction into account. We first evaluated the prior probability for reconstructing a presence of cuticular cavities in the ancestor of Zygaenoidea by searching for the most parsimonious explanation for the observed character pattern in 175,810 random trees assuming a monophyly of Zygaenoidea and Zygaenidae and considering cuticular cavities homologous within Zygaenidae: 172,270 (=97.99%) trees clearly contradicted an ancient origin of cuticular cavities and 3,510 (=2.01%) trees were ambiguous. By assigning equal weight to each of the two character states in case of ambiguous ancestral state reconstruction, we found 1.00% support for the presence of cuticular cavities in random trees (Table 8). Of the 180,000 trees that had been sampled in the Bayesian analysis, on the other hand, 175,810 trees were congruent with a monophyly of Zygaenoidea and Zygaenidae. Among these trees, 39,732 (=22.60%) suggested the absence of cuticular cavities in the most recent common ancestor whereas in the remaining 136,078 (=77.40%) trees character reconstruction was ambiguous. Assigning again equal weight to each of the two possible character states in case of ambiguous ancestral state reconstruction, we received 38.70% support for the presence of cuticular cavities (Table 8).

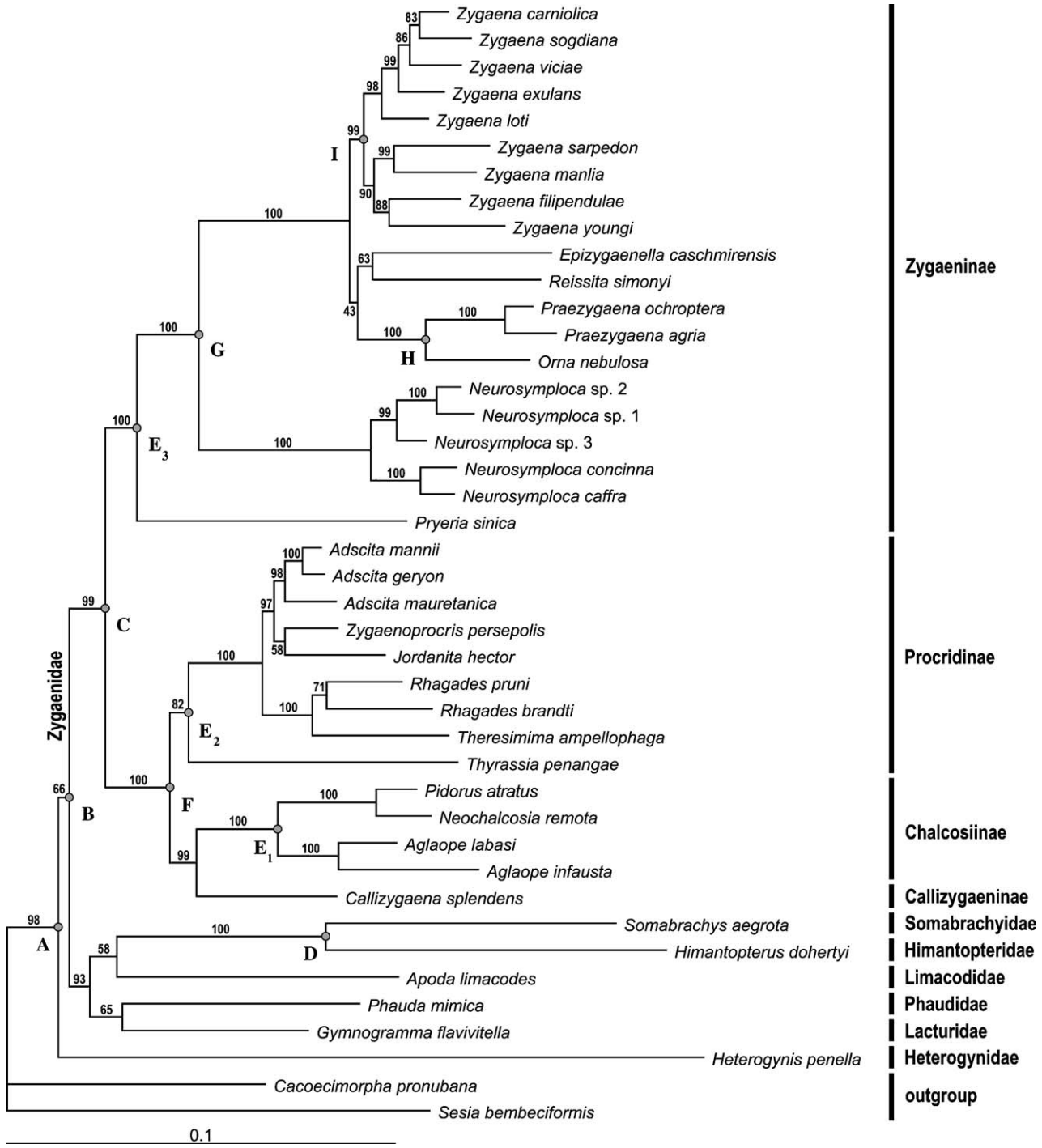

Fig. 3. Phylogenetic relationships of zygaenoid moths based on a Bayesian analysis of mitochondrial (ND1, tRNA-Leu, 16S rRNA, tRNA-Val, 12S rRNA) and nuclear (18S rRNA, 28S rRNA) markers. The topology shown is an extended majority rule consensus of 180,000 MCMC samples taken near the likelihood peak. Branch lengths are estimated using a maximum likelihood approach. Numerals on branches represent posterior probability values. Letters refer to nodes specifically addressed in the text.

4. Discussion

In the past, resolving phylogenetic relationships in the taxon Zygaenoidea has been notoriously difficult to assess with morphological data only (Epstein et al., 1999). Studying DNA sequences to elucidate the history of this group was therefore the consequent next step. We decided to focus on ribosomal genes, since the secondary structure of their RNA provides supplementary information (e.g., Billoud et al., 2000; Gillespie et al., 2004; Lydeard et al., 2000; Misof and Fleck, 2003; Niehuis et al., 2006, in press; Ouvrard et al., 2000). Additionally, we analyzed a protein coding gene to consider a different type of molecular marker as well. Despite their wide use in phylogenetic

Table 7
Estimated posterior probability of specific phylogenetic hypotheses

Hypothesis	Posterior probability
((Phaudidae, Zygaenidae), remaining Zygaenoidea)	0.0000
(((Chalcosiinae), (Zygaeninae)), remaining Zygaenoidea)	0.0000
(((Chalcosiinae, Callizygaeninae), (Zygaeninae)), remaining Zygaenoidea)	0.0000
((Heterogynidae, (Zygaenidae[a])), remaining Zygaenoidea)	0.0020
((Heterogynidae, Zygaenidae[a]), remaining Zygaenoidea)	0.0024

[a] Excluding Phaudidae.

Table 8
Prior and posterior probabilities for reconstructing a presence of cuticular cavities in the most recent common ancestor of Zygaenoidea assuming monophyly of Zygaenoidea and Zygaenidae and considering cuticular cavities to be homologous within Zygaenidae

Hypothesis	Prior probability (%)	Posterior probability (%)	B_{10}
H_1: cuticular cavities present in the most recent common ancestor of Zygaenoidea	1.00	38.70	~157
H_2: cuticular cavities absent in the most recent common ancestor of Zygaenoidea	99.00	61.30	

The Bayes factor quantifies the change of the odds of the hypotheses.

systematics, ribosomal RNAs have properties related to their secondary structure, which complicate a phylogenetic analysis (Higgs, 2000): hydrogen bonds in rRNA molecules are only thermodynamically stable between certain nucleotides (e.g., AU, GU, GC). The consequence is a correlation between paired sites in stem regions. Ignoring this correlation by applying DNA substitution models can cause misleading phylogenetic relationships and inflate measurements of tree robustness, particularly effective in likelihood approaches (see for example Galtier, 2004; Parsch et al., 2000; Rzhetsky, 1995; Stephan, 1996; Tillier and Collins, 1995, 1998). The same problem applies to tRNA molecules (Higgs, 2000). RNA substitution models (Higgs, 2000; Muse, 1995; Rzhetsky, 1995; Savill et al., 2001; Schöniger and von Haeseler, 1994; Tillier, 1994; Tillier and Collins, 1998) can meet these difficulties, but have rarely been considered. The present investigation is one of just few so far (e.g., Hudelot et al., 2003; Jow et al., 2002; Kjer, 2004), which deliberately utilizes these types of models in a Bayesian approach to estimate the posterior probability of phylogenetic hypotheses. However, our results point to problems restricting the application of these models; since the number of free parameters in RNA models is typically larger than those used to study DNA (Savill et al., 2001) and as the number of characters is virtually bisect, parameter estimation can be difficult when sequence variation is limited. Although our RNA stem data partitions consisted of 42 species taxa with 848 BP (mt) and 1,210 BP (nc) each, respectively, we found clear evidence for over-parameterization in most RNA models tested (RNA6A, RNA7D, RNA7A). Molecular systematists should be aware of this problem when considering sequencing smaller fragments of ribosomal RNA as is typically done.

The combined analysis of nuclear and mitochondrial markers provided us with a well supported phylogenetic hypothesis of major zygaenoid lineages. The inferred phylogeny confirmed the exclusion of Himantopteridae from the Zygaenidae as suggested by Fletcher and Nye (1982) using morphological evidence. The affinities of the Himantopteridae to other zygaenoid taxa remained unclear so far, however; the loss of a functional proboscis, the presence of an anal tuft in the females, and the presence of spinulate transverse bands on abdominal tergites suggested a close relationship to Somabrachyidae and Megalopygidae as well as to Anomoeotidae (Fänger et al., 1998; Minet, 1986) whereas a breeding record of Himantopteridae revealed morphological affinities with larvae of Megalopygidae (Barlow and Carter, 1996) and Phaudidae (see Fänger et al., 1998). Based on a cladistic analysis of larval morphology, Fänger (pers. comm.) in recent times assumed a closer relationship to Somabrachyidae. We already reported structural coincidence of helix 49 in the 12S rRNA between Himantopteridae and Somabrachyidae (Niehuis et al. (2006)). The Bayesian analysis of sequence data now provided convincing additional support for this opinion. Nevertheless, Anomoeotidae and Megalopygidae were not present in our taxon sampling, thus we cannot rule out that one or both of them belong to this clade, too. Future studies should address this issue.

The inclusion of the Phaudidae in the family Zygaenidae was first questioned by Fänger et al. (1998). The traditional assignment of the Phaudidae to this family is not supported since it lacks all of the characters considered to substantiate the monophyly of the Zygaenidae. Fänger et al. (1998) studied the external larval morphology of a *Phauda* species in detail and found almost exclusively derived characters shared with taxa of the limacodid family group. The molecular data in the present investigation independently confirmed that the Phaudidae and the remaining Zygaenidae do not form a natural group. Fänger et al. (1998) hypothesized Phaudidae as sister to the limacodid family group whereas the molecular data suggested a more close relationship to Lacturidae, although with poor support. Since some zygaenoid families that are important to address this

question (e.g., Dalceridae, Anomoeotidae) are missing in our taxon sampling, we refrain from a further discussion.

Regarding Phaudidae as a separate zygaenoid family, the Bayesian analysis recovered a monophyletic taxon Zygaenidae consisting of Callizygaeninae, Chalcosiinae, Procridinae, and Zygaeninae in 99% of the sampled trees. The inferred phylogeny is congruent with the assumption that each of the four currently recognized subfamilies constitutes a monophylum. The molecular data also confirmed the previous segregation of the genus *Callizygaena* from the Procridinae. The phylogenetic affinities among the four zygaenid subfamilies as inferred from the sequence data contradicted the currently accepted phylogeny of Zygaenidae (Naumann et al., 1999), however. We found no evidence for a sister group relationship of Chalcosiinae and Zygaeninae. Far from it, Callizygaeninae, Chalcosiinae, and Procridinae seem to be a well supported monophyletic group as it was once speculated by Alberti (1954), although one must bear in mind that Alberti (1954) included Anomoeotidae and Himantopteridae in this group, too. Anyway, since chalcosiine and zygaenine moths are not sister taxa, the evolutionary origin of larval cuticular cavities in the family Zygaenidae must be reconsidered.

The morphology and ultrastructure of cuticular cavities found in the subfamily Zygaeninae have been studied extensively by Povolný and Weyda (1981), Franzl and Naumann (1984, 1985), and Naumann and Povolný (1987). According to these studies, the zygaenine moth cuticle is much thicker than in other Lepidoptera (about 10 times that of a noctuoid or a geometric moth for example; Naumann et al., 1999) and shows two different types of cavities. The larger ones are located beneath pigment spots, which are arranged in the dorsal and subdorsal anterior and posterior regions of the second and third thoracic segments as well as the abdominal segments 1-10; the smaller cavities occupy the remaining cuticular surface except for the ventral region. Both types of cavities are connected with the exterior by complicated opening mechanisms illustrated by Povolný and Weyda (1981) and Naumann (1990). Epstein et al. (1999) and Naumann et al. (1999) reported an identical arrangement and differentiation of cuticular cavities and opening mechanisms in chalcosiine moths, although neither descriptions nor illustrations were provided. If this coincidence is correct, the structures seen in both taxa are likely homologous. Given the phylogenetic relationships of chalcosiine and zygaenine moths as inferred from the molecular data and assuming a homology of cuticular cavities in chalcosiine and zygaenine moths, we postulate a presence of cuticular cavities already in the most recent common ancestor of the Zygaenidae and suppose a multiple independent loss within this family. Accordingly, the lack of cuticular cavities in procridine moths as well as in the chalcosiine genus *Aglaope* must be regarded as derived condition. Whether cuticular cavities occur in larvae of the genus *Callizygaena* has not been investigated so far, but the present phylogeny lets a presence in this taxon appear reasonable.

The present phylogeny has given rise to the idea that cuticular cavities could be part of the ground plan of the superfamily Zygaenoidea. The Bayes factor, which was calculated to measure the amount in which our opinion has altered by a change of the odds of the hypotheses, indicated indeed a very strong positive (~157) influence of the molecular data on our confidence in this evolutionary scenario (Table 8). Nonetheless, the posterior probability for a presence of these structures in the most recent common ancestor of Zygaenoidea was only 38.70%. It has to be considered, however, that if the present topology were inferred even without uncertainties, one could not expect to find a value higher than 50%; this is because a parsimony reconstruction is ambiguous for this particular character distribution. To clarify the origin of cuticular cavities in zygaenoid moths it is therefore paramount to assess their homology now more directly by examining their histology, ultrastructure, and ontogenesis. Such investigations should also consider samples of Dalceridae, Lacturidae, and Limacodidae since defensive droplets have been observed in larvae of these taxa (Common, 1990; Epstein, 1996, 1997). We interpret this as an indication that cuticular cavities could be more widely distributed among zygaenoid moths than currently recognized. Verifying identical structures in other zygaenoid taxa could thus provide additional evidence for our hypothesis of an ancient origin of cuticular cavities in Zygaenoidea.

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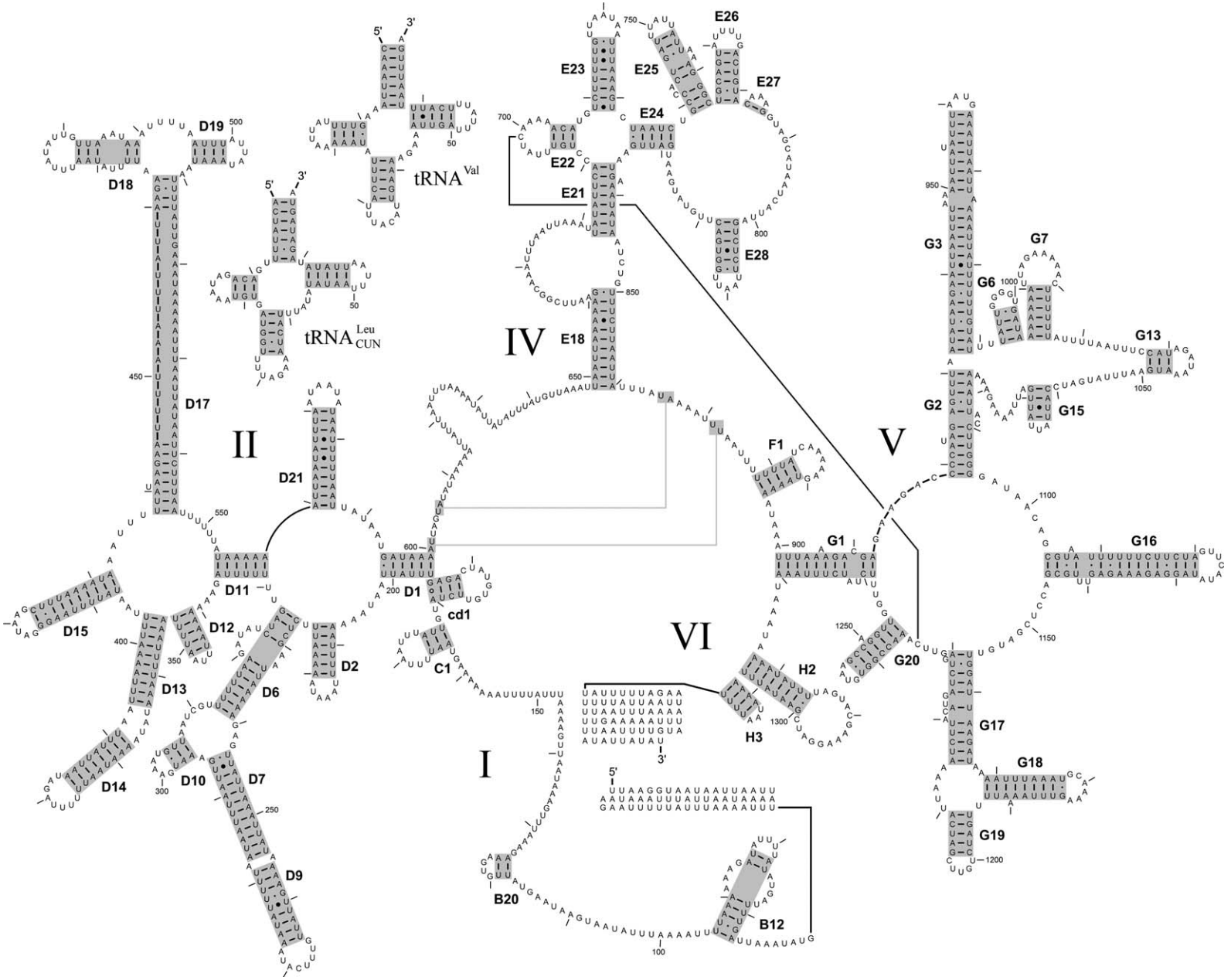
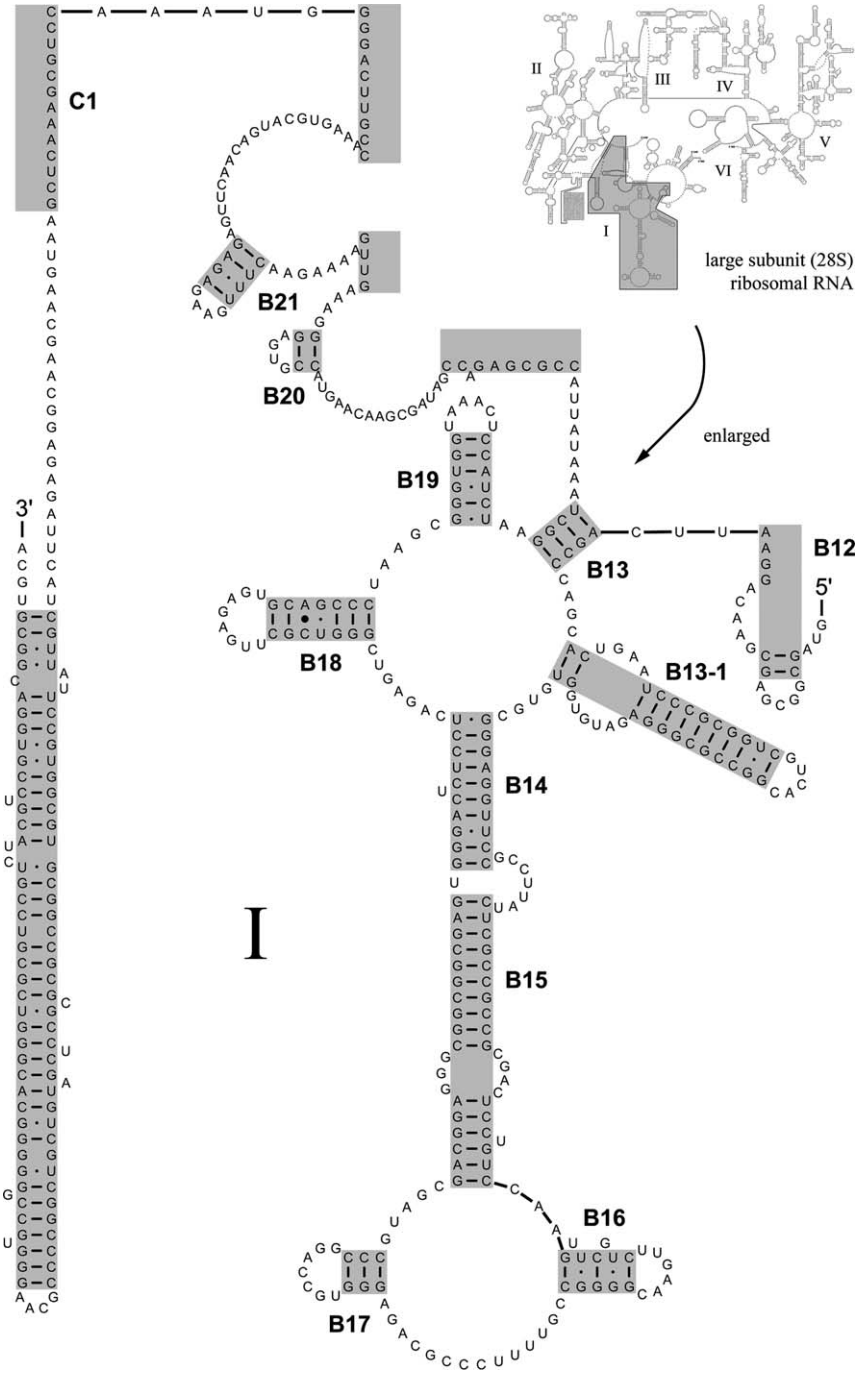
Appendix A

Models of mtRNA secondary structures in *Zygaena sarpedon lusitanica* (AJ831713): tRNA-Val, LSU rRNA (16S rRNA), and tRNA-Leu.



Appendix B

Secondary structure model of the 5′-end near part (domain I) of the nc LSU rRNA (28S rRNA) in *Zygaena sarpedon lusitanica* (AJ844136).



Appendix C. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.01.007.

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