In Search of the Origin of Twin Penises: Molecular Phylogeny of Earwigs (Dermaptera: Forficulina) Based on Mitochondrial and Nuclear Ribosomal RNA Genes

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Ann. Entomol. Soc. Am. 97(5): 903–912 (2004)

ABSTRACT Forficulina, the largest suborder of Dermaptera (earwigs), has eight families. In five families (Pygidicranidae, Diplatyidae, Anisolabididae, Apachyidae, and Labiduridae), the males have two penises, whereas the males of the other three families (Spongiphoridae, Chelisochidae, and Forficulidae) have a single penis. Several cladograms have been proposed for Forficulina. However, those systems are constructed mainly from observations of male genital morphology and outstandingly inconsistent. This study reconstructed an earwig phylogeny with representatives of seven families (excluding Apachyidae) by using partial sequences of the mitochondrial 16S and nuclear 28S rRNA genes, sequences that are independent of genital evolution. To avoid difficulties caused by outgroup-rooting for a deep phylogeny, ingroup relationships were first established as unrooted trees based on the 16S, 28S, and combined data sets. The resulting affinities of the earwig families supported several superfamilies, such as Forficuloidea (single-penis families) and Pygidicranoidea (Pygidicranidae + Diplatyidae). Inclusion of the outgroup (Glylloblattodea and Blattodea) did not distort the established ingroup relationships. However, the root position varied according to the genes and outgroup taxa used. Kishino–Hasegawa tests based on the maximum likelihood criterion suggested that the common ancestor of contemporary Forficulina had twin penises, each with one gonopore.

KEY WORDS Dermaptera, family relationships, genital evolution, molecular phylogeny, outgroup rooting

EARWIGS (DERMAPTERA) ARE orthopteroids and are characterized by their diversity in penis morphology of males (Popham 1965, Kamimura 2004). The order Dermaptera is divided into three suborders: Hemimerina, Arixeniina, and Forficulina. The first two suborders are epizoic parasites that live on giant rats and bats, respectively (Popham 1985). The third suborder is the largest and consists of typical earwigs belonging to eight families (Pygidicranidae, Diplatyidae, Anisolabididae, Apachyidae, Labiduridae, Spongiphoridae, Chelisochidae, and Forficulidae; according to Sakai 1982). The males of the first five families have paired penises, whereas the males of the last three families have a single penis (Burr 1915a,b, 1916; Popham 1965; Kamimura 2004). In several examples, male genital morphology is characterized by further modifications. Diplatyieds have two gonopores on each of their two penises; that is, a single male has two double-barreled penises and four gonopores (Popham 1965, Kamimura 2004). The two penises of the anisolabidid *Euborellia plebeja* (Dohrn) are as long as the body length, and they function as a device for removing rival sperm from the female sperm storage organ (Kamimura 2000). Other anisolabidids, such as *Anisolabis maritima* (Bonelli) have similar penises (Kamimura and Matsuo 2001, Kamimura 2004). The long, fragile penis sometimes breaks in the female sperm storage organ (spermatheca), and the remaining penis of the pair then function as a spare (Kamimura and Matsuo 2001, Kamimura 2003). In the species examined, males endowed with two penises use only one during mating, although both are functional (Kamimura and Matsuo 2001, Kamimura 2004). It is interesting to trace the diversification of penis morphology in earwig phylogeny together with functional analyses of these diverse earwig penises. To deduce the function of twin penises in ancestral earwigs, it is important to estimate the origin of the twin penis. Because the penis is termed the "virga" in dermapterology, virga is used in this article hereafter.

Although several authors have proposed cladograms for the suborder Forficulina based mainly on male genital traits, they are markedly inconsistent (Popham 1965, 1969, 1985; Sakai 1987; Haas 1995; Haas

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0013-8746/04/0903–0912$04.00/0

and Kukalová-Peck 2001). Using principally neck, leg, and male genital morphology, Popham (1965, 1969, 1985) classified earwigs (including Arixeniina) into three superfamilies: Pygidicranoidea, Labioidea, and Forficuloidea. In his system, Labioidea (Anisolabididae and Spongiphoridae of Forficulina, and Arixeniidae of Arixeniina) and Forficuloidea (Labiduridae, Chelisochidae, and Forficulidae) are arranged as sister groups, whereas Pygidicranoidea (Pygidicranidae and Diplatyidae) is basal for them. In this system, the presence of paired virgae is treated as an ancestral state; therefore, families with a single virga evolved by losing one of the paired virgae. Popham's system is characterized by the polyphyly of single-virga families. In other words, his system requires that loss of virgae occurred independently at least twice: once in Labioidea at the root of Spongiphoridae + Arixeniidae, and once in Forficuloidae at the root of Chelisochidae + Forficulidae. By contrast, the single-virga families (Spongipohridae, Chelisochidae, and Forficulidae) form a monophyletic group in the system of Sakai (1987), in which Diplatyidae is the most basal family of Forficulina. In this system, Pygidicranidae and Anisolabididae are the second and third earliest offshoots, respectively. Labiduridae and Apachyidae (the latter family is sometimes included in the former; Popham 1965, 1969, 1985) is a sister clade to the cluster of single-virga families (Spongiphoridae, Chelisochidae, and Forficulidae). In addition to the characters used in the previous studies, Haas (1995) examined many thoracic and wing characters under the principles of phylogenetic systematics and obtained a system similar to that of Sakai (1987). In Haas' system, the basal family is Karschiellidae, which is sometimes included in Pygidicranidae (Popham 1969; Sakai 1982; Steinmann 1986, 1989). Diplatyidae, Pygidicranidae, Apachyidae, and Labiduridae are the second, third, fourth, and fifth earliest offshoots, respectively. In contrast to the system of Sakai (1989), Anisolabididae is the sister group to single-virga families. More recently, by adding 18 new characters of wing venation and articulation to Haas' (1995) data set, Haas and Kukalová-Peck (2001) proposed a new hypothesis almost the same as Haas' (1995) system except that the sister clade to the cluster of single-virga families is Labiduridae and that Forficulidae is the basal to Spongiphoridae and Chelisochidae in the single-virga clade.

Despite the considerable incongruence among these systems, every system placed double-virga families basal to single-virga ones. However, this conclusion resulted from the analytical assumption that the double status is ancestral. To avoid this type of circular argument in an investigation of genital morphology, one must reconstruct an earwig phylogeny based on data that are independent of genital morphology. Molecular data, such as DNA and amino acid sequences, enable such analyses. To date, the only molecular phylogeny of earwigs has been one that Wirth et al. (1999) reconstructed for several earwig species based on the mitochondrial cytochrome oxidase II (COII) gene sequence. However their analysis was not performed with the intent of reconstructing family relationships of earwigs, and only six earwig species from three families (Anisolabididae, Labiduridae, and Forficulidae) were included in their study.

Compared with close relationships, such as intrageneric or intrafamily relationships, the resolution of deep phylogenies is generally more difficult. There are several reasons for this. First, it is often difficult to select genes with an appropriate evolutionary rate. Because genes differ extensively in their information content for reconstructing phylogeny (Russo et al. 1996), consensus or combined analyses based on multiple genes are often effective or even necessary to resolve deep relationships (Flook et al. 1999; Gatesy et al. 1999). Second, it is sometimes difficult (or practically impossible) to choose appropriate outgroup taxa that are not overly distant to the ingroup (focal taxa). Distant outgroups are likely to indicate incorrect positions of the root of ingroup trees, especially when the nucleotide composition (e.g., G + C content) varies extensively among taxa. This is because outgroup taxa are likely to be attracted to ingroup taxa that have similar nucleotide compositions, sometimes providing high bootstrap support for the incorrect topology (Lockhart et al. 1992, Hasegawa and Hashimoto 1993, Naylor and Brown 1998, Lin et al. 2002). Unfortunately, the order that is the nearest outgroup to earwigs among orthopteroids has not yet been determined. Analysis of the mitochondrial COII sequences of orthopteorids suggested that earwigs (Dermaptera) form the basal taxon (Maekawa et al. 1999). Recently, Plecoptera (stoneflies), Glylloblattodea, and Orthoptera have been suggested as the closest lineages, whereas Phasmida, Mantodea, Isoptera, and Blattodea (cockroaches) are the second closest group (Whiting et al. 2003). Therefore, several outgroup taxa from other orthopteroid orders, including the closest ones, should be examined to root the earwigs. Under these circumstances, it may be worthwhile to carry out both an unrooted analysis for establishment of ingroup relationships and a rooted analysis (Lin et al. 2002). For the latter, maximum likelihood (ML) methods provide probabilistic measures for each of the possible rooted topologies, enabling us to compare them in a statistical manner (Kishino and Hasegawa 1989, Kishino et al. 1990).

In this study, I constructed the phylogeny for 16 species representing seven earwig families, based on partial sequences of the mitochondrial 16S and nuclear 28S ribosomal RNA genes. Family relationships of earwigs were established as an unrooted tree by using 16S, 28S, and 16S + 28S combined analyses. Before merging the 16S and 28S data sets, the congruence between them was examined. In addition to standard outgroup-rooting, possible positions of the root (the origin of earwigs) were explored using maximum likelihood and bootstrapping methods. Based on the results using this approach, possible scenarios for the diversification process are discussed in comparison with previous works based on observations of morphology.

Table 1. Specimens used in the molecular analysis

Order	Family	Species	Locality in Japan
Dermaptera	Pygidicranidae	*P. infernalis*	Okinawa-jima,Okinawa
		Challia sp.	Yaku-shima, Kagoshima
	Diplatyidae	*D. flavicollis*	Iriomote-jima, Okinawa
	Anisolabididae	*A. maritima*	Kawasaki, Kanagawa
		G. marginalis	Komae, Tokyo
		E. plebeja	Komae, Tokyo
		Euborellia annulipes (Lucas)	Komae, Tokyo
	Labiduridae	*Labidura riparia* (Pallas)	Iriomote-jima, Okinawa
	Chelisochidae	*P. simulans*	Iriomote-jima, Okinawa
	Spongiphoridae	*N. lewisi*	Sagamiko, Kanagawa
		Labia minor (Linnaeus)	Hachioji, Tokyo
		Metalabella curvicauda (Motschulsky)	Kawasaki, Kanagawa
	Forficulidae	*Eparchus yezoensis* (Shiraki)	Tajima, Fukushima
		Forficula mikado (Burr)	Karuizawa, Nagano
		Forficula hiromasai (Nishikawa)	Nago, Okinawa
		Anechura harmandi (Burr)	Karuizawa, Nagano
Blattodea	Blattellidae	*Blattella nipponica*[a] (Asahina)	Hachioji, Tokyo
Grylloblattodea	Grylloblattidae	*Galloisiana nipponensis* (Caudell et King)	Niimi, Okayama

[a] Only the 16S rRNA gene fragment was amplified. The 28S sequence of a congener was obtained from GenBank (see text).

Materials and Methods

DNA Isolation, Polymerase Chain Reaction (PCR) Amplification, and DNA Sequencing. Sixteen species of earwigs, belonging to seven families, and two outgroup species were collected in Japan between September 1998 and January 2003 (Table 1). All specimens were in Y. Kamimura Collection. Total genomic DNA was extracted from single insects by using the phenol-chloroform extraction method (Sambrook et al. 1989). Total DNA was used as a template for PCR amplification of mitochondrial 16S rDNA fragments (362–407 bp, except for anisolabidids with large deletions [≈330 bp]) and nuclear 28S rDNA fragments (513–550 bp, except for *Prolabisca* and outgroup taxa in which a wider range was amplified; Appendices 1 and 2). The PCR primers were shown in Appendices 1 and 2. PCR reactions were performed with a thermal cycler (PTC-100; MJ Research, Watertown, MA) by using the following parameters: one cycle of 93°C denaturation (3 min), 47°C annealing (1 min), and 72°C extension (1 min 15 s); and 29 cycles of 93°C denaturation (30 s), 47°C annealing (1 min), and 72°C extension (1 min 15 s). Amplified DNA was sequenced directly on an ABI 377 or a 310 automated sequencer using a BigDye-Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). The sequences have been deposited in GenBank (accession nos. AB052800-AB052813, AB119536-AB119556). The 28S rDNA sequence of an outgroup taxon (*Blattella vaga* Hebard; accession no. AF321246) was obtained from GenBank.

Phylogenetic Analysis. Preliminary alignments of the sequences were made using the ClustalX program package (Thompson et al. 1997) with the default parameters. Several regions could not be aligned unambiguously, and these regions were excluded from the analyses. No manual modifications were made to the alignments to obtain the highest reproducibility. Elimination of ambiguous parts did not change the 16S or 28S branching patterns obtained in the neighbor-joining (NJ; Saitou and Nei 1987) or maximum parsimony (MP) analyses, except that those supported by low bootstrap values collapsed (data not shown). For the 16S and 28S rDNA sequences, 202 and 318 sites (total alignment length) were used for the subsequent analysis, respectively. Of these, 100 (95) and 243 (137) were variable and 80 (75) and 146 (85) were parsimony informative sites for 16S and 28S sequences, respectively (numbers in parentheses indicate sites when only earwig sequences were considered). The G + C content of each taxon is shown in Table 2. Alignments are available on request from kamimu@ris.ac.jp or ykamimu@hotmail.com.

I first established the ingroup relationships (unrooted trees) of the earwigs based on the 16S and 28S sequences, by using the NJ and unweighted MP methods with MEGA version 2.1 (Kumar et al. 2001) and 1000 bootstrap replications (Felsenstein 1985). In both analyses, gap positions were excluded completely. For the NJ analysis, the Jukes and Cantor (1969) distance was adopted, whereas the Kimura (1980) two-parameter distance and Tajima and Nei (1984) distance gave identical results, except for certain details concerning the branching patterns with low bootstrap support (data not shown). For the MP analysis, a min-mini search algorithm (with search factor of 2; Kumar et al. 1993, Nei and Kumar 2000) was adopted to reconstruct a 50% majority-rule bootstrap consensus tree. In addition to the consensus tree for the 16S and 28S trees, I performed a combined analysis of the 16S and 28S sequence data. Before the combined analysis, the partition homogeneity test (Farris et al. 1994, 1995) was conducted (1000 replication of randomization) to examine the incongruence of the 16S and 28S data sets, according to the protocol presented in O'Grady et al. (2002). This test was conducted using PAUP* 4.0b10 (Swofford 1999) and a heuristic search strategy of MP analysis with random addition of taxa (10 replicates) and the tree bisection-reconnection (TBR) option. The ML

Table 2. G + C content (%) of the taxa examined

Taxa	16S	28S	16S + 28S
Dermaptera			
Pygidicranidae			
P. infernalis	36.5	35.5	35.9
Challia sp.	36.5	39.2	38.2
Diplatyidae			
D. flavicollis	37.5	33.5	35.0
Anisolabididae			
A. maritima	31.5	38.0	35.4
G. marginalis	35.5	38.3	37.2
E. plebeja	33.0	37.7	35.8
Euborellia annulipes (Lucas)	37.0	37.3	37.2
Labiduridae			
Labidura riparia (Pallas)	38.5	37.3	37.8
Chelisochidae			
P. simulans	38.0	38.3	38.2
Spongiphoridae			
N. lewisi	33.7	39.4	37.1
Labia minor (Linnaeus)	32.0	37.7	35.5
Metalabella curvicauda (Motschulsky)	32.0	34.6	33.6
Forficulidae			
Eparchus yezoensis (Shiraki)	42.0	35.9	38.3
Forficula mikado (Burr)	39.5	38.3	38.8
Forficula hiromasai (Nishikawa)	40.0	38.5	39.0
Anechura harmandi (Burr)	38.5	37.7	38.1
Blattodea			
Blattellidae			
Blattella spp.[a]	36.5	65.7	54.0
Grylloblattodea			
Grylloblattidae			
Galloisiana sp. nipponensis (Caudell et King)	41.0	61.4	53.2

[a] *B. nipponica* for 16S and *B. vaga* for 28S.

method also was applied to the combined data set. The ML tree was drawn using the program Nucml (star-decomposition option) in the MOLPHY2.3b3 program package (Adachi and Hasegawa 1996). The local bootstrap probability (10,000 replicates) of each branch was estimated by using the resampling-of-estimated-log-likelihood (RELL) method (Kishino et al. 1990, Hasegawa and Kishino 1994). The ratio of transitions to transversions (R) was estimated to be ≈2.6 for 16S, 28S or 16S + 28S. This value and the HKY base substitution model (Hasegawa et al. 1985) were used for the analysis. The key results remained unchanged when R = 4.0 (the default value for the software) was used for the analysis (data not shown).

In my search of the origin of earwigs, I included two outgroup taxa in the ML analysis. The inclusion of the outgroup did not alter the ingroup relationships (see Results). The resulted rooted tree was statistically compared with all the other 28 topologies while fixing the ingroup relationships and varying the rooting positions. For this purpose, the significance between the likelihood values of the best and other possible topologies was examined by the Kishino–Hasegawa test (Kishino and Hasegawa 1989), by using the RELL method and parameters specified avobe.

Results

Standard NJ analysis and bootstrap consensus analysis (NJ or MP) of the 16S data set gave same topologies for the earwig ingroup relationships (Fig. 1, left). Earwigs were divided into four clusters: (Diplatyidae + Pygidicranidae), Anisolabididae, Labiduridae, and single-virga families as Forficulidae + Spongiphoridae + Chelisochidae (species in the shaded box of Fig. 1), although the support for the last cluster is rather weak.

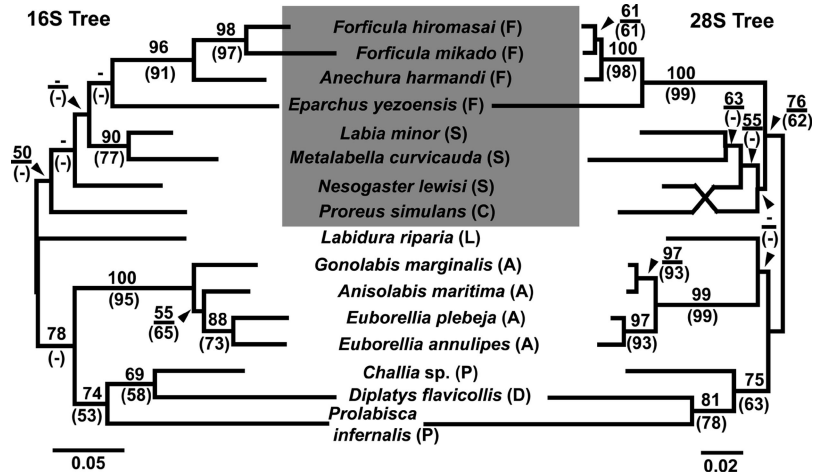

Fig. 1. NJ tree for 16 earwig species based on DNA sequences of parts of the 16S (left) and 28S (right) ribosomal RNA genes. The rooting positions are arbitrary. The bootstrap consensus trees reconstructed using either NJ or MP methods matched those of the standard NJ trees shown, except that several internal branches are not supported and condensed. The numbers above and below (in parentheses) the internal branches are the bootstrap probabilities of NJ and MP bootstrap consensus trees, respectively. Only values >50% are shown (− indicates <50%). Family names are indicated by the first letter in parentheses: F, Forficulidae; S, Spongiphoridae; C, Chelisochidae; L, Labiduridae; A, Anisolabididae; P, Pygidicranidae; D, Diplatyidae. Males of the species with names in the shaded box have a single virga, whereas the others have double virgae.

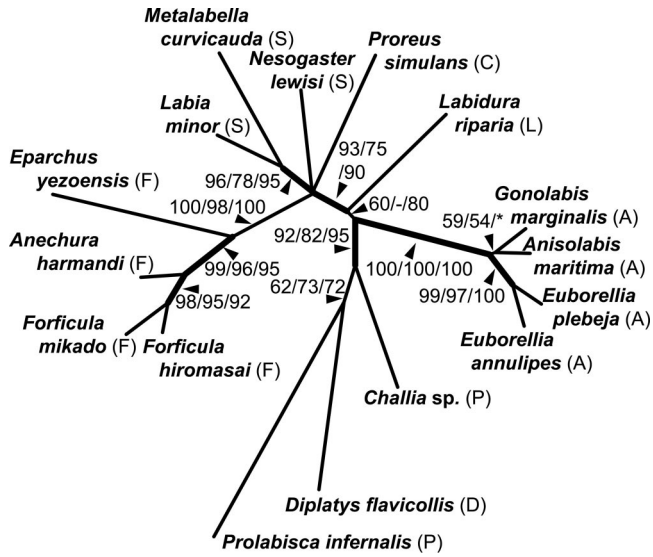

Fig. 2. Unrooted tree of earwigs based on the combined 16S + 28S analysis. On the topology yielded using the standard NJ method, branches are labeled with the NJ bootstrap consensus support/MP bootstrap consensus support/ML local bootstrap support (− indicates <50%, * indicates the branch that was not recovered in the ML analysis). Family names are as in Fig. 1. The branches drawn in thicker lines are supported in the consensus tree.

The first and last clusters correspond to superfamilies Pygidicranoidea and Forficuloidea (sensu Sakai 1982, Steinmann 1989), respectively. The analyses of the 28S data set recovered an almost identical topology (Fig. 1, right). Branches differing between the 16S and 28S trees were mainly those supported by low bootstrap values (<50%), including the sister group to Labiduridae (Forficuloidea and Anisolabididae in the 16S and 28S analyses, respectively). The relationships involving the diplatyid (*Diplatys favicollis* Shiraki) and pygidicranids (*Prolabisca infernalis* (Burr), *Challia* sp.) were exceptional. In general, the 28S data and the NJ method outperformed the 16S data and MP method in resolving the ingroup relationships (Fig. 1). For example, the bootstrap value supporting the clustering of single-virga species was 62% (<50%) in the MP analysis, compared with 76% (50%) in the NJ analysis based on 28S (16S) data. Members of the Forficulidae and Anisolabididae were each clustered into one group. The 50% majority-rule consensus of four trees (bootstrap consensus of the NJ and MP trees based on 16S or 28S data) confirmed the four-way split of earwigs circumscribed by branches with high support: Pygidicranoidea (Diplatyidae + Pygidicranidae), Anisolabididae, Labiduridae, and Forficuloidea (single-virga families as Forficulidae + Spongiphoridae + Chelisochidae) (Fig. 2).

As expected from the small differences between the 16S and 28S trees (Fig. 1), the partition homogeneity test revealed no significant incongruence between the 16S and 28S data sets ($P = 0.691$ and 0.155 when outgoup taxa were excluded and included, respectively). Standard NJ tree and bootstrap consensus trees (NJ and MP) of the combined data set were almost identical to the consensus topology obtained using separate data sets (Fig. 2). Bootstrap support for many nodes identified using the consensus strategy was enhanced in the combined analysis. Furthermore, a clustering of four forficulids that was not fully resolved using the consensus method earned high bootstrap supports. Maximum-likelihood analysis of the combined data set also recovered a similar topology (as the unrooted topology of that shown in Fig. 3), excepting the positions of ambivalent species *Nesogaster lewisi* (de Bormans) and *Gonolabis marginalis* (Dohrn). The four-way split of earwigs also was recovered with >90% local bootstrap supports, with the sister relationship between Labiduridae and single-virga clade (80% support; Fig. 2).

Addition of the two outgroup taxa into the ML analysis of the combined data set brought no rearrangements on the ingroup tree (Fig. 3). Bootstrap supports for internal branches were similar or slightly reduced compared with those in the unrooted analysis (Figs. 2 and 3). Therefore, the ingroup relationships shown in Fig. 3 were fixed for the subsequent comparison of alternative rooting positions. The standard out-group rooting procedure moved anisolabidids deepest in the earwigs (Fig. 3). However, the Kishino–Hasegawa test demonstrated that the ML root position varies with the genes or outgroup taxa used (Fig. 3; Table 3). This test compares differences (and associated standard errors) in log-likelihood between the ML topology and all other possible topologies, also providing with relative bootstrap probabilities of each topology. When the two outgroup taxa were used simultaneously, the 16S and combined analyses rejected several rooting positions, primarily those on external

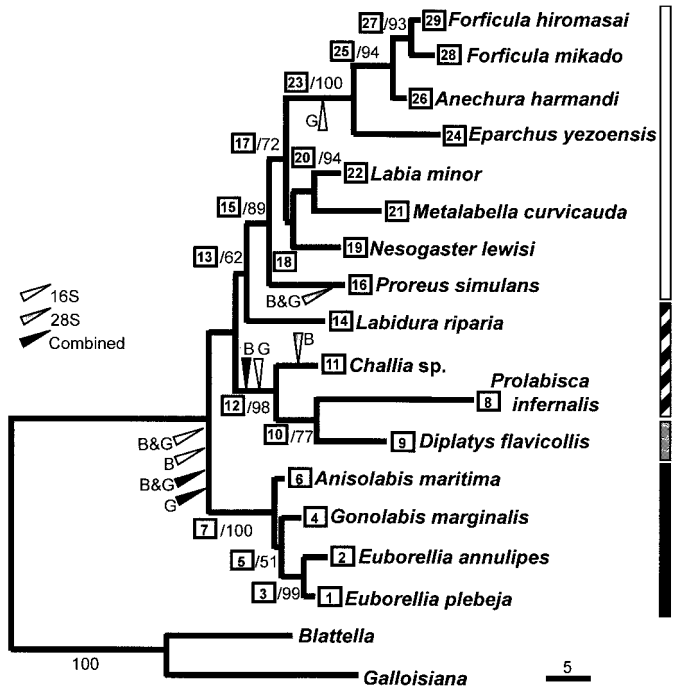

Fig. 3. Maximum likelihood rooted tree of earwigs based on the combined data set, with possible root positions for alternative data sets and outgroup taxa. The numbers (in squares) are the possible positions of the root and are also shown in Table 3. Internal branches also are labeled with the local bootstrap support (10,000 replications). Only values >50% are indicated. The open, shaded and black arrows indicate ML root positions based on the 16S, 28S, and 16S + 28S data sets, respectively. The outgoup taxa are indicated using the first letter of the name: G, Glylloblattodea; B, Blattodea. Male genitalia are categorized into four types as in Kamimura (2004) and indicated by the bars: open bar, one short single-barreled (*Forficula hiromasai*, etc.); striped bar, two short single-barreled (*Labidura riparia*, etc.); shaded bar, two short double-barreled (*Diplatys flavicollis*); and black bar, two long single-barreled (*Euborellia plebeja*, etc.). Long virgae are those longer than 50% of the male body length.

branches of the ingroup tree, whereas the 28S data rejected none of the 29 possible topologies (Table 3).

Discussion

In the analyses of the mitochondrial 16S, nuclear 28S and combined sequence data sets, the 16 earwigs examined clustered into four established groups: (Diplatyidae + Pygidicranidae), Anisolabididae, Labiduridae, and single-virga families (Forficulidae + Spongiphoridae + Chelisochidae) (Figs. 1 and 2). The first and last groups correspond to the superfamilies Pygidicranoidea and Forficuloidea (sensu Sakai 1982, Steinmann 1989). Except for Labiduridae, for which only one species was examined, the other three clusters were supported by moderate-to-high bootstrap probabilities in the combined analyses (75–100%; Fig. 2). Therefore, these groups are likely monophyletic or paraphyletic, depending on the root position. The polyphyletic nature of the single-virga families proposed by Popham (1965, 1969, 1985) was not supported by the molecular data sets. Popham's hypothesis originated from the idea that Spongiphoridae (with single virga, termed "Labiidae" in his articles) and Anisolabididae (with double virgae, termed "Carcinophoridae") are sister groups that share characteristics, such as the absence of auxiliary sclerites (reniform vesicles) at the base of the virga. However, at least some members of Anisolabididae and Spongiphoridae possess this sclerite (Kamimura and Matsuo 2001; unpublished data). Accordingly, these morphological characters should be subjected to further scrutiny.

Among the four clusters, Labiduridae seemed to be a sister clade to the single-virga families, as proposed in Sakai (1987) and Haas and Kukalová-Peck (2001) systems. Although this sister relationship lacks compelling support, molecular trees based on mitochondrial COII sequences also indicated that Labiduridae, rather than Anisolabididae (suggested by Haas 1995), is close to the single-virga clade (Wirth et al. 1999). Therefore, the molecular data support the system of Sakai (1987) as the most likely hypothesis with respect to the ingroup relationships. In the Haas (1995) system, the absence of reniform vesicles is one of the characteristics causing the close affinity between Anisolabididae and Spongiphoridae. As mentioned above, this trait should be reexamined in the future.

Given the ingroup relationships, the estimated root positions varied markedly with outgroup taxa

Table 3. Comparison among 29 alternative rooting positions of 16S, 28S, and combined 16S + 28S trees by the max likelihood method: outgroup taxa, Glylloblattodea + Blattodea

Topology[a]	16S		28S		Combined	
	Li - Lmax[b]	*Pi*[c]	Li - Lmax	*Pi*	Li - Lmax	*Pi*
1	(−34.5 ± 10.0)	0.0000	−6.2 ± 5.9	0.0261	(−26.1 ± 8.3)	0.0002
2	(−32.1 ± 10.7)	0.0003	−7.8 ± 5.6	0.0000	(−25.7 ± 8.3)	0.0002
3	(−31.2 ± 9.5)	0.0000	−5.0 ± 5.4	0.0007	(−20.4 ± 7.4)	0.0002
4	(−30.2 ± 9.5)	0.0003	−5.0 ± 5.4	0.0016	(−20.0 ± 7.3)	0.0003
5	(−29.0 ± 9.3)	0.0000	−4.8 ± 5.4	0.0047	(−19.0 ± 7.1)	0.0005
6	(−29.0 ± 9.3)	0.0000	−4.6 ± 5.4	0.0440	(−19.0 ± 7.1)	0.0005
7	0 (ML)	0.5045	−3.4 ± 4.3	0.0142	0 (ML)	0.3399
8	−9.7 ± 8.8	0.0405	−5.6 ± 4.7	0.0079	−11.3 ± 7.4	0.0061
9	−11.2 ± 8.5	0.0108	−5.6 ± 4.7	0.0009	−11.8 ± 7.4	0.0012
10	−6.1 ± 7.2	0.0451	−2.9 ± 4.2	0.0107	−1.9 ± 5.6	0.0397
11	−6.1 ± 7.2	0.0077	−1.9 ± 4.3	0.1189	−1.6 ± 5.8	0.1380
12	−0.8 ± 5.1	0.3238	−2.9 ± 4.2	0.0050	−0.1 ± 4.2	0.1761
13	−3.7 ± 3.4	0.0084	−3.9 ± 3.8	0.0005	−3.1 ± 2.9	0.0060
14	−9.9 ± 6.7	0.0201	−3.9 ± 3.8	0.0043	−6.2 ± 4.5	0.0019
15	−11.0 ± 6.3	0.0010	−1.3 ± 2.1	0.0173	−4.0 ± 5.5	0.0332
16	−15.2 ± 8.3	0.0048	0 (ML)	0.2319	−5.7 ± 7.6	0.0694
17	−15.6 ± 8.0	0.0004	−1.0 ± 2.5	0.0026	−7.6 ± 7.3	0.0010
18	(−23.3 ± 8.9)	0.0000	−1.0 ± 2.5	0.0164	−9.6 ± 8.0	0.0002
19	−17.1 ± 9.3	0.0100	−1.5 ± 3.1	0.0603	−6.2 ± 8.7	0.1008
20	(−23.3 ± 8.9)	0.0000	−2.1 ± 2.8	0.0032	−10.7 ± 8.2	0.0000
21	(−32.4 ± 9.8)	0.0000	−3.1 ± 3.3	0.0071	(−17.9 ± 8.9)	0.0000
22	(−32.4 ± 9.8)	0.0000	−2.8 ± 3.5	0.0278	(−17.7 ± 9.0)	0.0003
23	(−20.9 ± 9.3)	0.0002	−0.1 ± 2.4	0.0976	−6.8 ± 8.2	0.0381
24	(−22.5 ± 9.9)	0.0001	−2.3 ± 4.9	0.0540	−12.6 ± 9.3	0.0078
25	−18.9 ± 10.8	0.0161	−3.1 ± 4.7	0.0049	−11.9 ± 9.6	0.0147
26	(−22.8 ± 11.6)	0.0046	−4.6 ± 5.4	0.0001	−15.7 ± 10.7	0.0076
27	(−22.9 ± 11.5)	0.0013	−2.7 ± 5.6	0.0099	−15.7 ± 10.7	0.0037
28	(−31.9 ± 11.6)	0.0000	−1.2 ± 5.6	0.2014	−17.1 ± 11.0	0.0116
29	(−33.1 ± 11.5)	0.0000	−2.6 ± 5.6	0.0260	−18.7 ± 11.1	0.0008

[a] Root positions shown in Fig. 3.

[b] Difference and the associated SE in log-likelihood between the *i*th topology and the ML topology. The values for rejected (i.e., difference + 1.96SE < 0) topologies are shown in parentheses.

[c] Bootstrap probability (recovering probability) of the *i*th topology estimated by the RELL method (10,000 replications).

(Glylloblattodea, Blattodea, or both) and the genes (16S, 28S, 16S + 28S) analyzed (Fig. 3). In the rooting analysis based on 28S data, Glylloblattodea and Blattodea pointed to a root in the branches to Forficulidae (single virga) and *Challia* sp. (Pygidicranidae, double-virga), respectively. When both of these outgroup taxa were included simultaneously, the root was in the external branch to *Proreus simulans* (Stål) (Chelisochidae, single virga). However, Table 3 shows that the 28S analysis cannot reject any of the 29 possible root positions, suggesting that the outgroup taxa possess no reliable information to determine the root. One possible reason for this is the extremely high G + C content of the outgroup, compared with earwigs (Table 2). Consistent with this view, *P. simulans* and *Challia* sp. possess the highest and the second highest G + C contents among the earwigs, although these species are far apart in the ingroup tree (Figs. 2 and 3). Thus, as in the cases reported in Lockhart et al. (1992) and Naylor and Brown (1998), rooting based on the 28S data may be incorrect and unreliable. In contrast to the 28S data set, no extensive bias in nucleotide composition was observed in the 16S data set (Table 2). The analysis of the 16S data rejected 16 out of the 27 possible root positions (Table 3), indicating that the data contain significant information for rooting. Again, the root position varied with the outgroup taxa used. However, all three cases (Glylloblattodea, Blattodea, or both) indicate that the root is in the double-virga earwigs, at the branch to Pygidicranoidea (Pygidicranidae + Diplatyidae) or to Anisolabididae (Fig. 3). These two possibilities were also supported by the analysis of the combined 16S + 28S data sets.

If the root of earwigs resides in branch 1–14 in Fig. 3, the parsimony principle suggests that the common ancestor had two virgae and that one of them was lost at branch 15. By contrast, if the root is in branch 16–29, the likely scenario is that ancestral earwigs had a single virga, and it was duplicated at branch 15. When the root is at branch 15, the ancestral state, single or double, cannot be deduced using the parsimony principle. Figure 4 shows the cumulative bootstrap probabilities for each of these three hypotheses. Whereas the 28S data were inconclusive for determining the root position, the rooting using the 16S or combined data seemed more reliable. Overall, the common ancestor of earwigs likely had two virgae, supporting the hypotheses on earwig phylogeny of previous researchers based on morphology.

Because diplatyids formed a basal, paraphyletic family in his phylogenetic analysis based on morphology, Haas (1995) and Haas and Kukalová-Peck (2001) proposed that the last common ancestor of all contemporary Forficulina had two virgae, each with two

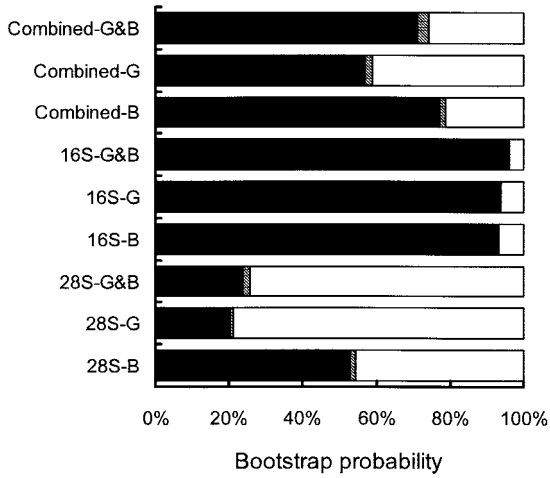

Fig. 4. Cumulative bootstrap probabilities estimated using the RELL method (10,000 replications) for each hypothesis on the evolution of earwig genitalia. Black and open columns represent the estimated possibilities that the common ancestor of earwigs had two or one virga(e), respectively. Striped columns represent indecisive cases. G, Grylloblattodea, and B, Blattodea, indicate outgroup taxa used.

gonopores, like present diplatyids. Although this hypothesis provides a fascinating way to illuminate the function of the twin virgae in ancestral earwigs (Kamimura 2004), the molecular analyses did not support this hypothesis, because the root is not likely in Diplatyidae (position 9 in Fig. 3 and Table 3).

In conclusion, the current study suggests two likely phylogenetic scenarios for Forficuline earwigs: (Pygidicranidae + Diplatyidae [Anisolabididae (Labiduridae, single-virga families)]) or (Anisolabididae [Pygidicranidae + Diplatyidae (Labiduridae, single-virga families)]). Either of these possibilities leads to the hypothesis that the common ancestor of earwigs had twin virgae with a single gonopore on each, which were as short as those in pygidicranids and labidurids, or as long as those of present-day anisolabidids.

Without incongruence between data sets, one strong point of the combined data strategy is that it enhanced the support for each node, increasing the resolution of the tree. In this study, there was reinforced support for Forficuloidea (the single-virga clade) and Pygidicranoidea (Diplatyidae + Pygidicranidae). Hillis (1987) discussed another advantage of combining multiple data sets for phylogenetic studies: some data are effective at resolving terminal relationships, whereas others may be better at elucidating basal relationships. This study is a good example of this result of combining data sets: that is, the 28S data set was superior for resolving ingroup relationships, and the 16S data set contained valuable information for rooting. By dividing the tree-building procedure into two steps, reconstruction of the ingoup tree(s) and rooting, this study illustrated this effect of combining multiple data sets.

With a few exceptions, researchers never know the true tree for their focal group. Therefore, every topology should be treated as a competing hypothesis subject to further verification. To predict the most likely topology, the rooting strategy based on the Kishino–Hasegawa test adopted in this study enables us to evaluate alternative hypotheses in probabilistic terms (but see Nei and Kumar 2000 for a criticism of likelihood ratio testing between different topologies).

I have proposed two essentially new hypotheses for earwig phylogeny as described above. Several other competing hypotheses were safely rejected using the Kishino–Hasegawa test. Nevertheless, outstanding difficulties remain for elucidating the family relationships of earwigs. The phylogeny within the Forficuloidea (single-virga families) is one example. In this group, Spongiphoridae contain many groups lacking established taxonomical status (Popham and Brindle 1967). The molecular data suggest that this family is possibly polyphyletic or paraphyletic (Fig. 1). Pygidicranoidea (Pygidicranidae and Diplatyidae) provide another example of an ambiguous relationship. The combined analysis suggested that the former family is paraphyletic and contains the latter. Because several taxonomists designate Diplatyidae as a subfamily (Diplatyinae) belonging to Pygidicranidae (Popham 1969, Steinmann 1986), the relationships suggested here may reflect genuine ones. Unfortunately, several earwig groups, both inside and outside Forficulina, were not included in this study. These groups include Karschiellinae (of Pygidicranidae or Karschiellidae), Apachyidae (or Apachyinae of Labiduridae), Hemimerina, and Arixeniina. The hypotheses presented here should be retested using larger data sets with additional taxa and molecules.

Acknowledgments

I thank Tadashi Suzuki, Tamotsu Kusano, Fumio Hayashi, Shin'ichi Katada, Hidetaka Ichiyanagi, and Yasuoki Takami for providing valuable advice during this study. Kina Hayashi, Fumio Hayashi, Masaaki Kimura, Hiroshi Yamaguchi, Gensaku Dobata, and Shin'ichi Katada gave me several of the samples used in the study. Seiroku Sakai and Karl M. Kjer provided valuable references and information on primers, respectively. Takayuki Nagashima kindly identified the glyllblattid. Fumio Hayashi and Akiko Takami helped me with the DNA sequencing. Thanks also go to Tadashi Suzuki, Fumio Hayashi, Rowan E. Hooper, and anonymous referees for comments on the earlier versions of the manuscript. This work was partly supported by a fellowship for young scientists (No. 02404) from the Japan Society for the Promotion of Science, and Open Research Center (Rissho University) Project subsidized by Ministry of Education, Culture, Sports, Science and Technology of Japan.

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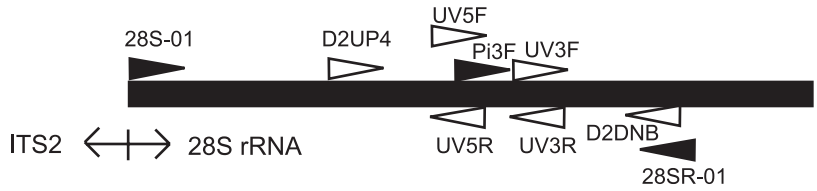
Received 15 September 2003; accepted 14 May 2004.



Appendix 1. Positions of the PCR primers used to amplify part of the 28S rRNA gene. The primers indicated with black arrows were used for Glylloblattodea and *Prolabisca infernalis*, whereas the other primers (open arrows) were used for earwigs other than *Prolabisca*.

Appendix 2. Sequences of the PCR primers used to amplify parts of the 28S and 16S rRNA genes

Gene	Primer name	Sequence (5′–>3′)
28S rRNA	Pi3F	ARC GTC GCG AYC CRT TGK
	28S-01	GAC TAC CCC CTG AAT TTA AGC AT
	28SR-01	GAC TCC TTG GTC CGT GTT TCA AG
	D2UP4	GAG TTC AAS AGT ACG TGA AAC YG
	UV5R	GKT WGA AAT GCG GTA AAC YA
	UV5F	TGG TTT ACC GCA TTT CWA CC
	UV3F	ACT GAT TAT TCG ATG GTA KC
	UV3R	GAT ACC ATC GAA TAA TCA GT
	D2DNB	CCT TGG TCC GTG TTT CAA GAC
16S rRNA	16F	TTA CGC TGT TAT CCC TAA
	16R	CGC CTG TTT ATC AAA AAC AT