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PARAPHYLY OF THE *ENOPLOGNATHA OVATA* GROUP (ARANEAE, THERIDIIDAE) BASED ON DNA SEQUENCES

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ABSTRACT: Five species of *Enoplognatha* Pavesi 1880 were recently recognized as a monophyletic *Enoplognatha ovata* group based on morphological data. We analyzed the *E. ovata* clade for monophyly using four species in the *E. ovata* group (*E. ovata* (Clerck 1757), *E. latimana* Hippa & Oksala 1982, *E. margarita* Yaginuma 1964 and *E. afrodite* Hippa & Oksala 1983) and three other closely related taxa (*E. japonica* Bösenberg & Strand 1906, *E. thoracica* (Hahn 1833), and *E. intrepida* Sørensen 1898). Two species of the presumed sister genus (*Steatoda* Sundevall 1833) were employed as outgroups. The results indicate that the ''*E. ovata* clade'' is not monophyletic.

The genus *Enoplognatha* Pavesi 1880 is characterized by the presence of a large colulus, a plesiomorphic character for the family; and accordingly, the genus is generally considered one of the more primitive groups in the Theridiidae. The spiders are medium-to-large sized with a subspherical abdomen. Females have a tooth on the posterior margin of the chelicerae; males usually have enlarged chelicerae, with enlarged teeth on the posterior margin, and have the paracymbium on the margin of the cymbium. The genus is very close to *Steatoda* Sundevall 1833, medium-to-large sized spiders, again characterized by a very large colulus (Levi 1962; Levy & Amitai 1981). The chelicerae are often enlarged in males, and have one or more teeth on the anterior margin, none on the posterior margin.

Enoplognatha is well known because of the striking color and pattern polymorphism exhibited by representative species in the genus, which has been most intensively studied in *E. ovata* (Clerck 1757). Three distinct morphs have been described in *E. ovata* (Locket & Millidge 1951; Hippa & Oksala 1979; Oxford 1976): *lineata* (all yellow), *redimita* (yellow with two dorsolateral carmine stripes on the abdomen), and *ovata* (yellow with a solid shield of carmine on the dorsal surface of the abdomen). The color pattern variation in *E. ovata* is genetically determined, and has been the subject of numerous studies on the genetics and evolution of the color polymorphism (Hippa & Oksala 1979, 1981; Oxford 1983, 1985, 1989, 1991, 1992; Oxford & Reillo 1993; Reillo & Wise 1988a, b). Consistent with most invertebrate color polymorphisms (Haldane 1939) the dominance hierarchy of the expression of morphs in *E. ovata* follows the inverse of morph frequencies in nature, i.e., the least dominant (or most recessive) allele is most frequent; the most dominant is the rarest. For the mode of inheritance of the polymorphism in *E. ovata*, Oxford (1983) has proposed a two locus model: one locus is concerned with pattern and color, the other with the regulation of this color locus during development. When red-pigmented alleles are linked to the late developing allele, the color morphs are sex-limited: males are *lineata* no matter which allele they carry. *Enoplognatha latimana* Hippa & Oksala 1982 shares color, regulatory, and black spotting polymorphisms with *E. ovata* (Oxford 1992), although *E. latimana* lacks the *ovata* color morph.

In the 1980s Hippa & Oksala (1982) erected the *E. ovata* group to include *E. ovata* sensu stricto, *E. latimana*, and *E. penelope* Hippa & Oksala 1982. Members of the group share the following characters: trichobothrium on the first metatarsus subapical; elongated, sclerotized and subtubular tip of conductor in male palp; female vulva with massive copulatory pockets and abdomen with sharply delimited dorsolateral black spots (Hippa & Oksala 1982). Further examination of material

from Europe and Japan added another two species to the *E. ovata* group (Hippa & Oksala 1983): *E. afrodite* Hippa & Oksala 1983 and *E. margarita* Yaginuma 1964. *E. margarita* shares the subapical trichobothria and sclerotized subtubular tip of the conductor with *E. ovata*, *E. latimana* and *E. penelope*. However, it lacks the massive copulatory pockets. Considering all of these characters as synapomorphies, Hippa & Oksala (1983) hypothesized that *E. margarita* was the closest sister to (*E. ovata* + *E. latimana* + *E. penelope*). *Enoplognatha afrodite* has a similar body shape, ground color and spotting pattern to (*E. ovata* + *E. latimana* + *E. margarita*) but lacks these synapomorphies. Accordingly, Hippa & Oksala considered *E. afrodite* as the most ancestral species in the group.

More recently, Oxford & Reillo (1994) questioned the phylogeny of the *E. ovata* group proposed by Hippa & Oksala. Their concern arose because *E. ovata*, *E. latimana*, *E. penelope* and *E. afrodite* all have European distributions (although the former two have been introduced into North America). All occur in the Mediterranean region; but only *E. latimana* and *E. ovata* occur further north, with *E. ovata* alone extending well into northern Europe. Based on this distributional information, Oxford & Reillo hypothesized a possible Mediterranean origin of the *E. ovata* group, suggesting that the Asian *E. margarita* may have been phylogenetically misplaced by Hippa & Oksala. Indeed, the phylogeny presented by Hippa & Oksala was open to criticism because of the lack of a suitable outgroup for character polarization, few (only nine) characters used, and because there was no quantitative assessment of phylogeny.

In the current study we examined four species in the *E. ovata* group, and three other species of *Enoplognatha*: *E. japonica* Bösenberg & Strand 1906 from Japan, *E. thoracica* (Hahn 1833) from England, and *E. intrepida* Sørensen 1898 from North America. As outgroups in the analysis we used two species of *Steatoda*: *S. grossa* (C.L. Koch 1838) and *S. bipunctata* (Linnaeus 1758). We examined the pattern of sequence evolution in the *E. ovata* group to ascertain the monophyly of the clade. In this way we can evaluate the hypothesis that the Mediterranean served as the center of origin for the group as suggested by Oxford & Reillo (1994).

METHODS

Spiders sequenced.—*Enoplognatha*: *E. ovata*, two individuals from two localities: Grimes Graves, Norfolk, U.K., collected by G.S. Oxford, June 1991; and Berceto, Italy, collected by G.S. Oxford & P.R. Reillo, August 1991. *E. latimana*, one individual: Grimes Graves, Norfolk, U.K., collected by G.S. Oxford, June 1991. *E. afrodite*, one individual: near Carcassonne, S. France, collected by S. Peet, July 1988. *E. margarita*, one individual: Nukabira, Kamishihoro-cho, Hokkaido, Japan, collected by M. Matsuda, August 1992. Other *Enoplognatha* species examined: *E. japonica*, one individual: Hokkaido, Japan, collected by M. Matsuda, July 1989; *E. thoracica*, one individual: Flatford Mill, Suffolk, U.K., collected by C.J. Smith, May 1978; *E. intrepida*, one individual: Third Hill Mountain, Berkeley County, West Virginia, USA, collected by P.J. Martinat, May 1986 (det. D.T. Jennings, deposited in Smithsonian, Museum of Natural History). We also extracted DNA from *E. penelope*, one individual: Sami, Kefallinia, Greece, collected by J. Murphy, May 1987. However, we were not successful in amplifying the product. Outgroups: We used two species of *Steatoda* as the outgroup: *Steatoda grossa*: Molokai, Hawaii, collected by A.-M. Tan & G.S. Oxford October 1993 and *S. bipunctata*: Yorkshire, U.K., collected by G.S. Oxford, January 1994. Voucher specimens for all species used are at the Center for Conservation Research and Training, University of Hawaii.

DNA extraction and sequencing.—DNA samples were prepared by the conventional SDS-NaCl-Ethanol method (Medrano et al. 1990; Tan & Orrego 1992). Tissues from the legs or prosoma were placed in a 1.5 ml tube and ground with a pipette tip. After adding 15 μl of proteinase K, the tissues were incubated at 55 °C overnight. Proteins were removed by salt precipitation. DNA was precipitated, washed in alcohol and preserved in 1× TE buffer (pH 8.0).

For both double and single stranded PCR amplification we used the following primers (Table 1): E and B2 for the less variable region of the 18S sequence; B and P for the more variable region of the 18S sequence; A and B2 for the 16S sequence. PCR amplification of double-stranded products was per-

Table 1.—Primers used. Position obtained refers to *Drosophila* (Clary & Wolstenholme 1985).

Gene primer	Primer sequence in *Drosophila*	Position obtained	# Base pairs	Reference
18S E	CTGGTTGATCCTGCCAGTAG	24–553	529	modified from
18S B2	GCTGGCACCAGACTTGCCCTCC			Hillis & Dixon 1991
18S B	TTCCAGCTCCAATAGCGTAT	606–916	325	W.C. Wheeler & C. Hayashi,
18S P	GTCTTGCGACGGTCCAAGA			pers. comm.
16S A	CGCCTGTTTATCAAAAACAT	12864–13417	450	S.R. Palumbi & T. Hsiao,
16S B2	CTCCGGTTTGAACTCAGATCA			pers. comm.

formed in 12.5 μl volume with 38 cycles using *Thermus aquaticus* DNA polymerase (Saiki et al. 1985). Amplification was done with the following profile: 93 °C, 50 °C and 72 °C each for 30 seconds. Single strand products were prepared by asymmetric PCR (Gyllensten & Erlich 1988) with 1:50 primer ratios in 50 μl volumes and the same reaction profiles as above. The products were assessed by mini-gel electrophoresis using 5 μl aliquots, and washed in sterilized distilled water with three cycles of dialysis using Millipore MC 30 (Amicon Corp.). Dideoxy chain termination sequencing (Sanger et al. 1977) was performed using the US Biochemicals Sequenase version 2.0 kit and ^{35}S labeled dATP. Negative controls were used in all PCR amplifications to make sure the sequences were not from contaminated sources. Sequences were confirmed by resequencing the same strand from another PCR product.

Phylogenetic analysis.—Ribosomal sequences were initially aligned using the program SeqEd 1.0.3 (Applied Biosystems 1995), after which alignment of multiple sequences was optimized in CLUSTAL W 1.4 (Higgins & Sharp 1988) in SeqPup 0.6 (Gilbert 1996). The entire first sequence is optimally aligned with the second entire sequence, with mismatches, gaps and insertions penalized equally, and with an additional gap length penalty for each residue in the insertion. Subsequent detailed alignment was by eye using the secondary structures (Kjer et al. 1994). The 18S sequences were aligned against the secondary structure of *Eurypelma californica* to match multiple sequences against conserved regions (Hendriks et al. 1988). The 16S sequences were aligned against *Drosophila yakuba* (Clary & Wolstenholme 1985), using the secondary structure of the region. Sequences were first analyzed using Maximum Likelihood (ML) in PHYLIP (version 3.5c, Felsenstein 1993), using a generalized Jukes & Cantor (1969) model to allow for unequal base frequencies (Felsenstein 1981) as well as different rates of transitions and transversions. Sequences were also analyzed by Maximum Parsimony (MP) in PAUP (version 3.1.1, Swofford 1993). In both analyses gaps were treated as missing data. Bootstrap analyses (Felsenstein 1985) were used to estimate the statistical confidence of the different nodes in the trees.

RESULTS

The aligned sequences of the 18S region (Fig. 1) and 16S region (Fig. 2) are shown for each species (the two specimens of *E. ovata* were identical in sequence). Except for *E. thoracica* (18S only) and *E. intrepida* (16S only) we obtained 18S and 16S sequence for all species used. The data were first analyzed separately to determine the degree of congruence. The 18S sequences showed little bias in base composition, and no evidence for a transition: transversion (TS:TV) bias. The ML tree (using a TS:TV ratio of 1:1) was similar to the MP tree (using a branch-and-bound search) (Fig. 3A): (*E. thoracica* + *E. margarita*) and (*E. latimana* + *E. ovata*) were both discrete clades, and *E. japonica* fell outside all other species of *Enoplognatha*. The only difference between the analyses was that *E. afrodite* was placed with (*E. thoracica* + *E. margarita*) in the ML tree, while its position relative to (*E. thoracica* + *E. margarita*) and (*E. latimana* + *E. ovata*) was unresolved in the MP tree. Constraining *E. ovata*, *E. latimana*, *E. margarita* and *E. afrodite* to be monophyletic increased the length of the MP tree by two steps. We tested the monophyly of *E. ovata*, *E. latimana*, *E. margarita* and *E. afrodite* by calculating likelihood values (Fel-

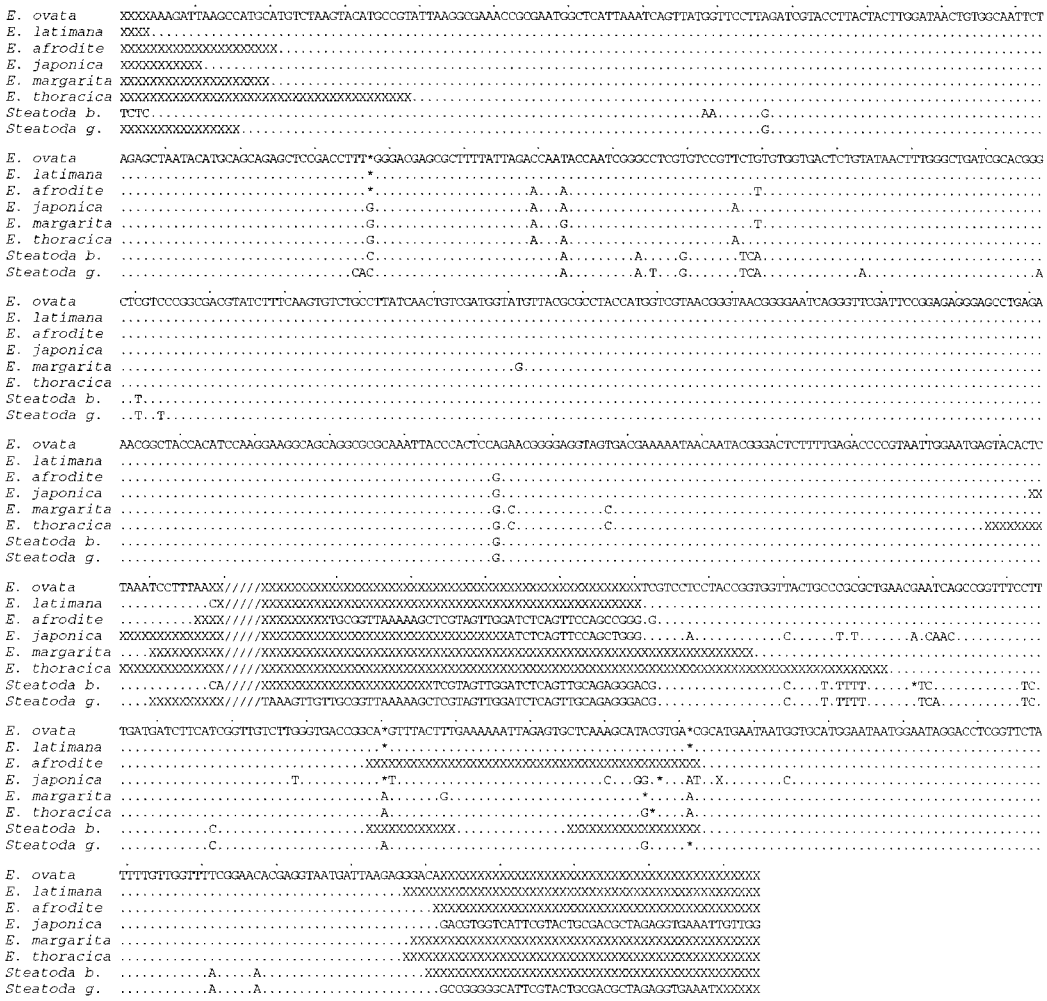

Figure 1.—Comparison of nuclear 18S ribosomal DNA sequences from 6 species of *Enoplognatha* and *Steatoda bipunctata* and *S. grossa*. Dots represent positions that are identical in sequence to the top sequence; asterisks represent gaps in the sequence required to maximize alignment; crosses indicate no data for a region. The sequence begins at position 24 in *Drosophila* and ends at position 916. The area marked by ///// indicates the end of the more conserved region of the 18S sequence (position 553 in *Drosophila*) and the beginning of the more variable region (position 606 in *Drosophila*).

senstein 1988) for phylogenies that forced these taxa to be monophyletic: PHYLIP was used to perform a statistical test of each of these trees against the one with highest likelihood. This test uses the mean and variance of log-likelihood differences between trees, taken across sites (Kishino & Hasegawa 1989); trees are considered significantly different if their means differ by more than 1.96 standard deviations. The log likelihood value for the best tree was −1574.9, and was not significantly higher than the value obtained when *E. ovata*, *E. latimana*, *E. margarita* and *E. afrodite* were constrained to be monophyletic (log likelihood −1577.8).

The 16S sequences show a heavy AT bias, and accordingly most of the changes were A<–>T transversions. The ML analysis was based on a model which uses the empirical frequencies of the bases observed in the input sequences, and thus accommodates biases in AT richness. Using TS:TV ratios of 1:1 and 2:1 we obtained a tree which was similar to that from MP analysis (Fig. 3B): (*E. latimana*

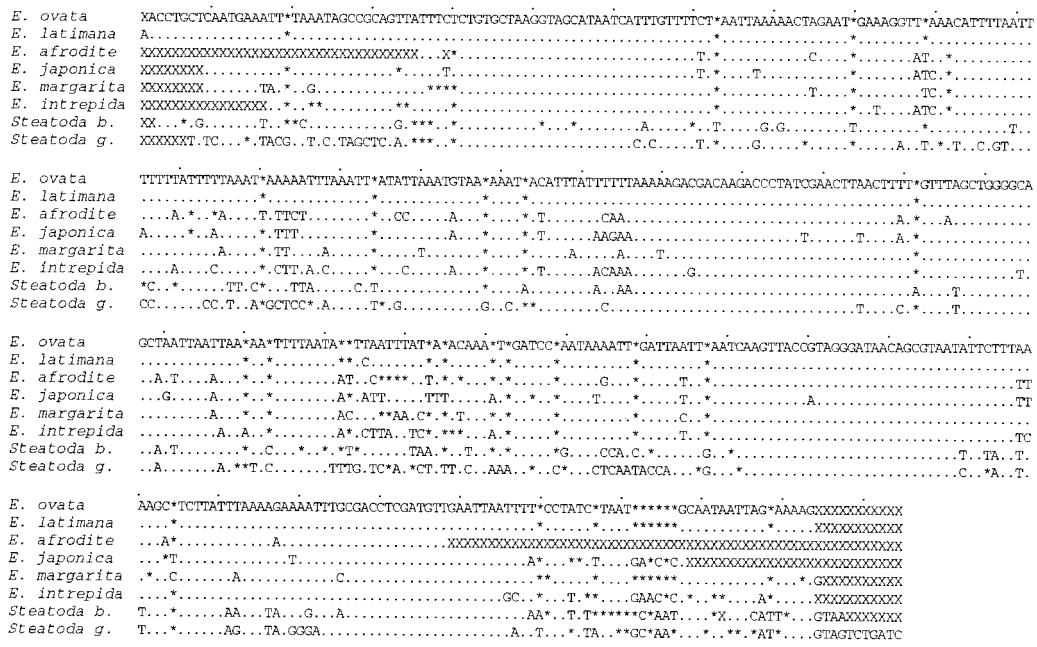

Figure 2.—Comparison of mitochondrial 16S ribosomal DNA sequences from six species of *Enoplognatha*, and *Steatoda bipunctata* and *S. grossa*. Terminology as in Figure 1.

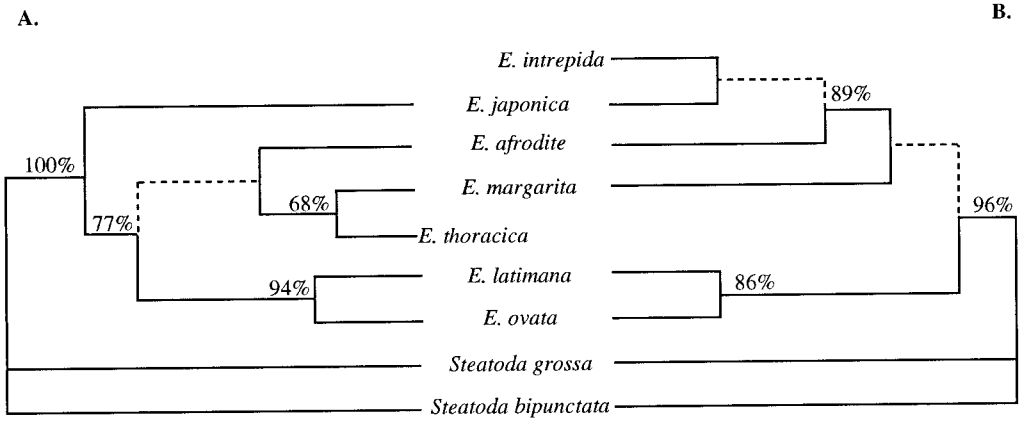

Figure 3.—Phylogeny of representatives of the genus *Enoplognatha* based on Maximum Likelihood using: (A.) 18S sequences. All branches are significant based on the approximation of the Likelihood Ratio Test (LRT, indicated by ∗ in PHYLIP, Felsenstein 1993). Parsimony analysis gave three trees with similar topologies, but with less resolution in the consensus: branches that were not supported by bootstrap values $>$ 50% are indicated as dashed lines; for branches that were supported, bootstrap values are given above nodes. Tree length 65, CI 0.923. (B.) 16S sequences. All branches are significant (approximate LRT, Felsenstein 1993). Parsimony analysis gave a single tree with similar topology (see text). Tree length 230, CI 0.798. The off-center positions of *E. thoracica* and *E. intrepida* indicate only 18S and only 16S sequence data obtained respectively for these two species.

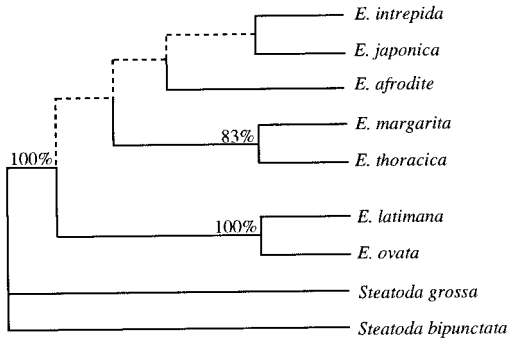

Figure 4.—Phylogeny of representatives of the genus *Enoplognatha* based on Maximum Likelihood using the combined data set of 16S and 18S sequences. All branches are significant (approximate LRT, Felsenstein 1993). Parsimony analysis gave a similar topology but with less resolution: branches that were not supported by Maximum Parsimony are indicated as dashed lines; for branches that were supported, bootstrap values are given above nodes.

+ *E. ovata*) and (*E. japonica*, *E. intrepida* and *E. afrodite*) formed discrete clades. The primary difference between the analyses was that *E. margarita* was placed with (*E. japonica*, *E. intrepida* and *E. afrodite*) on the ML tree, but with (*E. latimana* + *E. ovata*) on the MP tree. Constraining *E. ovata*, *E. latimana*, *E. margarita* and *E. afrodite* to be monophyletic increased the length of the MP tree by six steps and resulted in a significantly lower log likelihood value for the ML tree (−1491.8 for the best tree, −1518.1 for the constrained tree).

Because the results from the two data sets were largely in agreement the data sets were combined and analyzed together. The resulting ML tree differed from the MP tree only in the degree of resolution it provided (Fig. 4). In all analyses *E. ovata* fell with *E. latimana*, *E. intrepida* with *E. japonica* (and in most cases with *E. afrodite*), *E. margarita* with *E. thoracica*. The *E. ovata* + *E. latimana* clade fell outside all others. We concluded that *E. ovata*, *E. latimana*, *E. margarita* and *E. afrodite* are not monophyletic, and again tested the robustness of these conclusions. Constraining *E. ovata*, *E. latimana*, *E. margarita* and *E. afrodite* to be monophyletic increased the length of the MP tree by three steps and gave a significantly lower log likelihood value for the ML tree (−3244.9 for the best tree, −3290.5 for the constrained tree).

DISCUSSION

The species *E. latimana*, *E. penelope*, *E. afrodite*, and *E. margarita* are similar in gross morphology to the well-studied *E. ovata*, and this similarity appears to be the basis for grouping these species into what has been considered to be a monophyletic clade (Hippa & Oksala 1983). The phylogenetic analysis presented here based on both the 16S and 18S sequences does not support monophyly of the ''*E. ovata* group'' as described by Hippa & Oksala (1983).

The *E. latimana* + *E. ovata* clade is strongly supported, and is consistent with evidence from color polymorphism: *E. ovata* and *E. latimana* share color, regulatory, and black spotting polymorphisms (Oxford 1992), although the latter species lacks the *ovata* color morph. These genetic traits suggest a recent common ancestor for this species pair. Color polymorphism has never been reported in any other species in the ''*E. ovata* group''. However, the 18S and 16S data sets individually and combined consistently place *E. afrodite* and *E. margarita* outside the *E. latimana* + *E. ovata* clade, more closely associated with *E. japonica* and *E. intrepida*, and *E. thoracica* respectively. We have no molecular sequence data from *E. penelope*, and therefore cannot evaluate its position relative to others in the ''*E. ovata* group''.

The results do not refute the Mediterranean center of origin hypothesis of Oxford & Reillo (1994), although the lack of monophyly of the group indicated by the current results demands a considerably larger representation from the genus be surveyed before their origin can be identified with any degree of certainty.

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