Molecular Phylogeny of Vespidae (Hymenoptera) and the Evolution of Sociality in Wasps

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The oriental Stenogastrinae is a group in which there is considerable interest as regards the study of evolution of sociality in wasps, because they show broad diversity in social behavior. Using cladistic analysis on morphological and behavioral data, they have been grouped together with the social Vespinae and Polistinae in the family of Vespidae. This is not without dispute, because several other morphological and behavioral characters separate Stenogastrinae from the other Vespidae subfamilies. DNA sequences were obtained from nuclear 28S ribosomal DNA and the mitochondrial 16S ribosomal DNA of two Apis species and nine social and three solitary wasp species of the family Vespidae. Solitary wasps of the family Braconidae and Pteromalidae were used as outgroups. Parsimony, distance, and maximum-likelihood methods of both mitochondrial and nuclear DNA did not support the conventional phylogenetic position of Stenogastrinae. In all phylogenetic reconstructions, the solitary Eumeninae were a sister taxon to the Polistinae cluster. The analyzed sequences provide strong evidence that sociality has independently evolved twice in the Vespidae.

INTRODUCTION

Wasps of the family Vespidae are typically characterized by the extraordinary ability to longitudinally fold the forewings, which is why they have been coined "Faltenwespen" in German literature. Latreille (1817) proposed the name Diploptera for this characteristic. The Stenogastrinae (hover wasps) and the majority of Masarinae are exceptions to this rule (Wilson, 1971; Spradbery, 1973). Another remarkable aspect of the Vespidae is the abundance of social species, which are found in three subfamilies: the Stenogastrinae, the Polistinae, and the Vespinae. The two subfamilies Eumeninae and the Masarinae comprise only solitary species. West-Eberhard (1978) modeled a scenario for the evolution of sociality in wasps in which the Stenogastrinae represent a hypothetical phylogenetic link and an intermediate stage in behavior between the solitary Eumeninae and the social Polistinae and Vespinae. This view was in contrast to morphological data available at that time. Richards (1971) suggested that the Stenogastrinae are very different from other social wasps, due to a long pointed clypeus, long narrow mandibles lying alongside it, and abnormally placed first thoracic spiracles. He proposed a Eumenes-like solitary ancestor. Spradbery (1975) also argued that the Stenogastrinae originated from an early vespid ancestor. Van der Vecht (1977) concluded that the Stenogastrinae are more closely related to the Eumeninae than to the other social subfamilies, based on the analysis of eight morphological characters. However, West-Eberhard's concept gained strong support in more rigorous cladistic studies by Carpenter (1988, 1991). Carpenter (1988) criticized Van der Vecht (1977) for the missing polarization of the characters in derived and primitive forms. He reanalyzed the data using polarization and found that most of the characters used are in fact autapomorphies or convergences and, therefore, not informative for phylogeny. Carpenter (1988) concluded that there is no evidence indicating that Stenogastrinae and other eusocial wasps are not closely phylogenetically related.

He based his grouping of the Stenogastrinae as the sister group of Polistinae and Vespinae on three synapomorphies: (a) the forewing marginal cell is pointed onto the costa; (b) the larval labrum is not narrowed where it joins the clypeus, and it is narrower than the maximum width of the clypeus; and (c) the behavior of simultaneous progressive provisioning. He acknowledges that these characters may be prone to homoplasy, but lists some more behavioral features to consolidate the grouping (Carpenter, 1981).

The subfamily of the Stenogastrinae wasps is indeed intriguing. In contrast to the other subfamilies of the Vespidae, it comprises species with a high diversity in social behavior, ranging from subsocial to eusocial species. Therefore, they seem to be an ideal group to
Species of the genus Eustenogaster build only small nests with few individuals. Krombein (1976) observed in Eustenogaster eximia that the first female assists her mother by foraging and brood care. Hansell (1987) found elements of eusociality in Eustenogaster fraterma. Sociality is more conspicuous in the genera Parischnogaster and Liostenogaster. Parischnogaster melyi shows division of labor, with old and young females remaining on the nest, and middle-age females constituting the forager force (Turillazzi, 1991). Hansell et al. (1982) described complex dominance hierarchies and the cofounding of nests by multiple females of Liostenogaster flavolineata, similar to that observed in Polistes.

Although it is obvious that the Stenogastrinae display a wide range of sociality, it is less clear whether they can form a potential phylogenetic link between solitary and social wasps. In particular, the use of social behavior in cladistic studies may be difficult, since it is well known that sociality evolved independently at least eight times among bees (Wilson, 1971; Cameron, 1993) and the Vespinae (West-Eberhard, 1978). There seems to be a reasonable risk that one is trapped by a homoplasy if social behavior is used as aphylogenetic character in cladistic analysis of distantly related groups. Furthermore, behavioral traits may show great plasticity, rendering them less informative for phylogenetic studies.

In this molecular systematic study we reconstruct the Vespidae phylogeny on the basis of ribosomal DNA sequences from both the nuclear and the mitochondrial genomes. Ribosomal DNA (rDNA) is a classical tool in molecular phylogenetic reconstruction (Hillis and Dixon, 1991). The rDNA is ubiquitous and includes a mosaic of regions evolving at extremely different evolutionary rates (Solignac et al., 1991), providing regions of rDNA for almost any systematic question. The D2 domain in the nuclear 28S rDNA (Schmitz and Moritz, 1994) and part of the 16S rDNA of the mitochondrial genome. Amplification was done after designing primers correspond to nucleotide positions 427 and 736 of the 16S rDNA sequence (Schmitz et al., 1988). The mitochondrial ribosomal DNA (mt-rDNA) from the outgroup solitary wasp Cotesia glomerata are from the EMBL Data Library (Heidelberg, Accession No. U06958). The sequence of the 28S nuclear DNA (nu-rDNA) of Nasonia vitripennis can be retrieved by Accession No. U02952.

MATERIALS AND METHODS

Samples

The Vespinae Vespa crabro and Vespula germanica were collected in Germany, Provespa nocturna in Malaysia, the Polistinae Polistes dominulus in Greece, Polistes sagittarius in Malaysia, and Belonogaster petiolata in South Africa. The Eumeninae species (Ancistrocerus oviventris, Ancistrocerus nigricornis, Eumenes coarctatus) were collected in Germany and one Eumenes spp. was collected in Malaysia. The three Stenogastrinae (P. melyi, Liostenogaster vechti, E. fraterma) were collected in Malaysia. The Apinae species Apis mellifera was collected in Germany and Apis dorsata in Malaysia. Sequence data of the 16S mitochondrial DNA (mt-rDNA) from the outgroup solitary wasp Cotesia glomerata are from the EMBL Data Library (Heidelberg, Accession No. U06958). The sequence of the 28S nuclear DNA (nu-rDNA) of Nasonia vitripennis can be retrieved by Accession No. U02952.

DNA Extraction

Muscular larval tissue was homogenized in 300 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and treated with SDS (1% in the final concentration) and proteinaseK (50 µg/ml) for 2 h at 50°C. Proteins and cell debris were extracted with a buffer-saturated phenol/chloroform/isooamylalcohol mix (25/24/1) and precipitated in ethanol (Sambrook et al., 1989).

Gene Amplification with Polymerase Chain Reaction (PCR)

We chose the D2 part of the 28S rDNA (Schmitz and Moritz, 1994) and part of the 16S rDNA of the mitochondrial genome. Amplification was done after designing the following primer pairs: for nuclear ribosomal DNA, 5'-AAAGATCGAATGGGAGATTC-3' and 5'-CACC-GGGTCCGTACCTCC-3', and for mitochondrial ribosomal DNA 5'-TTGACTGTACAAAGGTAGC-3' and 5'-GATATACGCTGTATCCC-3'. The nuclear ribosomal DNA primers correspond to nucleotide positions 427 and 736 of the V. crabro 28S rDNA sequence (Schmitz and Moritz, 1994). The mitochondrial ribosomal DNA primers correspond to nucleotide positions 803 and 1221 of the honeybee 16S rDNA sequence (Crozier and Crozier, 1993).

FIG. 1. Sequence alignments of the nuclear rDNA fragment (a) and the mitochondrial rDNA fragment (b). Dots indicate identity to the bases presented in the first taxon (Vespa crabro). Dashes indicate gaps. Frames indicate the excluded highly variable regions of the mitochondrial rDNA. The lines above the sequences display the analyzed single-stranded and double-stranded regions. This structural elements were selected, when recorded, in all investigated species.
Amplifications were performed with the following parameters: initial step at 94°C (3 min), continued for 30 cycles of 94°C (30 s) and 55°C (30 s for nu-rDNA annealing) or 50°C (30 s for mt-rDNA annealing) and 72°C (30 s). An elongation of PCR products by 72°C for 3 min completed the reaction. We used about 10 ng template DNA, 400 nM primer, 1.25 mM dNTPs, 1.5 mM MgCl₂, and 2.5 U Taq polymerase in a total reaction volume of 50 µl.

Cloning

The PCR product was purified by gel elution and with a GeneClean II Kit (Bio 101, Inc.), inserted in the pUC19–Smal site, and cloned in Escherichia coli DH5α.

Gene Sequencing

Sequencing was performed by the Sanger dideoxy-chain termination technique (Sanger et al., 1977) with the Sequenase 2.0 sequencing kit (United States Bio-chemical Corp.). The α-35S-dATP-labeled sequence reactions were electrophoresed in 7% acrylamide, 7 M urea gels and visualized by autoradiography. At least two individuals per species from different populations were sequenced to detect potential intraspecific variation.

Data Analysis

Sequence data were aligned by using the CLUSTAL V program (Higgins and Sharp, 1989) and improved by comparison of the secondary structure of the rRNAs (Schmitz and Moritz, 1994) (Fig. 1). The secondary structures were fitted into available secondary structure models from HsuChen et al. (1984), Huber et al. (1993), and Schmitz and Moritz (1994). The resulting structures were verified by the PC/Gene program RNAFOLD (Freier et al., 1982) and examined for compensatory mutations (for an example see Fig. 2).

Hillis (1991) and Hillis and Huelsenbeck (1992) suggest that tree-length distribution can provide an accurate and sensitive indication of the presence of phylogenetic signal in comparative sequence data. To detect the presence of phylogenetic signal in our data, we used the measure of skewness of tree-length distribution (g₁ statistic), which is available in the PAUP 3.1.1 package (Swofford, 1993), to find the presence of phylogenetic signal in comparative sequence data. To find an optimal alignment of DNA sequences, the CLUSTAL V program yields reliable results if the distances are small between the compared species. Sequence similarities of less than 70% often produce ambiguous alignments (Hillis and Dixon, 1991). Therefore, we improved the CLUSTAL V alignments by comparison of the secondary folding of the rRNAs.

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As an example, the secondary structure of the nuclear 28S rRNA fragment of P. nocturna is shown in Fig. 2 (top). It is very similar to the secondary folding of V. crabo 28S rRNA (Schmitz and Moritz, 1994) and consists of 173 characters (92 variable) in double-stranded regions and 116 characters (60 variable) in single-stranded positions. At six nucleotide positions, compensatory mutations were found reestablishing the pairing potential.

Figure 2 (bottom) shows the mitochondrial 16S rRNA secondary structure of P. nocturna. We distinguish double-stranded positions with 187 nucleotides (133 variable) and single-stranded positions with 111 nucleotides (66 variable). We found 16 nucleotide positions with compensatory substitutions (excluding highly variable regions mentioned below).

Kraus et al. (1992) argued that sequences evolve more rapidly in single-stranded loops than in double-stranded stems. Taking variable characters as a param-
er for sequence evolution, we fail to find a significantly biased variability between single- or double-stranded regions ($\chi^2 = 2.97; P > 0.05$; Table 2). Perhaps functional constraints of the double-stranded stems favor compensatory mutations, which counteracts a potentially reduced frequency of nucleotide substitutions in double-stranded regions (Hillis and Dixon, 1991; Wheeler and Honeycutt, 1988).

Three highly variable regions (up to 60% divergence) are present in the mitochondrial 16S mt-rDNA fragment. One is the hairpin at nucleotide positions 152–195 (Fig. 1, right). This region is characterized by some deletions and insertions, and the derived secondary structure is very different among the various species. This is also true for nucleotide positions 239–247 and 268–276. These three regions gave poor alignments and were discarded from the phylogenetic analysis as proposed by Swoford and Olsen (1990).

**Phylogenetic Tree of 28S rDNA**

The tree-length distribution for all 15 species is significantly more skewed than the distribution from random data sets ($g_1 = -0.921; P < 0.01$). This suggests a strong phylogenetic signal in the analyzed 28S rDNA sequences.

Based on the alignment shown in Fig. 1 (left), Fig. 3 (top) shows the most parsimonious tree obtained from the nu-rDNA. The tree length is 256, including 125 informative positions (Table 2). The Vespiidae cluster with a bootstrap value of 68%. The data set is therefore not suitable for resolving the phylogeny inside the Vespiidae. Separation of the Vespiinae and Polistinae is supported by a bootstrap value of 91%. The monophyly of Polistinae is supported by 100% bootstrap confidence. The separation of Vespiinae + Polistinae and Eumeninae from a common ancestor is sustained by 99% bootstrap replicates based on 30 informative positions. The honeybees are the sister group to Eumeninae + (Vespiinae + Polistinae) with only 60% bootstrap and 15 informative positions. The Stenogastrinae are shown as the sister group to this cluster.

**Phylogenetic Tree of 16S rDNA**

The tree-length distribution for the 16S rDNA is significantly more skewed than the distribution from random data sets ($g_1 = -0.809; P < 0.01$). This suggests a strong phylogenetic signal.

Based on the alignment shown in Fig. 1 (right) and discarding the three highly variable positions mentioned above, Fig. 3 (center) shows the most parsimonious phylogenetic tree of the selected 252-character mt-rDNA fragment (Fig. 1, right) including 113 informative positions. The Vespiidae species cluster with a bootstrap value of 55%. The Polistinae form the sister group to the Vespiidae. The common ancestor is supported by 81% bootstrap replicates. The sister group to the Vespiinae + Polistinae is the Eumeninae wasps with a 64% bootstrap confidence value (7 informative positions). The honeybees form the sister group to Eumeninae + (Polistinae + Vespiinae) with a bootstrap value of 100% based on 21 informative positions. The Stenogastrinae is the sister group to this cluster.

**Phylogenetic Tree of the Combined Data Set**

The strong phylogenetic signal in the combined data set is shown by the $g_1$ statistic ($g_1 = -0.897; P < 0.01$). In Fig. 3 (bottom), we present the PAUP parsimony tree produced by analysis of both data sets together. The

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**TABLE 1**

| Base Composition | 28S rDNA | 16S rDNA |
|------------------|----------|----------|
|                  | Length (A (%) | T (%) | C (%) | G (%) | Length (A) | T (%) | C (%) | G (%) |
| Vespa crabo      | 288       | 39/13.5 | 63/21.9 | 93/32.3 | 93/32.3 | 298       | 130/43.6 | 125/42.0 | 14/4.7 | 29/9.9 |
| Provespa nocturna| 289       | 39/13.5 | 64/22.2 | 92/31.8 | 94/32.5 | 298       | 131/44.0 | 124/41.6 | 14/4.7 | 29/9.9 |
| Vespula germanica| 289       | 43/14.9 | 62/21.5 | 93/32.2 | 91/31.4 | 297       | 128/43.1 | 123/41.4 | 14/4.7 | 32/10.8 |
| Polistes dominulus| 292      | 39/13.4 | 74/25.3 | 88/30.1 | 91/31.2 | 307       | 137/44.6 | 125/40.7 | 14/4.6 | 31/10.1 |
| Polistes sagittarius| 292      | 40/13.7 | 71/24.3 | 89/30.5 | 92/31.5 | 298       | 130/43.6 | 126/42.3 | 15/5.0 | 27/9.1 |
| Belonogaster patiodelata| 295 | 39/13.2 | 70/23.8 | 93/31.5 | 93/31.5 | 298       | 137/46.0 | 124/41.6 | 13/4.4 | 24/8.0 |
| Eumenes coarctatus| 288      | 45/15.6 | 66/22.9 | 86/29.9 | 91/31.6 | 302       | 138/45.7 | 120/39.7 | 14/4.7 | 30/9.9 |
| Ancistrocerus oviventeris| 286 | 42/14.7 | 57/19.9 | 92/32.2 | 95/33.2 | 300       | 132/44.0 | 124/41.3 | 12/4.0 | 32/10.7 |
| Ancistrocerus nigricornis| 286    | 43/15.1 | 57/19.9 | 93/32.5 | 93/32.5 | 300       | 129/43.0 | 124/41.4 | 13/4.3 | 34/11.3 |
| Apis melifera      | 320       | 45/14.1 | 70/21.9 | 107/33.4 | 98/30.6 | 299       | 128/42.8 | 128/42.8 | 12/4.0 | 31/10.4 |
| Apis dorsata       | 320       | 45/14.1 | 70/21.9 | 107/33.4 | 98/30.6 | 299       | 125/41.8 | 128/42.8 | 13/4.4 | 33/11.0 |
| Parischnogaster melyi| 299     | 50/16.7 | 72/24.1 | 82/27.4 | 95/31.8 | 285       | 133/46.7 | 113/39.6 | 12/4.2 | 27/9.5 |
| Liostenogaster vechti| 295     | 49/16.6 | 63/21.4 | 90/30.5 | 93/31.5 | 282       | 136/48.2 | 108/38.3 | 11/3.9 | 27/9.6 |
| Eustenogaster frater|na | 295    | 46/15.6 | 66/22.4 | 91/30.8 | 92/31.2 | 286       | 129/45.1 | 120/42.0 | 11/3.8 | 26/9.1 |
| Nasonia vitripennis| 293      | 35/11.9 | 67/22.9 | 92/31.4 | 99/33.8 | 276       | 136/49.3 | 113/40.9 | 10/3.6 | 17/6.2 |
| Cotesia glomerata  | 276       | 45/14.1 | 70/21.9 | 107/33.4 | 98/30.6 | 299       | 125/42.8 | 125/42.8 | 12/4.0 | 32/10.7 |

**Notes:** *Eumenes* spp. indicates a common ancestor among the species; *Ancistrocerus nigricornis* and *A. melifera* and *A. dorsata* are found together in a large clade with a 64% bootstrap confidence value (7 informative positions). The honeybees are the sister group to *Eumeninae* + (Vespiinae + Polistinae) with only 60% bootstrap and 15 informative positions. The Stenogastrinae are shown as the sister group to this cluster. The separation of Vespiinae + Polistinae and Eumeninae from a common ancestor is sustained by 99% bootstrap replicates based on 30 informative positions. The honeybees are the sister group to *Eumeninae* wasps with a 64% bootstrap confidence value (7 informative positions). The honeybees form the sister group to *Eumeninae + (Polistinae + Vespiinae)* with a bootstrap value of 100% based on 21 informative positions. The Stenogastrinae is the sister group to this cluster. The strong phylogenetic signal in the combined data set is shown by the $g_1$ statistic ($g_1 = -0.897; P < 0.01$). In Fig. 3 (bottom), we present the PAUP parsimony tree produced by analysis of both data sets together. The
FIG. 2. Secondary structure of the ribosomal RNA for 28S nuclear rRNA (top) and mitochondrial 16S rRNA (bottom) of Provespa nocturna. Bars represent unsequenced complementary DNA chains.
combined data set comprises 238 informative characters out of 583 altogether. The tree length is 531, and the consistency index is 0.621. Monophyly of Vespinae and Polistinae is supported by 82 and 100% bootstrap values, respectively. Apinae and Stenogastrinae are monophyletic by a 100% bootstrap and Eumeninae by a 76% bootstrap. The monophyly of Vespinae plus Polistinae is supported by 100% of the bootstrap replicates (17 informative positions). The Eumeninae are the sister group to Vespinae + Polistinae confirmed by a 100% bootstrap (34 informative positions). The bees are placed as sister group to this cluster with one common ancestor (100% bootstrap, 41 informative positions).

The Stenogastrinae are the sister group to the Apinae + (Eumeninae + (Vespinae + Polistinae)) cluster. The Eumenes spp. are composed of an undefined Eumenes species for nu-rDNA and Eumenes coarctatus for mitochondrial DNA. Nasonia vitripennis and Cotesia glomerata were used as outgroups for nu-rDNA and mt-rDNA, respectively. The Tajima–Nei distances (Tajima and Nei, 1984) were calculated and used for reconstruction of the MEGA neighbor-joining tree (not shown), which has the same topology as the parsimony tree. Both the common ancestor of Eumeninae + (Vespinae + Polistinae) and the bifurcation of Apinae and Eumeninae + (Vespinae + Polistinae) are consilidated by a 100% bootstrap value. The PHYLIP maximum-likelihood tree further confirms the topology of the parsimony tree. The bootstrap values are identical to the neighbor-joining tree for the two nodes considered.

As mentioned above, there is some controversy about the reliability of double-stranded versus single-stranded regions in rRNA for phylogenetic analysis. For analyzing the effects of single-stranded and double-stranded regions on the phylogenetic reconstruction, we selected 161 single-stranded characters (55 informative) and 162 double-stranded characters (75 informative) out of the nuclear and mitochondrial rDNA pool (Table 2). Single- or double-stranded regions were selected only when recorded in all investigated species. The topology of the two resulting trees (not shown) is identical with the complete data set. There is occasionally poor resolution within some subfamilies, which we attribute to the reduced data set. Both analyses cluster Eumeninae + (Vespinae + Polistinae) with 87% bootstrap confidence for single-stranded regions and 88% bootstrap confidence for double-stranded regions. The cluster Apinae + (Eumeninae + (Vespinae + Polistinae)) was confirmed by 99 and 95% bootstraps, respectively (Table 2).

Both mitochondrial and nuclear rDNA trees independently result in a regrouping of the phylogenetic tree of Vespidae wasps (Carpenter, 1988, 1991). In the nuclear tree Eumeninae + (Vespinae + Polistinae) form a cluster confirmed by 99% bootstrap replicates, whereas the mitochondrial tree includes the bees by a 100% bootstrap value. In both trees, the Stenogastrinae are outside of the cluster, confirming the results of the combined tree.

Our data reject the view that social Vespidae wasps of the subfamilies Stenogastrinae, Polistinae, and Vespinae are descendants of a common ancestor (Carpenter, 1988, 1991). The alternative tree clustering of all Vespidae is 31 steps longer than the tree shown in Fig. 3 (bottom). This also means that sociality independently evolved at least twice in Vespidae: in the Stenogastrinae and the common ancestor of the Polistinae and the Vespinae. The closer relationship of honeybees to Eumeninae + (Vespinae + Polistinae) even suggests that the Stenogastrinae should be placed outside the other tested Aculeata (Hymenoptera having a sting).

The unusual position of the honeybees inside the Vespidae is a result of the unusual position of the Stenogastrinae outside of the remaining Vespidae. To clarify the exact position of the Stenogastrinae among the aculeate hymenoptera, a more extensive study, including a range of additional vespid and nonvespid members of the Vespioidea, is required. In spite of this, the presented analyses should be seen as preliminary, demanding further investigation into hymenopteran phylogeny.

Nevertheless, Stenogastrinae, although displaying a
wide range of social behavior, seems not to be a phylogenetic link between the solitary Eumeninae and the eusocial Polistinae and Vespinae. This reestablishes some of the older views of wasp phylogeny. Van der Vecht (1977) concluded from morphological and ethological differences between Stenogastrinae and Vespinae that a taxon comprising all three groups cannot be monophyletic. Furthermore, Van der Vecht (1977) assumed that Stenogastrinae evolved from a solitary cell-building ancestor with elongated gaster petiole and that this ancestor was closely related to the "Zethiniae," which Carpenter (1985) synonymized together with Raphiglossinae under Eumeninae. Our analysis of the nuclear and mitochondrial DNA regions supports a regrouping. We have no evidence for an Eumeninae-like ancestor because the Eumeninae are more closely related to honeybees than to Stenogastrinae. Our results are also in line with Spradbery (1975), who stated that "it would be unwise to look at the Stenogastrinae for examples illustrating intermediate steps in the achievement of social organization by higher Vespidae." It may be true that Spradbery and Van der Vecht did not present cladistically solid evidence to indicate that Stenogastrinae and other social wasps are not closely phylogenetically related (Carpenter, 1988). Nevertheless, our data support their theories by providing strong evidence for the sister group relationship of Eumeninae to Polistinae + Vespinae.

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