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 + Vespinae cluster. The analyzed sequences provide strong evidence that sociality has independently evolved twice in the Vespidae. © 1998 Academic Press

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

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study to understand the phases characterizing the early evolution of eusociality in wasps (West-Eberhard, 1978).

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 fraterna*. Sociality is more conspicuous in the genera *Parischnogaster* and *Liostenogaster*. *Parischnogaster mellyi* 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; Hölldobler and Wilson, 1995). 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 (Campbell et al., 1993) and the mitochondrial 16S rDNA (Dowton and Austin, 1994; Cameron, 1993) have proven to be excellent tools with which to reconstruct phylogeny in wasps and bees. The major functions of the rRNA to provide structure and to interact with other components of the ribosome. We can distinguish two structural elements: double-stranded stems and single-stranded loops. Both elements vary in their mode of substitution, depending on functional constraints. Double-stranded regions are prone to compensatory changes to maintain the functional structure (Hillis and Dixon, 1991). A point mutation in one strand selects for the complementary base in the other. In addition to analysis of the entire sequences, we treat stem and loop regions separately to reconstruct phylogenetic trees.

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 saggittarius in Malaysia, and Belonogaster petiolata in South Africa. The Eumeninae species (Ancistrocerus oviventris, Ancistrocerus nigricornis, Eumenes coarcta*tus*) were collected in Germany and one *Eumenes* spp. was collected in Malaysia. The three Stenogastrinae (P. mellyi, Liostenogaster vechti, E. fraterna) 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

Muscle or larval tissue was homogenized in 300 μ l TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and treated with SDS (1% in the final concentration) and proteinase K (50 μ g/ml) for 2 h at 50°C. Proteins and cell debris were extracted with a buffer-saturated phenol/chloroform/isoamylalcohol 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'-AAAGATCGAATGGGGAGATTC-3' and 5'-CACC-GGGTCCGTACCTCC-3', and for mitochondrial ribosomal DNA 5'-TTGACTGTACAAAGGTAGC-3' and 5'-GATATTACGCTGTTATCCC-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.

PHYLOGENY OF VESPIDAE

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	Vespa crabro	AGCGTCAGCG	GCGCTGGCTC	GGCCGAGT-G	AGCGATGTTG	CCGAC 50
	Provespa nocturna	• • • • • • • • • • •	C.			.G 50
	Vespula germanica	• • • • • • • • • •	· · · · · · · · · · · ·	· · · · <u>·</u> · · · · <u>-</u> ·	.AA	.G 50
	Polistes sanaittarius					.G
	Relanonaster petiolata	• • • • • • • • • • •	T.AA	G	T. TC. T	.GI 50
	Eumenes spec			GA.	TICI	.G 50
	Ancistracerus aviventris		π	GA -	A C	G 50
	Ancistrocerus nigricornis		Τ	GA -	GA C	G
	Apis mellifera	. T G A	.T	CGTT.GT.	CC.C	.G. TGAATC 50
	Apis dorsata	. T G A	. T	CGTT.GT.	CC.C	.G. TGAATC 50
	Parischnogaster mellyi	. Т	ΑΤ	CT.T.GT.	C	.AT.AT 50
	Liostenogaster vechti	. T	ACT	C.GAT.GT.	C	. AA . A T 50
	Eustenogaster fraterna	. T	A CT	CG.T.GT.	C A - CC	. TA . A C 50
	Nasonia vitripennis	. T	ΑΤΤ	CCGT.T.GAT	C GC	.G.GAC 50
	Vespa crabro	TACGGT - T	GGCGACA C	GTT-ATCACT	C-ATGCCT	 TGT-CCGGT 100
	Provespa nociurna		C	.T .		100
	Vespula germanica	A.	C	· · · · · · · · · · · · · · · · · · ·		C 100
	Polisies dominutus	IC.	T.G I		.C	TTC 100
	Polononastar patiolata	T	T.GG	· · A - · · · II ·		TTC 100
	Europagasier penotata		1.AG+G.			TC 100
	Ancistrocerus oviventris	G. IACCT.	AA	C	G	-C 100
	Ancistracerus nigricarnis	A	C		···	·C
	Anis mellifera	CTCGT TC	AT G GAGG	CAC C	TTCG TCGA	-CAC 100
	Apis dorsata	CTCGT TC	AT G GAGG	CAC C C	TTCG T GA	AC 100
	Parischnogaster mellvi	AT. TAAGG	-ATT TGA-G	ACGC TC	GC+C GTGTT	A 100
	Liostenogaster vechti	CT GC	• TTTTGA • G	ACGC TC		AC . 100
	Eustenogaster fraterna	CT GC	. TTT. GA-G	ACGC. TC	GC-C GTGTT	AC . 100
	Nasonia vitripennis	стс	TTC.GT-G	TCACGCGT . C	GC GGT	A AC 100
				<u></u>		
	Vespa crabro	GTCGTCGTCG	TGCACTTCTC	CTCTAGTAGA	ACGTCGCGAC	CCGTTGGGTG 150
	Provespa nociurna					
	Vespula germanica					150
	roustes dominulus	• • • • • • • • • • •	· · · · · · · · · T	.c	• • • • • • • • • •	150
	r outsies saggillarius	• • • • • • • • • • •	· · · · · · · · · · · <u>T</u>		• • • • • • • • • • •	150
	Euronogaster petiolala	• • • • • • • • • • • •	· · · · · · · · · · T		•••••	
	Ancietrocarue ovivortrie	• • • • • • • • • • • •	• • • • • • • • • • •		• • • • • • • • • • •	
	Ancistrocerus niaricamie		• • • • • • • • • • • •			
	Anis mellifera	A A				
	Apis dorsata	A A	•••••	C		T 150
	Parischnogaster mellyi			.C.		150
	Liostenogaster vechti			.C		150
	Eustenogaster fraterna			.C		150
	Nasonia vitripennis	AG		G		
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		111			111111111	
	Vespa crabro	CCGGTCTACG	GTCCGAGCGG	T-AGACTGTC	GCGTCGCTT-	CG-GCGCA 200
	Provespa noclurna	• • • • • • • • • • •	• • • • • • • • • • •	. •	• • • • • • • • • •	200
	vespuia germanica	<u>.</u>	• • • • • • • • • • •	••••••	· · · · · · · · · · · · •	200
	Pousies aominutus	1 <u>+</u>			· · · · · · · · · · · · · · ·	
	Polonooastar patiolata		·······		• • • • • • • • • •	200
	Fumenes spec	•••••	C		• • • • • • • • • • •	200 T 200
	Ancistrocerus oviventris	•••••	C GT	A	A	
	Ancistrocerus nigricornis		С от			
	Apis mellifera	Τ	.C	GC - C		
	Apis dorsata	τ	.CA	GC - C		
	Parischnogaster mellyi	A .	ACA. GAAA.	GT	GT	AAAAGT. 200
	Liostenogaster vechti	AA	ACA. GAAA.	GT		AAAACT. 200
	Eustenogaster fraterna	. A .	ACA. GAAA.	GT	GGT	AAAAC.T.T. 200
	Nasonia vitripennis	c	. C GTT	GTC	G.T	TAC.T 200
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	Vespa crabro	CGCGT - CAGA	CCCTCGGTCG	CCCGGCCGGC	TGCCCGGCGG	TCGAACTTAT 250
	Vespa crabro Provespa nocturna Vespula garmanica	CGCGT-CAGA	CCCTCGGTCG	CCCGGCCGGC	TGCCCGGCGG	TCGAACTTAT 250
	Vespa crabro Provespa nocturna Vespula germanica Polistes dominulus	CGCGT - CAGA	CCCTCGGTCG	CCCGGCCGGC	TGCCCGGCGG	TCGAACTTAT 250 250 250
	Vespa crabro Provespa nocturna Vespula germanica Polistes dominulus Polistes saggittarius	CGCGT - CAGA	CCCTCGGTCG	CCCGGCCGGC	TGCCCGGCGG	TCGAACTTAT 250
	Vespa crabro Provespa nocturna Vespula germanica Polistes dominulus Polistes saggittarius Belonogaster petiolata	CGCGT - CAGA	CCCTCGGTCG		TGCCCGGCGG	TCGAACTTAT 250
	Vespa crabro Provespa nocturna Vespula germanica Polistes dominulus Polistes saggitarius Belonogaster petiolata Eumencs spec.	CGCGT - CAGA	CCCTCGGTCG		TGCCCGGCGG	TCGAACTTAT 250
	Vespa crabro Provespa nocturna Vespula germanica Polistes dominulus Polistes saggittarius Belonogaster petiolata Eumence spec. Ancistrocerus oviventris	CGCGT - CAGA	CCCTCGGTCG		TGCCCGGCGG	TCGAACTTAT 250
	Vespa crabro Provespa nocturna Vespula germanica Polistes dominulus Polistes saggittarius Belonogaster petiolata Eumenes spec. Ancistrocerus nigricornis	CGCGT - CAGA	CCCTCGGTCG		TGCCCGGCGG	TCGAACTTAT 250
	Vespa crabro Provespa nocturna Vespula germanica Polistes dominulus Polistes saggittarius Belonogaster petiolata Eumenes spec. Ancistrocerus oviventris Ancistrocerus negricornis Apis mellifera	CGCGT - CAGA	CCCTCGGTCG A A A A T. T. T.		TGCCCGGCGG	TCGAACTTAT 250
	Vespa crabro Provespa nocturna Vespula germanica Polistes aggittarius Belonogaster petiolata Eumence spec. Ancistrocerus nigricornis Apis mellifera Apis dorsata	CGCGT - CAGA	CCCTCGGTCG		TGCCCGGCGG	ATTTAT 250 250 250 250 250 250 250 A
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	Vespa crabro Provespa nocturna Vespula germanica Polistes dominulus Polistes aggittarias Belonogaster petiolata Eumenes spec. Ancistrocerns nigricornis Ancistrocerns nigricornis Ancistrocerns nigricornis Apis mellijera Apis mellijera Apis actostar mellyi Liostenogaster vechti	CGCGT - CAGA	CCCTCGGTCG 	CCCGGCCGGC	TGCCCGGCGG	ALTITAT 250 TCGAACTTAT 250 250 250 250 250 A 250 AT
	Verpa caboro Provespa nocuma Vespula germanica Polistes dominulus Polistes saggiutarias Belonogaster petiolata Beumens spec. Ancistrocens noviventris Ancistrocens nicirconis Apis dengin Apis dengin Parischoogaster melhyi Lostonogaster melhyi Eutemogaster fratema	CGCGT - CAGA	CCCTCGGTCG A A A A A T T T C A A A A A A A A A A A A A	CCCGGCCGGC 	TGCCCGGCGG	1 250 1 250 250 250 250 250 250 250 1 250 1 250 1 250 1 -62 250 250 1 -62 2 - 2 - 2 - 2 - 2 - 2 - 2 - 2 - 2 - 2 - 2 - 2 - 3 - 3 - 4 - 4 - 4 - 5 - 4 - 4 - 5 - 4 - 5 - 6 - <t< td=""></t<>
	Vezpa czabro Provespa nociurna Vezpula germanica Polistes dominulus Polistes saggiturius Belonogaster petiolata Benones spec. Ancistrocerus owieneris Ancistrocerus negrecornis Apis mellifera Apis donata Parischoogaster mellyi Liostenogaster Pateiena Nasonia vitripennis	CGCGT - CAGA	CCCTCGGTCG 	CCC6GCCCGC	TGCCCGGCGG	CGAACTTAT 250
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	Vezpa czabro Prowszyn a nociuma Vezpuła germanica Polistes dominulus Polistes saggiturius Belonogaster petiolata Belonogaster petiolata Benenca spec Ancistrocerns owiventris Ancistrocerns noviventris Apis denata Patischongaster mellyi Liostongaster wechti Eutenogaster fratema Nasonia vitripennis Vezpa crabro Provezpa nocturna Yaupula germunica	CGCGT - CAGA	CCCTCGGTCG A A T T T T T 	CCC6GCCGGC	TGCCCGGCGG	TGAACTTAT 250
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	Vezpa człabro Prowszyn a nociuma Vezpiała germanica Polistes a dominulus Belonogaster peliolata Belonogaster peliolata Buenecs spec Ancistrocerus o wiveniris Ancistrocerus a niveniris Apis dengiał Patischoogaster meliyi Liostonogaster webii Eustenogaster fraterna Nasonia vitripennis Wezpa cnabro Proveszpa nociurna Perveszpa nociurna Polistes aggittraius Belonogaster peliolata Belonogaster peliolata Belonogaster peliolata Belonogaster peliolata Belonogaster peliolata Belonogaster peliolata Belonogaster peliolata Belonogaster peliolata Belonogaster peliolata Patischoogaster meliyi pis dorzata	CGCGT - CAGA	CCCTCGGTCG A A A A T T T T T T T T T T T T T T T T T 	CCC6GCCGGC .T .T .T .T .T .T .T .T 	TCGAAC	- TGCAACTTAT 250
	Vezpa czabro Prowszyn nociurna Vezpila germanica Polistes dominulus Polistes soggittarius Belonogaster petiolata Elemencs spec. Ancistrocerus owiventris Ancistrocerus owiventris Ancistrocerus angenerus Apis denata Patrischoogaster melhyi Liostenogaster melhyi Liostenogaster fraterna Nasonia vitripenais Vezpia crabro Provszpa nocturna Vezpia crabro Provszpa nocturna Polistes dominulus Polistes dominulus Polistes aggittarius Belonogaster petiolata Ancistrocerus nigricoruls tybi sendijera Apis mellijera	CGCGT-CAGA	CCCTCGGTCG 	CCC6GCCGGC .TT	TCGAAC TCGAAC TCGAAC AA. A. A. T	TGGAACTTAT 250
	Verpa caboro Provespa nocuma Vespula germanica Polites dominulus Polites aggiturius Belonogaster peitolata Belonogaster peitolata Belonogaster peitolata Ancistrocerns oviventris Ancistrocerns aviventris Apis densata Parischoogaster mellyi Liostonogaster tratema Nasonia vitripennis Vespa caboro Provespa nocurma Vespala germanica Polites dominulus Polites dominulus Polites aggiturius Belonogaster peitolata Belonogaster peitolata Belonogaster peitolata Belonogaster peitolata Belonogaster peitolata Parischoogaster mellyi Ingi dorsata Parischoogaster mellyi Ingi dorsata Parischoogaster mellyi Distonogaster mellyi Distonogaster finemani	CGCGT - CAGA	CCCTCGGTCG A A A T T TC. A. AC. AA. TC. AA. AC. AA. T GCCGCAATTA G G G G C 	T. T. T. T. T. T. T. T. TT. T. <tr< td=""><td>TCGAAC</td><td>- TGCAT-TTAT 250 </td></tr<>	TCGAAC	- TGCAT-TTAT 250
	Vezpa czabro Prowszyn a nociuma Vezpuła germanica Polistes dominulus Polistes sagitiarius Belonogaster petiolata Elemencs spec. Ancistrocerns owiventris Ancistrocerns owiventris Ancistrocerns noviventris Apis denata Patischoogaster mellyi Lastenogaster mellyi Lastenogaster pratema Nasonia vitripensis Vezpa crabro Provszpa nocturna Vezpa crabro Prosezpa nocturna Vezpa crabro Polistes aggitraius Belonogaster petiolata Belonogaster petiolata Belonogaster petiolata Belonogaster necliyi Lustenogaster mellyi Lustenogaster mellyi Lustenogaster mellyi Lustenogaster puelio	CGCGT - CAGA	CCCTCGGTCG	CCCGGCCGGC	TGCCGGGGGG 	TGCAACTTAT 250
	Verpa cabro Provespa nocuma Vespula germanica Politate dominulus Politate dominulus Politate saggittarius delonogaster peilolata Elemenes spec. Ancistrocerns oviventris Ancistrocerns nigricornis Apis dersata Parischongaster mellyi Lostonogaster mellyi Lostonogaster vechti Elettenogaster fratema Nasonia vitripennis Vespa crabro Provespa nocuma Vespala germanica Politate saggittarius Belonogaster peilolata Eumenes yoventris Ancistrocerus oviventris Ancistrocerus oviventris Ancistrocerus nigricornis Upis dorsata Parischongaster mellyi Lostenogaster fuelta	CGCGT - CAGA	CCCTCGGTCG AC 	CCCGGCCGGC 	TCGAAC	TCGAACTTAT 250
	Vezpa czabro Prowszyn a nociuma Vezpuła germanica Polistes sogiutraius Belonogaster petiolata Belonogaster petiolata Belonogaster petiolata Ancistrocerns owiventris Ancistrocerns noviventris Apis mellýera Apis dorzata Parischoogaster melhyi Loustenogaster melhyi Loustenogaster patema Nazonia vitripennis Vezpa crabro Provszpa nociurna Vezpa crabro Polistes dominulus Polistes dominulus Belonogaster petiolata Belonogaster petiolata Belonogaster petiolata Belonogaster melhyi Loustenogaster melhyi	CGCGT-CAGA	CCCTCGGTCG 	CCCGGCCGGC .T T TC TT TC TT TC TT TC TT TC TT TC TT TC TT TC TT TC TT TC TT TC TT TC TT TT TC TT TT TC TT TT TC TT TT TC TT TT TC TT TT TC TT TT TC TT TT TC TT TT TC TT TT TC TT TT TC TT TT TC TT	TCGAAC	TGCAACTTAT 250
	Verpa cabro Provespa nocuma Polistes dominius Polistes dominius Polistes dominius Polistes adminius Polistes dominius Ancistrocerns oviventris Ancistrocerns angitocomis Apis dorsata Parischongaster mellyi Liostenogaster mellyi Liostenogaster vechti Eustenogaster fraiema Nasonia vitripennis Verpa cabro Provespa nocuma Vespala germanica Polistes dominus Polistes aggittarius Belonogaster peilolan Eumenes spec- Macistrocerus oviventris Ancistrocerus oviventris	CGCGT - CAGA	CCCTCGGTCG 	T. T. T. <	TCGAAC TCGAAC AA . A. T AA . A. T AA T AA T T T T T T C T T T T A T A T A T A T A T A T A T C T S T S T S T S	TCGAACTTAT 250
	Vezpa czboro Prowszyn nociuma Vezpiła germanica Polistes dominulus Doliste saggiturius Belonogaster peliolata Elemencs spec Ancistrocerus owiventris Ancistrocerus owiventris Ancistrocerus noiventris Apis melligran Apis donzata Patischoogaster mellyi Liostonogaster webli Eustenogaster fraterna Nasonia vitripennis Powszyn nociuma Belonogaster peliolata Elemenes spec Holistes aggiturius Belonogaster mellyi Dolistes dominulus Polistes dominulus Polistes dominulus Polistes aggiturius Belonogaster mellyi Liostonogaster mellyi Iustenogaster mellyi Iustenogaster mellyi Iustenogaster mellyi Distoromy vechti Eustenogaster mellyi Liostonogaster webli Eustenogaster mellyi Liostonogaster webli Eustenogaster protema	CGCGT - CAGA	CCCTCGGTCG 	CCC6GCCGGC 	TCGAAC	TGCAACTTAT 250
	Vespa cabro Provespa nociuma Posistas dominius Polistas dominius Polistas dominius Polistas dominius Polistas dominius Polistas dominius Ancistrocents aviventris Ancistrocents aviventris Ancistrocents avientificat pais dorata Parischoogaster mellyi Lustenogaster mellyi Lustenogaster roterna Vespa crabro Provespa nociuma Vespa crabro Provespa nociuma Polistas dominus Polistas dominus Polistas dominus Polistas dominus Pais mellifera Ancistrocents apgeitarius Belonogaster mellyi Lustenogaster vechi Lustenogaster vechi Lustenogaster torienta Ancistrocents apgeitarius Pais mellifera Ancistrocents angricornis Apis mellifera Ancistrocents angricornis Apis mellifera Vespa crabro Polistas dominus	CGCGT - CAGA	CCCTCGGTCG 	CCC6GCCGGC .T T TT ACCATTTTT ACCATTTTT ACCATTTTT ACCATTTTT ACCATTTTT ACCATTTTTT ACCATTTTTT ACCATTTTTT	TCGAAC AA . A. TCGAAC AA T T T T. TGTGT TGTGT TGTGT TGTGT TGTGT TGT T. TTAAG L C	TGCAACTTAT 250
	Verpa cabro Provespa nocuma Polistes aggiturius Polistes aggiturius Bélonogaster peilolata Bélonogaster peilolata Bélonogaster peilolata Ancistrocerns oviventris Ancistrocerns oviventris Apis melligren Apis donzata Parischoagaster mellyi Liostonogaster mellyi Liostonogaster vechti Eutenogaster fraterna Nasonia vitripennis Verpa cabro Provespa nocurna Verpala gernanica Polistes aggiturius Bélonogaster mellyi Diatsen aggiturius Bélonogaster mellyi Diatsen aggiturius Belonogaster mellyi Diatsen aggiturius Polistes aggiturius Belonogaster mellyi Diatsenogaster mellyi Diatsenogaster mellyi Diatsenogaster mellyi Diatsenogaster mellyi Diatsenogaster fraterna Nasonia vitripennis	CGCGT - CAGA	CCCTCGGTCG 	CCC6GCCGGC 	TCGAAC	TGCAACTTAT 250
	Vezpa czabro Prowszen nociuma Vezpuła germanica Polistes dominulus Polistes saggiturius Belonogaster petiolata Eutomencs spec. Ancistrocerns owivenris Apis melifera Apis denzia Parischongaster melipi Liostonogaster melipi Liostonogaster vechti Eutomogaster fratema Nasonia vitripennis Vezpa crabro Provezpa nocturna Vezpa crabro Polistes aggiturius Belonogaster petiolata Belonogaster petiolata Apis melifera Apis nelifera Apis melifera Apis nelifera Apis nelifera Apis nelifera Apis nelifiera Apis melifiera Apis melifiera Apis melifiera Apis melifiera Apis melifiera Apis melifiera Apis melifiera Apis melifiera Apis nencia Polistes aggiturius Vezpula germanica Polistes aggiturius Velonogaster peliolata	CGCGT - CAGA	CCCTCGGTCG 	CCC6GCCGGC . T. . GGGC C. . T. T. T. 	TCGAAC TCGAAC TCGAAC AA. A TT TT TT TT TT TT TT TT TT TT TGGTT TGGTT TGGT TGGT TT. TGAG TT. TGAG TT. TGAG TT. TGAG TT GG. TTAAG L C 	TGCAACTTAT 250
	Verpa cabro Provespa nocuma Politaes dominulus Politaes adguitarius Belonogaster peilolata Belonogaster peilolata Belonogaster peilolata Antistrocerns oriventris Apis melligra Apis dorsata Parischongaster mellyi Liostonogaster vechi Eustenogaster fratema Nasonia vitripennis Verpa cabro Provespa nocuma Politaes dominulus Politaes dominulus Politaes dominulus Parischongaster mellyi liostonogaster peilolata Belonogaster peilolata Belonogaster peilolata Stastenogaster fueloma Nasonia vitripennis Vespa cabro Politaes dominulus Politaes dominulus Parischongaster mellyi Liostenogaster mellyi Liostenogaster mellyi Disotenata Parischongaster fueloma Nasonia vitripennis Vespa cabro	CGCGT-CAGA	CCCTCGGTCG AC 	CCC6GCCGGC .T T T TT CGGGCCGGCCGCGCCGC	TCGAAC TCGAAC AA . A. T AA . A. T AA T AA T AA T AA T.	TGCAACTTAT 250
	Vezpa czabro Prowszen nociuma Vezpuła germanica Polistes dominulus Polistes sogiturius Belonogaster peliolata Euenects spec Ancistrocerns owiyentris Ancistrocerns owiyentris Apis melligra Apis dorzata Parischoogaster mellyi Liostenogaster mellyi Liostenogaster vechti Eutenogaster fratema Nasonia vitripennis Vezpa crabro Provszpa nociurna Vezpa crabro Prosezpa nociurna Belonogaster mellyi Loistenogaster dominutus Polistes adgiturius Belonogaster peiolata Belonogaster peiolata Belonogaster peiolata Euenenes spec: Mediarocenta owienentis	CGCGT-CAGA	CCCTCGGTCG 	CCCGGCCGGC .T	TCGAAC	TGCAACTTAT 250
	Verpa cabro Provespa nocuma Positas dominius Politas dominius Politas dominius Politas dominius Politas dominius Politas dominius Ancistrocerns oviventris Ancistrocerns informita Parischongaster mellyi Liostongaster vechti Eutenogaster fratema Nasonia vitripennis Verpa cabro Provespa nocuma Vespala germanica Politas dominus Politas dominus Politas aggiitarius Belonogaster peilolan Eumenes spec- Macistrocerus oviventris Ancistrocerus nigricornis Yasonia vitripennis Vespa cabro Politas dominus Politas dominus Politas dominus Politas peilolan Eumenes spec- Macistrocerus nigricornis Vespa cabro Politas dominus Politas dominus	CGCGT - CAGA	CCCTCGGTCG 	CCC6GCCGGC .T T TT CGGGCCGGCCGGCCGGCCGGCCGGCCGCGCCGC	TCGAAC	TCGAACTTAT 250
	Vezpa czabro Prowszen nociuma Vezpiała germanica Polistes adguitrius Belonogaster peliolata Elemence spec Ancistrocerus owiventris Ancistrocerus owiventris Ancistrocerus anguitrio Apis donzia Parischoogaster mellyi Liostonogaster mellyi Liostonogaster vechi Eutenogaster fratema Nazonia vitripennis Vezpiała germanica Polistes adguitrius Belonogaster peliolata Belonogaster peliolata Belonogaster mellyi Jostasta Polistes adguitrius Belonogaster mellyi Jostasta Parischoogaster mellyi Liostenogaster mellyi Distes doninulus Polistes adguitrius Belonogaster peliolata Belonogaster peliolata Parischoogaster mellyi Jostastonogaster mellyi Distostonogaster mellyi Distostonogaster mellyi Distostonogaster polista Nazonia vitripennis Vezpiała germanica Polistes adguitruis Belonogaster polistas Selonogaster peliolata Belonogaster peliolata	CGCGT - CAGA	CCCTCGGTCG 	CCC6GCCGGC .T	TCGAAC	TGCAACTTAT 250
	Vespa cabro Provespa nociuma Vespula germanica Polistes dominulus Polistes dominulus Polistes dominulus Polistes dominulus Ancistrocents oriventris Ancistrocents oriventris Ancistrocents oriventris Apis dorsaia Parischoogaster mellyi Lustenogaster traierna Nasonia virtipennis Vespa crabro Provespa nocturna Vespia germanica Polistes dominulus Polistes dominulus	CGCGT - CAGA	CCCTCGGTCG 	CCC6GCCGGC . T. . T. T. T. T. T. 	TCGAAC TCGAAC AA. A. T. AA. A. T. TCGAAC AA. T. T. T. T. T. T. T. T. T. T	TGCAACTTAT 250
	Verpa cabro Provespa nocuma Politaes dominulus Politaes adguitarius Belonogaster peilolata Belonogaster peilolata Belonogaster peilolata Maistorcerns oviventris Ankistrocerns oviventris Apis adrata Parischoogaster mellyi Liostonogaster tratema Nasonia vitripennis Politaes dominulus Politaes dominu	CGCGT-CAGA	CCCTCGGTCG 	T.	TCGAAC	TGCAACTTAT 250
	Vezpa czabro Prowszen nociuma Vezpila germanica Polistes dominulus Polistes sogitivrius Belonogaster peliolata Elemencs spec. Ancistrocerns oviyentris Ancistrocerns oviyentris Ancistrocerns noviyentris Apis denzia Parischoogaster melhyi Liostenogaster peliolata Elettenogaster fratema Nasonia vitripennis Vezpa crabro Provszpa nociurna Vezpa crabro Polistes aggittrisus Belonogaster peliolata Belonogaster melhyi Liostenogaster peliolata Pelistes aggittarius Belonogaster melhyi Liostenogaster peliolata Belonogaster	CGCGT-CAGA	CCCTCGGTCG A A A A A T T T T 	CCCGGCCGGC . T. . T. T. 	TCGAAC TCGAAC TCGAAC TCGAAC T.	TGCAACTTAT 250
	Verpa cabro Provespa nocuma Politate dominulus Politate gormanica Politate gormanica Politate saggittarius Belonogaster peilolata Elemenes spec: Ancistrocerns oriventris Apis mellifera Apis dorsata Parischongaster mellyi Lostonogaster vechi Eutenogaster fratema Nasonia vitripennis Verpa cabro Provespa nocuma Politase dominulus Politase dominulus Politase angentaria Eunenes spec: Mesi dorsata Elenongaster peilolata Eunenes spec: Mesi dorsata Politase angentaria Politase angentaria Politase angentaria Politase angentaria Politase angentaria Parischongaster mellyi Distorogaster vechi Eutenogaster fratema Vasonia vitripennis Vespa carbo Provespa nocuma Politase dominulus Politase angentaria Politase angentaria Politase angentaria Politase dominulus Politase angentaria Politase dominulus Politase dominulus Politase dominulus Politase dominulus Politase angentaria Politase politaria Politase	CGCGT - CAGA	CCCTCGGTCG	T.	TCGAAC TCGAAC TCGAAC AA T	TCGAACTTAT 250

			TTTTTUU			
b	Vespa crabro	AAAATCATTT	GTTTTTTAAT	TGAAAACTGG	AATGAAAGAG	TGCATGAAAT 50
	Provespa nocturna	.т				. TA 50
	Vespula germanica	A.A		G		A
	Polistes aominuus Polistes saggittarius			т.	A	. TG A 50
	Belonogaster petiolata	A.A		A.	A	. TAA 50
	Eumenes coarctatus	A		· · · · · · · · · <u>-</u> ·		. TA
	Ancistrocerus oviventris Ancistrocerus nigricornis	. Т А			TA	. TG G 50
	Apis mellifera	.TA.A	c	G.A.T.	ΤΑ	. TA 50
	Apis dorsata	. T A . A	c	G.A.T.	CA	. TA 50
	Parischnogaster mellyi Liostenonaster vechti	. IA.A T A A	.A	.AIA. GTT	GGA GA	GTA
	Eustenogaster fraterna	.TA.A	C	.AG.TT.A.	GT.G.	ATAG 50
	Cotesia glomerata	. T A		A .	<i></i> . T T	. AA 50
						TTT:
	Vespa crabro	ATAAGTTGTC	TCAATATAAT	TAATTGAA	ΤΤΤΑΑΑΤΤΑΑ	AAGTTAAAAT 100
	Provespa nocturna	A	A	AT		100
	Vespula germanica	A	GG.C	••A		
	Polistes aominuus Polistes savaittarius	AC	AT	Τ-Α	· · · · · · · · · · · · · · ·	100
	Belonogaster petiolata	A	AT	Τ-ΑΑ	. A	100
	Eumenes coarctatus	.AA	T-TTA	A-AT		
	Ancistrocerus oviventris Ancistrocerus ninricornis	.AAI	G GTTA	A-AI	TT	. G 100
	Apis mellifera	.AT.AC		T-A	TT	Т 100
	Apis dorsata	. AT . AC	TGA T	Τ-Α	тт	Т 100
	Parischnogaster mellyi	.A. TAC. T.A	. T. T. TA	AAA		G 100
	Eustenogaster vecnii Eustenogaster fraterna	.A. TC.T.A	. TTT. TA	A	A	
	Cotesia glomerata	ACA	AT.T.A	A.A.A.	TTA.TT	A.AA 100
		TTTTTT				
	Vespa crabro	GCTTTTATAT	ATTTATAAGA	CGATAAGACC	CTATAGAATT	TAATAAAATT 150
	Provespa nocturna	TA	. A			GT 150
	Vespula germanica	G.T.	. A <u>T</u>	G		
	roustes dominulus Polistes sanaittarine	А	т., т			. G.A II 150
	Belonogaster petiolata	TA	ΤΑΤ	G		.TT.A 150
	Eumenes coarctatus	ΑΑΑΤ.	тт	G		. т 150
	Ancistrocerus oviventris	AA	r <u>T</u>	A	• • • • • • • • • • •	. T 150 T 150
	Ancistrocerus nigricornis Apis mellifera	T AAA T.	. A GG			TT 150
	Apis dorsata	TAAAT.	. A GG			.TTTA 150
	Parischnogaster mellyi	AAG.	ΤΑΤ.Τ	. A . G	G	T.A 150
	Liosienogasier vechli Fusienogasier fraterna	. I AA I. T AA	T TA	G A	G	AT. TA 150
	Cotesia glomerata	AT AA A	ΤΤΑ	G	A	.T.ATTAA 150
		-				
	Vespa crabro		AATTAATAT-	ΤΤΤΤΑΑΤΑΑΑ	ΑΤΤΤΑΑΤΑΑΑ	TAA - TTATAT 200
	Provespa nocturna	GT	T.T	A	AT	A
	Vespula germanica		. TA T	G T	TATT	AT.T 200
	Polistes dominulus Polistes sanoittarius	AA.T.	GG.AT.T	A.GATT.	.AII	. T.A.AT.T. 200 T.TAAT.T. 200
	Belonogaster petiolata	ATAA A	. TAATTAT.A	A TAGT .	ΤΤΤΑΤΤ.	A
	Eumenes coarctatus	AAA.G	.GAT	G.AAT	AATT.C.T	AT.A 200
	Ancistrocerus oviventris	A.GG.AAAGT	AA	G.AAT.T	ATT.T.T	. TTT. 1 200
	Apis mellifera	.T. AAA T.	TT.A-TAT	A. TAT		A 200
	Apis dorsata	.T.AT.	ΤΤΑΑ.ΤΑΤ	AA.T.TGT	. AA G . TG	A 200
	Parischnogaster mellyi	AT A T.	TTAAT	AAT	T.AA.TA	TAT. 200
	Liosienogasier vechii Eustenopasier fraterna	ATA . AA	. T TTA . A -		T. ATTGT. T	A.TT-ATAT. 200
	Cotesia glomerata	AT.AA		····TT	TA	-TTTA. TATA 200
					1.	т
	Vespa crabro	TTTATTGGGA	GGATAGTTAA	ΑΤΤΑΑΤΤΑΑΑ	CTTT-AATA-	ATTAGTTAAC 250
	Provespa nocturna		A	A.G	• •	.AATA 250
	Vespula germanica		T	TT	T A	. AATA 250
	Polistes aominutus Polistes saggittarius		GA		т	TA . TA 250
	Belonogaster petiolata			TT	т.	TAAA 250
	Eumenes coarctatus		· · · · · A · · · ·	T.AA	· · · · · · · · · · · · · · · · · · ·	A T T A A T 250
	Ancistrocerus oviventris Ancistrocerus niericomis	AG	TA.A	T.A. I		T.ATA.AG. 250
	Apis mellifera	G	T.G	T.A	т.та	.GATTT 250
	Apis dorsata	G	T.A	· · · · · · · · · · · · · · · · · · ·	• . T . TA	ATT T 250
	rarischnogaster mellyi Liostenogaster vechti	AGG	ATA			AAT-AAL . 250
	Eustenogaster fraterna	A G	ΑΤΑ	T.A.G.	татт	- AT . A. T. 250
	Cotesia glomerata	A G	AATT.A	T.AA	тт <mark>-т</mark>	.AA • • AA 250
						
	Vespa crabro	ATAGATT	AATGAATTA	TTTG-AT	TATT-ATTAT	AATTATTA-G 300
	Provespa nocturna	TG		<u>T</u>	- C	
	Vespula germanica Polistas dominutur	TA	AGT	.A.T	A -	
	Polistes saggittarius	.CA	т	. AAT A	ΑΤ	
	Belonogaster petiolata	TA	•.т	AAAAT	ΑΤ	A 300
	Eumenes coarciatus	· · · · · · · · · · · · · · · · · · ·		A.GAGA	· · · · · · · · · ·	
	Ancistrocerus oviventris Ancistrocerus nivricornis			AA		
	Apis mellifera	Т.ТА	T.A. TAAT	TT		AA 300
	Apis dorsata	Τ.Τ	ТАТAG.	. A . T A	AAT	
	Parischnogaster mellyi	. A TA	- TT	AA	A	AA. 300
	Lustenogaster vechti Eustenogaster fraterna		. т <u> </u> .^.	<u></u>	A.G	T.A. AATA. 300
	Cotesia glomerata	TA	-TT	T. A	т.	AA.AATAA 300
		TTTTT.	ттт —			
	Vesna crabro		CTTA			314
	Provespa nocturna					314
	Vespula germanica					314
	Polistes dominulus	•••• <u>•</u> ••••••				314
	Polistes saggittarius Belongeaster patiolete	T T AT	• • • •			314
	Eumenes coarciatus	A				314
	Ancistrocerus oviventris	A				314
	Ancistrocerus nigricornis	.GA				314
	Apis memjera Apis dorsata	.G				314
	Parischnogaster mellyi					314
	Liostenogaster vechti	•••				314
	Eustenogaster fraterna	 т.т				314
	concant gromeran					514

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 μ l.

Cloning

The PCR product was purified by gel elution and with a GeneClean II Kit (Bio 101, Inc.), inserted in the pUC19–*Sma*I site, and cloned in *Escherichia coli* DH5 α .

Gene Sequencing

Sequencing was performed by the Sanger dideoxychain termination technique (Sanger *et al.*, 1977) with the Sequenase 2.0 sequencing kit (United States Biochemical 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 phylgenetic signal in comparative sequence data. To detect the presence of phylogenetic signal in our data, we used the measure of skewness of tree-length distributions (g_1 statistic), which is available in the PAUP 3.1.1 package (Swofford, 1993), by generating 10⁶ random sampling trees.

Parsimony analyses were conducted with the computer program PAUP 3.1.1 (Swofford, 1993) by including all characters. Heuristic searches using accelerated transformation were used to find the most parsimonious tree. The taxa were added by random addition and 20 repeats. Bootstrap values were determined from 1000 replications. Gaps were treated as missing data.

Distance analyses were conducted with the computer program MEGA (Kumar *et al.*, 1993) using the Tajima and Nei distance method (Tajima and Nei, 1984). Tree construction was performed by the neighbor-joining method (Saitou and Nei, 1987), with bootstrap values on 1000 replications. Gaps were excluded by pairwise deletion.

Maximum-likelihood trees were constructed with the PHYLIP-DNAML program (Felsenstein, 1993). Bootstraps (100 replicates) were performed using SEQ-BOOT and CONSENSE of the PHYLIP 3.3c package. Comparison of tree length was done with the MacClade 3.01 program (Maddison and Maddison, 1992).

RESULTS AND DISCUSSION

Base Composition

We could not detect any intraspecific sequence variability in the target DNA regions. The analyzed 289-bp fragment, e.g., of *P. nocturna* nu-rDNA, is about 13.5% adenine (A), 21.9% thymine (T), 32.3% cytosine (C), and 32.3% guanine (G). This nucleotide ratio is almost constant in all species studied (Table 1) and similar to that found in mice (Hassouna *et al.*, 1984). The extreme AT content in *Drosophila melanogaster* (79%) seems to be an exception for eukaryotic species (Tautz *et al.*, 1988).

The length of the analyzed mt-rDNA fragment is, e.g., 298 bp in *P. nocturna* with 43.6% A, 42% T, 4.7% C, and 9.7% G (Table 1). The extreme AT bias (85.6%) is nearly constant in all analyzed samples and seems to be characteristic for insect mitochondrial DNA. Crozier and Crozier (1993) found an AT bias of 84.9% for the total mitochondrial genome of *A. mellifera*.

Secondary Structure of the rRNA

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.

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. crabro* 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 doublestranded stems. Taking variable characters as a param-

base Composition										
	28S rDNA				16S rDNA					
	Length	A (%)	T (%)	C (%)	G (%)	Length	A (%)	T (%)	C (%)	G (%)
Vespa crabro	288	39/13.5	63/21.9	93/32.3	93/32.3	298	130/43.6	125/42.0	14/4.7	29/9.7
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	29/9.7
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 saggittarius	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 petiolata	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						302	138/45.7	120/39.7	14/4.7	30/9.9
Eumenes spp.	288	45/15.6	66/22.9	86/29.9	91/31.6					
Ancistrocerus oviventris	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 mellifera	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 mellyi	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 fraterna	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					
Cotesia glomerata						276	136/49.3	113/40.9	10/3.6	17/6.2

TABLE 1

Base Composition

eter for sequence evolution, we fail to find a significantly biased variability between single- or doublestranded 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 Swofford 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 Vespinae cluster with a bootstrap value of 68%. The data set is therefore not suitable for resolving the phylogeny inside the Vespinae. Separation of the Vespinae and Polistinae is supported by a bootstrap value of 91%. The monophyly of Polistinae is supported by 100% bootstrap confi-

dence. The separation of Vespinae + 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 + (Vespinae + 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 Vespinae species cluster with a bootstrap value of 55%. The Polistinae form the sister group to the Vespinae. The common ancestor is supported by 81% bootstrap replicates. The sister group to the Vespinae + Polistinae is the Eumeninae wasps with a 64% bootstrap confidence value (7 informative positions). The honeybees form the sister group to Eumeninae + (Polistinae + Vespinae) 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



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.

						Bootstrap values			
	Characters	Variable	Informative	Tree length	CI	E + (P + V)	A + (E + (P + V))		
nu-rDNA	331	167	125	256	0.703	99	60		
mt-rDNA	252	143	113	270	0.556	64	100		
nu + mt-rDNA	583	310	238	531	0.621	100	100		
ss-region	160	73	55	127	0.567	87	99		
ds-region	163	89	75	159	0.610	88	95		

TABLE 2

Supplementary Information on the Parsimony Trees

Note. CI is the consistency index by excluding uninformative characters. ss-region, single-stranded region; ds-region, double-stranded region. The first bootstrap values are for the ancestral node for Eumeninae + (Polistinae + Vespinae), the second bootstrap values are for the ancestral note for Apinae + (Eumeninae + (Polistinae + Vespinae)).

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 consolidated 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 Vespoidea, 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

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FIG. 3. The most parsimonious trees inferred by the heuristic search and confirmed by 1000 bootstrap replications of the PAUP program for the 331 characters of the 28S nuclear rDNA (top), the 252 selected characters of mitochondrial rDNA (center), and the 583 characters of the combined sequence (bottom). Outgroup contains *Nasonia vitripennis* for the nuclear sequence and *Cotesia glomerata* for the mitochondrial sequence part. *Eumenes* spp. in the combined tree denotes *Eumenes coarctatus* for the nuclear and a undefined *Eumenes* species for the mitochondrial part. Numbers above the branches indicate the inferred branch length (excluding uninformative characters). Numbers in circles denote the bootstrap values.

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 + Polistinae 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 gastral petiole and that this ancestor was closely related to the "Zethinae," 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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