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Research article

Phylogeny and phylogeography of the Mediterranean species of the parasitic ant genus *Chalepoxenus* and its *Temnothorax* hosts

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Abstract. We analysed the phylogenetic and phylogeographic relationships of four Mediterranean species of the rare slave-making ant genus *Chalepoxenus* and eleven of its about 20 *Temnothorax* host species by sequencing the mitochondrial Cytochrome Oxidase I and II genes. Neighbour-Joining, Maximum Parsimony and Bayesian analyses based on 1320 bp indicate that the genus *Chalepoxenus* constitutes a monophylum. In all three analyses, *C. kutteri* from Southwest Europe and the workerless, "degenerate slavemaker" *C. brunneus* from North Africa form a monophyletic group. *C. muellerianus* and *C. tauricus*, distributed in Southern Europe and Ukraine, respectively, form a monophylum in the Neighbour-Joining and the Maximum Parsimony analysis. In our limited set of only 11 of several hundred *Temnothorax* species, *T. flavicornis* forms the sister group of *Chalepoxenus*. Our study further indicates paraphyly of the genus *Temnothorax* with respect to *Chalepoxenus*. Moreover, the results suggest that speciation in this slave-making genus is possibly caused by the formation of host races as different *Chalepoxenus* species use different hosts, and some samples seem to cluster by host species rather than by geographical distance.

Keywords: Social parasitism, slave-making ants, inquilinism, Formicoxenini, *Chalepoxenus*, *Temnothorax*.

Introduction

Of the roughly 14500 described species and subspecies of ants, a minority of about 3 % are social parasites, which depend on workers from other ant species throughout or at least during part of their life cycle (Hölldobler and Wilson, 1990). The mated queens of permanent social parasites search for and enter suitable host colonies. Whereas the queens of some workerless "inquilines" seek to be adopted in the colony and live alongside the host queen, those of other inquilines and, in particular, those of slavemakers kill or expel the resident queen and, in some species, also all adult workers. Host workers that emerge from the conquered brood care for the parasite queen and her offspring. While inquiline queens solely produce sexual offspring, slavemaker queens also produce workers, which, however, are incapable of performing colony maintenance tasks. Instead, they specialise on raiding neighbouring host colonies for worker pupae that, after their emergence, serve as slaves (Buschinger et al., 1980; Buschinger, 1986; D'Ettorre and Heinze, 2001).

The evolution of social parasites from non-parasitic ancestors and the interrelations among the different types of social parasitism have been discussed extensively for almost 150 years (Darwin, 1859; Wheeler, 1907, 1910; Emery, 1909; e.g., Wasmann, 1909; Viehmeyer, 1910a, b). Thorough molecular phylogenies of social parasites, which allow the elucidation of their evolutionary pathways, have only recently become available (Baur et al., 1993, 1995, 1996; Savolainen and Vepsäläinen, 2003; Steiner et al., 2005; Beibl et al., 2005). The myrmicine tribe Formicoxenini is particularly rich in permanently social parasites, workerless "inquilines", active slavemakers and degenerate slavemakers, workerless species that presumably have evolved from active slavemakers (Buschinger, 1986, 1989; Hölldobler and Wilson, 1990; Stuart, 2002). Whereas several clades of formicoxenine slavemakers are monotypic (*Protomognathus americanus* (Emery, 1895), *Temnothorax duloticus* (Wesson, 1937), *Temnothorax* undescribed species, Beibl et al., 2005) or consist of only two or three species (*Harpagoxenus* Forel,

1893), eight species of active or degenerate slavemakers are currently recognized in the genus *Chalepoxenus* Menozzi, 1923 (Bolton, 1995).

The members of the genus *Chalepoxenus* are distributed in Southern Europe, North Africa, and Western and Central Asia and parasitise colonies of a number of species of the formicoxenine genus *Temnothorax* Mayr, 1861 (Buschinger et al., 1988a; Radchenko, 1989; Buschinger, 1997). Several *Chalepoxenus* species are known only from type material or scattered findings (*C. spinosus* (Arnol'di, 1968), *C. tarbinskii* (Arnol'di, 1976), *C. tauricus* Radchenko, 1989, *C. tramieri* Cagniant, 1983, *C. zabelini* Radchenko, 1989), and only *C. muellerianus* (Finzi, 1922), *C. kutteri* Cagniant, 1973, and *C. brunneus* Cagniant, 1985 have been studied in more detail (Buschinger et al., 1988a, b; Buschinger, 1997). *C. muellerianus* is known from Spain to Turkey. This slave-making ant species predominantly utilizes *T. unifasciatus* (Latreille, 1798), but has also been found with slaves belonging to almost a dozen other *Temnothorax* host species, with different populations specializing mostly on one particular host (Buschinger et al., 1988a). *C. kutteri* is known from sites in France and Spain and predominantly parasitises *T. massiliensis* (Bondroit, 1918) and a few other congeneric species. As in *C. muellerianus*, mixed colonies with workers from different host species are very rare. *C. brunneus* is a workerless species known only from nests of *T. marocana* (Santschi, 1909) at its type locality at Tizi n'Test in Morocco (Buschinger et al., 1988a).

The phylogeny and phylogeography of *Chalepoxenus* has as yet not been investigated in detail. Previous investigations suggested that *Chalepoxenus* is an old genus (Beibl et al., 2005) that forms a monophylum with its formicoxenine host genus *Temnothorax* (Baur et al., 1995, 1996). The aim of our present study therefore was to describe the phylogenetic relationships among different species and populations of the genus *Chalepoxenus* and between *Chalepoxenus* and their various host species by the help of molecular markers. In addition, by contrasting the molecular phylogeny with the host species utilized by the sampled parasite population we wanted to determine whether host races exist in *Chalepoxenus*.

Table 1. Social parasites and their *Temnothorax* (*T.*) host species (modified after Buschinger et al., 1988a).

Chalepoxenus species	Host species
C. muellerianus (Finzi, 1922)	*T. unifasciatus* (73.6%) (Latreille, 1798) *T. recedens* (10.0%) (Nylander, 1856) *T. nigriceps* (6.3%) (Mayr, 1855) *T. flavicornis* (1.3%) (Emery, 1870) *T. exilis* (0.8%) (Emery, 1869) *T. tuberum* (1.0%) (Fabricius, 1775) *T. affinis* (0.3%) (Mayr, 1855) *T. semiruber* (André, 1881) *T. interruptus* (Schenck, 1852) *T. racovitzai* (Bondroit, 1918) / *T. luteus* (Forel, 1874) *T. pyrenaeus* (Bondroit, 1918) *T.* cf. *rottenbergii* (Emery, 1870)
C. kutteri Cagniant, 1973	*T. massiliensis* (Bondroit, 1918) *T. exilis* (Emery, 1869) / *T. specularis* (Emery, 1916) *T. niger* (Forel, 1894) *T. racovitzai* (Bondroit, 1918) *T. berlandi* (Bondroit, 1918) *T. rabaudi* (Bondroit, 1918) *T. unifasciatus* (Latreille, 1798) *T. recedens* (Nylander, 1856)
C. brunneus Cagniant, 1985	*T. marocana* (Santschi, 1909)
C. tauricus Radchenko, 1989	*T. unifasciatus* (Latreille, 1798)
C. tramieri Cagniant, 1985	*T. spinosus* (Forel, 1909)
C. zabelini Radchenko, 1989	?
C. spinosus (Arnol'di 1968)	?
C. tarbinskii (Arnol'di, 1976)	?

Methods

DNA isolation, amplification and sequencing

Our analysis includes a total of 39 specimens (32 haplotypes) from four *Chalepoxenus* species (14 *C. muellerianus*, 1 *C. tauricus*, 3 *C. kutteri,* and 1 *C. brunneus*) and 11 *Temnothora*x host species, which were collected in 10 countries, from Spain and Morocco to Cyprus and Ukraine (Figure 1; Tables 1, 2). *Crematogaster smithi* Creighton, 1950, an ant from outside the Formicoxenini, but within the formicoxenine tribe group (Bolton, 2003), served as outgroup.

High molecular weight DNA was extracted from individual, frozen or ethanol-conserved ants by grinding them in liquid nitrogen and subsequently following a cetyltrimethyl ammonium bromide protocol (Hamaguchi et al., 1993). The dried pellet was dissolved in 40µl purified water (Sigma) and stored at 4°C until analysis. PCR amplifications were conducted in a total volume of 25µl using the primers C1-J-2195 and C2-N-3661 (Simon et al., 1994), MIBI and CW-3031rev (Beibl et al., 2005), and four self-designed primers: CO-684for (5′-CTA ATA TTT ATT ATT TGA GAA GC-3′), CO-841for (5′-GGA CTT AAA CCC CTC TTA-3′), CO-1055for (5′-CAT ACT ATT GAA CTA ATC TGA-3′) and CO-1075rev (5′-TCA GAT TAG TTC AAT AG-3′), which amplify overlapping PCR products of a 1430 bp fragment of the subunits I and II of the mitochondrial gene cytochrome c oxidase (CO I/II). Each reaction mixture contained 1–50 ng DNA, 2.5 µl 10x polymerase buffer (without $MgCl_2$), 2.8 mM $MgCl_2$, 1.4 µM of each primer, 400 µM of each dNTP and 1 unit of *Taq* polymerase (MBI Fermentas). DNA was amplified with a Biometra T1 Thermocycler with the following temperature profile: an initial denaturation step of 4 min at 94°C, followed by 40 cycles at 94°C for 1.15 min, 50°C for 1.15 min, and 68°C for 1.30–2.30 min. A final extension at 72°C for 5 min was then conducted, followed by a soak at 6°C. PCR products were either purified from 1% agarose gels after separation by electrophoresis for 45 min at 100 mA, using NucleoSpin® Extract columns (Macherey-Nagel), or directly using Montage™PCR Centrifugal Filter Devices (Millipore). Sequencing reactions were conducted in a total volume of 20 µl using the Big Dye Terminator Cycle sequencing kit from Applied Biosystems. Each cycle sequencing reaction mixture contained 20–100 ng DNA, 3 µl 5x sequencing buffer, 0.5 µM primers and 2 µl Big Dye ready reaction mix. The cycle sequencing reactions were incubated for 30 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, and stopped by cooling to 6°C. After amplification, the sequencing products were

Table 2. Overview of the sampled specimens, their collection sites, and their CO I/II GenBank accession numbers. *Chalepoxenus* (*C.*) and *Temnothorax* (*T.*) host species were sampled from the same communities when co-occurring. Locality designations correspond to those in Figure 1.

Species	Locality	Designation	Haplotype	Accession Number	Slave species
C. brunneus	Tizi n'Test, Great Atlas, Morocco	1	h14	DQ989251	*T. marocana*
C. kutteri	Sitges, Catalonia, Spain	2	h11	DQ989256	*T. specularis*
	La Selva de Mar, Catalonia, Spain	4	h12	DQ989254	*T. racovitzai*
	El Port de la Selva, Catalonia, Spain	5	h13	DQ989263	*T. racovitzai*
C. muellerianus	Caldes, Catalonia, Spain	3	h7	DQ989255	*T. rabaudi*
	Vaison la Romaine, Provence, France	7	h6	DQ989243	*T. unifasciatus*
	Collet Blanc, Provence, France	8	h3	DQ989262	*T. unifasciatus* (and possibly *T. rabaudi*)
	Mont Ventoux, Provence, France	9	h1	DQ989264	*T. unifasciatus*
	Savoillan, Provence, France	10	h4	AY909573	*T. unifasciatus*
	Calino, near Rovato, Lombardy, Italy	14	h2	DQ989260	*T. unifasciatus*
	Gargnano, Lago di Garda, Lombardy, Italy	15	h1	DQ989257	*T. unifasciatus*
	Tignale, Lago di Garda, Lombardy, Italy	16	h1	DQ989265	*T. unifasciatus*
	Marniga, Lago di Garda, Lombardy, Italy	17	h1	DQ989259	*T. unifasciatus*
	Manerba, Lago di Garda, Lombardy, Italy	18	h1	DQ989258	*T. unifasciatus*
	Baška, Krk, Croatia	20	h5	DQ989249	*T. recedens*
	Colle della Croce, near Barrea, Abruzzi, Italy	21	h1	DQ989261	*T. unifasciatus*
	Anogia, Crete, Greece	22	h8	DQ989252	*T.* cf. *rottenbergii*
	Troodos mountains, Cyprus	23	h9	DQ989284	similar to *T. tuberum* or *T. nigriceps*
C. tauricus	Yalta, Crimea, Ukraine	24	h10	DQ989247	*T. unifasciatus*
T. affinis	Manerba, Lago di Garda, Lombardy, Italy	18	h16	DQ989242	
	Medea, Friuli Venezia Giulia, Italy	19	h17	DQ989278	
T. flavicornis	Manerba, Lago di Garda, Lombardy, Italy	18	h15	DQ989276	
T. luteus	Savoillan, Provence, France	10	h30	DQ989268	
T. nigriceps	Waldenhausen, Baden-Wuerttemberg, Germany	13	h21	AY909567	
T. rabaudi	Villes sur Auzon, Provence, France	11	h18	DQ989279	
T. racovitzai	El Port de la Selva, Catalonia, Spain	5	h32	DQ989270	
	Colle della Croce, near Barrea, Abruzzi, Italy	21	h31	DQ989269	
T. recedens	El Port de la Selva, Catalonia, Spain	5	h27	DQ989275	
	Savoillan, Provence, France	10	h28	DQ989273	
	Manerba, Lago di Garda, Lombardy, Italy	18	h27	DQ989272	
	Baška, Krk, Croatia	20	h26	DQ989271	
T. cf. *rottenbergii*	Anogia, Crete, Greece	22	h19	DQ989280	
T. specularis	Sitges, Catalonia, Spain	2	h29	DQ989281	
T. tuberum	Binntal, Swiss Valley, Switzerland	12	h20	DQ989282	
T. unifasciatus	Savoillan, Provence, France	10	h22	AY909570	
	Calino, near Rovato, Lombardy, Italy	14	h23	DQ989283	
	Gargnano, Lago di Garda, Lombardy, Italy	15	h24	DQ989239	
	Manerba, Lago di Garda, Lombardy, Italy	18	h24	DQ989240	
	Colle della Croce, near Barrea, Abruzzi, Italy	21	h25	DQ989241	
Crematogaster smithi	Chiricahua Mountains, Arizona, USA	–	h33	EF488233	

precipitated, dried, dissolved in 20 µl H_2O, and run on an ABI Prism 310 genetic analyzer.

Phylogenetic analyses

Our study sequences consisted of 789 bp CO I coding region including the stop codon (3′ end of the cytochrome c oxidase subunit I), and 531 bp CO II coding region (5′ end of the cytochrome c oxidase subunit II). The non-coding region including the leucine-tRNA locus between the two subunits CO I and CO II varied in length, could not be aligned with confidence, and for this reason was excluded from the analyses. This intergenic region was considerably longer in *T. racovitzai*, *T. luteus* and *T. specularis*. Sequences of these samples were double-checked and yielded the same results in both cases. CO I and CO II sequences were of same length for all species. The continuous nucleotide sequences were compiled, edited, and aligned using Bioedit 7.0.5.2 (Hall, 1999), adjusted by eye and truncated at the edges to a standard length of the shortest sequence. Nucleotide composition was calculated using MEGA 3.1 (Kumar et al., 2004). The final sequence alignment of

Table 3. Mean distances (Kimura-2) between species groups (below the diagonal) and standard error (above the diagonal) based on CO I/II sequence data.

	C. muellerianus (h1–h9)	*C. tauricus* (h10)	*C. kutteri* (h11–h13)	*C. brunneus* (h14)
C. muellerianus	–	0.0035	0.0068	0.0069
C. tauricus	0.0276	–	0.0075	0.0075
C. kutteri	0.0779	0.0788	–	0.0037
C. brunneus	0.0732	0.0759	0.0187	–

both genes consisted of 1320 base pairs. Haplotypes and GenBank accession numbers are available in Table 2. One double peak was substituted by "Y". Nonetheless, the data appeared to be mitochondrial DNA sequences and not nuclear integrated pseudogene copies, as the CO I and CO II sequences contained no introns, gaps, or stop codons (except the regular CO I stop codon).

Phylogenetic relationships among *Chalepoxenus* and their host species were inferred by a distance method, Maximum Parsimony, and Bayesian analysis. Neighbour-Joining (NJ) trees were constructed in PAUP 4.0b10 (Swofford, 2002) using Kimura's two-parameter model (Kimura, 1980). Bootstrap values were estimated from 5000 replicates. Maximum Parsimony (MP) analysis was conducted using the program PAUP 4.0b10. Trees were found in a heuristic search using default parameters. Branch-swapping was performed by the tree-bisection–reconnection (TBR) method. Deviating from the default settings we used a random addition sequence with ten replications and chose outgroup rooting with the specification that the ingroup was monophyletic. Clade support was evaluated with nonparametric bootstrapping (Felsenstein, 1985) with 2000 pseudoreplicates. The consistency index (CI) and the retention index (RI) are traditionally used to test the robustness of the most parsimonious tree (Farris, 1989a). The values range from 0 to 1 and higher values indicate better fit. These indices were calculated as implemented in PAUP. The RI is independent of tree length (Farris, 1989b), whereas the CI is highly correlated with tree length (Archie, 1989), which is dependent on the number of characters and taxa. In Modeltest 3.7 (Posada and Crandall, 1998), the GTR+I+G model of sequence evolution (a general time reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites) was determined the best-fit evolutionary model for the Bayesian analysis. Bayesian analysis was carried out using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003, 2005). Studies have shown that partitioning data can produce less biased posterior probability estimates and provide a better fit between model and sequence data (e.g. Castoe et al., 2004). In our analysis, data were partitioned by gene and by codon position. Default priors were used and two separate runs were carried out with four simultaneous Markov chains, each starting from a random tree. The analysis ran for 2,000,000 generations to allow both runs to converge, and the chain was sampled every 500th generation (with a total of 4,001 saved trees each run). The first 1,000 trees (25 % as recommended in the manual) were discarded as the "burn-in" before the chains converged on a stable value and the posterior probabilities of tree topology were determined from the remaining 3,001 trees.

A statistical parsimony network based on CO I/II sequences of *C. muellerianus* individuals from 14 localities was constructed using the program TCS 1.21 (Clement et al., 2000). TCS calculates the probability of parsimony for all haplotype pairwise differences until the probability exceeds 95 %. The program generates a network linking closely related haplotypes by the maximum number of mutational differences or steps and leaves all other haplotypes as outgroups. In this way haplotypes are grouped into separate clusters.

Results

Sequence statistics

The sequences of both cytochrome c oxidase subunits could be combined for further analysis, as previously done in other ants, including several Formicoxenini (e.g., Wetterer et al., 1998; Savolainen and Vepsäläinen, 2003; Janda et al., 2004; Heinze et al., 2005). This is justified by similar nucleotide composition (CO I: T 39.5 %; C 18.2 %; A 30.9 %; G 11.3 %; CO II: T 39.2 %; C 19.4 %; A 33.6 %; G 7.9 %) and Modeltest 3.7 yielding GTR+I+G (general time reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites) as best-fit substitution model equally for the CO I data, the CO II data, as well as for the data of both genes combined. The complete 1320 bp fragment of the CO I and CO II gene contained T 39.4 %; C 18.7 %; A 32.0 % and G 9.9 %. Of the 789 nucleotide sites of CO I, 500 characters were constant, 34 were uninformative, and 255 were informative. Of the 531 nucleotide sites of CO II, 263 characters were constant, 52 were uninformative, and 216 were informative. The 1320 bp fragment of CO I and CO II combined contained 763 constant characters, 86 uninformative characters and 471 informative characters.

Among the CO I/II sequences of *Chalepoxenus* samples, the mean distance within the *C. kutteri* haplotypes was 0.004 (± 0.002 SE; Kimura-2 distance). The mean distance within the *C. muellerianus* haplotypes was 0.021 (± 0.002 SE; Kimura-2 distance), whereas the two most distant samples of *C. muellerianus*, h7 and h8, showed a sequence divergence of 0.048 (Kimura-2 distance). Mean distances between species groups are shown in Table 3.

Phylogenetic and phylogeographic analyses

Figure 2A shows a Neighbour-Joining tree based on the combined CO I and CO II sequences with all nodes supported by bootstrap values greater than 80 %. The haplotypes of the four studied *Chalepoxenus* species form a rather well-supported monophylum, and further, a well-supported monophyletic group with the *T. flavicornis* (Emery, 1870) haplotype. The genus *Chalepoxenus* is situated amidst *Temnothorax* and splits into two well-supported sister groups, one comprising *C. tauricus* and all

Figure 1. Map showing sampling localities. For locality names see Table 2.

C. muellerianus haplotypes, the other containing *C. kutteri* and the degenerate slavemaker *C. brunneus*. Within *C. muellerianus*, a substructure seems to exist, with one group comprising haplotypes from Italy and France, which all co-occurred with *T. unifasciatus* hosts, a second group consisting of haplotypes from Croatia, France and Spain, and finally separate haplotypes from Greece, Cyprus, and *C. tauricus* from Ukraine. The phylogenetic relationships towards the host species and within the host species are poorly resolved. The investigated host species form four well-supported groups, one comprising the haplotypes of *T. affinis*, *T. rabaudi,* and *T.* cf. *rottenbergii*, one those of *T. unifasciatus*, *T. nigriceps*, and *T. tuberum*, one only those of *T. recedens*, and one those of *T. specularis*, *T. luteus*, and *T. racovitzai*. Analysing CO I and CO II sequences separately (data not shown), gave a similar, albeit less well supported tree morphology due to shorter sequence length, with the following two, well-supported deviations from the combined tree. In the NJ-tree based on CO I only, the haplotype of *T.* cf. *rottenbergii* was situated within *T. affinis* (bootstrap support value 96), in the NJ-tree based on CO II, the *T. flavicornis* haplotype was grouped with haplotypes h1, h2, h3 and h4 of *C. muellerianus* (bootstrap support value 99).

The MP analysis of all characters resulted in three best trees (Length = 1641; CI = 0.4796; RI = 0.7763). Figure 2B shows the 80% majority-rule consensus tree with bootstrap values estimated from 2000 pseudoreplicates. This tree shows the same topology as the NJ-tree based on CO I/II, except the fact that the group of *T. affinis*, *T. rabaudi* and *T.* cf. *rottenbergii* haplotypes falls apart. In MP analyses based on CO I (Length = 870; CI = 0.4632; RI = 0.7920; 24 trees) and CO II (Length = 713; CI = 0.5386; RI = 0.7907; 4 trees) separately, tree topology was incompletely resolved (data not shown). The major differences in the CO I based consensus tree were that, first, the *T. flavicornis* haplotype did not form a monophylum with *Chalepoxenus*, and second, the sequences of *T. affinis*, *T. rabaudi*, *T.* cf. *rottenbergii*, *T. nigriceps*, *T. tuberum* and *T. unifasciatus* formed a monophyletic group (bootstrap support value 92). The resolution of a MP consensus tree based on CO II was even worse, and the *T. flavicornis* sequence grouped within *Chalepoxenus*, next to h1, h2, h3 and h4 (bootstrap support value 98).

Figure 2C depicts the majority rule consensus tree recovered in the Bayesian analysis. The tree is based on the 1320 bp CO I/II dataset and data were partitioned by codon. As in the other trees, all *Chalepoxenus* sequences form a monophylum, with *T. flavicornis* as sister group. The remaining host species form similar groups as in the other analyses. Further, *C. brunneus* and *C. kutteri* sequences are monophyletic. However, compared to the NJ and MP analysis, the *C. muellerianus* and *C. tauricus* sequences group differently and do not form a monophylum, though these groupings are only supported by posterior probability values of 0.93 in both cases. In a Bayesian analysis based on CO I only, partitioned by codon (data not shown), *C. muellerianus* and *C. tauricus* formed a monophyletic clade (posterior probability 0.76). When the CO I/II data were partitioned by gene (data not shown), tree topology was basically the same, but posterior probability values for the relationships between host species groups were considerably lower. In this analysis, *T. specularis*, *T. luteus*, and *T. racovitzai* formed the sister clade to *Chalepoxenus*, *T. flavicornis* and *T. recedens* with a very low posterior probability value of only 0.55.

For *Chalepoxenus muellerianus*, we constructed a haplotype network using the program TCS, which is especially useful for closely related sequences (Fig. 3). The analysis identified several clusters, most of which were unconnected due to the large genetic distance. One cluster contained h1, h2, h3 and h4 from Italy and France, all from *C. muellerianus* colonies using *T. unifasciatus* as host; another cluster contained h6 and h7 from France and Spain, from colonies parasitising *T.*

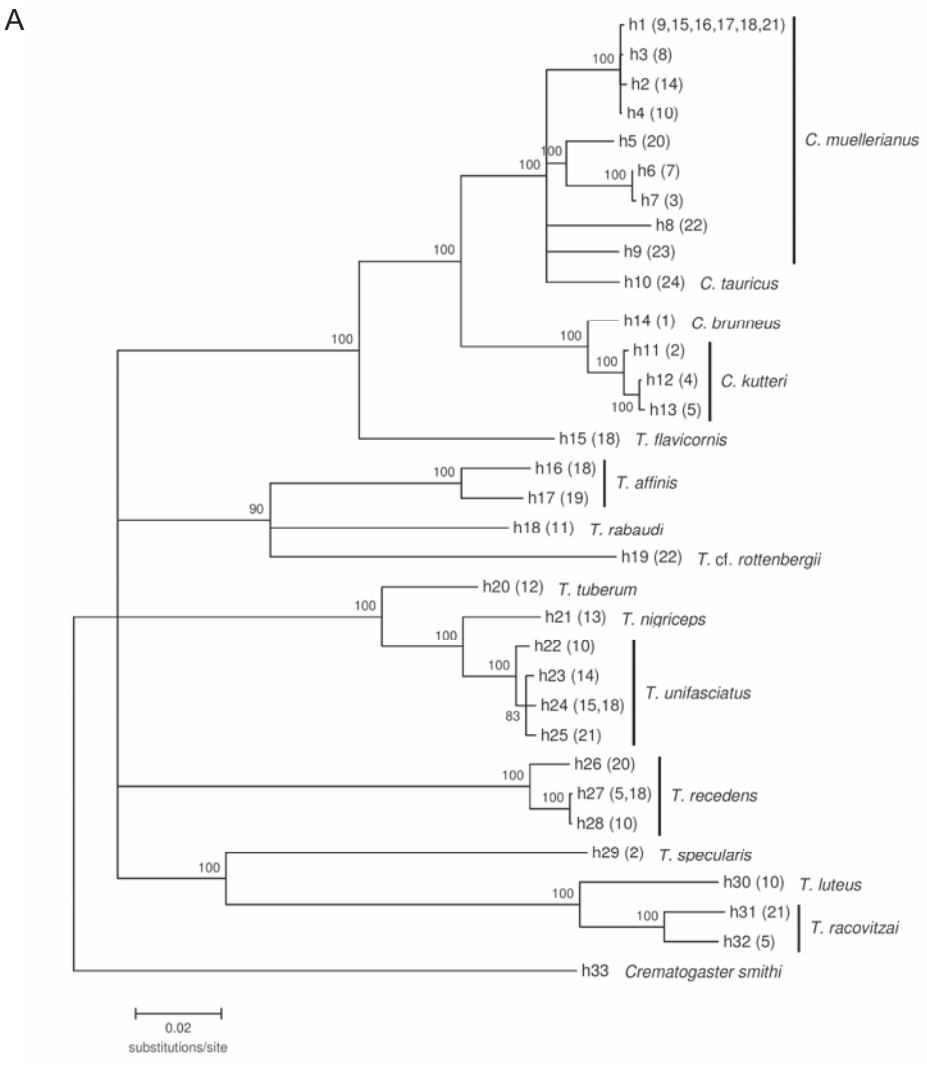

Figure 2. Phylogenetic trees of haplotypes of *Chalepoxenus* (*C.*) and its *Temnothorax* (*T.*) host species, based on 1320 base pairs of the mitochondrial cytochrome c oxidase I and II gene. Sample information is given in Table 2. Locality codes are given in parentheses and refer to Figure 1 and Table 2. A. Neighbour-Joining tree with bootstrap values estimated from 5000 replicates. Bootstrap percentages with values greater than 80 are shown on nodes. B. Maximum Parsimony consensus tree found by heuristic search, and shown with bootstrap percentages (2000 pseudoreplicates) greater than 80%. C. Majority rule consensus tree recovered in a Bayesian analysis (2,000,000 generations, partitioned by codon position). Numbers represent clade credibility values.

unifasciatus or *T. rabaudi,* respectively. The remaining haplotypes h5, h8 and h9 occurred together with three different host species. Kimura-2 distances and geographical (great circle) distances between individual *C. muellerianus* haplotypes are given in Table 4 and Figure 4. A simple Mantel test performed with the program zt (Bonnet and Van de Peer, 2002) shows that in *C. muellerianus* genetic distances are significantly correlated to geographical distances ($r = 0.606239$, $p = 0.001200$ (one-tailed), 10000 randomizations). In a Mantel test based on all *Chalepoxenus* samples (h1–14), genetic distance is also linked to geography ($r = 0.365422$, $p = 0.018798$ (one-tailed), 10000 randomizations). However, most *Chalepoxenus* in our analysis that use different host species are often also geographically distant, and the *C. muellerianus* haplotype network appears to be not only structured by geography. For example, some sample pairs are geographically quite distant and have the same or a similar sequence (e.g. h1; h6–h7), whereas others that are geographically closer are separated by a high number of mutations (e.g. h1–h6; h1–h5).

Discussion

On the whole, all three tree reconstruction methods yielded similar results with only minor deviations. In all three analyses, the sequences of the four *Chalepoxenus* species form a very well-supported monophyletic group with *Temnothorax flavicornis. T. flavicornis* is the only European *Temnothorax* with 11-jointed antennae and only rarely serves as host of *C. muellerianus* (Buschinger et al., 1988a). In previous studies, in which *T. flavicornis* and several species of our study were not included, *C. muellerianus* grouped with *T. interruptus* (Schenck, 1852), *T. unifasciatus*, and *T. nigriceps* (Mayr, 1855) based on 360bp of the mitochondrial cyt b gene (Baur et al., 1995), and with *T. interruptus* based on 380bp of ITS-1 (Baur et al., 1996). The genus *Temnothorax* probably

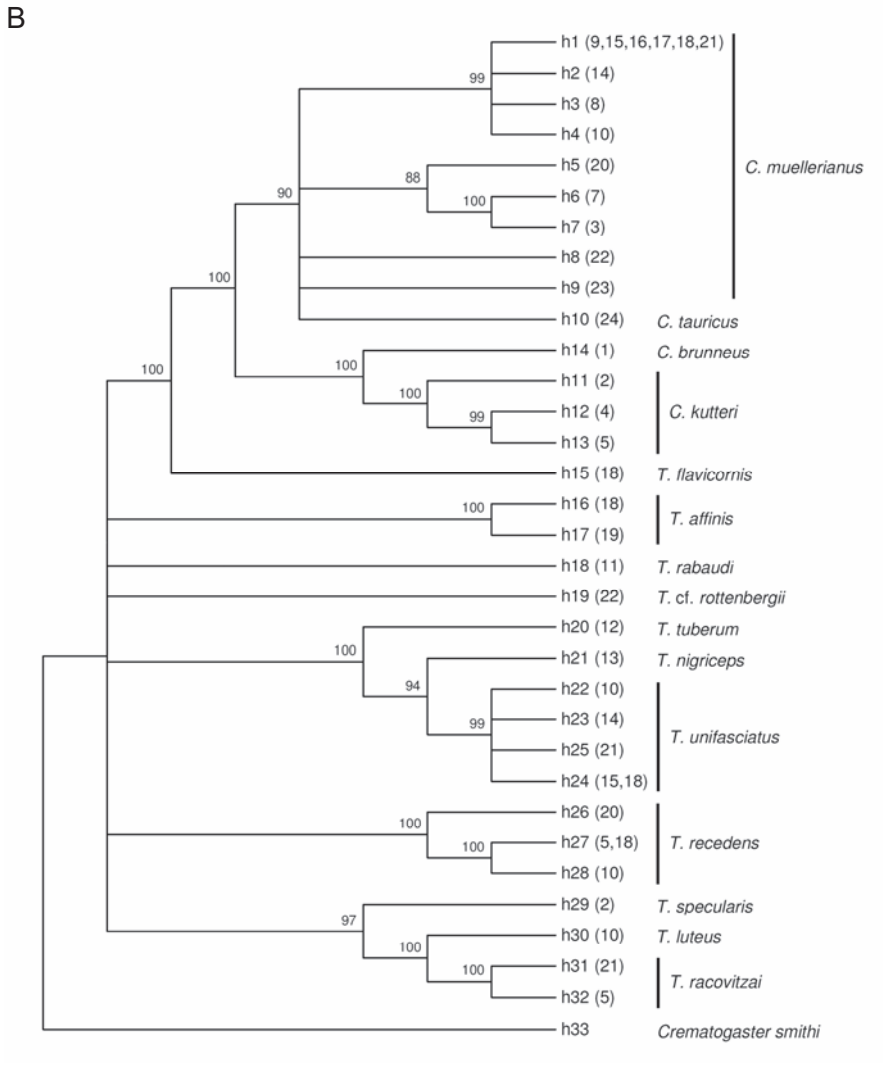

Figure 2. (continued)

Table 4. Kimura-2 distances based on CO I/II sequence data (below the diagonal) and geographical (great circle) distances in kilometres (above the diagonal) between individual *C. muellerianus* haplotypes. Locality codes are given in parentheses and refer to Figure 1 and Table 2.

	h1 (9)	h1 (15)	h1 (16)	h1 (17)	h1 (18)	h1 (21)	h2 (14)	h3 (8)	h4 (10)	h5 (20)	h6 (7)	h7 (3)	h8 (22)	h9 (23)
h1 (9)	–	461	468	470	447	760	407	12	10	758	17	378	1935	2553
h1 (15)	0	–	7	10	18	513	54	450	450	330	470	819	1660	2211
h1 (16)	0	0	–	5	25	516	60	455	455	328	475	826	1661	2208
h1 (17)	0	0	0	–	24	511	62	458	458	324	477	827	1656	2206
h1 (18)	0	0	0	0	–	508	43	438	438	335	456	805	1656	2215
h1 (21)	0	0	0	0	0	–	534	747	751	364	777	984	1184	1805
h2 (14)	0,0008	0,0008	0,0008	0,0008	0,0008	0,0008	–	397	397	379	416	768	1695	2257
h3 (8)	0,0008	0,0008	0,0008	0,0008	0,0008	0,0008	0,0015	–	7	749	30	384	1930	2550
h4 (10)	0,0008	0,0008	0,0008	0,0008	0,0008	0,0008	0,0015	0,0015	–	750	25	387	1930	2550
h5 (20)	0,0279	0,0279	0,0279	0,0279	0,0279	0,0279	0,0287	0,0287	0,0287	–	771	1085	1377	1900
h6 (7)	0,0351	0,0351	0,0351	0,0351	0,0351	0,0351	0,0359	0,0359	0,0359	0,0263	–	374	1954	2576
h7 (3)	0,0359	0,0359	0,0359	0,0359	0,0359	0,0359	0,0367	0,0367	0,0367	0,0271	0,0008	–	2090	2750
h8 (22)	0,0366	0,0366	0,0366	0,0366	0,0366	0,0366	0,0374	0,0374	0,0374	0,0415	0,0472	0,0480	–	730
h9 (23)	0,0327	0,0327	0,0327	0,0327	0,0327	0,0327	0,0335	0,0335	0,0335	0,0384	0,0425	0,0433	0,0358	–

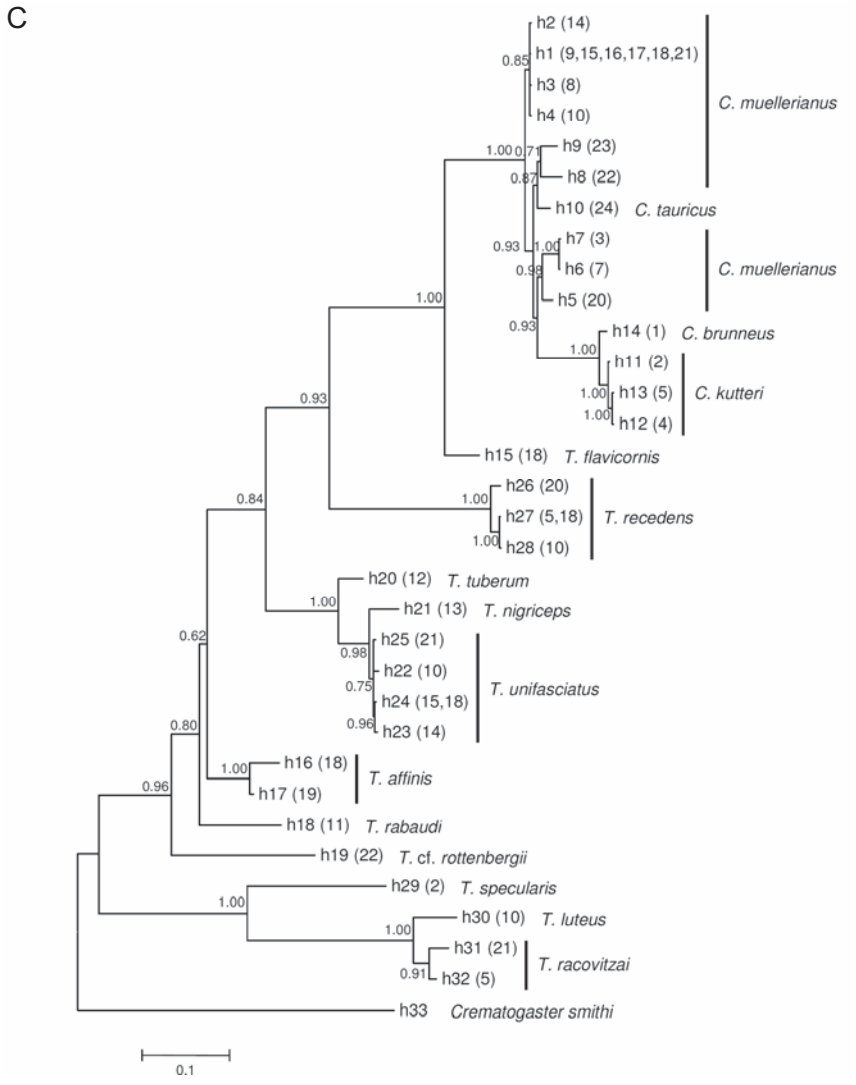

Figure 2. (continued)

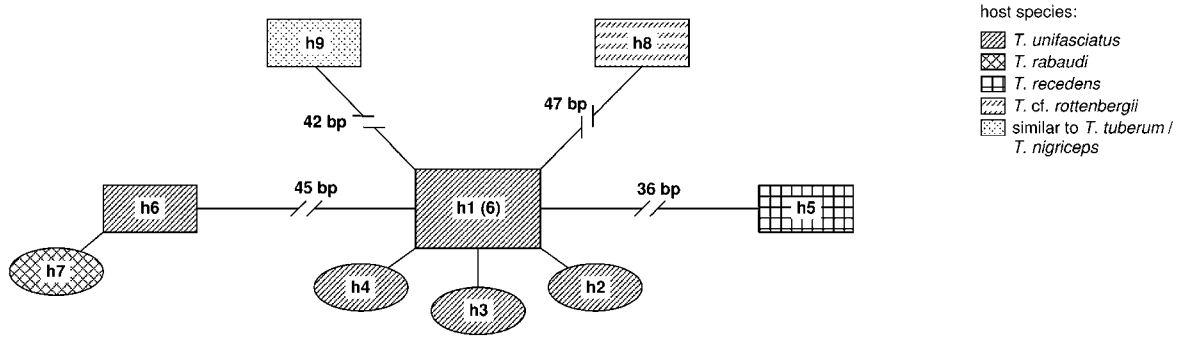

Figure 3. Statistical parsimony network of *C. muellerianus* haplotypes calculated by TCS 1.21 using mitochondrial CO I/II sequences. The haplotype with the highest ancestral probability is displayed as a square, while other haplotypes are displayed as ovals. The identity of the haplotypes is indicated in Figure 2 and Table 2. Numbers in parentheses indicate the observed number of haplotypes. The boxes are shaded according to host species used.

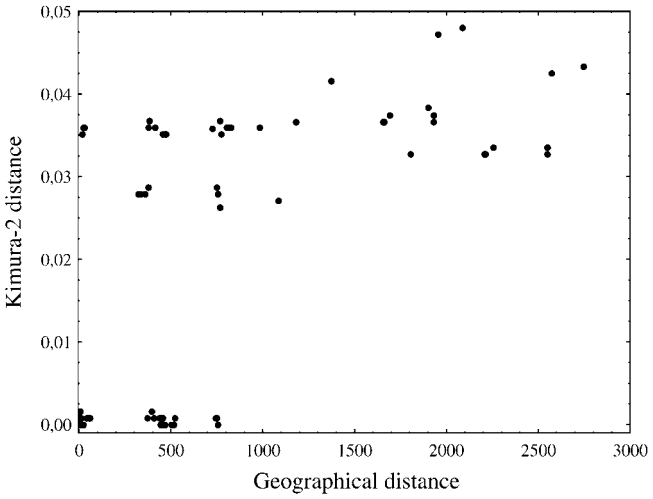

Figure 4. Kimura-2 distances and geographical (great circle) distances (in kilometres) between individual *C. muellerianus* haplotypes.

comprises several hundred species in the Mediterranean area alone (Schulz et al., submitted; A. Schulz, pers. comm.) and any statement on the sister taxon of *Chalepoxenus* is premature as long as only a small percentage of these are included. In the analyses based on CO II alone, *T. flavicornis* is situated within *Chalepoxenus*, but due to short sequence length this result is doubtable. In any case, our study confirms that *Temnothorax* is paraphyletic and that *Chalepoxenus* is nested within this genus (Baur et al., 1996). Given the highly specialised life history of *Chalepoxenus* and the similar paraphyly of the genera *Protomognathus* and *Myrmoxenus* Ruzsky, 1902 within *Temnothorax*, we strongly suggest keeping the social parasites as separate genera.

In all analyses, *Chalepoxenus* constitutes a monophylum with 100 percent support. In the NJ and the MP analysis, this monophylum contains two sister groups, one comprising *C. muellerianus* and *C. tauricus* with a more Central and Eastern Mediterranean distribution, and the other with the degenerated slavemaker *C. brunneus* from Morocco and *C. kutteri* from Spain and France. In the Bayesian tree, *C. brunneus* and *C. kutteri* are also monophyletic, whereas *C. muellerianus* and *C. tauricus* are not monophyletic apparently due to sequence divergence in the CO II region. The genetic distance between *C. tauricus* and *C. muellerianus* is smaller than between the two most divergent samples presently recognized as *C. muellerianus*. *C. tauricus* might therefore either be synonymous to *C. muellerianus* or *C. muellerianus* might in fact consist of several independent taxa. Experiments showed that mating between sexuals of *C. muellerianus* and *C. kutteri* is possible, and that crossbred queens produce viable hybrid offspring (Ehrhardt, 1987, 2004). Further, in crossbreeding experiments, *C. muellerianus* females mated with males of workerless *C. brunneus* produced female sexuals, males, and even workers (Ehrhardt, pers. comm.). These studies confirm that the *Chalepoxenus* species are indeed very closely related, and that genetic isolation between species is not yet very pronounced. Undoubtedly, the genus is in the process of speciation.

Our samples of *C. muellerianus* reflect a certain substructure and division into genetically different clades. One group is distributed in Italy and Southern France, another one in Northern Spain, Southern France and Croatia. Kutter (1973) and Buschinger et al. (1988a) considered *Chalepoxenus gribodoi* Menozzi, 1923, *C. insubricus* Kutter, 1950, and *C. siciliensis* Kutter, 1973 as junior synonyms of *C. muellerianus*, but future research might reveal that *C. muellerianus* is indeed a group of a number of closely related, perhaps incipient species.

Population structure is the result of both present processes and past history. The range of palearctic species has repeatedly undergone contractions and expansions during the course of Pleistocene climate changes, and their present distribution and genetic variation reflects re-colonisation of glaciated areas from southern refugia (Hewitt, 1996, 1999, 2004). The haplotype distribution of *C. muellerianus* might therefore reflect historic patterns, but might also result from the formation of host races. Mixed slave stocks have been found in only 3.4 % of the *C. muellerianus* colonies and 2.4 % of the *C. kutteri* nests, although both species parasitise several potential host species and different populations seem to specialize on different hosts (Buschinger et al., 1988a). This preference for a single host species probably is caused by imprinting of young slavemaker queens and workers on the odour of the host present in their nests (Schumann and Buschinger, 1994, 1995). We have recently shown that rearing sexual pupae of *C. muellerianus* with different host species negatively affects the frequency of interactions among adult male and female sexuals. Imprinting on a particular host species might therefore lead to decreased gene flow and eventually to speciation (Beibl et al., in press).

On a first glance, our sequence data appear to support such a pathway: *C. brunneus* parasitises *T. marocana*, *C. kutteri* parasitises *T. massiliensis*, *T. specularis* (Emery, 1916) and *T. racovitzai* (Bondroit, 1918), *C. tauricus* from Crimea and *C. muellerianus* from Italy and France parasitise *T. unifasciatus*, *C. muellerianus* samples in this study from Krk *T. recedens* (Nylander, 1856) (but also see Buschinger et al., 1988a for Croatia), and the remaining *C. muellerianus* samples three further host species. However, a sample of *C. muellerianus* from Vaison la Romaine contains *T. unifasciatus* but otherwise clusters with specimens using other host species. This might indicate that rare host switches are possible, although adaptation to local host species is the rule. Our results therefore suggest that speciation in this slave-making genus is possibly caused by host race formation. In contrast to *Chalepoxenus*, the species-poor slavemaker clades *Harpagoxenus* from Eurasia and North America and *Protomognathus americanus* from North America simultaneously parasitize a small number of host species over a large geographical range and mixed slave stocks

are more common (e.g., Buschinger et al., 1988a; Heinze et al., 1992; Foitzik et al., 2003; Fischer-Blass et al., 2006). Host races have neither been found in *H. sublaevis* (Nylander, 1849) nor in *P. americanus* (Brandt and Foitzik, 2004; Brandt et al., 2007). The European *H. sublaevis* shows pronounced geographical structuring and low genetic variation (Brandt et al., 2007), whereas we found large genetic differences within *C. muellerianus*.

Mitochondrial genes certainly reveal only one facet of evolution (Avise, 2000) and mtDNA analysis may be flawed due to incomplete lineage sorting, hybridisation, introgression (Shaw, 2002; Machado and Hey, 2003; Ballard and Whitlock, 2004), and copies integrated into the nuclear genome (Numts, e.g., Bensasson et al., 2001). We did not discover any evidence for Numts. Furthermore, hybridisation or incomplete lineage sorting are unlikely because our samples formed groups in accordance with morphology. Nevertheless, our study provides only a first step towards a complete phylogeny and phylogeography of this fascinating ant genus.

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