RADIATION IN SOCIALLY PARASITIC FORMICOXENINE ANTS

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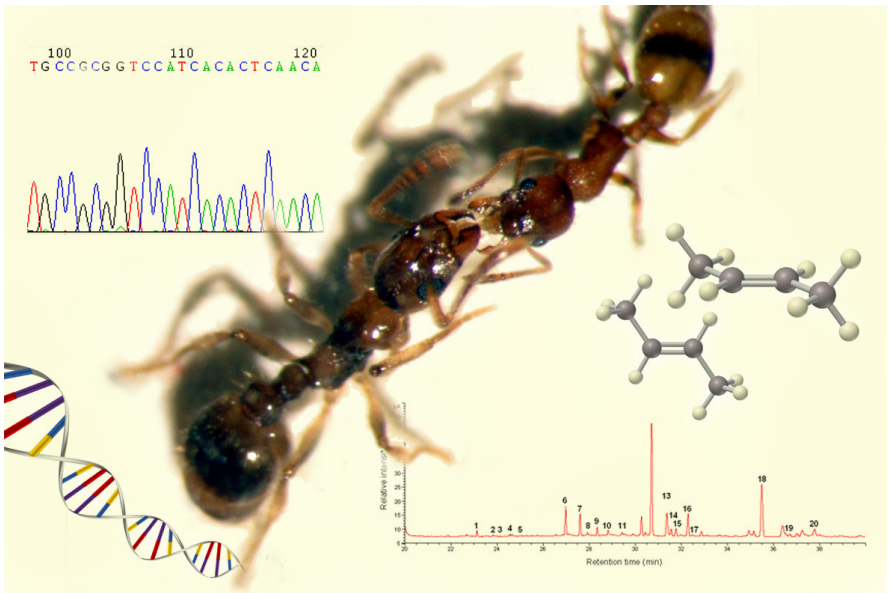

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GENERAL INTRODUCTION

Parasitism is an extremely successful mode of life and is considered to be one of the most potent forces in evolution. As many degrees of symbiosis, a phenomenon in which two unrelated organisms coexist over a prolonged period of time while depending on each other, occur, it is not easy to unequivocally define parasitism (Cheng, 1991). In biology, the term has been used do describe an intimate relationship between two species, in which one, the parasite, lives on or at the expense of another, the host. This implies that one of the partners benefits while the other is harmed, with the effects on the host ranging from slight injury to complete destruction. Most animals and plants harbour a number of parasites, and depending on the definition used, parasites are viruses, bacteria, protozoa, fungi, metazoa or even genetic elements (e.g. bacteriophages, plasmids, ultraselfish genetic elements) (Toft et al., 1991; Schmid-Hempel, 1998; Poulin and Morand, 2000; Majerus et al., 1996; Freeman and Herron, 1998).

A special case of parasitism is the exploitation of the work of social animals. "Social parasites" take advantage of interactions between members of a social host species to their detriment. Social parasitism has been defined as the coexistence of two species of eusocial insects in the same nest, one of which is parasitically dependent on the other, at least during part of its life (Buschinger, 1986; Hölldobler and Wilson, 1990). Social parasitism can predominantly be found in the Hymenoptera, in bees (e.g. *Psithyrus*), wasps (*Sulcopolistes* and others), and ants. In ants, this way of life is especially widespread and occurs in a variety of manifestations (Buschinger, 1994).

In the family Formicidae, several hundreds of the almost 12000 described species exhibit a parasitic lifestyle and depend on the help of already established colonies. Four basic types of parasitic relations between ants are distinguished: xenobiosis, temporary parasitism, permanent parasitism without dulosis, and permanent parasitism with dulosis. In Hölldobler and Wilson (1990), xenobiosis is hypothesized to be a possible intermediate stage to inquilinism, a form of permanent parasitism. The so-called guest ants live together with usually unrelated host species in the same nest, keeping their own brood strictly segregated from the host's brood. They depend on the host only with respect to nutrition and use the host's social system in order to steal food, usually by soliciting regurgitation. The formicoxenine genus *Formicoxenus* is the classic example of xenobiosis and comprises several species of guest ants, which live within the nest material of their much bigger hosts belonging to the genera *Myrmica* and *Formica*. In temporary parasitism, a symbiosis that was

first recognized by Wheeler (1904), the parasitic species is dependent upon its host only during colony foundation. Usually, after her nuptial flight, the fecundated parasite queen tries to enter a host colony by force or conciliatory behaviour. Upon entry, she kills or in another way replaces the resident queen and starts to reproduce. Her worker force develops, and gradually the host workers die out, until finally a pure colony of the parasitic species is formed. Temporary parasitism occurs in the subfamily Dolichoderinae, in the Myrmicinae (but not in the Formicoxenini), and most frequently in the Formicinae (e.g. several species of wood ants of the *Formica* group). The third type, inquilinism or permanent parasitism without dulosis, includes a wide range of lifestyles which may have different evolutionary origins. Inquilinism clearly is the most frequent form of social parasitism and has been found in the subfamilies Myrmeciinae, Formicinae, Myrmicinae, and only recently in the Ectatomminae (Hora et al., 2005). Common to all inquilines is the fact that they spend their entire life in the nest of the host species. The queens of some inquiline species kill the host queen(s) or replace her otherwise, whereas others permit the host queen to stay alive and produce the workers of the colony. Parasite workers may be present, but usually they are rare or completely absent, and the parasitic queen only or predominantly produces sexual offspring. In cases where the host queen is killed, the colony logically perishes with the last host workers (Buschinger, 1986, 1989, 1994; Hölldobler and Wilson, 1990).

As this work primarily deals with dulotic species, this lifestyle is discussed more thoroughly in the following. Dulosis or slavery is a form of permanent parasitism that combines parasitic colony foundation and slave raiding. Thus, slave-making ants are parasitic and dependent upon the host species throughout their whole lifetime. Young mated slave-maker queens establish a new colony similarly to temporary parasites. After their mating flight, they penetrate the nest of a suitable host species, kill or expel the resident queen and in most cases also dispose of the adult workers. From the captured brood, their first slave workers emerge and then care for the parasitic queen and her brood. First, a number of slavemaker workers is produced. These workers are often unable to forage, to feed larvae, to maintain the nest, or even to eat by themselves. On the other hand, they are specialized in fighting: during highly organized slave raids, the slavemaker workers go out, localize and attack neighbouring nests of their host species, capture brood stages, and bring them back to their own nest, where those raided pupae later eclose and become functional members of the slavemaker colony, performing all tasks in the nest. Usually, the slavemaker workers are differently equipped depending on the species, either with sabre-shaped mandibles (e.g. *Polyergus*, *Strongylognathus*), broad heads with strong mandibles (e.g. *Harpagoxenus*,

Protomognathus), a stout and well developed sting (e.g. *Chalepoxenus*), or special glandular secretions for confusing and chasing away opponents (*Raptiformica*). The organisation of slave raids is highly specific and different in the various genera. Single scouts search for suitable host nests. Then they recruit other slavemaker workers from their maternal nest, either individually, for example by tandem-running (e.g. *Harpagoxenus*, *Chalepoxenus*), or in groups by placing an odour trail (e.g. *Myrmoxenus*, *Polyergus*). In this way, the slave stock may be replenished and enlarged a number of times a year, and thus, a slavemaker colony can survive up to 15 years and produce young parasite queens and males besides the workers. Dulosis has evolved independently several times among the Formicinae (*Rossomyrmex*, *Polyergus*, *Raptiformica*) and especially among the Myrmicinae: the genus *Strongylognathus* in the tribe Tetramorini and six genera in the tribe Formicoxenini. Further, so-called degenerate slavemakers exist; these are dulotic species with a worker caste that is reduced or completely absent (e.g. some species of the genus *Myrmoxenus*, *Chalepoxenus brunneus*) (Buschinger, 1986, 1989, 1994; Hölldobler and Wilson, 1990; D'Ettorre and Heinze, 2001; Beibl et al., 2005).

Parasitic forms represent only a small fraction among the approximately 12000 described species of ants. However, it is expected that the number of detected parasites will continue to increase as most of them are rare and only locally distributed. This holds true especially for the advanced social parasites, namely dulotic and inquiline species. Most of the known parasitic species that have been recorded stem from the temperate areas of Europe, North America and South America. On the one hand, this might reflect a certain bias in ant collecting because the tropical ant fauna is still poorly known. On the other hand, several factors possibly favour the evolution of social parasitism; in general, those factors may include cooler temperatures, polygyny (the occurrence of multiple laying queens in one nest), polydomy (the spread of a colony to multiple nest sites), a high population density, or the flexibility of early learning of brood labels (Hölldobler and Wilson, 1990; Buschinger, 1986). Concerning dulosis, three behavioural traits have been suggested to have led to the evolution of slavery: predation (Darwin, 1859), territoriality (Wilson, 1975; Alloway, 1980; Stuart and Alloway, 1982, 1983), and polydomy with brood transport combined with polygyny (Buschinger, 1970). Starting with Darwin (1859), the origin and evolution of social parasitism as well as the connections between the various parasitic life histories have been debated expansively for nearly 150 years now (e.g. Viehmeyer, 1910a, b; Wasmann, 1909; Wheeler, 1907, 1910). In 1909, Emery formulated what was later called "Emery's rule": he suggested that "the dulotic ants and the parasitic ants, both temporary and permanent, generally originate

from the closely related forms that serve them as hosts." The parasite may either have evolved from the host species directly, or it may parasitize a very closely related species with a compatible communication, similar feeding behaviour and so on. Emery's generalization seems correct above all for the intimate forms of social parasitism, and exceptions to this rule exist mostly in rudimentary associations like xenobiosis (Hölldobler and Wilson, 1990). In the recent past, it has been discussed whether social parasites more likely originate by geographic speciation (Wilson, 1971) or by sympatric speciation (Buschinger, 1986; Bourke and Franks, 1991). Moreover, Hölldobler and Wilson (1990) present a scheme of the evolutionary pathways of social parasitism in ants, compiled from contributions by Wheeler (1904, 1910), Emery (1909), Escherich (1917), Stumper (1950), Dobrzański (1965), Wilson (1971), Buschinger (1986) and themselves (Figure 1).

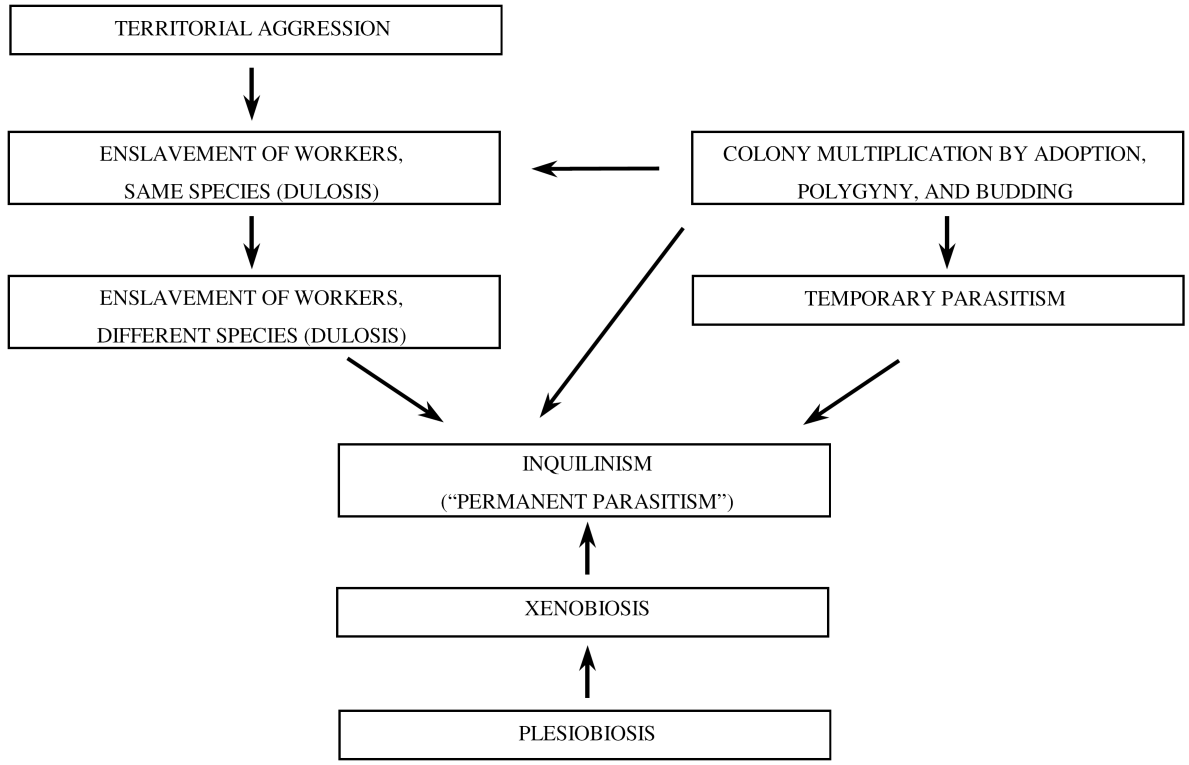

Figure 1. Hypothetical evolutionary pathways of social parasitism in ants (modified after Hölldobler and Wilson, 1990, p. 450).

Despite a great interest in the evolution of social parasitism in ants, comparatively little has been done to investigate the ecological, behavioural and genetic factors involved, and only recently, through improvements of the methodology, detailed molecular phylogenies of social parasites have become available (e.g. Baur et al., 1993, 1995, 1996; Savolainen and Vepsäläinen, 2003; Beibl et al., 2005; Steiner et al., 2005). The phylogenetic distribution of

the advanced forms of social parasitism across the subfamilies is surprising. Interestingly, these social parasites are mainly known in the Formicinae and Myrmicinae, and they are concentrated in particular genera, among them *Leptothorax* and *Temnothorax*. Until now, it is unknown why social parasites thrive in certain clades whereas they lack in others, for instance in the large subfamily Ponerinae. The myrmicine tribe Formicoxenini (formerly Leptothoracini Emery, 1914) apparently represents one of the hot spots of social parasite radiation. It is extremely rich in social parasites and contains about 15% of all known parasitic ants (Hölldobler and Wilson, 1990). Among the formicoxenine lineages, slavery has evolved six times independently (Beibl et al., 2005), inquilinism a number of times (Buschinger, 1981; Hölldobler and Wilson, 1990), and xenobiosis once (Francoeur et al., 1985). The Formicoxenini have a global distribution and include about 500 species in 22 genera (plus two genera incertae sedis). The tribe comprises five genus groups: the *Leptothorax* group (*Cardiocondyla*, *Formicoxenus*, *Harpagoxenus*, *Leptothorax*), the *Temnothorax* group (*Chalepoxenus*, *Myrmoxenus*, *Protomognathus*, *Temnothorax*, *Ochetomyrmex*), the *Nesomyrmex* group (*Atopomyrmex*, *Gauromyrmex*, *Nesomyrmex*, *Xenomyrmex*), the *Podomyrma* group (*Dilobocondyla*, *Peronomyrmex*, *Podomyrma*, *Teratataner*), and the *Romblonella* group (*Poecilomyrma*, *Romblonella*, *Rotastruma*, *Vombisidris*, *Stereomyrmex*) (Bolton, 2003). Formicoxenine colonies are usually small, containing up to 400 adult individuals. Queens and workers are tiny and measure about 2-5 mm. Their nests often consist of one-chamber cavities in rock crevices or wood and are accessible without difficulty. The population density of the host species can be high, and parasites can occur in up to 10% (although often much less) of the host colonies in a given locality (e.g. Buschinger, 1968c, 1987).

Within the Formicoxenini, surprisingly different degrees of diversification exist. This work mainly focuses on the six slave-making genera in the tribe. The monophyletic groups *Protomognathus americanus* (Emery, 1895), *Temnothorax duloticus* (Wesson, 1937) and a yet undescribed *Temnothorax* species consist of only one taxon each. *P. americanus* is distributed in North America and enslaves three host species, *Temnothorax longispinosus*, *T. curvispinosus* and *T. ambiguus*. *T. duloticus* from North America parasitizes the same three hosts as *P. americanus*. And the undescribed Nearctic *Temnothorax* slavemaker uses *T. longispinosus* and *T. ambiguus* as hosts. In contrast to the single-species monophyla, the genus *Harpagoxenus* Forel, 1861 comprises three species, *Chalepoxenus* Menozzi, 1922 includes eight species of active or degenerate slavemakers, and in the genus *Myrmoxenus* Ruzsky, 1902 even twelve species of active and degenerate slavemakers are currently

recognized (Bolton, 2003). Two *Harpagoxenus* species, *H. sublaevis* (Nylander, 1849) and *H. zaisanicus* Pisarski, 1963 are distributed in Eurasia, parasitizing colonies of the *Leptothorax* species *L. acervorum*, *L. muscorum* and *L. gredleri*, whereas *H. canadensis* Smith, 1939 has been found in North America and Canada with its host species *L. canadensis* and *L.* species A. As this work primarily deals with *Chalepoxenus* and *Myrmoxenus*, these two genera are described in detail later. Numerous studies, for instance by Buschinger and co-workers, tried to shed light on the special life histories of socially parasitic Formicoxenini. In addition, several authors tried to unveil the phylogenetic relationships of formicoxenine parasites and their hosts by the use of molecular methods (Douwes and Stille, 1987; e.g. Baur et al., 1995; Heinze, 1991, 1995). But still, at the start of this project, the causes underlying the variation of intra-lineage diversity of socially parasitic formicoxenine ants could not be clarified. Thus, the availability of qualitatively better genetic markers, ameliorated molecular methods and improved general concepts on radiation (e.g. Schluter, 2000) today holds out the prospect of elucidating the question of the origin of formicoxenine parasite diversity after all. Speciation in the context of radiation is regarded as a key process in the creation of organismic diversity, and radiations are an important source of biodiversity. In biology, radiation describes the near-synchronous divergence from a common ancestor into many species of divergent forms (Carlquist, 1974).

Several factors might influence the species diversity among socially parasitic taxa in the tribe Formicoxenini. In this work, I concentrated on the history of the association between parasite and host as well as on the age of several parasitic lineages, on the geographical distribution patterns of several parasitic lineages, and on the chemical basis of colony odour and its role in the parasite's mate choice and in the formation of host races. One hypothesis is that the various taxa of social parasites might differ in their age. Thus, monophyla that consist of only one single species which is morphologically similar to its hosts and has a limited geographical distribution might have evolved more recently than taxa that contain several species which are more widely dispersed and possess special morphological features. Another hypothesis is that the diversity of social parasites might result from the diversity and geographical distribution of their host or ancestor species. Taxa parasitizing a high number of host species with particular parasitic species being specialized on a small subset of the potential host species of the entire genus might show higher degrees of diversification than taxa that are dependent on a small number of host species over large geographical areas where no other suitable hosts are present. A third hypothesis is that imprinting of the parasite on the odour of a particular host species might drive the radiation of this parasite taxon. The

preference for a certain host might lead to the formation of host races with several parasitic species specializing on single host species, followed by restricted gene flow and diversification of the parasite. In order to investigate the variation in diversity in the dulotic Formicoxenini, I established a phylogeny of formicoxenine social parasites, *Chalepoxenus*, and *Myrmoxenus* based on mitochondrial DNA sequences (chapters 1-3), and used behavioural studies and gas chromatography / mass spectrometry to investigate the influence of the host odour on the mate choice of *Chalepoxenus* sexuals as well as the influence of the slaves on the cuticular profile of *Chalepoxenus* workers and vice versa (chapters 4, 5).

As already mentioned, this work mostly focuses on the genus *Chalepoxenus* (chapters 2, 4, 5), and to a lesser extent on the genus *Myrmoxenus* (chapter 3). Therefore, these two genera will be introduced in the following. *Myrmoxenus* is essentially a dulotic genus with active and degenerate slavemakers. It presently comprises twelve species: *M. adlerzi* (Douwes, Jessen & Buschinger, 1988), *M. africanus* (Bernard, 1948), *M. algerianus* (Cagniant, 1968), *M. bernardi* (Espadaler, 1982), *M. birgitae* (Schulz, 1994), *M. corsicus* (Emery, 1895), *M. gordiagini* Ruzsky, 1902, *M. kraussei* (Emery, 1915), *M. ravouxi* (André, 1896), *M. stumperi* (Kutter, 1950), *M. tamarae* (Arnol'di, 1968), *and M. zaleskyi* (Sadil, 1953). Figure 2 exemplarily depicts two species of *Myrmoxenus*, *M. ravouxi* and *M. kraussei*.

Figure 2. *M. ravouxi* queen, *M. ravouxi* worker, and *M. kraussei* queen (from left to right).

The known range of *Myrmoxenus* is widely coincident with that of *Chalepoxenus*. The *Myrmoxenus* species are all distributed in the south-western part of the Palaearctic region, above all around the Mediterranean, throughout Central and Southern Europe, to Georgia (*M. tamarae*) and Kazakhstan (*M. gordiagini*), in North Africa (*M. africanus*, *M. algerianus*, *M. kraussei*) and on the Canary Islands (*M. birgitae*). They are relatively host-specific and parasitize species of the genus *Temnothorax*. A summary of the most relevant aspects of the genus *Myrmoxenus* is given by Buschinger (1989), and the biology and behaviour of these

species were studied primarily by Buschinger and co-workers. Apart from that, the works of Cagniant (1968a, b), Mei (1992), Espadaler and Restrepo (1983) and Espadaler (1997) deserve mention. Species of this genus exhibit a stepwise transition from active slavery with organized slave raids, group recruitment and sting fighting (e.g. *M. ravouxi*) to workerless degenerate dulosis (e.g. *M. corsicus*), including several intermediate stages (e.g. *M. kraussei*). The young *Myrmoxenus* queens always strangle the host queen during colony foundation instead of co-existing with her, as is usual in true inquilinism. In parallel, a reduction of normal swarming behaviour to intranidal mating and continuous inbreeding has been observed.

Chalepoxenus (Greek: "nasty guest") is a genus of parasitic ants with several closely related species of active slavemakers and one degenerate slavemaker. After the first observation of parasitic behaviour (Menozzi, 1922), all *Chalepoxenus* species described later on (with exception of *C. spinosus* where only sexuals have been collected) were found to live with one or more host species. Le Masne (1970b) showed the slave-making behaviour in *C. kutteri*, and the dulotic status of *C. muellerianus* was demonstrated by Ehrhardt (1982) in laboratory experiments, and later observed in the field by Schumann (1992). Buschinger et al. (1988a) provide a detailed review and summary of the literature, range and slave species of the genus. At present, eight species of *Chalepoxenus* are recognized (Bolton, 1995). They occur around the Mediterranean (e.g. in Southern Europe, North Africa, Western Asia) and Central Asia, where they parasitize a number of potential host species of the formicoxenine genus *Temnothorax*, including some which also serve as hosts for *Myrmoxenus* (Buschinger et al., 1988a; Buschinger, 1997; Radchenko, 1989). Several *Chalepoxenus* species are only rarely found, represented by one or two records each, and very little is known about their life histories: *C. spinosus* (Arnol'di, 1968) from Kazakhstan, *C. tarbinskii* (Arnol'di, 1976) from Kyrgyzstan, *C. tauricus* Radchenko, 1989 from Ukraine, *C. tramieri* Cagniant, 1983 from Algeria and Morocco, and *C. zabelini* Radchenko, 1989 from Turkmenistan. In contrast, *C. brunneus* Cagniant, 1985, *C. kutteri* Cagniant, 1973, and especially *C. muellerianus* (Finzi, 1922) have been collected and studied more extensively. Colonies with queens and/or sexuals of *C. brunneus* have been collected at Tizi-n'Test, a small site in the Great Atlas of Morocco. This parasitic species apparently has reached the stage of a workerless, degenerate slavemaker, convergently to some *Myrmoxenus* species. *C. brunneus* parasitizes *Temnothorax maroccanus*. Buschinger et al. (1988b) showed that the *C. brunneus* queen stings to death the host queen and part of the host workers, and afterwards is accepted by the residual workers of the colony. In the laboratory, the production of sexuals was female-biased, and mating is

suggested to take place near the maternal nest without the involvement of a female sexual pheromone. *C. kutteri* is known from France and Southern Spain, where it mainly parasitizes *T. massiliensis*, with *T. exilis* (including *T. specularis*) being a second important host species. Most colonies contain just one slave species and mixed slave populations are rare in *C. kutteri*. This species has been examined by several authors (e.g. Le Masne, 1970a; Cagniant, 1973; Buschinger et al., 1988a; Espadaler and Restrepo, 1983; Ehrhardt, 1987; Tinaut et al., 2005). *C. muellerianus*, of which several hundred colonies have been collected, is examined best and occurs in Spain, France, Switzerland, the former Yugoslavia, Bulgaria, Greece and Turkey. This yellowish-brown species has a surprisingly wide range of at least 12 host species but various populations are obviously specialized on one particular slave species each. The predominant host *Temnothorax unifasciatus* was found in about ¾ of the samples, while slaves of the second most important host species *T. recedens* were present in about 10% of all colonies investigated. As in *C. kutteri*, mixed colonies with slaves from different host species are extremely rare (Buschinger et al., 1988a). This kind of host specificity is suggested to be mediated at least to some extent by imprinting (Schumann and Buschinger, 1994, 1995). Figure 3 shows a *C. muellerianus* worker and workers of the two main host species *T. unifasciatus* and *T. recedens*.

Figure 3. Workers of *C. muellerianus*, *T. unifasciatus*, and *T. recedens* (from left to right).

Different *Chalepoxenus* species and populations have been found from elevations near sea level up to high altitudes (2000 m) (Buschinger et al., 1988a). They nest mostly in cavities of dry stone walls, in crevices of limestone rocks, in rotten sticks of wood or sometimes under the bark of pine trees. Figure 4 shows various types of habitats.

Figure 4. Different *Chalepoxenus* habitats: dry stone walls and pine trees on the island of Krk (Croatia), limestone rocks in the Abruzzi mountains (Italy), and a stony slope in Sitges (Spain) (from left to right).

In chapters 1 to 3, mitochondrial DNA sequences serve to establish phylogenies of formicoxenine parasites, and of the genera *Chalepoxenus* and *Myrmoxenus*. Animal mitochondrial DNA (mtDNA) is a small, circular molecule, usually about 16 kb long. It generally contains 13 protein-encoding genes, two ribosomal RNA genes, 22 tRNA genes, and a control region with the origin of replication (Avise et al., 1987; Moritz et al., 1987; Harrison, 1989; Crozier, 1990). The characteristics that have attracted evolutionists to mitochondrial DNA are its presence in high copy numbers, its rapid evolution, its maternal and clonal inheritance, and its lack of recombination (Avise, 1991, 1994; Simon et al., 1994). Another advantage is that many regions are conserved so that primers work in different insect taxa (Roderick, 1996). Among the protein-encoding genes of the mtDNA, there are genes for cytochrome oxidase subunits I and II (CO I and CO II). Analyses indicated that their A+T content is higher in the honeybee *Apis mellifera* than in *Drosophila*, and that the long-term evolutionary rates differ in hymenopteran and dipteran mtDNA (Crozier et al., 1989). In numerous studies, CO I and CO II sequences have been used to study phylogenetic relationships in insects (e.g. Beckenbach et al., 1993; Brower, 1994; Brown et al., 1994; Heinze et al., 2005). In chapter 1, a 1386 bp sequence of the mitochondrial cytochrome oxidase subunits I and II was used in phylogenetic analyses to document different degrees of genetic divergence between different monophyla of slavemakers and their host species. Our study also revealed a sixth independent origin of slavery in a yet undescribed Nearctic *Temnothorax* ant. This species is morphologically similar to *T. duloticus*. Both slavemakers are depicted in Figure 5.

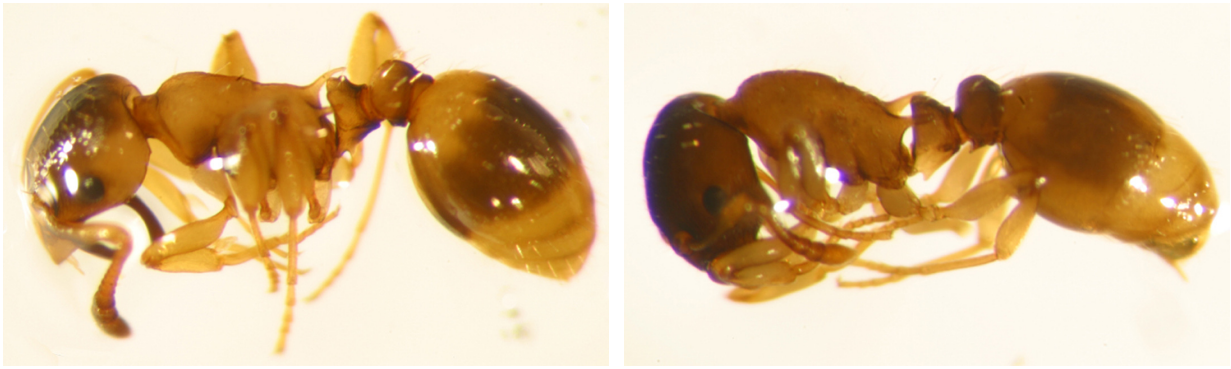

Figure 5. *Temnothorax duloticus* (on the left) and *Temnothorax* sp. (on the right).

In chapter 2, the aim was to analyse the phylogenetic relationships of the available Mediterranean species of the genus *Chalepoxenus* and its *Temnothorax* hosts by sequencing 1320 bp of the mitochondrial genes CO I and CO II. In chapter 3, a phylogeny of several representatives of the genus *Myrmoxenus* and a number of host and non-host species based on a fragment of the cytochrome oxidase subunit I is presented.

In chapters 4 and 5, the chemical basis of colony odour in parasite colonies was investigated. An insect's body surface is covered by the cuticle, a multi-layered structure outside the epidermis that forms an exoskeleton. The outermost layer is composed of a mixture of lipids containing linear and branched, saturated and unsaturated hydrocarbons, fatty acids, alcohols, alkyl esters, glycerides, sterols and aldehydes (Hackman, 1984; Lockey, 1988). It is well known that in insects, hydrocarbons are synthesized by oenocytes associated with either fat body or epidermal tissue (Romer, 1991), and are secreted during cuticle deposition after separation of the old and new cuticle (Lockey, 1988). Insect hydrocarbons have chain lengths of 11-43 carbon atoms and have evolved primarily to protect the animal from desiccation (Hadley, 1984) and as a barrier to microorganisms. Moreover, enormous advances during the last 20 years made clear that a major function of cuticular hydrocarbons in arthropods is to serve as recognition cues between two or more individuals (Howard, 1993; Smith and Breed, 1995). Especially social insects have evolved a highly developed recognition system. That way, they are able to identify friend from foe, recognize potential mates, or distinguish between natal or alien nest. Discrimination is based on the colony odour, a common chemical bouquet shared by members of the same colony (Soroker et al., 1998). As already mentioned, cuticular hydrocarbons are considered to be the main chemical cues responsible for recognition and discrimination in ants and wasps (Lorenzi et al., 1996, 1997; Singer, 1998; Lahav et al., 1999). These hydrocarbons are mainly linear and branched alkanes with chain lengths ranging from C23 to C32, sometimes accompanied by alkenes and

alkadienes. Studies have demonstrated that the hydrocarbons are mixed and stored in the post-pharyngeal gland (PPG) and exchanged between colony members by grooming, trophallaxis and physical contact (Soroker et al., 1994). In slavemaker colonies, the cuticular profile is influenced among others by the slaves present in the colony (see Lenoir et al., 2001). Thus, in chapter 4, I investigated how the host species affects the cuticular hydrocarbon profile of winged *C. muellerianus* sexuals and whether it has an effect on the sexuals' mate choice. Finally in chapter 5, the goal was firstly to examine whether and how quickly the cuticular profile of *C. muellerianus* changes after an exchange of the slave species, and secondly to determine the impact of *Chalepoxenus* on the hydrocarbon composition of two different slave species.

Chapter 1

Six origins of slavery in formicoxenine ants*

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ABSTRACT

Slave-making (dulotic) ants have long fascinated biologists because of their intriguing behaviour and highly specialized lifestyle. Dulosis evolved convergently several times within the two ant subfamilies Myrmicinae and Formicinae. Here, we demonstrate that it originated at least six times independently within the small myrmicine tribe Formicoxenini alone. Our phylogenetic trees, based on 1386 base pairs of the mitochondrial cytochrome oxidase gene, document different degrees of genetic divergence between different monophyla of slave-makers and their host species, which suggests that they evolved from non-parasitic Formicoxenini at different times. Two Nearctic slavemakers, *Temnothorax duloticus* and a new species still to be formally described, appear to be of particularly recent origin. In contrast, the other parasitic monophyla clearly diverged much earlier from their non-parasitic ancestors and have a much longer evolutionary history.

Keywords: Social parasitism, slave-making ants, phylogeny, dulosis, Formicoxenini

INTRODUCTION

Slave-making in ants (dulosis) has fascinated scientists and the public ever since its first detailed description almost 200 years ago (Huber, 1810). For example, Charles Darwin dedicated several pages of his groundbreaking book 'On the origin of species' to the complex behaviour of slave-making ants during slave raids (Darwin, 1859). However, despite much debate, the evolutionary origins of slave-making are still not well understood.

Slavemakers are socially parasitic ants, which permanently depend on the help of already established colonies of related ant species for all tasks in the nest (Buschinger, 1986; Hölldobler and Wilson, 1990; Stuart, 2002). Their colonies are predominantly initiated in summer by young, mated slavemaker queens, which usurp a host colony by killing or expelling the resident queen and, in some genera, also all adult workers. The first host workers to emerge from the conquered brood then care for the slavemaker queen and her offspring. In subsequent years, the slavemaker workers produced by the queen attack neighbouring host nests and capture their pupae in order to replenish the stock of host workers.

Slavery is believed to have evolved independently at least nine times within two of the 21 known ant subfamilies, the Formicinae and the Myrmicinae (Buschinger, 1986; Hölldobler and Wilson, 1990; Stuart, 2002). The small myrmicine tribe Formicoxenini appears to be a particular hot spot in slavemaker evolution: five independent origins of slave-making ants (*Chalepoxenus* spp.; *Harpagoxenus* spp.; *Myrmoxenus* spp. – formerly *Epimyrma*, Schulz and Sanetra, 2002; *Protomognathus americanus*; *Temnothorax duloticus*, formerly *Leptothorax duloticus*, Bolton, 2003) have previously been identified (Buschinger, 1990; Baur et al., 1995). All these taxa occur throughout temperate and boreal habitats of Eastern North America, Eurasia, the Mediterranean and North Africa and parasitize various *Temnothorax* and *Leptothorax* species (Buschinger, 1986, 1990; Hölldobler and Wilson, 1990).

The different lineages of slave-making Formicoxenini exhibit strikingly different degrees of species richness. For example, whereas *Myrmoxenus* comprises eight or more morphologically similar species, suggesting an adaptive radiation, other monophyla, such as *Protomognathus americanus*, consist of only a single taxon. One reasonable hypothesis for this varying diversity is that different lineages evolved at different times in the past, with old lineages being richer in species than young lineages. Our attempt to quantify variation within extant slavemaker species and their host species revealed a sixth independent origin of slavery in a yet undescribed Nearctic *Temnothorax* ant. This species shares its host species

T. longispinosus and *T. ambiguus* with *P. americanus* and *T. duloticus*, but, as we show here, is clearly distinct from the other Nearctic social parasites.

MATERIAL AND METHODS

Species of all presently known lineages of socially parasitic Formicoxenini and their dominant host species were collected in Europe and North America. In addition, we included the workerless inquiline *T. minutissimus* (Buschinger and Linksvayer, 2004). In Table 1-1, the social parasites and their respective host species are listed. Genus names correspond to Bolton's new classification of Formicidae (Bolton, 2003). Details on collection sites are given in Table 1-2.

Table 1-1. Social parasites and their host species. *T. minutissimus* is not a slave-making ant, but a workerless inquiline parasite.

Parasite species	**Host species**
Chalepoxenus muellerianus	*Temnothorax unifasciatus*
Myrmoxenus ravouxi	*Temnothorax nigriceps*
Harpagoxenus sublaevis	*Leptothorax acervorum*
Harpagoxenus canadensis	*Leptothorax canadensis*
Protomognathus americanus	*Temnothorax longispinosus* *Temnothorax curvispinosus* *Temnothorax ambiguus*
Temnothorax sp.	*Temnothorax longispinosus* *Temnothorax ambiguus*
Temnothorax duloticus	*Temnothorax longispinosus* *Temnothorax curvispinosus* *Temnothorax ambiguus*
Temnothorax minutissimus	*Temnothorax curvispinosus*

High molecular weight DNA was extracted from individual, frozen, female ants by grinding them in liquid nitrogen and subsequently following a cetyltrimethyl ammonium bromide protocol (Hamaguchi et al., 1993). We amplified a 1430 bp fragment of the subunits I and II of the mitochondrial gene cytochrome oxidase (CO I/II) using the primers C1-J-2195 and C2-N-3661 (Simon et al., 1994) and three primers designed by M. Brandt, B. Fischer-Blass, and A. Trindl: MIBI (5'-AGA TTT ATT CAC TGA TTC CC-3'), CW-3031rev (5'-TTT GCM CTW ATC TGC CMT ATT-3') and CO1-516 for (5'-ATT TTT YTC TAT ATT TAT YGG A-3'). The 25 µl PCR 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 Thermo-cycler with the following temperature profile: 4 min at 94°C, 41 cycles of 94°C for 1.15 min, 50°C for 1.15 min, 68°C for 1.30 – 2.30 min, and a final extension at 72°C for 5 min. PCR products were either purified from 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). The 20 µl cycle sequencing reaction mixture contained 20 - 100 ng DNA, 3 µl 5x sequencing buffer, 0.5 µM primer and 2 µl ready mix (Big dye Terminator Cycle sequencing kit, Applied Biosystems). Both strands were sequenced on an ABI Prism 310 Genetic Analyzer.

Table 1-2. Overview of the sampled specimens, their collection sites and the GenBank accession numbers. In all instances, we sequenced the gene COI/COII, with the interjacent leucine tRNA. *Temnothorax* (*T.*) and *Leptothorax* (*L.*) host species were sampled from the same communities as their parasites. Some slavemaker species have a broader distribution than their host species and consequently co-occur with various hosts at different sites.

Species	Locality	Accession Number
Chalepoxenus muellerianus	Savoillan, Provence, France (F)	AY909573
Myrmoxenus ravouxi	Waldenhausen, Bavaria, Germany (D)	AY909575
Harpagoxenus sublaevis	Abensberg, Bavaria, Germany (D)	AY754680
Harpagoxenus canadensis	Tadoussac, Quebec, Canada (CN)	AY909574
Protomognathus americanus	Huyck Preserve, New York, USA	AY754775
Protomognathus americanus	Harpersfield, Ohio, USA	AY754785
T. sp.	Huyck Preserve, New York, USA	AY909557
T. sp.	Huyck Preserve, New York, USA	AY909558
T. sp.	Huyck Preserve, New York, USA	AY909559
T. duloticus	Columbus, Ohio, USA	AY909560
T. duloticus	Columbus, Ohio, USA	AY909561
T. duloticus	Columbus, Ohio, USA	AY909562
T. duloticus	Columbus, Ohio, USA	AY909563
T. minutissimus	Bloomington, Indiana, USA	AY909564
T. minutissimus	Watoga State Park, West Virginia, USA	AY909566
T. minutissimus	Columbus, Ohio, USA	AY909565
T. unifasciatus	Savoillan, Provence, France (F)	AY909570
T. nigriceps	Waldenhausen, Bavaria, Germany (D)	AY909567
L. acervorum	Abensberg, Bavaria, Germany (D)	AY909571
L. canadensis	Tadoussac, Quebec, Canada (CN)	AY909572
T. longispinosus	Huyck Preserve, New York, USA	AY754805
T. longispinosus	Harpersfield, Ohio, USA	AY754798
T. longispinosus	Watoga State Park, West Virginia, USA	AY754797
T. ambiguus	Huyck Preserve, New York, USA	AY909568
T. curvispinosus	Harpersfield, Ohio, USA	AY754754
T. curvispinosus	Watoga State Park, West Virginia, USA	AY909569
Cardiocondyla mauritanica	La Gomera, Spain (E)	AY909576

The 27 sequences were compiled, edited and aligned in the program Bioedit 5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html, Hall, 1999) and by eye. Two tree-building methods were used to reconstruct the phylogeny of the formicoxenine slavemakers and their host species, with an ant from the same tribe, *Cardiocondyla mauritanica*, serving as outgroup. Both phylogenetic trees are based on 1386 bp of the CO I/II sequence (789 bp CO I coding region, 54 bp intergenic region with a leucine tRNA motif, 543 bp CO II coding

region). Eight to 13 bp in the non-coding region could not be aligned and were removed in frame. Three double peaks and four missing bases at the end of single sequences were substituted by “N”s. The sequences used in the final analysis were without gaps, frameshifts, unexpected stop codons, insertions, deletions, or rearrangements. We also did not find any evidence of pseudogenes or gene duplications. The AT content of the selected region was 71.4%. The sequences comprised 551 variable sites, of which 457 were parsimony-informative (DnaSP 4.00.5, Rozas et al., 2004). We found 501 transitions and 288 trans-versions. Sequences are deposited in the GenBank database under accession numbers shown in Table 1-2.

Maximum parsimony analysis was conducted using the program PAUP 4.0 (Swofford, 2000) at default settings in a heuristic search to find the most parsimonious tree. Deviating from these settings we used a random addition sequence with ten replications and the specification that the ingroup was monophyletic. We found a single tree and support for individual branches were assessed by 2000 bootstrap replicates. A Bayesian tree was generated using MRBAYES 3.0b3 (Huelsenbeck, 2000) with the general time reversible model with invariable sites and γ-distribution (GTR+I+G), calculated by Mr.Modeltest as included in the MRBAYES program. We used this model in the in MRBAYES implemented Monte Carlo algorithm with four Markov chains over two million generations, generating a tree each 500 generations. We excluded all trees generated within the first 10,000 generations before the chains converged to a stable value.

For scanning electron microscopy, specimens were fixed, washed in distilled water, dehydrated through a graded ethanol series, dried, coated with gold-palladium and examined in a Zeiss DSM950 scanning electron microscope.

RESULTS

Assuming that slave-making is a derived state, both phylogenetic trees shown in Figures 1-1A and 1-1B clearly show six independent origins of slavery in the Formicoxenini. The inquiline *T. minutissimus* forms a lineage separate from the other studied social parasites. Our research therefore supports previous claims that slave-making evolved convergently in *Chalepoxenus*, *Harpagoxenus*, *Myrmoxenus*, *P. americanus* and *T. duloticus* and beyond that reveals a sixth slave-making lineage, an as yet undescribed, Nearctic *Temnothorax* parasitizing *T. longispinosus* and *T. ambiguus.*

Palaearctic *H. sublaevis* and Nearctic *H. canadensis* are presumably sister taxa, although branch lengths representing 127 mutations (9.2% sequence divergence) suggest an early separation and a long independent history of these two species, and *H. sublaevis* is grouped with *Leptothorax* in the Bayesian consensus tree. *Harpagoxenus* forms a robust monophyletic group with their host species *L. acervorum* and *L. canadensis* (bootstrap support value 100, posterior probability 1.00).

Similarly, the Nearctic parasites of *Temnothorax* and their hosts form a rather well supported monophylum (bootstrap support value 86, posterior probability 0.99). However, the four social parasites show very different divergence patterns. The long branch length and high intraspecific variability of *P. americanus* suggests a long, independent history, with its closest living relative being yet unknown. The two other Nearctic slavemakers clearly cluster with one of their respective host species each and apparently have split from it or a related species much more recently. *T. duloticus* is close to *T. curvispinosus*, and both form a well-supported monophyletic group with the inquiline *T. minutissimus* (bootstrap support value 96, posterior probability 0.97).

The presently undescribed slavemaker, *Temnothorax* sp., is very close to *T. longispinosus* (bootstrap support value 92, posterior probability 1.00). It differs from *T. duloticus* in its larger and more pointed ventral postpetiolar sternite, its shorter and more robust epinotal spines and its more triangular mandibles (Fig. 1-2). The new species will be described in detail by R.J. Stuart and S.P. Cover.

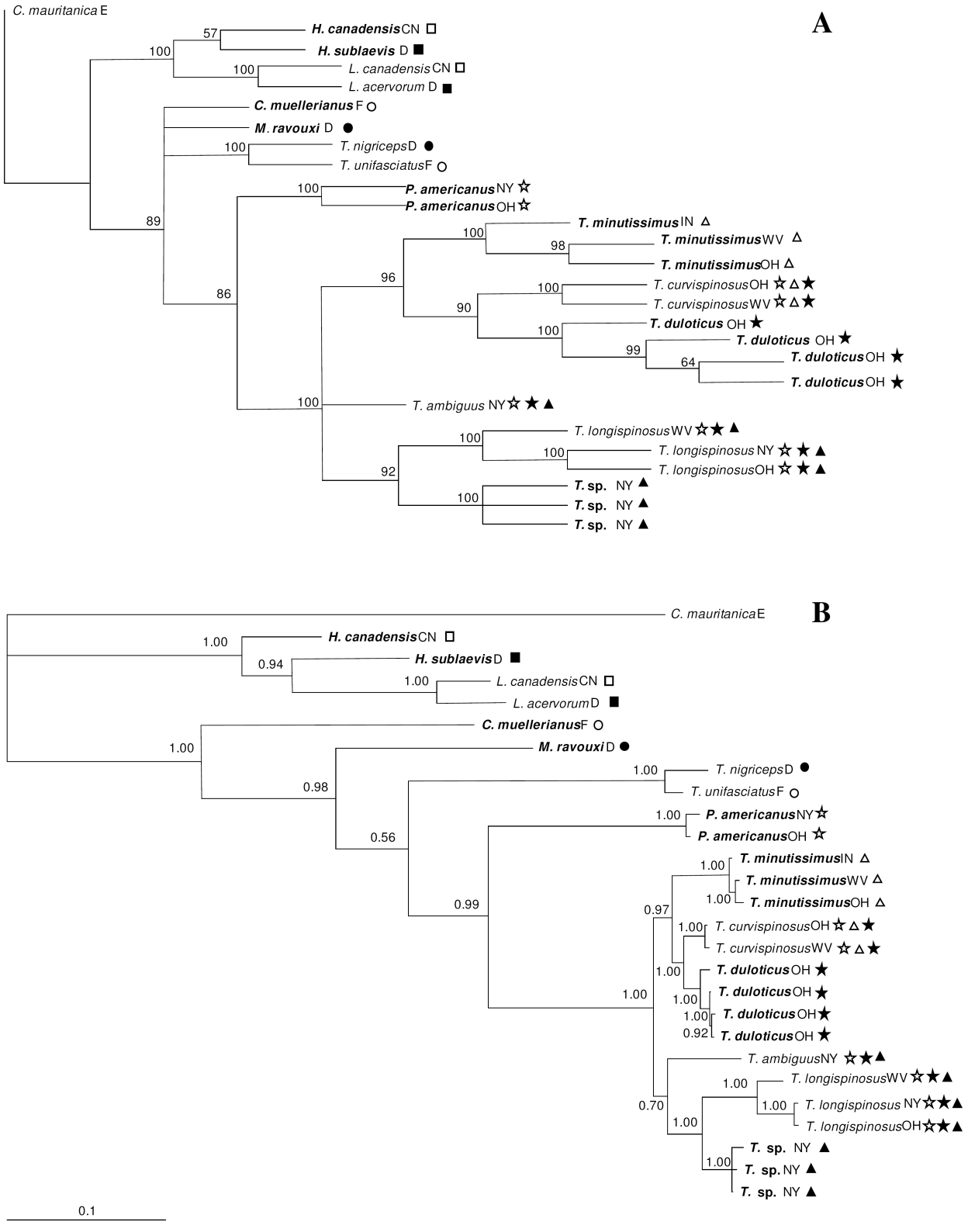

Figure 1-1. A. Bootstrap 55% majority-rule consensus tree for the ant taxa based on 1386 base pairs of the mitochondrial gene cytochrome oxidase I and II as calculated by PAUP 4.0 by heuristic search. Support for the branches are based on 2000 bootstrap replicates. Parasitic taxa are shown in bold. Parasites and their respective host(s) are marked with the same symbol. **B**. Majority rule consensus tree of 3980 Bayesian trees. Numbers above branches represent the posterior probability that the clade is correct given the model of evolution.

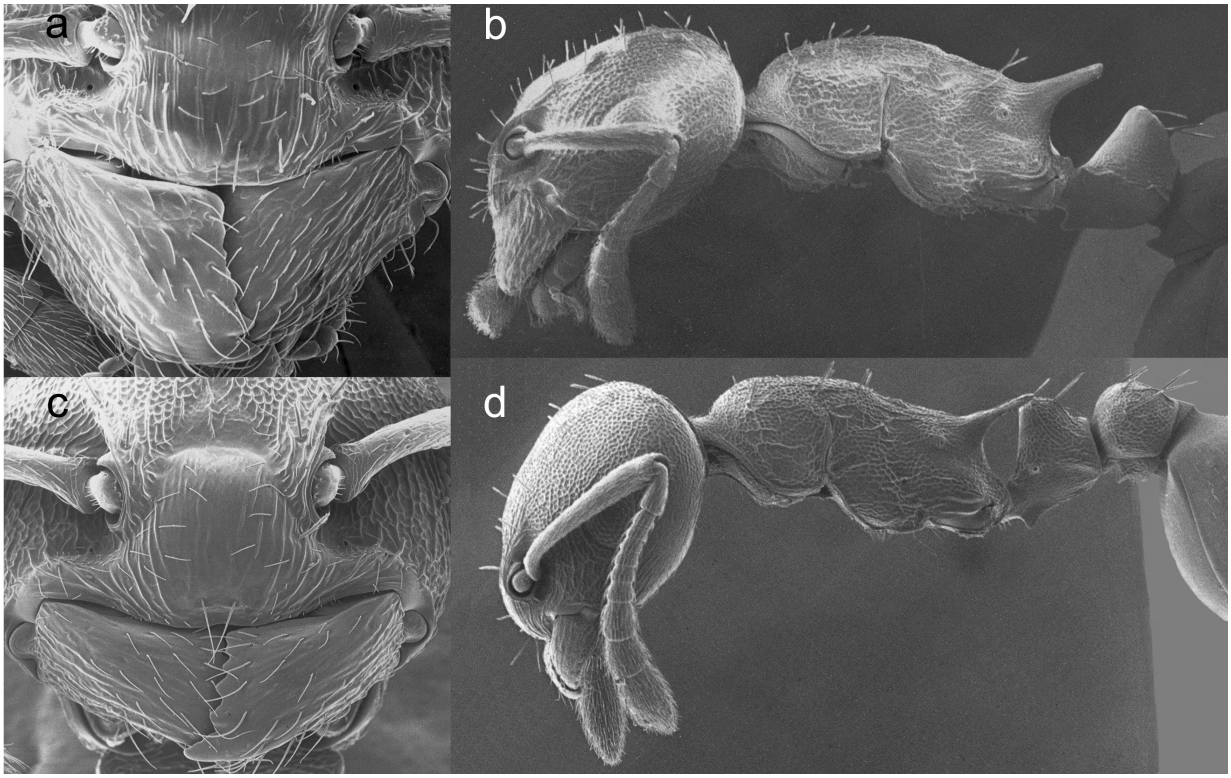

Figure 1-2. Scanning electron microscope pictures showing morphological differences between the mandibles and the front part of a worker from *Temnothorax* sp. (a, b) and *T. duloticus* (c, d).

DISCUSSION

Our analysis revealed six independent evolutionary origins of slavery within the Formicoxenini, which is one more than previously thought. The very robust branching pattern of our phylogenetic tree suggests three convergent origins within the North American *Temnothorax* alone. The slavemakers *T. duloticus* and *T.* sp. cluster with their host species, which suggests a close phylogenetic relationship and a recent evolution from non-parasitic *Temnothorax* ancestors. This is in conflict with earlier suggestions that *T. duloticus* is a close relative of *Leptothorax muscorum* (Wesson, 1937; Wilson, 1975) but supports conclusions from a previous allozyme study (Heinze, 1991) and justifies the recent transfer of this species into the genus *Temnothorax* (Buschinger, 1990; Bolton, 2003).

Phylogenetic relationships between social parasites and their host(s) have been long debated. According to Emery (1909), social parasites are typically closely related to their hosts, with both species evolving from a common ancestor. In the strict form of Emery's rule, each parasite is the sister species of its host, whereas, in its looser version, the non-parasitic clade most closely related to the parasite contains all the parasite's host species and parasites can radiate to less related host species (Buschinger, 1990). Too few of the hundreds of *Temnothorax* species have been investigated to determine whether the evolution of socially parasitic Formicoxenini matches Emery's rule, and it is also not our aim to do so. Nevertheless, our data support previous reports on a more or less close relationship between parasites and hosts (Buschinger, 1990; Heinze, 1991, 1995; Baur et al., 1995). For example, *Harpagoxenus* forms a well-supported monophylum with its *Leptothorax* hosts. Though *H. canadensis* constitutes the outgroup to *H. sublaevis* and *Leptothorax* in the Bayesian analysis, the two *Harpagoxenus* species clearly form a monophylum in the maximum parsimony analysis and in an earlier tree based on enzyme electromorphs (Heinze, 1995). Monophyly is also suggested by morphology and karyotype (Buschinger and Alloway, 1978, 1979; Fischer, 1987; Buschinger, 1990).

A close relationship between parasite and host is also suggested for *T. duloticus* and the new dulotic *Temnothorax* species, among whose hosts are what seem to be their closest free-living relatives. The comparatively short branch lengths suggest a recent evolution of both species and also *T. minutissimus* from the clade to which their host species belongs. In contrast, *P. americanus* appears to be phylogenetically old and only distantly related to its present hosts. Similarly, the Palaearctic, species-rich genera *Myrmoxenus* and *Chalepoxenus*

are all well separated from their *Temnothorax* hosts and their long branch lengths suggest a long independent evolutionary history (Buschinger, 1990; see also Baur et al., 1995).

Though *T.* sp. and *T. duloticus* are morphologically very similar, they differ in their geographic range and slave raiding behaviour. *T.* sp. was first discovered by R.J. Stuart in Southern Ontario and has subsequently been collected in Michigan, Vermont, and New York (Alloway, 1997; Herbers and Foitzik, 2002; R.J. Stuart, unpubl.), while *T. duloticus* occurs in Illinois, Ohio, and Michigan. The more north-eastern distribution of *T.* sp. is also reflected in its usage of *T. ambiguus* as a second, and in some places even the main host. While *T. duloticus* conducts processions during slave raids (Alloway, 1979; Buschinger, 1986), scouts of *T.* sp. recruit nestmates by tandem running (R.J. Stuart, unpubl.). Colonies of *T.* sp. appear to be highly polydomous, because most nests are queenless and many contain less than six slavemaker workers (R.J. Stuart, unpubl.).

The small tribe Formicoxenini, with less than 4% of the presently known ant species, contains 60% of all independent origins of slavery. In addition, a number of inquilines (Buschinger, 1982, 1990; Heinze, 1989a; Heinze and Alloway, 1991), as well as the guest ant genus *Formicoxenus* (Francoeur et al., 1985) all have arisen in this group. Why are the Formicoxenini such a peculiar hot spot in the evolution of social parasitism? Three different behavioural traits have been suggested as starting points in the evolution of slave-making: predation on other ant species (Darwin, 1859), territoriality (Alloway, 1980; Stuart and Alloway, 1982, 1983), and polygyny, usually combined with polydomy (Buschinger, 1970, 1990; for recent reviews see D'Ettorre and Heinze, 2001; Stuart, 2002). The evolution of slavery from specialized predation on ant larvae for food is not likely in the Formicoxenini as they are food generalists and do not prey on other ants. Territorial disputes lead to raiding and intraspecific slavery in *Myrmecocystus* (Hölldobler, 1976b; Kronauer et al., 2003), and similar results have been obtained in laboratory studies with *Temnothorax* (Alloway, 1980; Alloway et al., 1991; Stuart and Alloway, 1982). However, formicoxenine ants typically do not engage in fights with neighbouring colonies in the field (Dobrzañski 1965, 1966; Heinze et al., 1996), and molecular analyses have not revealed the genetic heterogeneity that should result from frequent intraspecific slavery (Foitzik and Herbers, 2001b; Foitzik et al., 2004). Nevertheless, the fact that some sort of slave raiding can be elicited documents that the repertoire for this behaviour is present also in non-parasitic species.

Finally, polygyny and polydomy were suggested as starting points for the evolution of slave-making (Wasmann, 1909; Buschinger, 1970, 1990; Elmes, 1973, 1978; Bourke and Franks, 1991). Parasitic colony founding might have originated from the return of newly

mated young queens into their maternal colonies, and slave raiding might have evolved from brood transport. Though the transport of brood between neighbouring nests of a polydomous colony resembles brood transport during slave raids (Buschinger, 1986; 1990), it completely lacks the scouting and fighting behaviour exhibited by slavemaker workers. And although the host species of *Harpagoxenus*, *T. duloticus*, *T.* sp. and *P. americanus* are all facultatively polygynous, most *Temnothorax* hosts of *Chalepoxenus* and *Myrmoxenus* are monogynous and monodomous (see also D'Ettorre and Heinze, 2001). However, it must be recognized that the latter often live in ephemeral nests and might be frequently forced to move and transport brood.

Other aspects of the behavioural ecology and population biology of the Formicoxenini might therefore have contributed to the evolution of slavery in this group. Slavemakers can only be successful when the nests of their hosts are relatively dense and cannot easily be defended (Alloway et al., 1982; D'Ettorre and Heinze, 2001; Stuart, 2002). This certainly applies to formicoxenine ants more than to many other ant taxa. *Leptothorax* and *Temnothorax* are among the most common ants in boreal and temperate forests, locally reaching densities of 10 nests/m^2 and more, and their colonies typically contain only a few dozen individuals and nest in often fragile, preformed cavities in wood and nuts or under stones. The large effective population sizes and the ubiquity of nesting sites have probably facilitated the high rate of diversification and speciation in Formicoxenini. There are several hundred *Temnothorax* species (Schulz and Verhaagh, 1999; MacKay, 2000; Bolton, 2003), and the diversity of *Leptothorax* is presumably also considerably higher than previously thought, with numerous Nearctic taxa still unnamed (Heinze, 1989b) and several Eurasian taxa only recently recognized as valid species (Heinze et al., 1993; Radchenko and Heinze, 1997). The occurrence of large numbers of closely related sympatric species with asymmetries in their fighting abilities and in their discrimination abilities between nestmates and non-nestmates might similarly have facilitated the repeated evolution of slavery (Alloway, 1997; Stuart, 1988, 1993, 2002).

Detailed investigations of the behaviour and ecology of non-parasitic formicoxenine species and slavemakers at an early stage in their parasitic evolution, such as *T. duloticus* and *T.* sp., might shed light on the evolutionary origin and development of slave-making in this fascinating group of ants.

ACKNOWLEDGEMENTS

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Chapter 2

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, *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 parasitize 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 parasitizes *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*.

MATERIAL AND 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 2-1; Tables 2-1, 2-2). *Crematogaster smithi* Creighton, 1950, an ant from outside the Formicoxenini, but within the formicoxenine tribe group (Bolton 2003), served as outgroup.

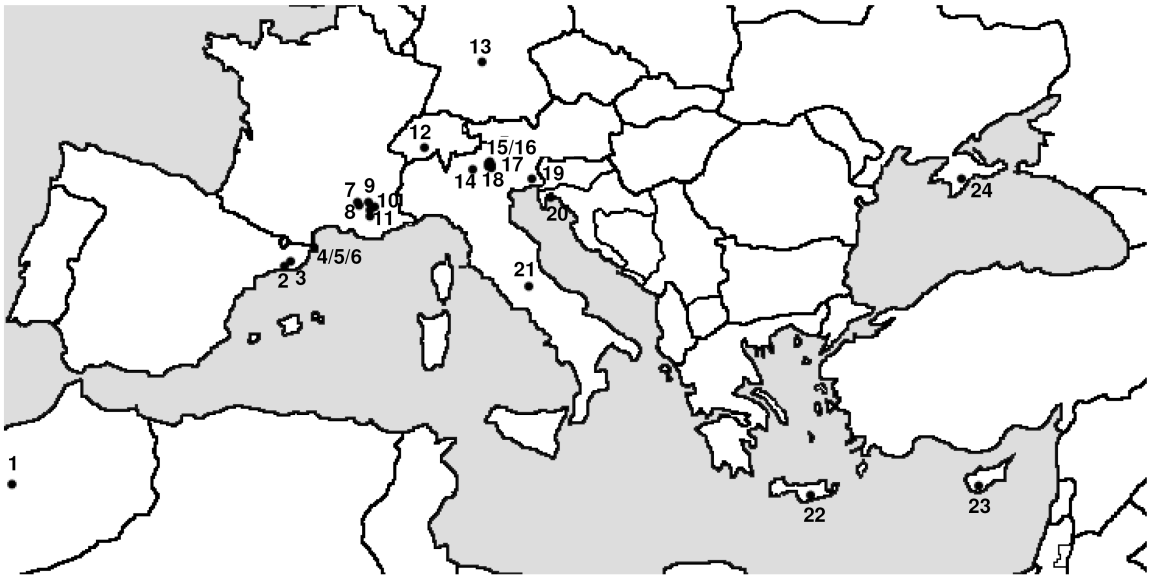

Figure 2-1. Map showing sampling localities. For locality names see Table 2-2.

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 40 µl purified water (Sigma) and stored at 4°C until analysis. PCR amplifications were conducted in a total volume of 25 µl 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).

Table 2-1. 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)	?

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

Table 2-2. 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 2-1.

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	

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 both genes consisted of 1320 base pairs. Haplotypes and GenBank accession numbers are available in Table 2-2. 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 2-3.

Table 2-3. 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	-

Phylogenetic and phylogeographic analyses

Figure 2-2A 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 *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 2-2B 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).

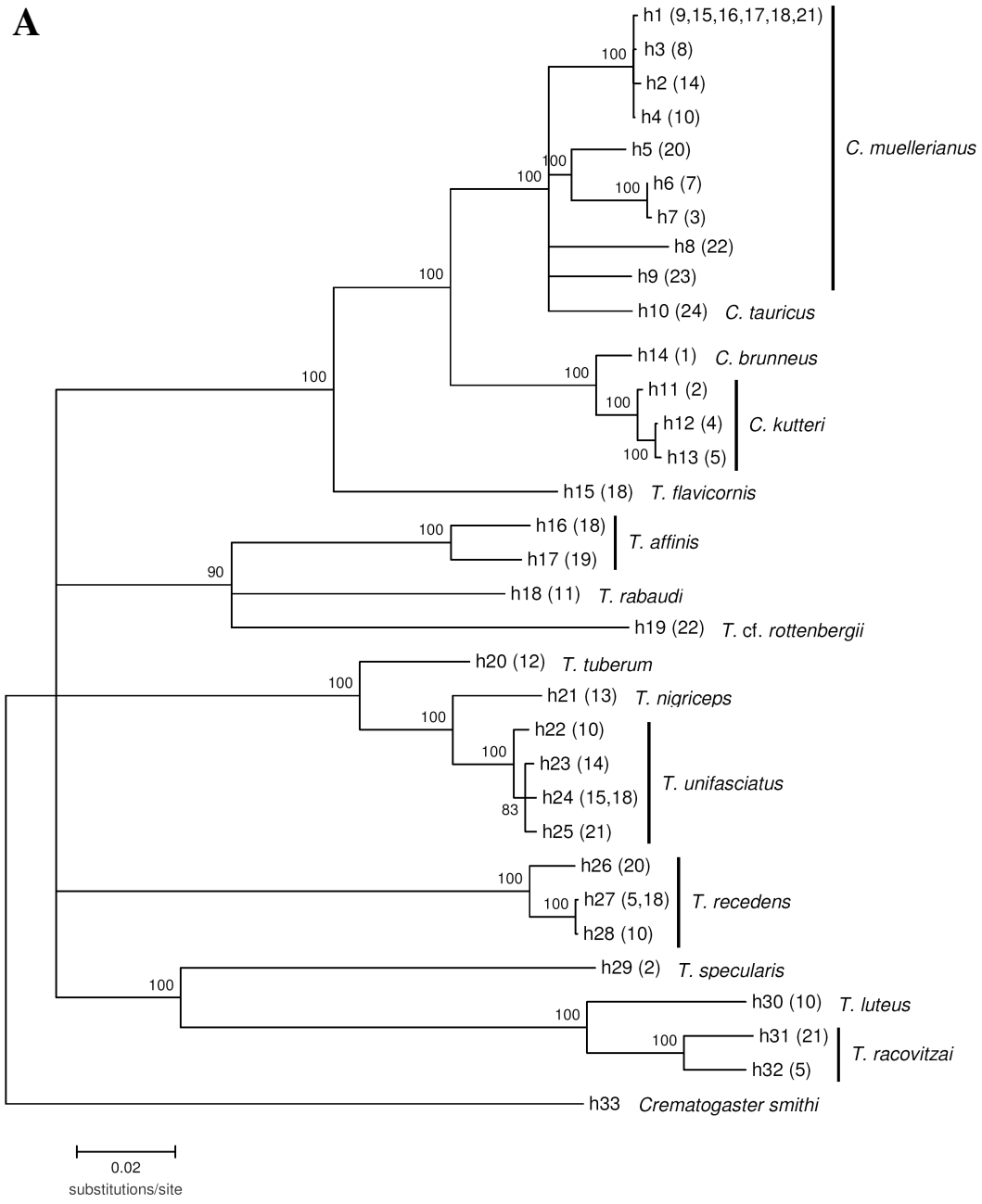

Figure 2-2. Phylogenetic trees of haplotypes of *Chalepoxenus* (*C.*) and its *Temnothorax* (*T.*) host species, based on 1320 base pairs of the mitochondrial cytochrome c oxidase I and II gene. Sample information is given in Table 2-2. Locality codes are given in parentheses and refer to Figure 2-1 and Table 2-2. **A.** Neighbour-Joining tree with bootstrap values estimated from 5000 replicates. Bootstrap percentages with values greater than 80 are shown on nodes.

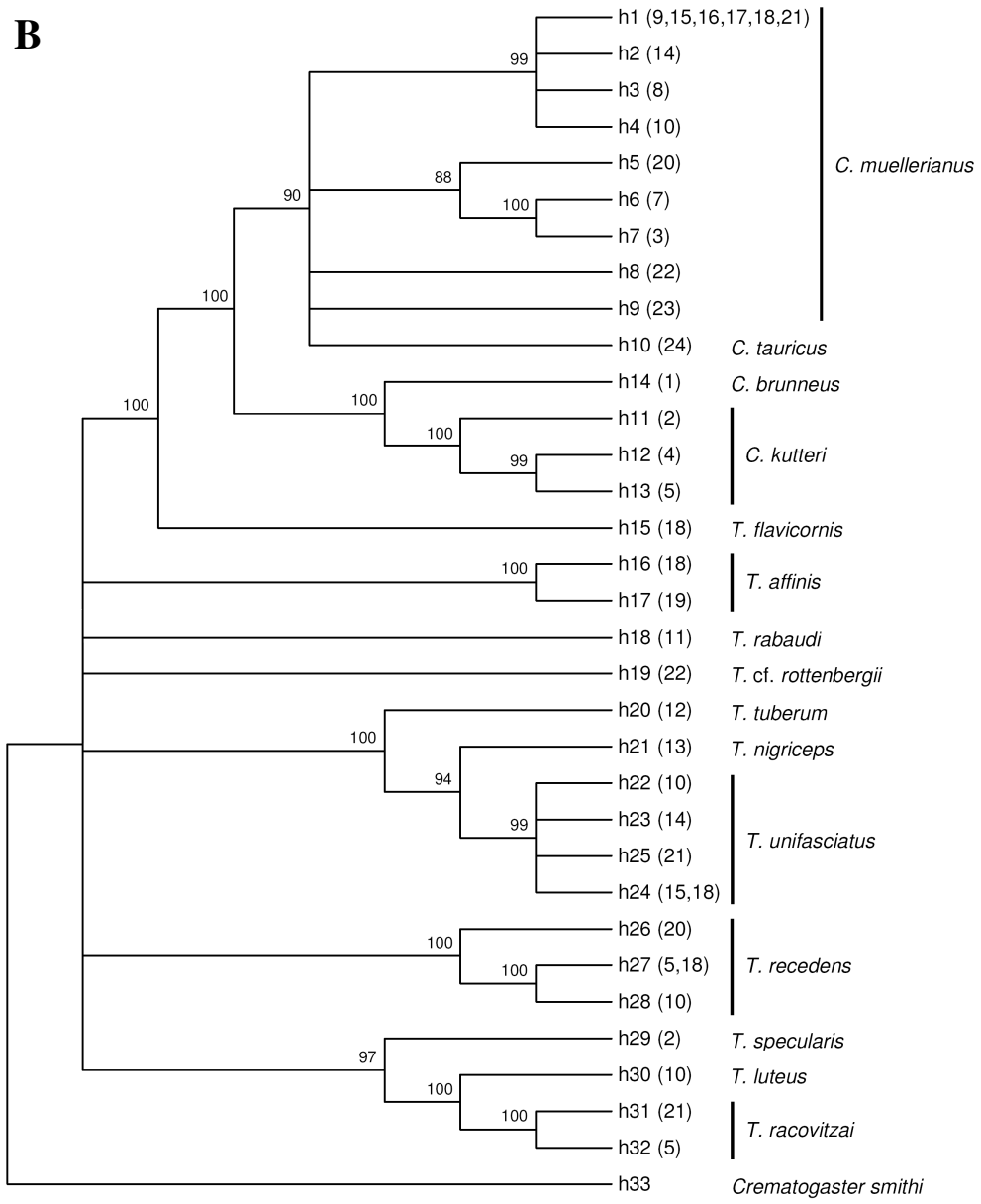

Figure 2-2. (continued) **B.** Maximum Parsimony consensus tree found by heuristic search, and shown with bootstrap percentages (2000 pseudoreplicates) greater than 80%.

Figure 2-2C 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.

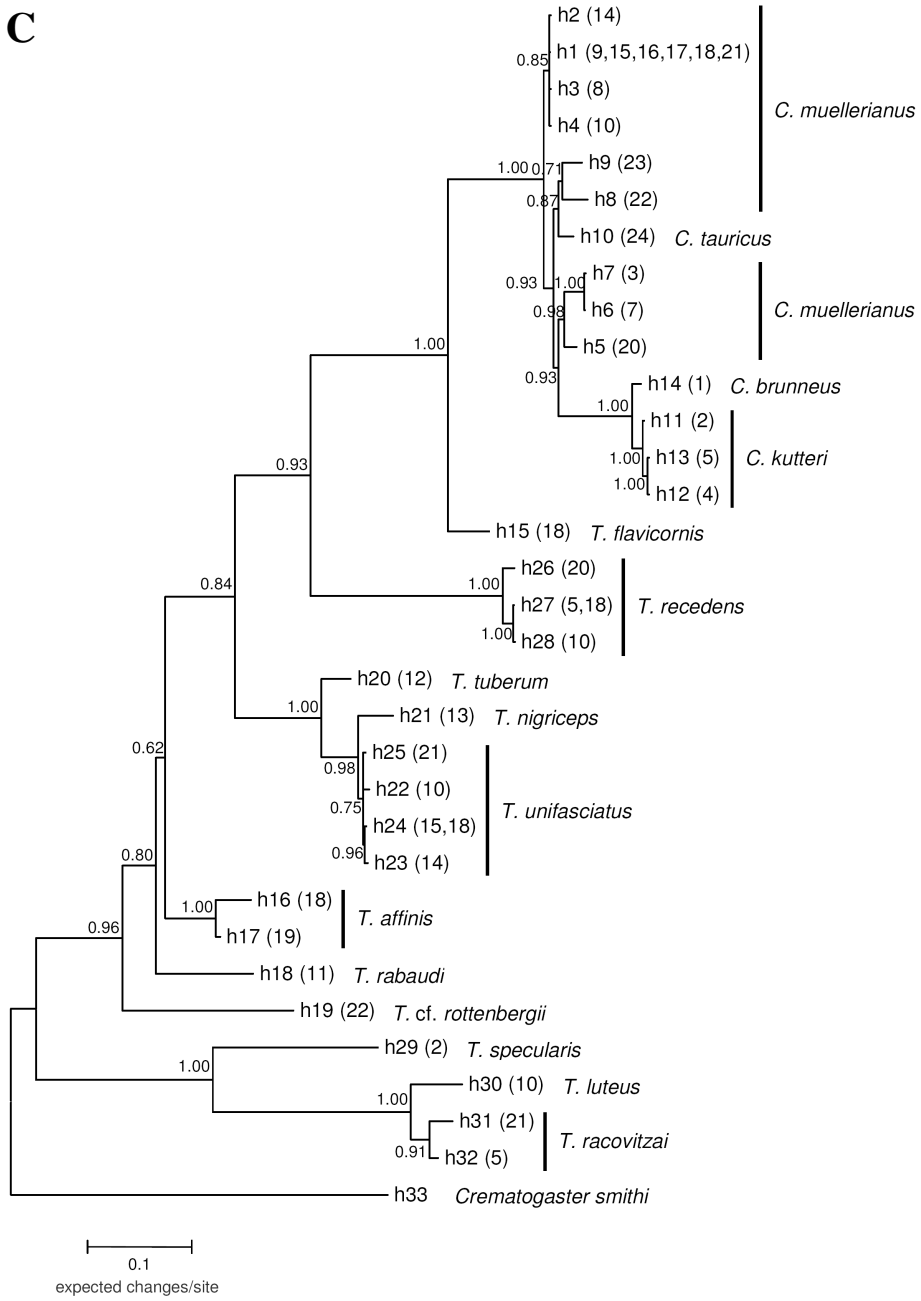

Figure 2-2. (continued) **C.** Majority rule consensus tree recovered in a Bayesian analysis (2,000,000 generations, partitioned by codon position). Numbers represent clade credibility values.

For *Chalepoxenus muellerianus*, we constructed a haplotype network using the program TCS, which is especially useful for closely related sequences (Figure 2-3). 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. unifasciatus* or *T. rabaudi,* respectively. The remaining haplotypes h5, h8 and h9 occurred together with three different host species.

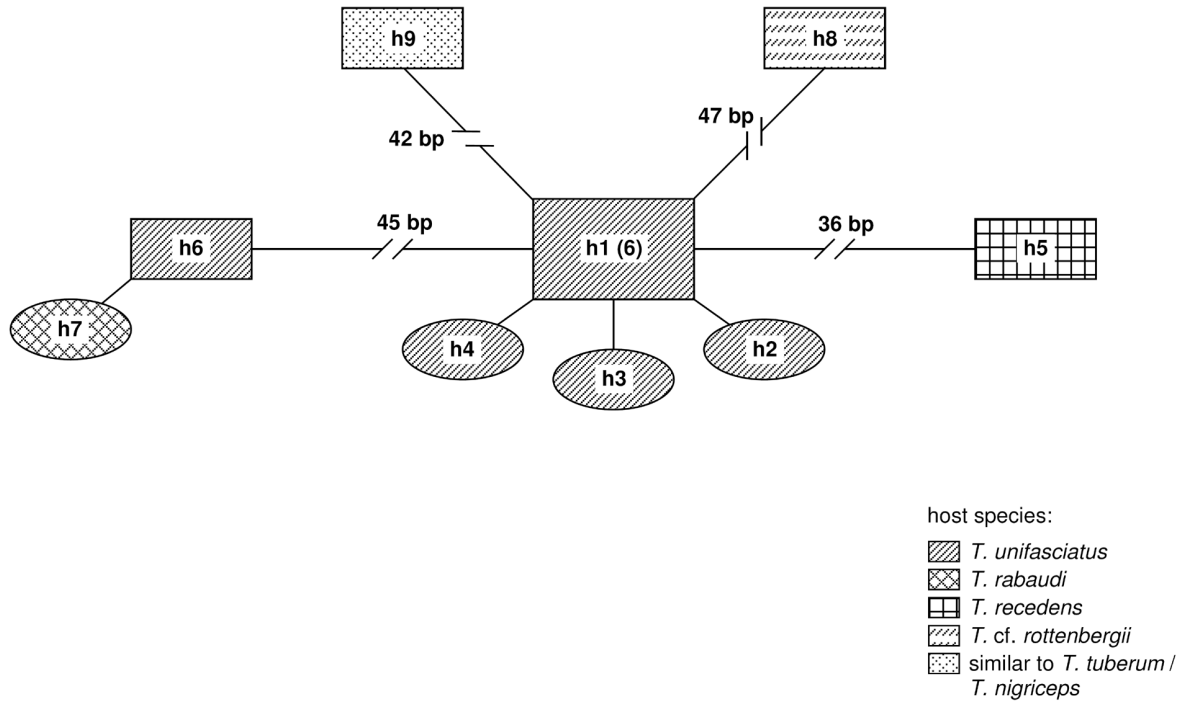

Figure 2-3. Statistical parsimony network of *C. muellerianus* haplotypes calculated by TCS 1.21 using mitochondrial CO I/II sequences. The haplotype with the highest ancestral probability is displayed as a square, while other haplotypes are displayed as ovals. The identity of the haplotypes is indicated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

Kimura-2 distances and geographical (great circle) distances between individual *C. muellerianus* haplotypes are given in Table 2-4 and Figure 2-4. 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-h14), 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).

Table 2-4. 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 2-1 and Table 2-2.

	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	-

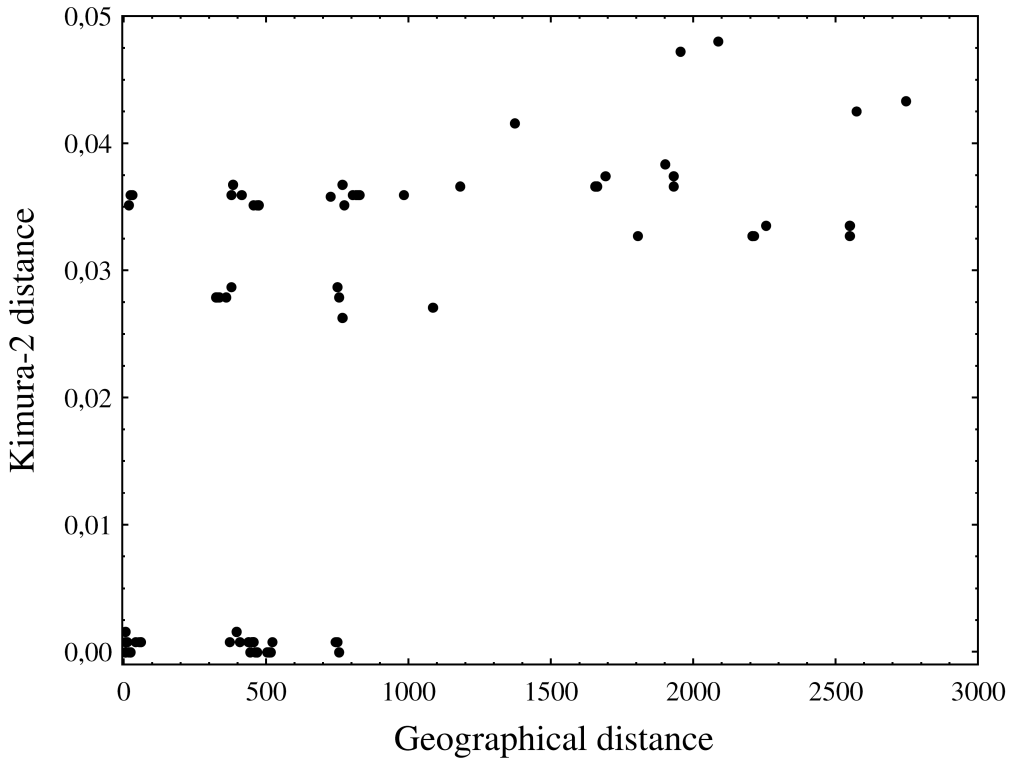

Figure 2-4. Kimura-2 distances and geographical (great circle) distances (in kilometres) between individual *C. muellerianus* haplotypes.

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 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 parasitize 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* parasitizes *T. marocana*, *C. kutteri* parasitizes *T. massiliensis*, *T. specularis* (Emery, 1916) and *T. racovitzai* (Bondroit, 1918), *C. tauricus* from Crimea and *C. muellerianus* from Italy and France parasitize *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.

ACKNOWLEDGEMENTS

W. Ehrhardt kindly contributed results from his crossbreeding experiments. We thank R. Blatrix and C. Wanke for providing samples of *C. muellerianus* and *T. tuberum*, respectively, and A. Trindl for providing the *Crematogaster* sequence. P. D'Ettorre, K. Pusch, C. Wanke, M. Schiwek, R. Blatrix, and X. Espadaler helped collecting ants in the field and two referees made helpful comments on the manuscript. Financial support came from the German Science Foundation (He1623/13).

Chapter 3

Phylogenetic analyses of the parasitic ant genus *Myrmoxenus**

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ABSTRACT

In this study, phylogenetic trees of some representatives of the formicoxenine ant genus *Myrmoxenus* and several of its host species are presented. A gene fragment of the subunit I of the mitochondrial cytochrome oxidase (CO I) was sequenced in five species of the parasitic genus *Myrmoxenus*, seven *Temnothorax* host species and eleven additional ant species of this tribe. Three tree-building methods were used to describe the phylogenetic relationships between the parasites and their hosts. Neighbour-Joining, Maximum Parsimony and Bayesian analyses based on 651 base pairs indicate that *Myrmoxenus* is an old monophylum, where *M. gordiagini* was the first species to branch off. Furthermore, a Neighbour-Joining tree based on 399 base pairs was constructed including GenBank-sequences of three additional *Myrmoxenus* species, providing supplementary insights into *Myrmoxenus* phylogeny.

Keywords: Social parasitism, slavery, dulosis, inquilinism, Formicoxenini, *Temnothorax*

INTRODUCTION

Ants are among the most dominant and ecologically most important species of the world. Presently, the world ant fauna comprises approximately 12000 described species, and some species implement fascinating life strategies. As highlighted in previous studies, the myrmicine tribe Formicoxenini is especially rich in social parasites and contains more than 10% of all known socially parasitic ant species. It furthermore appears to be a hot spot in slavemaker evolution (Beibl et al., 2005, in press).

Myrmoxenus is one dulotic genus in this tribe. It is distributed throughout Central and Southern Europe, Bulgaria, the former Czechoslovakia, Georgia, Kazakhstan, and North Africa, and comprises 12 species: *M. adlerzi* (Douwes, Jessen & Buschinger, 1988), *M. africanus* (Bernard, 1948), *M. algerianus* (Cagniant, 1968), *M. bernardi* (Espadaler, 1982), *M. birgitae* (Schulz, 1994), *M. corsicus* (Emery, 1895), *M. gordiagini* Ruzsky, 1902, *M. kraussei* (Emery, 1915), *M. ravouxi* (André, 1896), *M. stumperi* (Kutter, 1950), *M. tamarae* (Arnol'di, 1968), and *M. zaleskyi* (Sadil, 1953). These species all parasitize *Temnothorax* hosts, and their behaviour and biology have been investigated in numerous studies (summarized in Buschinger, 1989), except for *M. africanus*, *M. tamarae*, and *M. zaleskyi*, which are still poorly known. Like several other social parasites, the *Myrmoxenus* species are more or less host-specific (Buschinger, 1989).

In the genus *Myrmoxenus*, a remarkable evolutionary trend from fully developed slavery to a completely workerless parasitic condition (degenerate slave making) can be observed. A transition exists from species such as *M. algerianus* (with more than 200 workers), *M. stumperi*, *M. ravouxi* (with more than 75 workers), and *M. gordiagini* (with up to 40 workers), which can perform well organised slave raids with group recruitment and sting fighting in nature, over species with a low number of workers or none at all like *M. bernardi* (with up to 24 workers) and *M. kraussei* (up to 30 workers), which are able to carry out slave raids in the laboratory but not very successfully, to completely workerless species such as *M. corsicus*, *M. birgitae* and *M. adlerzi* (Buschinger, 1989, 2001). In contrast to true inquilines, the *Myrmoxenus* queen always kills the host queen(s) by throttling during colony foundation, even in the case of workerless species, and consequently, the lifespan of colonies without slavemaker workers is reduced compared to parasitic species with workers which repeatedly replenish the slave stock (Buschinger and Winter, 1982, 1983). Regarding the sexual behaviour of *Myrmoxenus*, there are two different groups, one with mating flights (e.g. *M. gordiagini*, *M. ravouxi*, *M. stumperi*) followed by colony foundation during

summer/autumn, and another one with intranidal mating and thus inbreeding (e.g. *M. adlerzi*, *M. corsicus*, *M. kraussei*) with young queens overwintering in the maternal nest before dispersing the following spring (Buschinger and Winter, 1982; Buschinger, 1989).

By constructing a phylogeny based on mitochondrial DNA sequences, we investigated the phylogenetic relationships between different species of the genus *Myrmoxenus*, and between *Myrmoxenus*, their *Temnothorax* hosts and several formicoxenine non-host species at a molecular level.

MATERIAL AND METHODS

The taxon sample includes five species of the genus *Myrmoxenus*, seven *Temnothorax* host species and nine non-host species collected in Europe. In addition, one *Chalepoxenus* sample was included, and *Cardiocondyla mauritanica* from Spain served as an outgroup species. Further, three sequences of *M. corsicus*, *M. algerianus* and *M. bernardi* were obtained from GenBank (AF096126, AF096132 and AF096127). In Table 3-1, the *Myrmoxenus* species and their respective hosts are listed, whereas Table 3-2 shows the collecting sites of the samples investigated in this study. A map showing sampling localities is presented in Figure 3-1. All in all, our own material contained a total of two samples of *M. gordiagini*, one sample each of *M. adlerzi, M. kraussei* and *M. stumperi*, and eleven samples of *M. ravouxi*, as well as 35 samples of 18 other formicoxenine species from different collecting sites. The material therefore contained a total of 51 individuals from 34 populations. Colonies, colony fragments or single ants were stored in ethanol or kept alive at the laboratory until the time of DNA extraction.

Table 3-1. *Myrmoxenus* and their *Temnothorax* host species (compiled from Buschinger, 1989, 1995, 1997).

Parasite species	**Type**	**Host species**
M. adlerzi	degenerate slavemaker	*L.* cf. *exilis*
M. africanus	?	?
M. algerianus	active slavemaker	*L. spinosus* 3 others
M. bernardi	degenerate slavemaker	*L. gredosi*
M. birgitae	degenerate slavemaker	*L. nivarianus*
M. corsicus	degenerate slavemaker	*L. exilis*
M. gordiagini	active slavemaker	*L. lichtensteini* *L. serviculus*
M. kraussei	degenerate slavemaker	*L. recedens*
M. ravouxi	active slavemaker	*L. unifasciatus* *L. nigriceps* *L. interruptus* *L. affinis* others
M. stumperi	active slavemaker	*L. tuberum*
M. tamarae	?	?
M. zaleskyi	?	?

Table 3-2. Overview of the sampled specimens of *Myrmoxenus* (*M.*), *Temnothorax* (*T.*), *Chalepoxenus* (*C.*) and *Cardiocondyla* and their collection sites. Locality designations correspond to those in Figure 3-1.

Species	Locality	Designation
M. ravouxi	near Millau, Midi-Pyrénées, France	5
	Ste Enimie, Languedoc-Roussillon, France	6
	Mont Ventoux, Provence, France	7
	Savoillan, Provence, France	8
	near Sault, Provence, France	9
	Les Sausses, Provence, France	10
	Bodenreich/Konstanz (Wollmatt. Ried), Baden-Wuerttemberg, Germany	12
	Bodenreich/Konstanz (Eberfall), Baden-Wuerttemberg, Germany	13
	Sipplingen, Baden-Wuerttemberg, Germany	14
	Waldenhausen, Bavaria, Germany	15
	Kallmünz, Bavaria, Germany	17
M. kraussei	Sulzano, Lago d'Iseo, Lombardy, Italy	21
M. gordiagini	near Baška, Krk, Croatia	26
	Baška, Krk, Croatia	27
M. adlerzi	Greece	-
M. stumperi	Switzerland	-
T. unifasciatus	Ste Enimie, Languedoc-Roussillon, France	6
	Savoillan, Provence, France	8
	Taubertal, Bavaria, Germany	16
	Schönhofen, Bavaria, Germany	18
	Gargnano, Lago di Garda, Lombardy, Italy	22
	Manerba, Lago di Garda, Lombardy, Italy	23
	Colle della Croce, near Barrea, Abruzzi, Italy	28
T. recedens	Llança, Catalonia, Spain	3
	El Port de la Selva, Catalonia, Spain	4
	Savoillan, Provence, France	8
	Manerba, Lago di Garda, Lombardy, Italy	23
	near Baška, Krk, Croatia	26
	Baška, Krk, Croatia	27
T. nigriceps	Waldenhausen, Bavaria, Germany	15
	Taubertal, Bavaria, Germany	16
T. tuberum	Binntal, canton Wallis, Switzerland	11
T. affinis	near Sault, Provence, France	9
	Weltenburg, Bayern, Germany	19
	Manerba, Lago di Garda, Lombardy, Italy	23
T. lichtensteini	Caldes, Catalonia, Spain	2
	near Bogino, Lago di Garda, Lombardy, Italy	24
	near Baška, Krk, Croatia	26
	Baška, Krk, Croatia	27
T. gredosi	Spain	-
T. albipennis	Great Britain	31
T. crassispinus	Škocjan, Slovenia	25
T. nylanderi	Brittany, France	-
T. sordidulus	Lovere, Lago d'Iseo, Lombardy, Italy	20
T. flavicornis	Manerba, Lago di Garda, Lombardy, Italy	23
T. laestrygon	Malta	29
T. luteus	Savoillan, Provence, France	8
T. specularis	Sitges, Catalonia, Spain	1
T. cf. *sylvanus*	near Sault, Provence, France	9
C. muellerianus	Gargnano, Lago di Garda, Lombardy, Italy	22
Cardiocondyla mauritanica	Gomera, Canaries, Spain	30

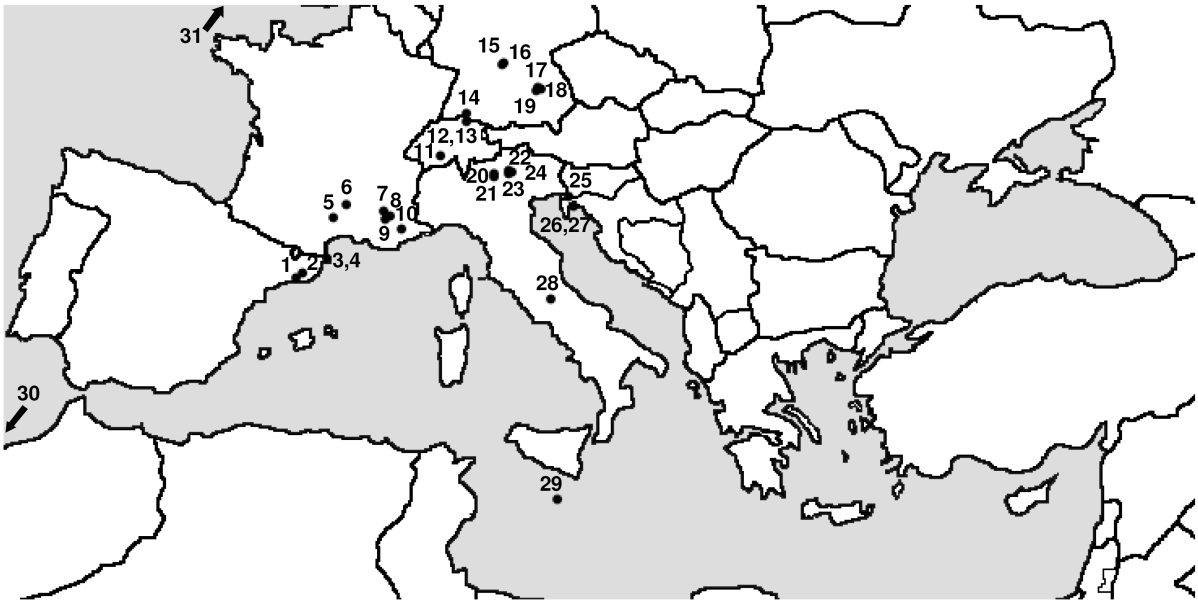

Figure 3-1. Map showing sampling localities of *Myrmoxenus*. For locality names see Table 3-2.

Total genomic DNA was isolated from individual female ants by grinding them in liquid nitrogen, followed by purification using a cetyltrimethyl ammonium bromide protocol (Hamaguchi et al., 1993). For each specimen, a 810 bp fragment of the subunit I of the mitochondrial gene cytochrome oxidase (CO I) was amplified via PCR using the primer pair C1-J-2195 (Simon et al., 1994) and CW-3031rev (Beibl et al., 2005). Double-stranded DNA was amplified in 25 µl volume reactions. 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). The CO I fragment was amplified in a Biometra T1 Thermocycler in 41 cycles of 94°C for 1.15 min, 50°C for 1.15 min, 68°C for 1.30 min, with an initial denaturation step of 4 min at 94°C and a final extension step at 72°C for 5 min. PCR products were either extracted from a 1% agarose gel after separation by electrophoresis using the NucleoSpin® Extract Kit (Macherey-Nagel), or directly using Montage™PCR Centrifugal Filter Devices (Millipore). Cycle sequencing was also performed in a Biometra T1 Thermocycler, using the Big dye Terminator Cycle sequencing kit (Applied Biosystems). Primers used for amplification served as sequencing primers. The 20 µl cycle sequencing reaction mixture contained 20 - 100 ng DNA, 3 µl 5x sequencing buffer, 0.5 µM primer and 2 µl Ready Mix (Applied Biosystems). The reactions were incubated for 30 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C, and stopped by cooling to 6°C. Both strands were sequenced on an ABI Prism 310 genetic analyzer.

After nucleotide sequences were collected, they were compiled, cut to size, edited and aligned in the program Bioedit 7.0.5.2 (Hall, 1999) and adjusted by eye. To infer phylogenetic

relationships among the parasite *Myrmoxenus* and its host species, several analyses were performed using PAUP 4.0b10 (Swofford, 2002) and MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003, 2005). Data were subjected to Neighbour-Joining, Maximum Parsimony, and Bayesian analysis, and phylogenetic trees are based on 651 and 399 base pairs (bp) respectively of the CO I coding region. The sequences used in the final analyses were without gaps, frameshifts, unexpected stop codons, insertions, deletions, or rearrangements. One double peak in the *M. kraussei* sequence was substituted by "N".

Neighbour-Joining analysis was conducted in PAUP using Kimura's two-parameter model (Kimura, 1980). Bootstrap values were estimated from 5000 replicates. Maximum Parsimony searches were also performed in PAUP using the heuristic search option with tree bisection-reconnection (TBR) branch swapping, a random addition sequence with ten replications, and the specification that the ingroup was monophyletic. Clade support was assessed by 2000 bootstrap replicates. A Bayesian analysis was carried out using the program MrBayes with the general time reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites (GTR+I+G), as calculated by Modeltest 3.06 (Posada and Crandall, 1998). The default value of four Markov chains was used, the Monte Carlo Markov chain (MCMC) length was 4,000,000 generations, and the chain was sampled every 500 generations. The first 2000 trees were discarded as "burn-in" and the posterior probabilities of tree topology were estimated from the remaining trees.

RESULTS

Sequences of our own samples produced a final aligned 651 bp fragment which contained 241 sites that were variable and 213 sites that were phylogenetically informative (PAUP 4.0b10). Examinations of base composition in the data set resulted in the following for the entire data set: A: 29.8%, C: 17.6%, G: 12.4%, T: 40.2% (Mega 3.1; Kumar et al., 2004).

In all analyses, only groups with a frequency of more than 50% are shown. Figure 3-2A shows a Neighbour-Joining tree based on 651 bp, Figure 3-2B a Neighbour-Joining tree with three supplementary species based on 399 bp, both with bootstrap support values estimated from 5000 replications. In Figure 3-2C, the majority rule consensus tree from the Bayesian analysis is depicted. The Maximum Parsimony analysis resulted in one single tree (L = 1103), shown in Figure 3-2D.

In all cases, *Myrmoxenus* constitutes an old and very well supported monophylum (bootstrap support values 98, 100 and 97, posterior probability 0.99). *M. gordiagini* from Croatia, Bulgaria and Kazakhstan represents the oldest splitting-off, forming the sister clade to all other *Myrmoxenus* species, formerly known as *Epimyrma*. *M. adlerzi* clusters next to *M. ravouxi* in all analyses, then *M. kraussei* splits off, followed by *M. stumperi*. The Kimura-2 distances between *M. ravouxi* (from Mont Ventoux, France) and the particular *Myrmoxenus* species are: *M. adlerzi*: 0.008, *M. kraussei*: 0.016, *M. stumperi*: 0.033, and *M. gordiagini* (from Baška, Croatia): 0.086. Figure 3-2B including three GenBank sequences indicates that *M. algerianus* forms a monophylum with *M. kraussei*, that *M. bernardi* clusters next to *M. stumperi* but with a low bootstrap support value, and that *M. corsicus* groups with *M. ravouxi*, *M. adlerzi*, *M. algerianus* and *M. kraussei*. Besides the outgroup *Cardiocondyla*, *Chalepoxenus* lies outside the group of *Myrmoxenus* and the sampled *Temnothorax* species in the Neighbour-Joining and the Bayesian analysis. The sister taxon of *Myrmoxenus* could not be identified. The study shows that *Myrmoxenus* is situated amidst the genus *Temnothorax*.

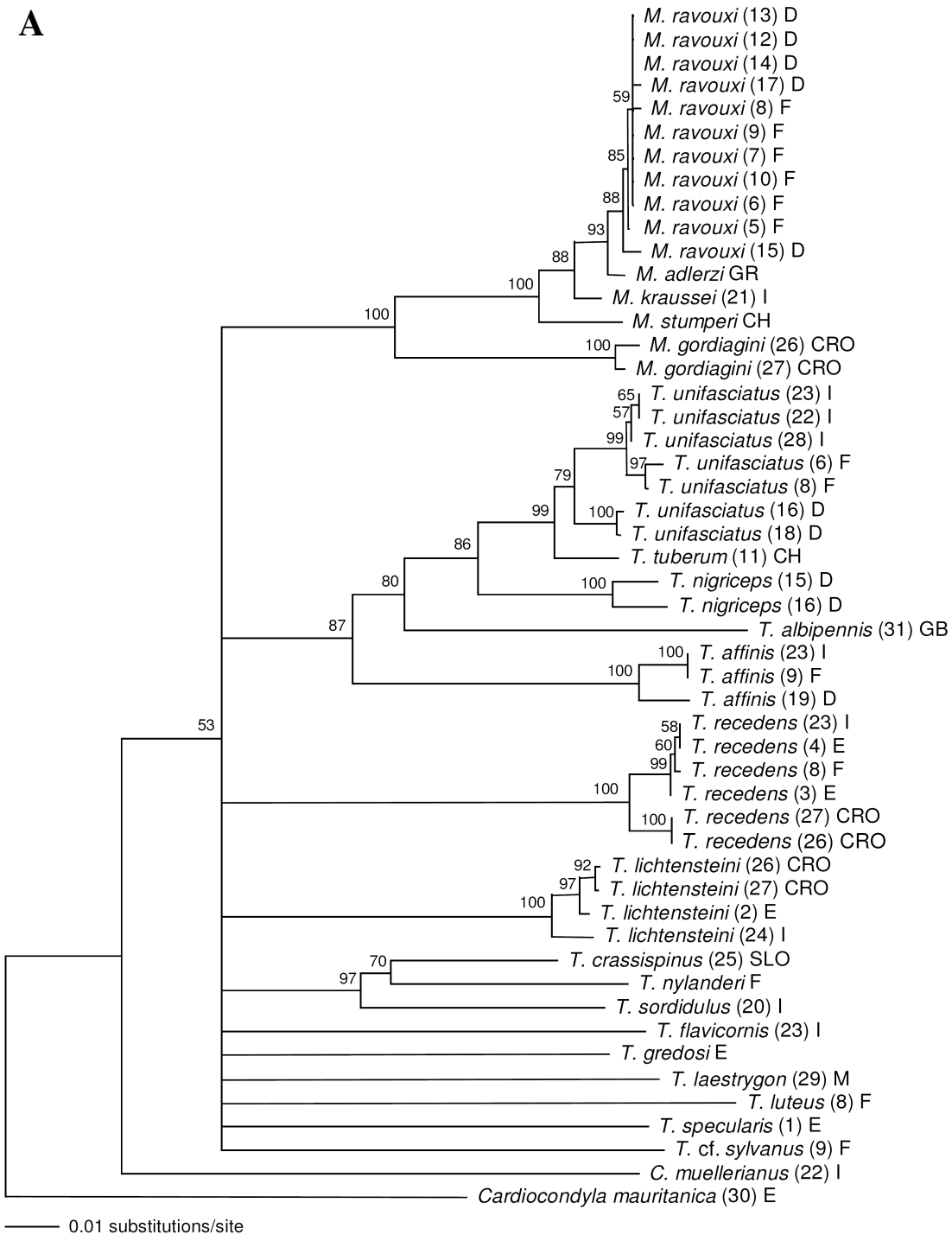

Figure 3-2. Phylogenetic trees of *Myrmoxenus* (*M.*) and other formicoxenine species from different collecting sites, amongst several *Temnothorax* (*T.*) host species. Trees are based on DNA sequences of the mitochondrial CO I gene. Numbers in parentheses correspond to those of localities in Figure 3-1 and Table 3-2. Abbreviations: D, Germany; F, France; GR, Greece; I, Italy; CH, Switzerland; CRO, Croatia; GB, Great Britain; E, Spain; SLO, Slovenia; M, Malta. **A.** Neighbour-Joining tree based on 651 base pairs with bootstrap values estimated from 5000 replicates.

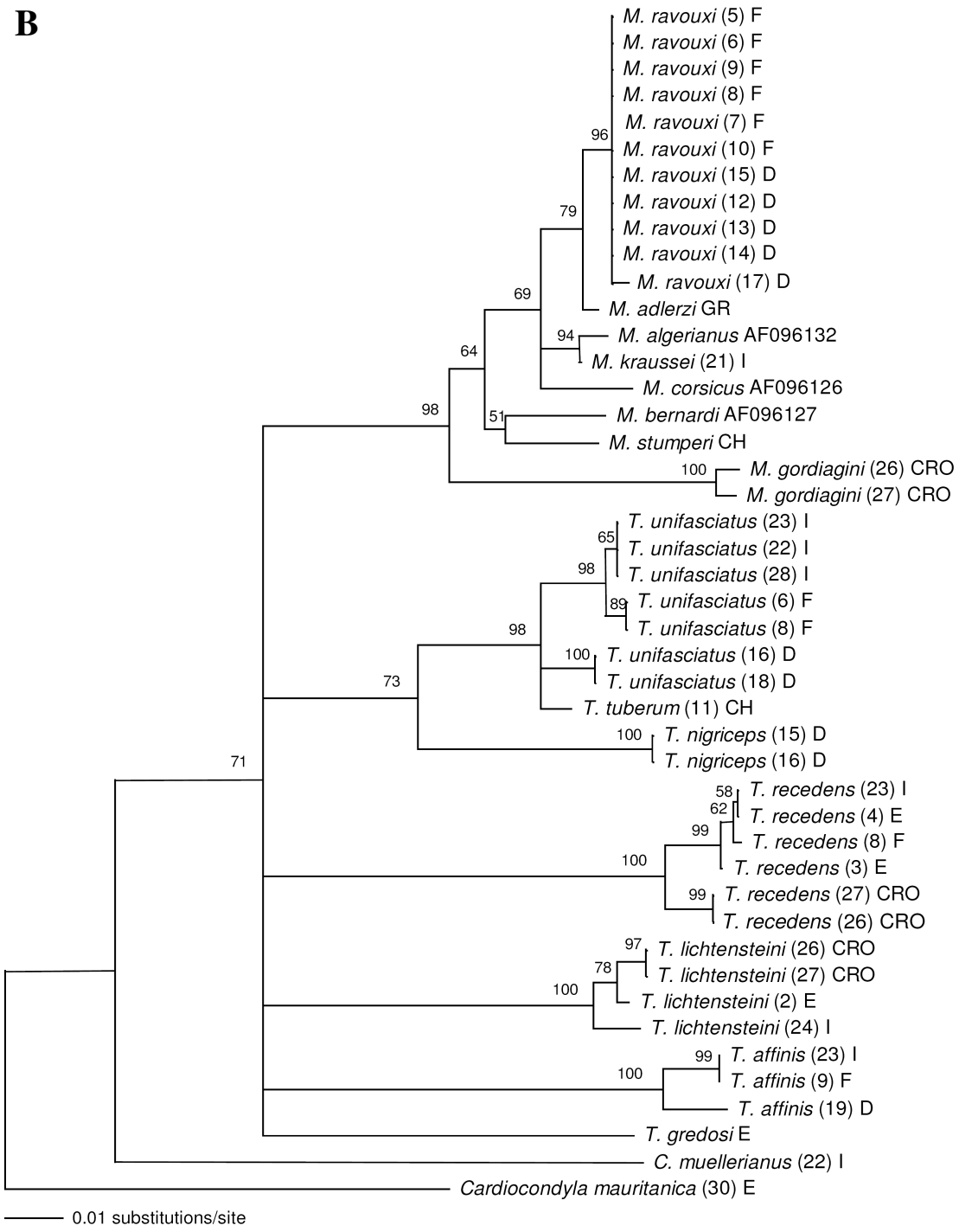

Figure 3-2. (continued) **B.** Neighbour-Joining tree with three additional *Myrmoxenus*-GenBank-sequences, based on 399 base pairs and with bootstrap values estimated from 5000 replicates.

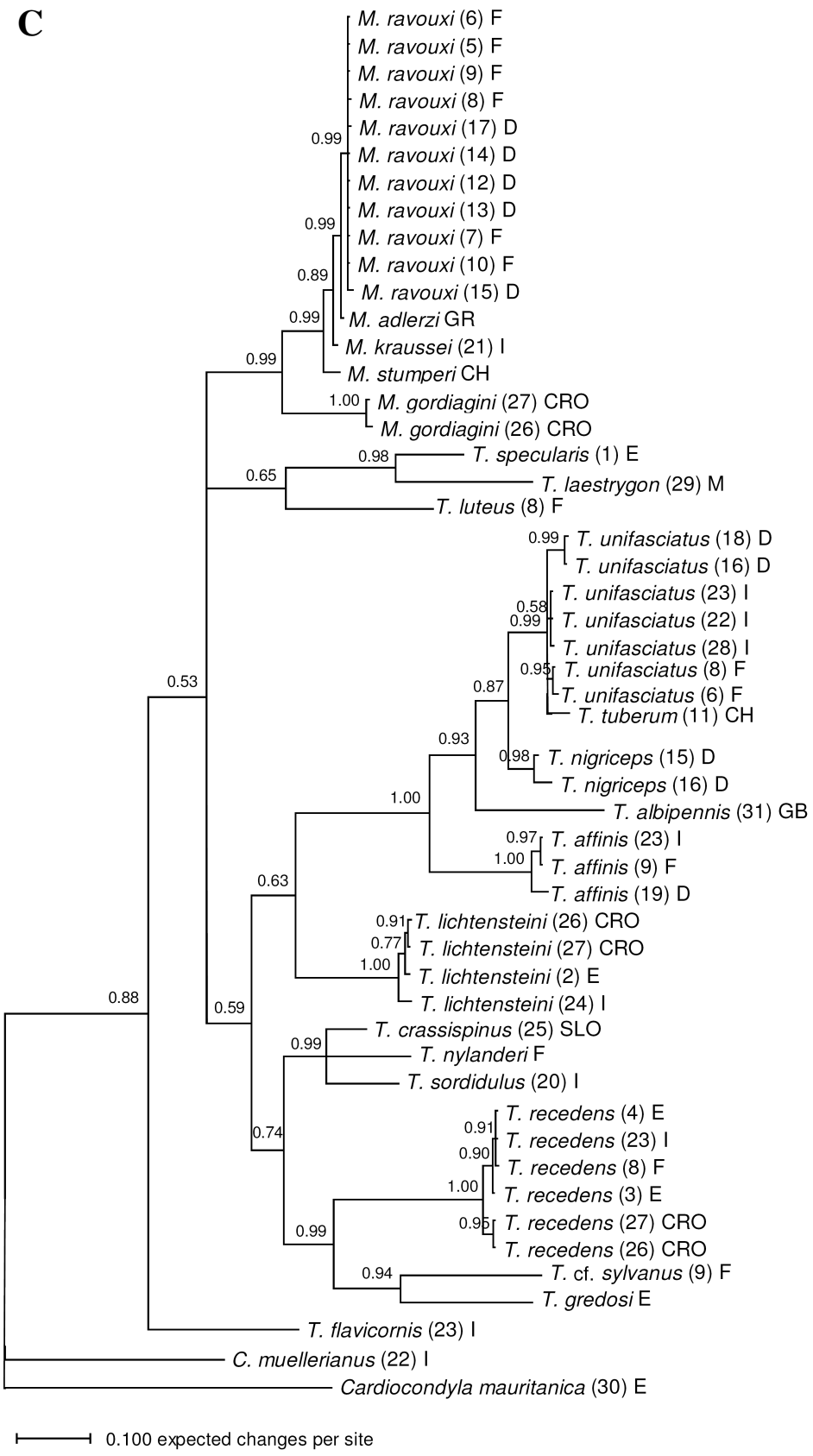

Figure 3-2. (continued) **C.** Majority rule consensus tree recovered in a Bayesian analysis (4,000,000 generations) with the GTR+I+G model. The tree is based on 651 base pairs and numbers represent clade credibility values.

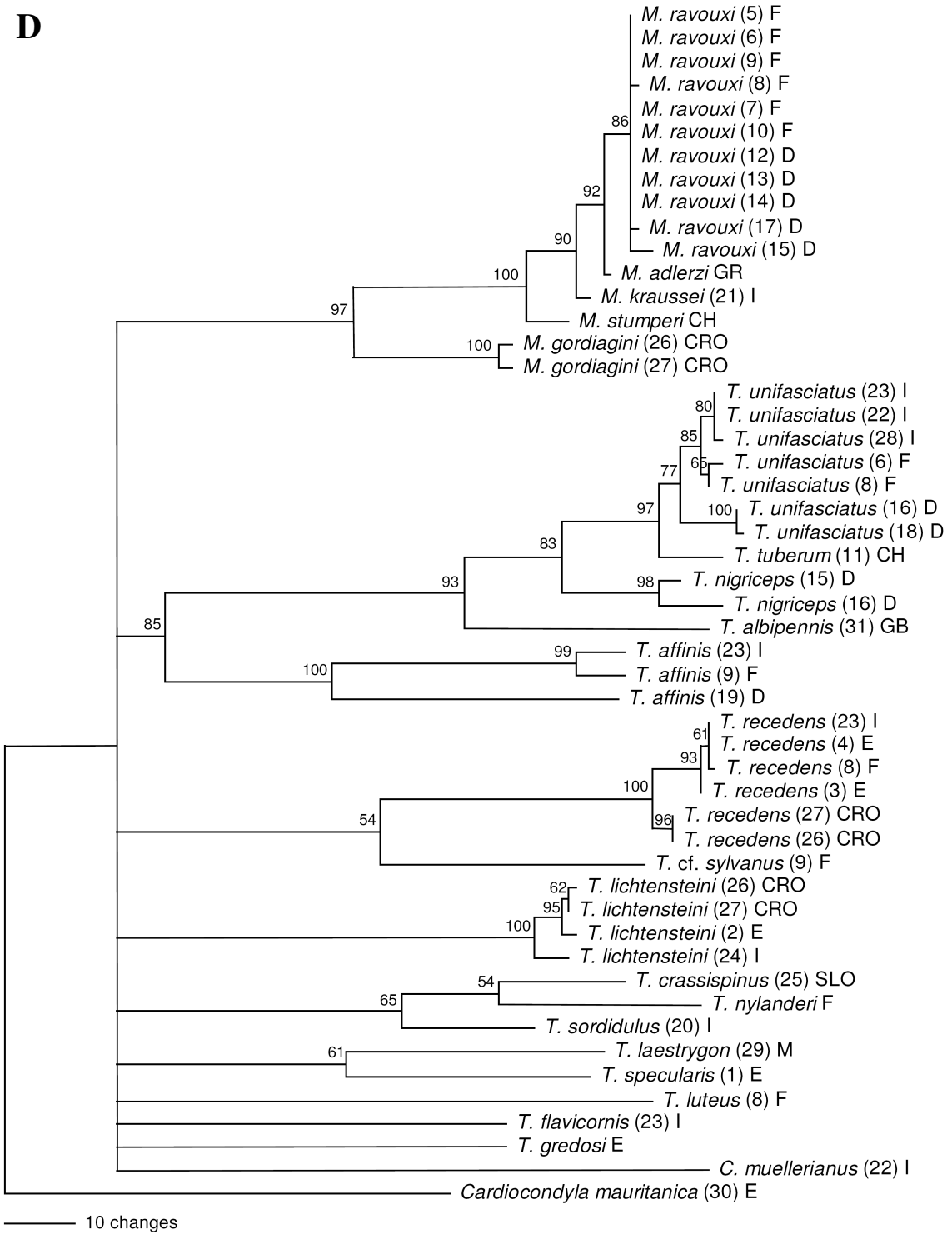

Figure 3-2. (continued) **D.** Single tree based on 651 base pairs, calculated in a Maximum Parsimony analysis by heuristic search, and shown with branch lengths. Support for the clades (>50%) were evaluated with 2000 replicates.

DISCUSSION

Among few molecular studies about formicoxenine ants, Beibl et al. (2005) included one sample of *M. ravouxi* in their analysis and showed that the genus *Myrmoxenus* is well separated from its *Temnothorax* hosts and has a long independent evolutionary history. Our results confirm these findings and provide further insights into *Myrmoxenus* phylogeny. *Myrmoxenus* forms a monophylum, with *M. gordiagini* being the sister group to all other *Myrmoxenus* species examined. It has been shown that all *Myrmoxenus* species share an identical karyotype of n = 10 chromosomes, but the number of antennomeres differs in *M. gordiagini* (12 in females; just as many as most of its host species) and the other *Myrmoxenus* species (usually 11 in females). Buschinger (1989) suggested that *M. gordiagini* might represent the state that is closest to the original, and that further speciation occurred after reduction of the antennal segments. The results of this study support this assumption. Furthermore, the throttling behaviour of young *Myrmoxenus* queens, the group-recruitment during slave raids in species with workers, and successful crossbreedings between several *Myrmoxenus* species investigated in numerous crossbreeding experiments have been suggested to indicate monophyly and a close relationship between the species (Buschinger, 1989, 2001; Buschinger et al., 1986; Jessen, 1987; Jessen and Klinkicht, 1990). Again, our results obtained from mitochondrial DNA sequences corroborate such close relationships. However, the closest relative of the *Myrmoxenus* clade could not be identified.

Interestingly, slavemakers and degenerate slavemakers cluster next to each other in different clades (*M. ravouxi* and *M. adlerzi*, *M. algerianus* and *M. kraussei*, *M. stumperi* and *M. bernardi*, respectively), although one has to admit that the tree is based on only 399 base pairs. However, it seems that degenerate slavemakers evolved from slave-making ancestors several times in *Myrmoxenus*. This is in accordance with Buschinger (1989), who hypothesized that populations of a widely distributed slave-making and outbreeding species specialized on different hosts or were isolated geographically, before some populations developed a tendency to mate near or in the nest and reduce slave raiding, thus becoming degenerate slavemakers.

To further clarify the origin and relationships of the genus *Myrmoxenus*, more material from additional populations and species, as well as more sequence information should be included in future studies.

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Chapter 4

Cuticular profiles and mating preference in a slave-making ant*

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ABSTRACT

The remarkable ability of slave-making ants to integrate chemically in the colonies of their host species makes them useful model systems for investigating the role of cuticular hydrocarbons in chemical recognition. The purpose of our study was to examine the influence of the rearing host species on the cuticular hydrocarbon profile and on the mating behaviour of sexuals of the slave-making ant *Chalepoxenus muellerianus*. Sexuals from a population parasitizing exclusively the host species *Temnothorax unifasciatus* were reared in the laboratory either with their natural host or another potential host species, *Temnothorax recedens. C. muellerianus* males reared with *T. unifasciatus* investigated and mounted female sexuals reared with the same host significantly more often than female sexuals reared with *T. recedens*. Similarly, *C. muellerianus* males reared with *T. unifasciatus* discriminated against female sexuals from natural *T. recedens* colonies. Males experimentally or naturally reared with *T. recedens* did not clearly discriminate between female sexuals reared by the two host species and only rarely engaged in mating attempts with either type of female sexuals. Chemical analyses showed that host species affect the chemical profile of *C. muellerianus* sexuals and vice versa. Our findings indicate that cuticular hydrocarbons might be important in the mating success of this ant species.

Keywords: *Chalepoxenus muellerianus*, social parasitism, mating success, cuticular hydrocarbons, Formicoxenini

INTRODUCTION

Individuals in sexually reproducing species need to locate and recognize appropriate mating partners and therefore have to collect information about their potential mates' species, gender, and quality. Social Hymenoptera (bees, ants, and wasps) are no exception, though sex plays a role only during a very limited period of their lives and is therefore rarely studied (Boomsma et al., 2005). Sexual communication in ants is mostly mediated by volatile pheromones, which are produced in the mandibular glands of the males of some species and in various glands in the female sexuals of others and serve to attract mating partners from a distance (reviewed by Ayasse et al., 2001). Ant males intensively antennate female sexuals before mating (e.g. Hölldobler and Bartz, 1985), suggesting that additional signals are involved in short-range communication. Cuticular hydrocarbons, a blend of surface chemicals known to be crucially important in nestmate recognition and other aspects of interindividual communication (e.g., Soroker et al., 1994, 1995; Vander Meer and Morel, 1998; Lahav et al., 1999; D'Ettorre and Heinze, 2005), might provide such signals. In many insect species, cuticular hydrocarbons play indeed a fundamental role in the context of mating, e.g., the recognition of sex (Carlson et al., 1984; Ginzel et al., 2003; Howard, 1998), mate choice (Howard et al., 2003), and as sex pheromones and male attractants (Blomquist et al., 1993, 1998; Ayasse and Dutzler, 1998; Ayasse et al., 1999; Schiestl et al., 1999). However, detailed experiments on near-range communication among ant sexuals are lacking.

The special lifestyle of slave-making ants and the way in which they are chemically integrated in the colonies of their hosts allows investigating, which role cuticular hydrocarbons play in courtship and mating (D'Ettorre and Heinze, 2001). Slave-making ants are social parasites that depend on the help of host workers from other ant species for all routine tasks in their nests. For the cohesion of their mixed colonies it is necessary that slavemakers and hosts possess a common colony odour (Lenoir et al., 2001). It has previously been shown that the cuticular hydrocarbon profiles of slave-making ants closely resemble those of their hosts, probably because slavemakers synthesize the host chemicals and/or acquire cuticular hydrocarbons from their hosts through grooming and other interactions (Franks et al., 1990; Yamaoka, 1990; Bonavita-Cougourdan et al., 1997, 2004; D'Ettorre et al., 2002; Kaib et al., 1993; Lenoir et al., 2001). Slave-making ants are therefore a particularly useful model system for studying interactions between social environment, chemical signature, and behaviour.

The slave-making ant *Chalepoxenus muellerianus* (Finzi, 1922) enslaves 12 or more species of the related genus *Temnothorax*. In contrast to several other formicoxenine slavemakers (Buschinger, 1966; Buschinger and Alloway, 1977; Buschinger and Winter, 1983), colonies and also whole populations of *C. muellerianus* typically parasitize only a single of several suitable host species present. For example, 96.6% of all *C. muellerianus* colonies investigated by Buschinger et al. (1988a) contained only one of several sympatric *Temnothorax* host species. About 3/4 of the colonies utilised exclusively *T. unifasciatus* (Latreille, 1798), 10% contained *T. recedens* (Nylander, 1856), and only 3.4% had a mixture of several host species. Schumann and Buschinger (1994, 1995) suggested that this preference for a single host species results from imprinting of young slavemakers on the odour of the host present in their nests: for colony foundation, young parasite queens preferentially invade colonies of the host species they are familiar with and likewise, during slave raids slavemaker workers pillage brood only of the species already present as hosts in their maternal colonies. The importance of imprinting and early learning of olfactory stimuli in host choice has previously been demonstrated also in the Nearctic slave-making ant *Polyergus lucidus* (Goodloe and Sanwald, 1985; Goodloe et al. 1987), and imprinting appears also to be important in other contexts, such as brood acceptance (Jaisson, 1975; Le Moli and Passetti, 1977; Le Moli and Mori, 1982) and habitat choice (e.g., Jaisson, 1980; Dijeto-Lordon and Dejean, 1999).

The chemical basis for the imprinting effect in *C. muellerianus* is unknown, neither for workers nor for sexuals. It has also not yet been studied whether imprinting affects mate choice and in this way might eventually lead to the formation of host races. Goodloe et al. (1987) already suggested determining whether mate choice by slavemaker sexuals, in particular those of *Polyergus lucidus*, is influenced by the host species in their partner's nest of origin. As cuticular hydrocarbons play an important role in interindividual recognition and in the mating process of many insect species, we investigated how the host species affects the cuticular hydrocarbon profile of *C. muellerianus* male and female sexuals and conducted mating experiments with slavemaker sexuals reared with two different host species, *T. unifasciatus* and *T. recedens.*

MATERIAL AND METHODS

Ant collecting and culture

In March 2003, colonies of the myrmicine slavemaker *Chalepoxenus muellerianus* and the two host species *Temnothorax unifasciatus* and *T. recedens* were collected from dry walls and rotten sticks at Tignale and Rovato at Lago di Garda, Italy. In these populations, *C. muellerianus* exclusively utilizes *T. unifasciatus* as host, and mixed colonies only contained the slavemaker and *T. unifasciatus* slaves. *T. recedens*, which is used as host species by *C. muellerianus* in other populations, occurs in the same sites and with the same density as *T. unifasciatus* (Schumann and Buschinger, 1994). In late April 2005, *Chalepoxenus muellerianus* colonies were collected near Baška on the island of Krk, Croatia. Colonies from Baška use only *T. recedens* as host species and *T. unifasciatus* was not found at this site.

The ants were housed in the laboratory in artificial nests in plastic boxes (10 x 10 x 3 cm³) with three connected chambers and a regularly moistened plaster floor (Buschinger, 1974; Heinze and Ortius, 1991). Twice a week all colonies were fed with diluted honey and pieces of cockroaches. Ants were kept in an incubator under semi-natural conditions with an annual cycle of artificial seasons with daily temperature variations and a natural photoperiod.

Behavioural experiments

Rearing of sexuals

Of the 23 *C. muellerianus* laboratory colonies from Italy with *T. unifasciatus* as host, 65% produced sexual pupae (a total of 146 female sexuals and 117 males in 2003, 45 female sexuals and 167 males in 2004, and 24 female sexuals and 66 males in 2005). As *C. muellerianus* sexuals reared with *T. recedens* were not available from the field in 2003 and 2004, about half of the unpigmented, young male and female pupae were removed from their mother colonies and transferred into free-living Italian *T. recedens* colonies (n = 19). Pupae were used because they can easily be transferred between nests of different ant species (Schumann and Buschinger, 1991, 1994; D'Ettorre et al., 2002; Errard et al., 2006a). *C. muellerianus* sexuals eclosed about three weeks later. After a short period, the callows start to synthesize their own cuticular hydrocarbons and to adsorb chemicals from their nestmates to integrate into the colony (Lenoir et al., 2001; Morel and Blum, 1988; Dahbi et al., 1998). We thus obtained male and female sexuals of Italian *C. muellerianus* that had either eclosed

in nests of their original host *T. unifasciatus* or in *T. recedens* nests. Female sexuals of *C. muellerianus* reared by *T. unifasciatus* workers are hereafter referred to as FU, female sexuals reared by *T. recedens* workers as FR. Similarly, *C. muellerianus* males reared by *T. unifasciatus* are referred to as MU and males reared by *T. recedens* workers as MR.

Additionally, of the 15 *C. muellerianus* colonies from Croatia with *T. recedens* as original host species, 53% produced sexual pupae in 2005 (a total of 62 female sexuals and 48 males). *C. muellerianus* female sexuals from Krk are referred to as FRK, males as MRK. *C. muellerianus* from Krk could not be transferred into sympatric *T. unifasciatus* colonies, as this species was not found at the collecting site.

Mating experiments

Behavioural experiments were carried out after eclosion of the sexuals between June and August 2003, between July and September 2004, and in August and September 2005, when the ants experienced summer conditions (28°C, 12 h light and 17°C, 12 h dark). Though there are no reports about the mating behaviour of *Chalepoxenus* in the field, it is known that young sexuals, as in most formicoxenine ants, leave the nest in summer to engage in mating flights (Buschinger et al., 1988b). In the field, winged and dealate *C. muellerianus* females have been collected in July and August 1986 near Rome (Mei, 1992). When ready to mate, young *Chalepoxenus* sexuals leave their nests about 2 weeks after eclosion and crawl or flutter around (Schumann and Buschinger, 1994; Buschinger et al., 1988b). Though female sexuals do not perform any visible sexual calling behaviour, they appear to produce sexual pheromones in the poison gland, which attract males (Buschinger et al., 1988b).

A total of 132 behavioural experiments were conducted in plastic flight cages of 35 x 22 x 29 cm^3 in 2003, and 24 x 16 x 17 cm^3 in 2004 and 2005 with a wire mesh on two sides to allow an air flow (Table 4-1). Trials were carried out on a meadow at the university campus at Regensburg or in the laboratory when the weather was cold and unstable. Since swarming behaviour naturally occurs between 4 and 9 pm (Schumann and Buschinger, 1994), the experiments were carried out in the afternoon, mostly around 2 to 8 pm. Only sexuals that had left the nest chambers of the formicaries were used for the mating experiments. For each trial, one female and one male *C. muellerianus* sexual were placed together in a flight cage. Although *C. muellerianus* form loose mating swarms in the field, only two individuals were used per trial, because marking these small, winged ants individually would have had a negative effect on their condition and presumably influenced mating behaviour. Females and males inside a flight cage were unrelated and each ant was used only in one experiment. The

frequency of the social interactions between each pair was recorded for 90 to 150 minutes, depending on the weather conditions. However, most of the trials (88%) lasted 120 minutes. The duration of the trials did not influence the activity of sexuals.

As complete mating sequences are often difficult to obtain in flight cages, we in particular focussed on the gentle, inspective antennation of the other individual's body, which typically precedes copulation attempts and in *C. muellerianus* reliably occurred in flight cages, and on mating attempts, i.e., males trying to mount the female sexuals and to insert their genitalia into the female cloaca. Complete copulations, during which the male tilted backwards with inserted genitalia, were rarely observed.

Behavioural data were analysed by Mann-Whitney U-tests, χ^2-tests, and, in the case of smaller data sets, two-sample permutation tests. The occurrence of antennations differed between 2003 and 2004, probably due to differences in cage size and/or environmental conditions (Mann-Whitney U-test: antennal contacts/min performed, males: $U = 93.0$; $p = 0.0001$; female sexuals: $U = 176.0$; $p = 0.0001$) and data from the two years were therefore analysed separately. The number of mating attempts/min did not differ between 2003 and 2004 ($U = 458.5$; $p = 0.382$) and therefore could be pooled for a χ^2-test. Data from 2005 with *C. muellerianus,* which naturally parasitized *T. recedens*, were again analysed separately. Combined probabilities were obtained using Stouffer's method, which is superior to Fisher's combined probability test (e.g., Whitlock, 2005).

Chemical analysis

We investigated the cuticular hydrocarbons of 100 *C. muellerianus* sexuals from ten *T. recedens* and ten *T. unifasciatus* colonies (26 FU; 21 FR; 33 MU; 20 MR), 10 slaves each from two colonies with *T. unifasciatus* (SU) and two with *T. recedens* (SR), and 10 workers each from three unparasitized *T. unifasciatus* (WU) and three unparasitized *T. recedens* (WR) colonies from Lago di Garda. Ants were killed by freezing and cuticular compounds were extracted by immersing them individually in 25 µl of pentane for 15 min. After evaporation of the solvent, the residues were re-dissolved in 10 µl of pentane and 2 µl of this solution were then injected into an Agilent Technologies 6890N gas chromatograph equipped with a flame ionisation detector and a capillary column. The injector was split-splitless, and the carrying gas was helium at 1ml/min. For half of the samples, a Rtx-5 capillary column (30 m x 0.25 mm x 0.50 µm, Restek, Bellefonte, USA) and a temperature program of 1 min at 80°C, from 80°C to 180°C at 30°C/min, from 180°C to 280°C at 4°C/min, and then held at 280°C for 22 min was used. The other half of the samples was analysed on a HP-5 capillary column

(30 m x 0.32 mm x 0.25 µm, J&W Scientific, USA) with a temperature program of 1 min at 80°C, from 80°C to 180°C at 30°C/min, from 180°C to 300°C at 4°C/min, and then held at 300°C for 12 min. The resulting chromatograms obtained with both methods were comparable and yielded the same pattern of peaks. Gas chromatography gave consistently 33 peaks, of which 21 could be identified by comparison with standards and from their mass spectra, produced by electron ionisation mass spectrometry using a Hewlett Packard (Palo Alto, CA, USA) 5890A gas chromatograph coupled to an HP 5917A mass selective detector. The analysis was performed on a HP-5MS column (30 m x 0.247 mm x 0.25 µm) with a temperature program of 1 min at 80°C, from 80°C to 180°C at 30°C/min, from 180°C to 280°C at 4°C/min, and then held at 280°C for 22 min. Electron impact mass spectra were obtained with an ionisation voltage of 70eV and a source temperature of 230°C.

Chemical profiles were compared by multivariate statistical analysis (Statistica 6.0, Statsoft, and SPSS 13.0, SPSS Inc.). The proportions of the 33 consistently detectable peaks were first analysed by principal component analysis (PCA) to reduce the number of variables used in the following discriminant analysis (DA). The standardized discriminant function coefficients and the factor loadings (> 0.7) were used to assess the importance of single compounds. DA was used to determine whether defined groups could be distinguished on the basis of their cuticular profiles and to assess the degree of similarity between groups. The correct classification of individuals to the respective groups was verified. Groups were also compared by calculating the squared Mahalanobis distances between the group centroids.

RESULTS

Behaviour

We investigated whether the species of the host that cared for the eclosing *C. muellerianus* sexuals had an influence on their interactions during courtship and mating. In particular, we analysed the number of antennal contacts per minute of males towards female sexuals and vice versa. Results for male sexuals are shown in Fig. 4-1 (a, b: 2003; c, d: 2004). Male and female sexuals did not differ in their antennation rates in 2003 (two-sample permutation test, $n_{FU} = 18$, $n_{MU} = 23$, $p = 0.212$; $n_{FR} = 17$, $n_{MR} = 12$, $p = 0.378$), but males were significantly more active in 2004 ($n_{FU} = 14$, $n_{MU} = 13$, $p = 0.022$; $n_{FR} = 16$, $n_{MR} = 17$, $p = 0.007$). Although there was a significant difference in the antennation rate of MU and MR towards FU in 2003 ($n_{MU} = 12$, $n_{MR} = 6$, $p = 0.043$), differences in activity were only marginally significant in 2004 ($n_{MU} = 6$, $n_{MR} = 8$, $p = 0.080$) and not significant concerning the antennation of MU and MR towards FR (2003: $n_{MU} = 11$, $n_{MR} = 6$, $p = 0.140$; 2004: $n_{MU} = 7$, $n_{MR} = 9$, $p = 0.904$).

Males reared by a particular host seemed to be more interested in female sexuals reared by the same host than those reared by the other host. In 2003, MU antennated FU significantly more often than FR ($n_{FU} = 12$, $n_{FR} = 11$, $p = 0.027$; Fig. 4-1a), but a difference in the same direction was not significant in the second year with a smaller sample size ($n_{FU} = 6$, $n_{FR} = 7$, $p = 0.799$; Fig. 4-1c). Similarly, in both years the average antennation rate of MR towards FR was higher than towards FU, though not significantly so (2003: $n_{FU} = n_{FR} = 6$, $p = 0.228$, Fig. 4-1b; 2004: $n_{FU} = 8$, $n_{FR} = 9$, $p = 0.087$, Fig. 4-1d). In 2005, MU also antennated FU significantly more often than female sexuals from natural colonies from Krk with *T. recedens* as slaves ($n_{FU} = 16$, $n_{FRK} = 20$, $p = 0.002$; Fig. 4-1e). The antennation rate of MRK did not differ between FRK and FU ($n_{FU} = 29$, $n_{FRK} = 2$, $p = 0.477$ Fig. 4-1f). Taking the results from the three years together, MU males appeared to be significantly more attracted to virgin queens reared by *T. unifasciatus* (Stouffer's method, $Z_S = -2.292$, $p = 0.0017$), while males reared by *T. recedens* did not choose ($Z_S = -1.247$, $p = 0.107$). Female sexuals did not preferentially contact a certain type of male (FU, 2003: $n_{MU} = 12$, $n_{MR} = 6$, $p = 0.427$; 2004: $n_{MU} = 6$, $n_{MR} = 8$, $p = 0.146$; 2005: $n_{MU} = 16$, $n_{MRK} = 2$, $p = 0.727$, $Z_S = -0.367$, $p = 0.358$; FR, 2003: $n_{MU} = 11$, $n_{MR} = 6$, $p = 0.686$; 2004: $n_{MU} = 7$, $n_{MR} = 9$, $p = 0.362$, FRK: $n_{MU} = 20$, $n_{MRK} = 29$, $p = 0.628$, $Z_S = 0.262$, $p = 0.603$).

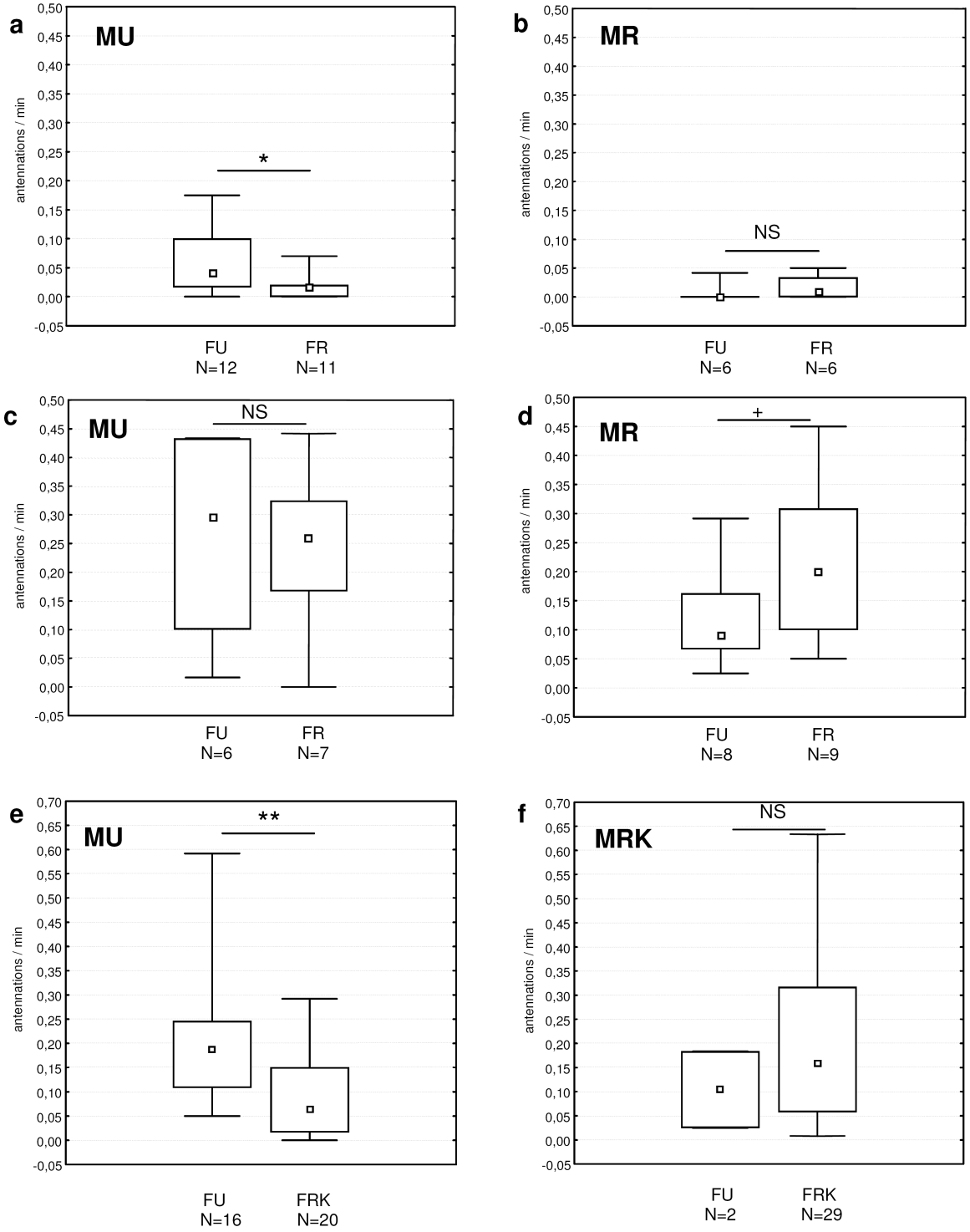

Figure 4-1. Antennal contacts per minute performed by male sexuals. a, b: 2003. c, d: 2004. e, f: 2005. In the upper left corner the actor is indicated, the receiver is given below the bars. Sample size is given below. Box plots show the median (□), 25% and 75% quartiles and minimum and maximum values. Significance levels from permutation tests are indicated as follows: ** < 0.01; * $p < 0.05$, + $0.05 < p < 0.1$, NS $p > 0.1$

Summed over 2003 and 2004, significantly more MU-FU pairs than MU-FR pairs showed mating activity, while there was no significant difference between MR-FR and MR-FU pairs. Only FU-MU pairs succeeded in copulating (Table 4-1). Concerning mating activity, neither MU nor MRK showed a significant preference for a certain kind of female (χ^2-test, MU: $\chi^2 = 2.56$, $p = 0.109$; MRK: $\chi^2 = 1.44$, $p = 0.230$; Table 4-1). Again, the only pair that succeeded in copulating consisted of a male and a female sexual reared with *T. unifasciatus* (Table 4-1).

Table 4-1. Number of trials, number of successful copulations, and number of pairs of the slave-making ant *Chalepoxenus muellerianus* showing mating activity ; FU stands for *C. muellerianus* female sexuals reared by *T. unifasciatus*, FR for *C. muellerianus* female sexuals reared by *T. recedens,* and MU and MR are *C. muellerianus* males reared by *T. unifasciatus* and *T. recedens*, respectively. FRK and MRK are *T. recedens* reared sexuals from Krk.

	Male	**Female**	**Number of trials**	**Number of successful copulations**	**Total number of pairs showing mating activity**	
Years 2003 and 2004	MU	FU	18	4	10 (55.6%)	$\chi^2 = 6.13$, $p = 0.013$
	MU	FR	18	0	2 (11.1%)	
	MR	FU	14	0	1 (7.1%)	$\chi^2 = 0.00$, $p = 0.949$
	MR	FR	15	0	2 (13.3%)	
Year 2005	MU	FU	16	1	5 (31.3%)	$\chi^2 = 2.56$, $p = 0.109$
	MU	FRK	20	0	2 (10.0%)	
	MRK	FU	2	0	0 (0.0%)	$\chi^2 = 1.44$, $p = 0.230$
	MRK	FRK	29	0	2 (6.9%)	

Chemical analysis

C. muellerianus sexuals and their host workers show a complex cuticular profile characterized by linear and methyl-branched alkanes with chain lengths from C25 to C31 (Fig. 4-2). PCA performed on the 33 compounds of *C. muellerianus* sexuals, their *T. unifasciatus* (SU) and *T. recedens* slaves (SR) and workers from unparasitized host colonies (WU, WR) produced 8 principal components with eigenvalues greater than 1, explaining 76% of the total variance. A subsequent discriminant analysis performed on 9 variables (factor loading > 0.7) significantly separated the eight groups (Wilks' $\lambda = 0.0013$, $F_{(56,678)} = 29.53078$, $p < 0.0001$; Fig. 4-3) and correctly classified 87.1% of all individuals. The classification was particularly good for MU (93.9%), FR (95.2%), SU (90%), SR (100%), WU (90%) and WR (90%), while only 84.6% of FU and 60% of MR were correctly classified. Two of 26 FU were classified each as MU and FR, and of the 20 MR four were classified as MU, two as SU, and one each as FU and FR. Function 1 accounted for 67%, function 2 for 18% of the total variance.

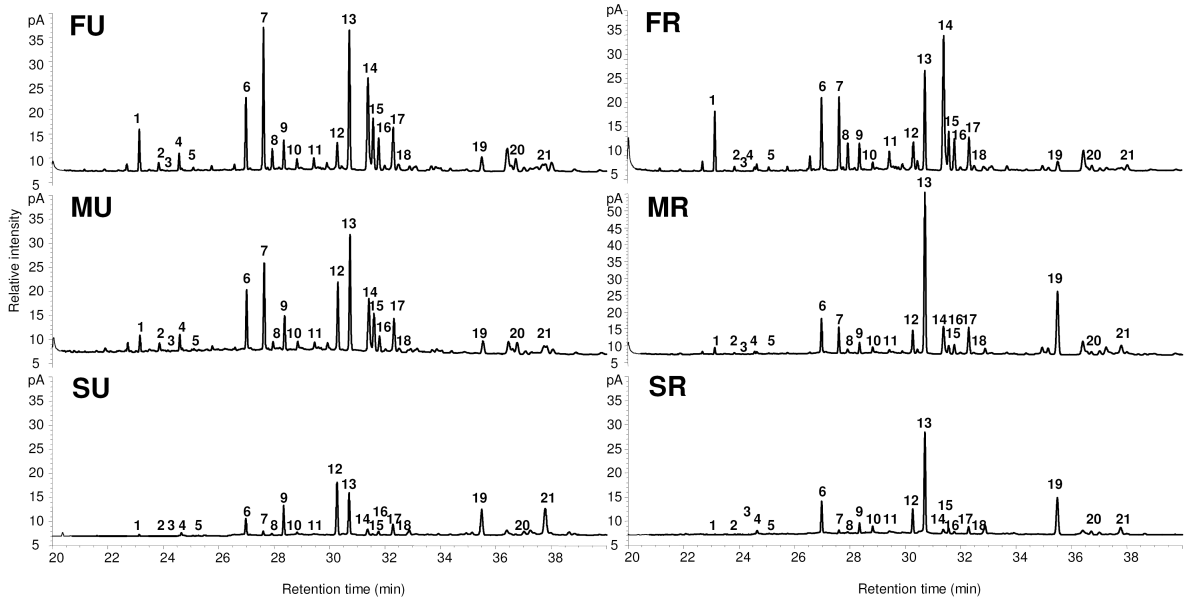

Figure 4-2. Representative gas-chromatograms of female sexuals (F) and males (M) of *Chalepoxenus muellerianus* reared with *Temnothorax unifasciatus* (U) and with *Temnothorax recedens* (R), and of *T. unifasciatus* (SU) and *T. recedens* (SR) slaves, with indication of the identified peaks: (1) C_{25}; (2) 11-Me-C_{25} + 13-Me-C_{25}; (3) 5-Me-C_{25}; (4) 3-Me-C_{25}; (5) C_{26}; (6) 4-Me-C_{26}; (7) C_{27}; (8) 11-Me-C_{27} + 13-Me-C_{27}; (9) 7-Me-C_{27} + 5-Me-C_{27}; (10) 3-Me-C_{27}; (11) C_{28}; (12) 2-Me-C_{28} + 4-Me-C_{28}; (13) C_{29}; (14) 11-Me-C_{29} + 13-Me-C_{29} + 15-Me-C_{29}; (15) 7-Me-C_{29}; (16) 11,15-diMe-C_{29} + 13,17-diMe-C_{29}; (17) 3-Me-C_{29}; (18) 5,y-diMe-C_{29}; (19) C_{31}; (20) x-Me-C_{31}; (21) 5-Me-C_{31}.

All squared Mahalanobis distances between the eight groups were statistically significant. The distance between *C. muellerianus* sexuals and SU was always smaller than that between *C. muellerianus* and SR. This means that the hydrocarbon pattern of the *C. muellerianus* sexuals was more similar to *T. unifasciatus* slaves than to *T. recedens* slaves. However, sexuals reared with *T. unifasciatus* clearly differed from those reared with *T. recedens*, i.e., the rearing host species influenced the chemical profile of *Chalepoxenus*. Comparing the distance between FU/FR and MU/MR, the influence of *T. recedens* appeared to be stronger in *Chalepoxenus* female sexuals than in males, because in females the difference was larger. Furthermore, slaves were considerably more similar to *C. muellerianus* than to unparasitized workers, suggesting that they acquire substances from their parasites. Interestingly, the distance between workers from unparasitized colonies of *T. unifasciatus* and *T. recedens* was considerably smaller than that between parasitized workers of both species, i.e., cuticular profiles of unparasitized workers of both host species were much more similar to each other than the profiles of slaves from both host species (Table 4-2).

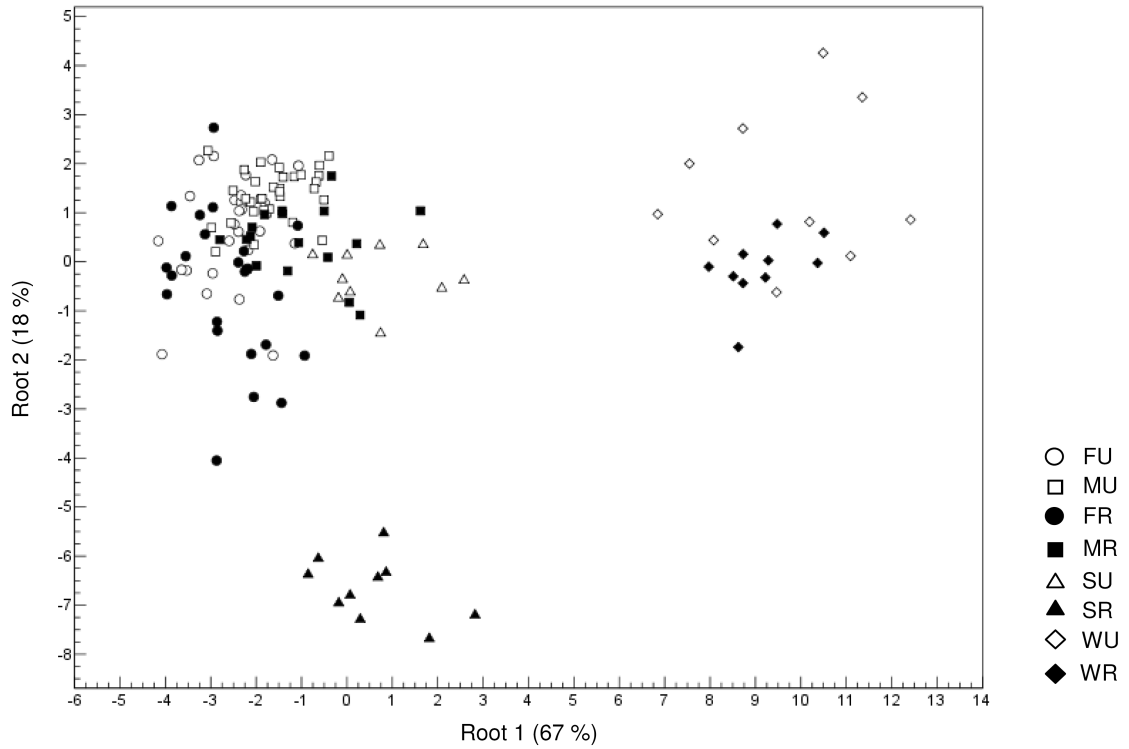

Figure 4-3. Plot of the first two functions of the discriminant analysis of cuticular hydrocarbon profiles of female sexuals (F) and males (M) of *Chalepoxenus muellerianus* reared with the host species *Temnothorax unifasciatus* (U) and *T. recedens* (R), including slaves (S) and unparasitized workers (W) of the two species. Due to the long distance between unparasitized host workers and *Chalepoxenus* sexuals, the slavemakers are clumped on the left side of the graph.

Table 4-2. Matrix of the squared Mahalanobis distances between the cuticular profiles of *C. muellerianus* female sexuals (F) and males (M) reared with the host species *Temnothorax unifasciatus* (U) and *T. recedens* (R), including slaves (S) and unparasitized workers (W) of the two species (referring to discriminant analysis, Figure 4-3).

	FU	**MU**	**FR**	**MR**	**SU**	**SR**	**WU**	**WR**
FU	-							
MU	**9,91** F=16.46 p<0.001	-						
FR	**10,31** F=13.56 p<0.001	**23,67** F=34.48 p<0.001	-					
MR	**9,80** F=12.53 p<0.001	**3,41** F=4.82 p<0.001	**13,23** F=15.25 p<0.001	-				
SU	**28,84** F=22.59 p<0.001	**12,13** F=10.09 p<0.001	**43,80** F=32.18 p<0.001	**14,42** F=10.42 p<0.001	-			
SR	**72,02** F=56.41 p<0.001	**76,75** F=63.82 p<0.001	**61,51** F=45.19 p<0.001	**62,04** F=44.8 5p<0.001	**51,17** F=27.26 p<0.001	-		
WU	**159,11** F=124.64 p<0.001	**134,44** F=111.78 p<0.001	**175,73** F=129.11 p<0.001	**129,61** F=93.70 p<0.001	**97,16** F=51.76 p<0.001	**163,27** F=86.97 p<0.001	-	
WR	**144,80** F=113.43 p<0.001	**136,15** F=113.21 p<0.001	**144,63** F=106.26 p<0.001	**119,83** F=86.62 p<0.001	**101,78** F=54.21 p<0.001	**134,35** F=71.57 p<0.001	**15,91** F=8.47 p<0.001	-

In a complementary analysis, only the four groups of *Chalepoxenus* sexuals were compared. The PCA performed on the chemical data produced 7 principal components with eigenvalues greater than 1, explaining 75% of the total variance. A subsequent discriminant analysis performed on 11 variables (factor loading > 0.7) significantly separated the four groups of males and female sexuals (MU, MR, FU and FR, Wilks' $\lambda = 0.0779$; $F_{(21,258)} = 17.667$, $p < 0.0001$; Fig. 4-4). The three groups MU, MR and FU overlapped, whereas function 1 clearly separated FR from the other groups. Discriminant analysis correctly classified 92% of FU, 91% of MU, 95% of FR and 75% of MR. The results of this second analysis again confirm that the host species clearly influences the hydrocarbon pattern of the *C. muellerianus* sexuals. For the sake of completeness, a PCA on only the 21 identified peaks produced 6 principal components (eigenvalues > 1), which explained 79% of the total variance. DA performed on 11 variables (factor loading > 0.7) also significantly differentiated the four groups of female and male sexuals (Wilks' $\lambda = 0.1089$; $F_{(18.257)} = 17.04818$, $p < 0.0001$).

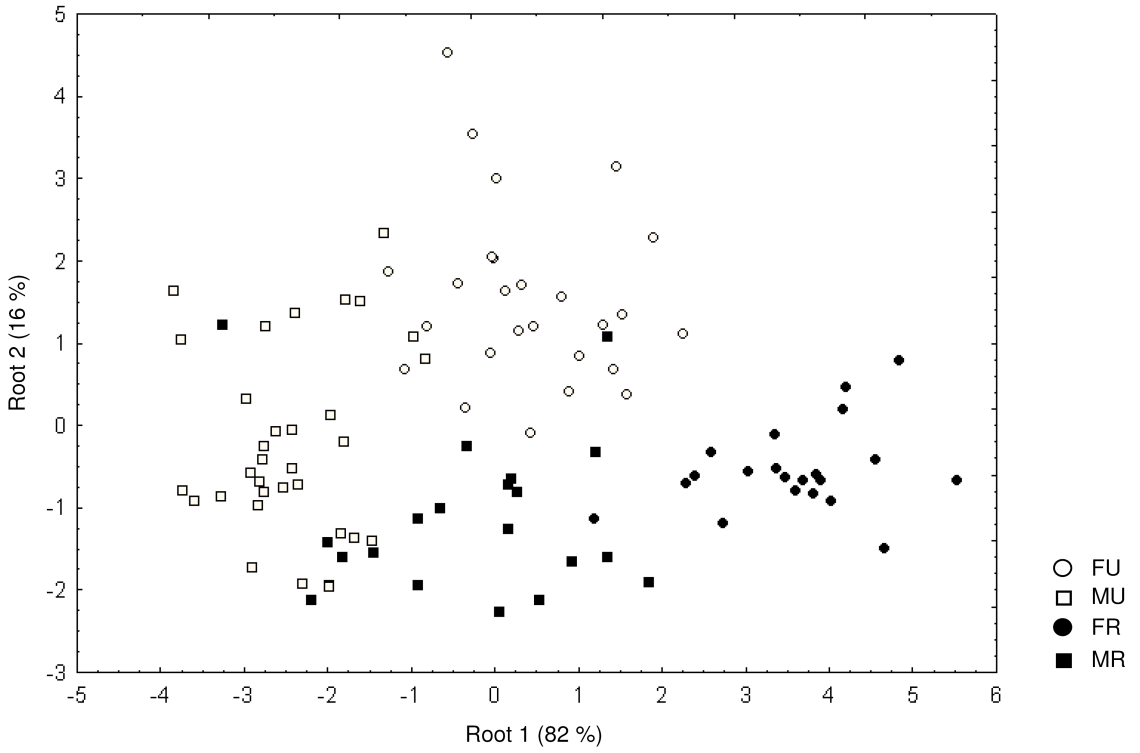

Figure 4-4. Plot of the first two functions of the discriminant analysis of cuticular hydrocarbon profiles of female sexuals (F) and males (M) of *Chalepoxenus muellerianus* reared with the host species *Temnothorax unifasciatus* (U) and *T. recedens* (R).

DISCUSSION

Our study documents that, on the one hand, the presence of *Chalepoxenus muellerianus* changes the chemical profile of the slaves present in the nest, and, on the other hand, the host species present in nests of the slave-making ant influences the cuticular hydrocarbon pattern of slavemaker sexuals. Chemical camouflage and mimicry have previously been shown also in a few other slave-making ants and are therefore not unexpected (Franks et al., 1990; Yamaoka, 1990; Kaib et al., 1993; Bonavita-Cougourdan et al., 1997, 2004; Lenoir et al., 2001; D'Ettorre et al., 2002).

The cuticular hydrocarbon profile of *C. muellerianus* consists of linear and methyl-branched hydrocarbons with chain lengths ranging from C25 to C31. A similar predominance of alkanes was previously also found in the related Nearctic slavemaker *Protomognathus americanus* (Brandt et al., 2005), whereas other related species additionally possess alkenes or alkadienes (Kaib et al., 1993; Tentschert et al., 2002). In our study, though the cuticular profile of the slavemaker sexuals was close to that of their *T. unifasciatus* slaves, those of *Chalepoxenus* reared with *T. recedens* showed similarities to the cuticular pattern of their foster parents. The difference between both rearing types was more pronounced in *Chalepoxenus* female sexuals than in males, perhaps due to the greater attention female sexuals receive.

Although we cannot show a direct causal correlation between the changes in the hydrocarbon pattern of *Chalepoxenus* sexuals and their mating preference, the host-induced modifications of the cuticular hydrocarbon profiles appear to be associated with variation in the interactions among slavemaker sexuals. *Chalepoxenus* males reared by *T. unifasciatus* appeared to be consistently more attracted to and engaged significantly more often in mating activities with female sexuals reared by the same host species than to female sexuals, which had been transferred to colonies of *T. recedens* as pupae or came from natural colonies with *T. recedens*. In 2005, the stronger reaction of males reared by *T. unifasciatus* to female sexuals reared by *T. unifasciatus* might have been caused in part by genetic differences between *C. muellerianus* from the two study populations. However, genetic differences cannot explain the results obtained during the first two years of our experiment, when all sexuals came from colonies from Lago di Garda. Unfortunately, the number of trials was restricted by the number of sexuals available and in particular males reared by *T. recedens* often did not show any interest in the female sexuals provided. The difference in the antennation rates of males reared by *T. recedens* towards female sexuals reared by the two

host species was therefore not statistically significant, though in all years males showed a slightly increased activity towards female sexuals reared experimentally or naturally by *T. recedens*.

The low antennation rate between sexuals reared by *T. recedens* might be explained by larval learning. Isingrini et al. (1985) suggested that colony-brood recognition may start during larval life and persist till the first days after emergence. *C. muellerianus* sexuals reared by *T. recedens* in the first two years of our experiments might already in the larval stage have been influenced by the odour of *T. unifasciatus*. However, mating activity was similarly low when sexuals came from natural colonies containing *T. recedens* as host. Our data are thus probably better explained by host-derived cuticular hydrocarbons influencing sexual communication. Ant sexuals typically attract their mates by volatile pheromones (Hölldobler and Bartz, 1985; Hölldobler and Wilson, 1990), but cuticular hydrocarbons are known to play a role in courtship and mating in bees (Ayasse and Dutzler, 1998; Ayasse et al., 1999; Schiestl et al., 1999). Furthermore, species-specific surface pheromones appear to prevent hetero-specific mating in *Pogonomyrmex* harvester ants (Hölldobler, 1976a).

If the low interest of *C. muellerianus* males in female sexuals reared by another host species indeed resulted from mismatches between the cuticular hydrocarbon profiles, the progeny of a *Chalepoxenus* queen, which accidentally usurped the nest of a new host species, would have a reduced mating success. Thus, natural host switches would not easily lead to the stable exploitation of a previously unparasitized species and were initially selected against. Males reared by *T. recedens* did not clearly prefer female sexuals reared by *T. recedens* and did not engage in mating activities in our flight cages. Nevertheless, the occasional occurrence of *Chalepoxenus* populations with this or other unusual host species indicates that host switches do occur and are successful on the long run.

Colonies of the species-poor slavemaker clades *Harpagoxenus* and *Protomognathus* may simultaneously contain workers from two or three host species (Buschinger, 1966; Buschinger and Alloway, 1977; Heinze et al., 1992), and at least *Harpagoxenus* queens do not preferentially invade nests of one particular host (Buschinger, 1966, 1974). In contrast, colonies of the more species-rich genera *Myrmoxenus* and *Chalepoxenus* usually contain workers from only a single host species (Buschinger et al., 1988a; Buschinger, 1989; but see Buschinger and Winter, 1983 for *M. ravouxi*). In *Chalepoxenus*, this preference for a certain host species is probably based on imprinting. Schumann and Buschinger (1994, 1995) demonstrated that *C. muellerianus* queens and workers imprint on the odour of the host species present in their maternal nests and therefore prefer the familiar host species during

colony founding and slave raids. If, in addition to colony-founding queens and raiding workers, the rearing slave species also influences the mate choice of slavemaker sexuals in such a way that sexuals reared by different host species are less attractive for each other, this might promote the formation of host races with limited gene flow and possibly lead to speciation. Selecting a mate that developed in the same host species might improve the adaptation to a well suited host and thus be advantageous, in particular when a second potential host species is already parasitized by another slavemaker (e.g., *Myrmoxenus kraussei* at Tignale, Buschinger et al., 1988a). Speciation through host race formation is thought to be an important process explaining parasite diversity, for example in phytophagous insects (Tauber and Tauber, 1989). Thus, host specificity, associated with incompatibility of sexuals reared by different host species, might therefore underlie the radiation of these slavemaker taxa.

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Chapter 5

Influence of the slaves on the cuticular profile of the slave-making ant *Chalepoxenus muellerianus* and vice versa*

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* Manuscript, unpublished

ABSTRACT

Due to their special life style, slave-making ants are useful model systems for studying the mutual influences on cuticular hydrocarbon profiles between a slavemaker and its hosts. The purpose of the first experiment in this study was to examine the impact of the slave species on the cuticular hydrocarbon profile of adult *Chalepoxenus muellerianus* workers. While exchanging the *Temnothorax* slave species in a slavemaker nest, we investigated by gas chromatography if and how fast the slavemakers' profile has changed. In a second experiment, we used gas chromatography to test the influences of two different *Temnothorax* host species on eclosing *C. muellerianus* workers, and the slavemakers' influence on the slaves' profiles. The two experiments suggest that on the one hand, the slave species has an influence on the slavemakers' profile, and on the other hand, the slavemaker workers also change the chemical profile of their slaves.

Keywords: Social parasitism, dulosis, Formicoxenini, *Temnothorax*, cuticular hydrocarbons

INTRODUCTION

In general, chemicals play a very important role in insect communication (Howard and Blomquist, 2005), and work on solitary and social insects has shown that in many cases, cuticular hydrocarbons provide the chemical basis for discrimination (reviewed by Vander Meer and Morel, 1998; Lahav et al., 1999). In ants, inter- and intraspecific recognition is thought to be based on the perception of colony odours, complex chemical bouquets derived from both genetic and environmental sources, and shared by members of the same colony (Soroker et al., 1998). These chemicals are usually stored and mixed in the postpharyngeal gland and are typically distributed via trophallaxis, self- and allogrooming, and physical contact (Soroker et al., 1994). Furthermore, it has been shown that the cuticular profile of an ant can vary, depending on its diet or nesting material (Liang and Silverman, 2000; Le Moli et al., 1992; Crosland, 1989; Heinze et al., 1996), physiological state (Cuvillier-Hot et al., 2001; Bonavita-Cougourdan et al., 1987; Dahbi et al., 1998; Morel et al., 1988; Dietemann et al., 2003; Heinze et al., 2002; Liebig et al., 2000; Monnin et al., 1998), task (Wagner et al., 1998; Kaib et al., 2000) or social environment (Bonavita-Cougourdan et al., 1989; Franks et al., 1990; Kaib et al., 1993). Several studies investigated the influence of slave workers on the cuticular profile of certain species of slave-making ants; slave-making ants are able to adopt the odour of their slaves (reviewed by Lenoir et al., 2001) and to adjust their profile depending on the host species (D'Ettorre et al., 2002). Furthermore, Alloway and Keough (1990) observed that the slavemaker *Protomognathus americanus* chemically marks its slaves to prevent them from returning to their parental nest.

In a slavemaker colony, the slave-making queen, her offspring and all the host workers live together in one nest and share a common colony odour. Thus in this case, chemical communication is particularly important for social integration, and for the cohesion of mixed societies (Lenoir et al., 2001). Many formicoxenine slavemakers simultaneously enslave a number of host species, and slave-making colonies may contain slaves of one or several different species together in one nest (see Buschinger, 1966 for *Harpagoxenus sublaevis*; Buschinger and Winter, 1983 for *Epimyrma ravouxi*; Buschinger and Alloway, 1977 for *Harpagoxenus americanus*; Buschinger et al., 1988a for *Chalepoxenus*). This makes them a handy system for studying the influences of the social environment on the ants' chemical profiles. The formicoxenine slavemaker *Chalepoxenus muellerianus* from the Mediterranean area uses at least 12 host species of the genus *Temnothorax*. *Temnothorax unifasciatus* seems to be the most important host species as it was present in about 74% of the examined colonies.

As second most important host species, *T. recedens* was found in about 10% of all colonies (Buschinger et al., 1988a).

In this study, we examined by gas chromatography, firstly how an exchange of the host species affects the cuticular profile of adult *C. muellerianus* workers in a nest, and secondly how the chemical signatures of previously unparasitized host workers react to the addition of *C. muellerianus* pupae that were left with them until they were 5 days old.

MATERIAL AND METHODS

Ant collecting and housing

In March 2003, colonies of the slavemaker *Chalepoxenus muellerianus* with only *Temnothorax unifasciatus* slaves in the nest, and of a second potential host species *T. recedens* were collected from dry walls at Lago di Garda, Italy. Colonies from Lago di Garda were used in experiment 1.

In late April 2004, colonies of *C. muellerianus*, and of the host species *T. unifasciatus* and *T. racovitzai* were collected at Colle della Croce (near Barrea), Abruzzi, Italy. *C. muellerianus* colonies from this site that were used in experiment 2 contained only *T. unifasciatus* slaves, although slavemaker colonies with *T. unifasciatus* and *T. racovitzai* slaves together in the same nest have also been found at this collecting site.

The ants were housed in the laboratory in artificial nests in plastic boxes (10 x 10 x 3 cm^3) with three connected chambers and a plaster floor (Buschinger, 1974; Heinze and Ortius, 1991). Twice a week all colonies were fed with diluted honey and pieces of cockroaches. Ants were kept in an incubator under semi-natural conditions with an annual cycle of artificial seasons with daily temperature variations and a natural photoperiod. Behavioural experiments were carried out from August 2003 to January 2004 (experiment 1) and in August 2004 (experiment 2).

Experiment 1: Exchange of slaves in C. muellerianus *colonies*

In the first experiment, we investigated if and how quickly the chemical profile of *C. muellerianus* workers in a slavemaker colony reacts to the exchange of the slave species.

Therefore, we transferred *T. recedens* worker pupae from different colonies (n = 7) into *C. muellerianus* colonies (n = 3) which only contained *T. unifasciatus* slaves in the beginning. When the *T. recedens* workers started to eclose, all *T. unifasciatus* workers were removed and frozen (timepoint T0). Cuticular hydrocarbons of the *C. muellerianus* workers were extracted by solid-phase microextraction (SPME) over a period of 24 weeks, until the *C. muellerianus* workers died a natural death or due to injuries (timepoints T1/2/3/4 = 1/8/16/24 week(s) after removal of *T. unifasciatus*). At the end, also *T. recedens* slaves from the examined colonies were frozen for analysis. Colony composition at different time points is shown in Table 5-1.

We investigated the cuticular profiles of the *C. muellerianus* workers by SPME, and of 15 *T. unifasciatus* (n = 5 of each colony) and 14 *T. recedens* workers (n = 4 from colony A, n = 6 from colony B, n = 4 from colony C) by pentane extraction.

Table 5-1. Experiment 1. Colony composition at different time points. (*C. m.* = *Chalepoxenus muellerianus*; *T. u.* = *Temnothorax unifasciatus*; *T. r.* = *Temnothorax recedens*).

	colony A	**colony B**	**colony C**
start of the experiment	1 *C. m.* queen, 3 *C. m.* workers, 10 *T. u.* workers, 43 *T. r.* pupae of 4 different colonies	5 *C. m.* workers, 46 *T. u.* workers, 47 *T. r.* pupae of 2 different colonies	3 *C. m.* workers, 23 *T. u.* workers, 30 *T. r.* pupae of 5 different colonies
eclosion of *T. recedens* workers and removal of *T. unifasciatus* (T0)	1 *C. m.* queen, 3 *C. m.* workers, 7 *T. u.* workers, 27 *T. r.* workers	5 *C. m.* workers, 35 *T. u.* workers, 23 *T. r.* workers	2 *C. m.* workers, 22 *T. u.* workers, 11 *T. r.* workers
1 week after removal of *T. unifasciatus* (T1)	1 *C. m.* queen, 3 *C. m.* workers, 35 *T. r.* workers	5 *C. m.* workers, 29 *T. r.* workers	2 *C. m.* workers, 23 *T. r.* workers
8 weeks after removal of *T. unifasciatus* (T2)	1 *C. m.* queen, 2 *C. m.* workers, 32 *T. r.* workers	5 *C. m.* workers, 27 *T. r.* workers	2 *C. m.* workers, 17 *T. r.* workers
16 weeks after removal of *T. unifasciatus* (T3)	1 *C. m.* queen, 2 *C. m.* workers, 32 *T. r.* workers	5 *C. m.* workers, 27 *T. r.* workers	2 *C. m.* workers, 14 *T. r.* workers
24 weeks after removal of *T. unifasciatus* (T4)	1 *C. m.* queen, 1 *C. m.* workers, 29 *T. r.* workers	5 *C. m.* workers, 27 *T. r.* workers	14 *T. r.* workers

Experiment 2: Rearing of C. muellerianus *workers with two different host species*

In the second experiment, we tested how the rearing slave species influences the chemical pattern of eclosing *C. muellerianus* workers and vice versa.

Therefore, *C. muellerianus* worker pupae from 3 different slavemaker colonies were divided up and transferred into 3 *T. unifasciatus* colonies or 3 *T. racovitzai* colonies respectively. So, *C. muellerianus* sisters were reared either with *T. unifasciatus* slaves or with *T. racovitzai* slaves. Before addition of the *C. muellerianus* pupae, 4 *Temnothorax* workers each were removed from the 6 “receptor”-colonies and frozen for analysis. Surviving *C. muellerianus* pupae were individually marked by tarsal clipping at eclosion, removed at the age of 5 days and frozen for pentane extraction. At the end of the experiment, 5 *Temnothorax* workers each were removed from the experimental colonies and frozen. The colony size and

composition of the "receptor"-colonies is shown in Table 5-2. Data of *T. unifasciatus* and *T. racovitzai* colonies where no *Chalepoxenus* could be reared (colonies E and I) were excluded from the analysis.

Cuticular profiles of 15 surviving *C. muellerianus* workers, 18 *T. unifasciatus* workers (n = 9 form each colony), and 18 *T. racovitzai* workers (n = 9 from each colony) were analysed by pentane extraction.

Table 5-2. Experiment 2: Composition of the "receptor"-colonies.

"receptor" colony, species	**queen**	**workers**	***C. muellerianus* pupae added**	**surviving *C. muellerianus***
colony D, *T. racovitzai*	1	55	8	3
colony E, *T. racovitzai*	1	36	7	0
colony F, *T. racovitzai*	1	54	7	3
colony G, *T. unifasciatus*	1	72	5	5
colony H, *T. unifasciatus*	0	84	4	4
colony I, *T. unifasciatus*	1	129	4	0

Chemical analysis

We investigated the cuticular hydrocarbon profiles of single ants either by solid-phase microextraction to keep the ants alive, or by pentane washes. For SPME, single ants were picked out of the nest, held gently with a forceps, and rubbed for 10 minutes with a SPME fibre at the thorax and the front part of the abdomen. Afterwards, the fibre was placed into an Agilent Technologies 6890N gas chromatograph equipped with a flame ionisation detector and a capillary column for 5 minutes. The injector was split-splitless, and the carrying gas was helium at 1ml/min. A Rtx-5 capillary column (30 m x 0.25 mm x 0.50 µm, Restek, Bellefonte, USA) and a temperature program of 1 min at 100°C, from 100°C to 180°C at 30°C/min, from 180°C to 280°C at 4°C/min, and then held at 280°C for 20 min was used.

For pentane extraction, ants were killed by freezing and cuticular compounds were extracted by immersing them individually in 25 µl of pentane for 15 min. After evaporation of the solvent, the residues were re-dissolved in 10 µl of pentane and 2 µl of this solution were then injected into the same gas chromatograph. The pentane extracted samples of experiments 1 and 2 were analysed on a HP-5 capillary column (30 m x 0.32 mm x 0.25 µm, J&W Scientific, USA) with a temperature program of 1 min at 80°C, from 80°C to 180°C at 30°C/min, from 180°C to 300°C at 4°C/min, and then held at 300°C for 12 min.

All resulting chromatograms were comparable. Chemical profiles were compared by multivariate statistical analysis (Statistica 6.0, Statsoft). In experiment 1, 19 consistently

detectable peaks were used for analysis, in experiment 2 we used 23 peaks. The proportions of the peaks were first analysed by principal component analysis (PCA) to reduce the number of variables used in the following discriminant analysis (DA). The standardized discriminant function coefficients and the factor loadings (> 0.7) were used to assess the importance of single compounds. DA was used to determine whether defined groups could be distinguished on the basis of their cuticular profiles and to assess the degree of similarity between groups. The correct classification of individuals to the respective groups was verified. Groups were also compared by calculating the squared Mahalanobis distances (SMD) between the group centroids.

RESULTS

Experiment 1: Exchange of slaves in C. muellerianus *colonies*

The PCA performed on the chemical data of *C. muellerianus* workers and their *T. unifasciatus* and *T. recedens* slaves produced 6 principal components with eigenvalues greater than 1, explaining 86.5% of the total variance. A subsequent discriminant analysis performed on 15 variables (factor loading > 0.7) significantly separated the 7 groups (Wilks' $\lambda = 0.0092845$, $F_{(36,270)} = 14.30283$, $p < 0.0001$; Figure 5-1) and correctly classified 79.5% of all individuals. The classification was particularly good for *T. unifasciatus* (93.3%) and *T. recedens* (100%), while *C. muellerianus* were correctly classified in 55.6-80% at different time points.

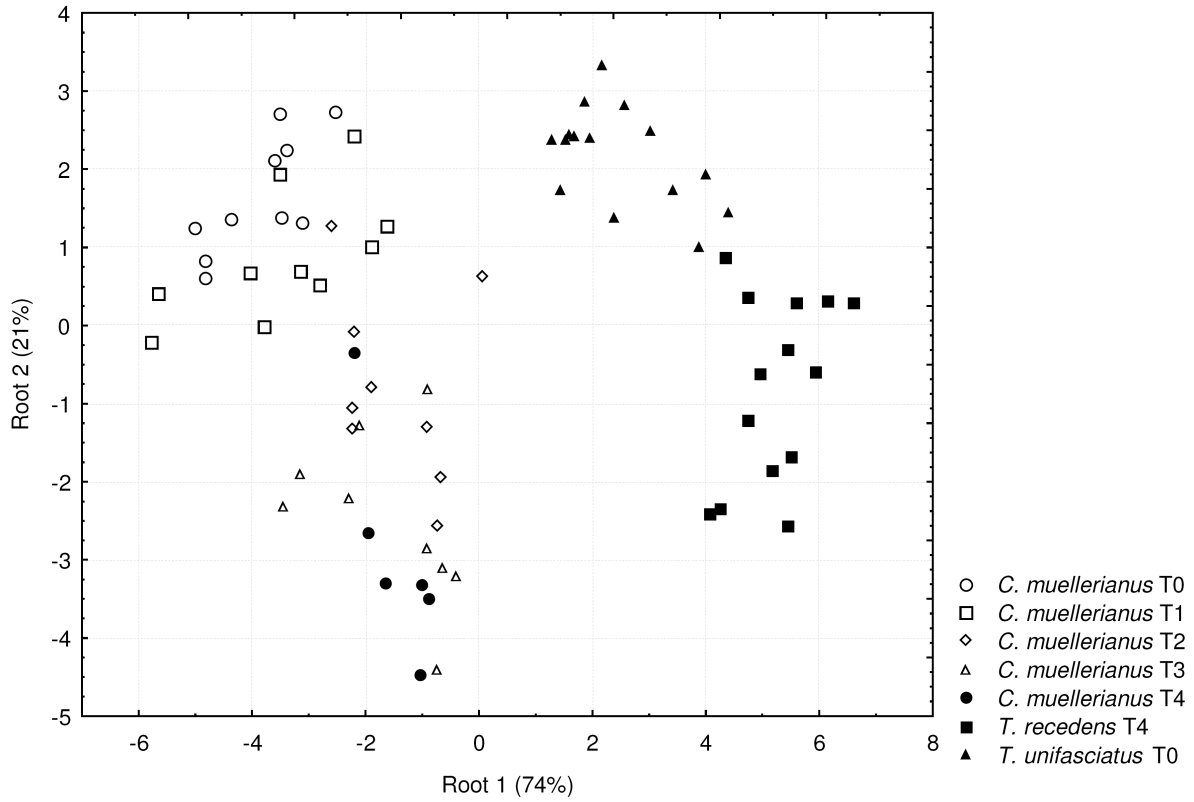

Figure 5-1. Experiment 1. Discriminant analysis of cuticular hydrocarbon profiles of *Chalepoxenus muellerianus* workers from 3 colonies at different timepoints (T0 = eclosion of *Temnothorax recedens* workers and removal of *Temnothorax unifasciatus*; T1/2/3/4 = 1/8/16/24 week(s) after removal of *T. unifasciatus* workers), and of *T. unifasciatus* and *T. recedens* slaves.

Function 1 accounted for 74%, function 2 for 21% of the total variance. Further, function 1 clearly separated *Chalepoxenus* and its *Temnothorax* hosts. The squared Mahalanobis distances between *C. muellerianus* workers of timepoints T0 and T1, T2 and T3, and T3 and T4 were not statistically significant (p-levels: $p = 0.440$, $p = 0.087$, and $p = 0.771$,

respectively), all other squared Mahalanobis distances between groups were significant. At T0, the squared Mahalanobis distance between *C. muellerianus* workers and *T. recedens* (SMD = 97.955) was larger than between *C. muellerianus* and *T. unifasciatus* (SMD = 48.297), whereas at T4, the difference between squared Mahalanobis distances was much smaller (*C. muellerianus* and *T. recedens*: SMD = 56.655; *C. muellerianus* and *T. unifasciatus*: SMD = 46.889).

Experiment 2: Rearing of C. muellerianus *workers with two different host species*

Based on the chemical profiles of 5 day old *C. muellerianus* workers and their *T. unifasciatus* and *T. racovitzai* hosts, a PCA produced 5 principal components with eigenvalues greater than 1, explaining 80.6% of the total variance. The 6 groups were significantly separated in a subsequent discriminant analysis performed on 10 variables (factor loading > 0.7) (Wilks' λ = 0.0021212, $F_{(25,153)}$ = 26.11060, $p < 0.0001$; Figure 5-2). 80.4% of all individuals were correctly classified, 66.7% of *C. muellerianus* reared by *T. racovitzai* and *T. unifasciatus* respectively, 75.0% and 100% of *T. unifasciatus* before addition of *C. muellerianus* and at the end of the experiment respectively, 62.5% and 100% of *T. racovitzai* before and after rearing of *C. muellerianus* respectively. Function 1 accounted for 85%, function 2 for 12% of the total variance. The squared Mahalanobis distances between groups significantly differed, except for the *C. muellerianus* workers of both rearing types (p-level: p = 0.516), and for *T. unifasciatus* and *T. racovitzai* before addition of *Chalepoxenus* (p-level: p = 0.405), where groups overlapped. The squared Mahalanobis distances between both *Temnothorax* species before the addition of *Chalepoxenus* was only 1.637. After rearing the *C. muellerianus* workers, *T. unifasciatus* and *T. racovitzai* workers are separated by function 1 from *Chalepoxenus* and also from their original state, with a squared Mahalanobis distance of 38.748 between them. The squared Mahalanobis distances between both *Temnothorax* species and *Chalepoxenus* became smaller, compared to their original state without *Chalepoxenus* workers (*T. unifasciatus* and *Chalepoxenus* before and after rearing the slavemaker: SMD = 214.541 and SMD = 64.485 respectively; *T. racovitzai* and *Chalepoxenus* before and after rearing the slavemaker: SMD = 243.047 and SMD = 95.557 respectively).

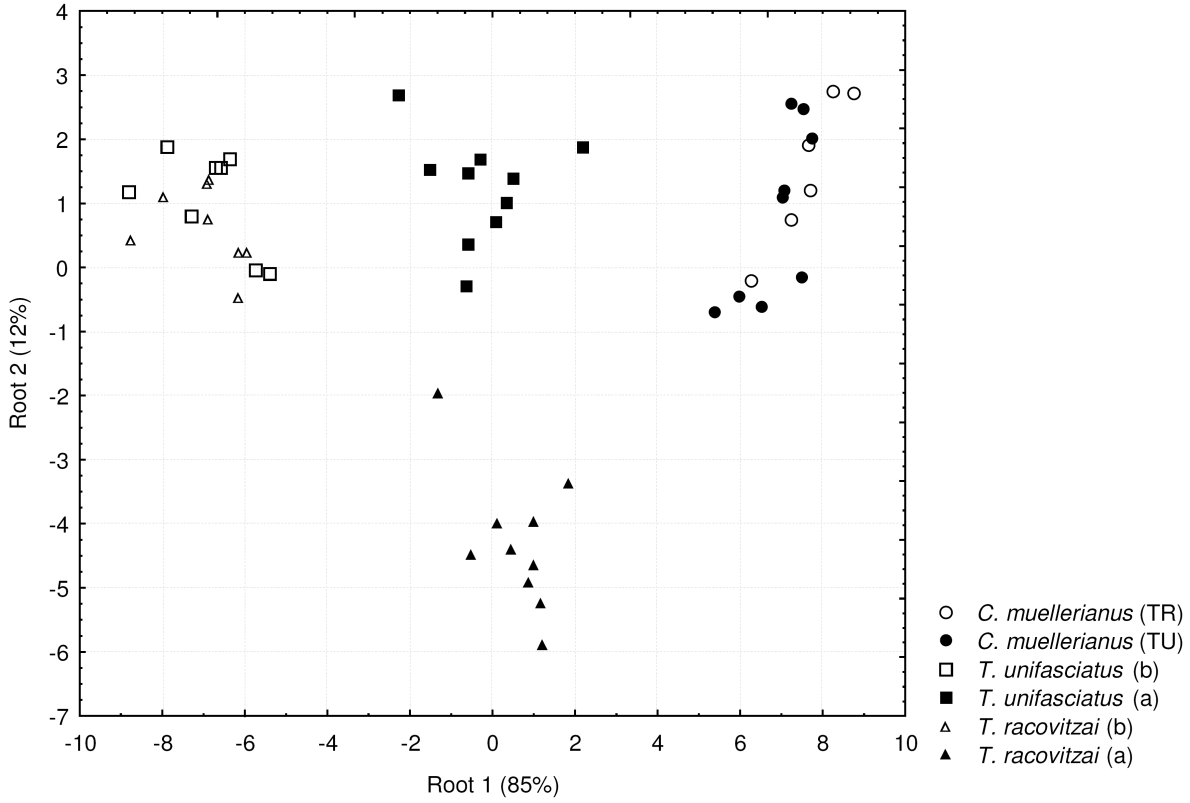

Figure 5-2. Experiment 2. Discriminant analysis of cuticular hydrocarbon profiles of 5 day old *C. muellerianus* workers, reared either by *T. racovitzai* (TR) or *T. unifasciatus* (TU), and of *T. unifasciatus* and *T. racovitzai* workers, before (b) and after (a) rearing the slavemaker.

DISCUSSION

This study is based on the cuticular hydrocarbon profiles of *C. muellerianus* and of certain *Temnothorax* host species, which have been shown to consist of linear and methyl-branched hydrocarbons with chain lengths ranging from C25 to C31 (Beibl et al., in press).

Our study documents that on one hand, the chemical profile of adult *Chalepoxenus muellerianus* workers changes over time when the host species in the nest is exchanged for another one. It seems that the slaves present in nests of the slave-making ant influence the cuticular hydrocarbon pattern of slavemaker workers. As slave-making ant species are typically tended by their heterospecific host workers, an influence of the slaves on the chemical signature of the slavemaker is not unexpected and has previously been shown in several studies (Beibl et al., in press; Bonavita-Cougourdan et al., 1997, 2004; D'Ettorre et al., 2002; Franks et al., 1990; Kaib et al., 1993; Lenoir et al., 2001; Yamaoka, 1990). The analysis of the chemical profile of *Chalepoxenus muellerianus* workers revealed that their cuticular hydrocarbons have changed, but they did not adopt the odour of their host workers. While the distance between *C. muellerianus* and the host *Temnothorax unifasciatus* remains more or less constant, the distance to the second host species *T. recedens* becomes much smaller by exchanging the slaves. However, cuticular hydrocarbon profiles of ants may also vary with time (Provost et al., 1993; Vander Meer et al., 1989b) and, although diet and nesting material remained unchanged, we can not exclude such effects in our experiment.

On the other hand, the second experiment in our study demonstrates that the presence of young *Chalepoxenus muellerianus* workers changes the chemical profiles of the hosts. Interestingly, only very few slavemaker workers that only stayed in the nests until the age of 5 days were able to change the slaves' cuticular profiles. The chemical profiles of both host species overlap before they are in contact with *Chalepoxenus*. After rearing some few *C. muellerianus* workers, their hydrocarbon profiles develop into different directions, and the distance towards *Chalepoxenus* diminishes in both cases. It seems as if the components they acquire from the slavemakers lead to different chemical bouquets in both host species. As the eclosing *Chalepoxenus muellerianus* workers do not completely adopt their slaves' chemical profiles, and as the profiles of *Chalepoxenus* reared with both host species markedly overlap, their chemical signature also seems to have an evident genetic component. In the slavemaker *Polyergus rufescens* for example, the chemical profile of workers is very flexible, but also genetically mediated: the parasite develops the chemical signature of its most important host species in the absence of social interactions with slaves (D'Ettorre et al., 2002).

Although our results are based on only few individuals due to difficulties in rearing, they can be a starting point for future studies providing useful information about the mutual influences of slavemakers and their hosts on each other's chemical profile.

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GENERAL DISCUSSION

The myrmicine ant tribe Formicoxenini is especially rich in social parasites. These parasites show surprisingly different degrees of diversification. The aim of this work was to investigate the variation in diversity in the dulotic formicoxenine species. For this purpose, phylogenies of social parasites, and of the genera *Chalepoxenus* and *Myrmoxenus* were established based on mitochondrial cytochrome oxidase sequences. Furthermore, the impact of the host odour on the chemical bouquet and on the mate choice of *C. muellerianus* sexuals, as well as the mutual influences of slaves and *Chalepoxenus*-slavemakers on each other's cuticular profile were investigated by gas chromatography and behavioural studies.

Chapter 1 contains phylogenetic analyses of six dulotic and one inquiline formicoxenine lineage and their dominant host species. First of all, this study uncovered a sixth origin of dulosis within the Formicoxenini, which is one more than previously known (Buschinger, 1986; D'Ettorre and Heinze, 2001). The phylogenetic trees show three well supported convergent origins in the North American *Temnothorax* group (*Protomognathus americanus*, *Temnothorax duloticus* and an undescribed *Temnothorax* slavemaker), two convergent origins in the Eurasian *Temnothorax* (*Chalepoxenus* and *Myrmoxenus*), and one origin in the *Leptothorax* group with representatives in North America and Eurasia (*Harpagoxenus*). The intention was not to investigate whether the evolution of slave-making Formicoxenini fulfil Emery's rule. For that purpose, too few of all known *Temnothorax* and *Leptothorax* species have been analysed, and the mutation rate of the cytochrome oxidase gene is too high to resolve the basal nodes. Nevertheless, the data support the loose form of Emery's rule stating that the parasites are close relatives of their hosts, with the sister group including the parasite's host species (e.g. Ward, 1989). However, the cytochrome oxidase gene is well suited to reflect the genetic diversity within the different monophyla. Based on the phylogenetic analyses, the hypothesis that parasitic lineages that comprise fewer species are always phylogenetically younger than more diverse taxa with a higher number of species has to be overruled. On the one hand, the single-species monophyla *T. minutissimus*, *T. duloticus* and the undescribed *Temnothorax* species display short branch lengths and they cluster right next to their hosts bearing the same genus name. This suggests that they are phylogenetically comparatively young and evolved only recently from their ancestor species. In contrast, the genera *Protomognathus* and *Harpagoxenus*, which also comprise only one and three species respectively, have long branch lengths and are thus phylogenetically old clades. *P. americanus* is much less related to its hosts and shows a high amount of intraspecific

variation. But the small F_{ST}-value between populations from New York and Vermont calculated from microsatellite markers (F_{ST} = 0.005 ± 0.011; Foitzik and Herbers, 2001a) gives no evidence for subdivision into different taxa. Similarly, *H. sublaevis* and *H. canadensis* seem to have split long ago without further speciation. The species-rich genera *Chalepoxenus* and *Myrmoxenus* appear to have on their part a long independent evolutionary history. To summarize, it can be concluded that the differences in species-richness of the various parasitic lineages is not only the consequence of a different age.

In chapter 2, a phylogeny of the Mediterranean species of *Chalepoxenus* and their hosts is presented. First, the phylogenetic analyses confirmed the paraphyly of the genus *Temnothorax* (Baur et al., 1996). Furthermore, the genus *Chalepoxenus* displays an early division into two lineages, one comprising *C. muellerianus* and *C. tauricus* distributed in the Central and Eastern Mediterranean region, and the other one containing the degenerate slavemaker *C. brunneus* from the High Atlas in North Africa and *C. kutteri* from the Western Mediterranean area. As viable hybrids have been produced in crossbreeding experiments between different *Chalepoxenus* species (Ehrhardt, 1987, 2004), it can be concluded that the genus *Chalepoxenus* consists of several closely related species, which are in the process of speciation. The host species *T. flavicornis* appears to be most closely related to *Chalepoxenus* but in this case a clear statement is problematic because too few of the hundreds of *Temnothorax* species were included in the study. The samples of *C. muellerianus* reflected a certain division into genetically different clades. That pattern may be the result of historic processes as Europe's species have undergone many range contractions and expansions in and out of refugia during the Pleistocene ice ages (e.g. Hewitt, 1996, 1999). However, the *C. muellerianus* haplotype network seems to be not only structured by geography. The phylogenetic branching pattern of *C. muellerianus* may also result from the formation of host races. Hypothetically, a parasite's preference for a certain host species might lead to decreased gene flow and thus to speciation of the slavemaker. In general, parasites of animals and plants are often highly specialized on a certain host. If a subgroup of a parasite population occupies a new host it can become isolated from the ancestral population, and thus adaptation to a new host is potentially linked to a reduction in gene flow between the new and the parental population (Berlocher, 2003; Kunz, 2002). Indeed, speciation through host race formation is thought to be an important process explaining parasite diversity, for example in phytophagous insects (Tauber and Tauber, 1989). The classic example of this type of divergence is the host race formation in the frugivorous haw fly *Rhagoletis pomonella*. After the introduction of domestic apples (*Malus pumila*) to the USA, a host shift occurred from the original hawthorn

host (*Crateagus* spp.) to the apple host (Bush, 1969). Later, studies demonstrated genetic differentiation between *R. pomonella* populations occurring on hawthorn and populations on apple hosts (Feder, 1988), and Bush (1993) hypothesized that the six or more members of the *R. pomonella* group radiated by means of host-plant shifts. A coral-dwelling fish of the genus *Gobiodon* is an analogous example for speciation by host shift in the sea (Munday et al., 2004). Like for instance phytophagous insects, parasitic ants are also able to actively choose their hosts. Further characteristics make them ideal candidates for speciation by host shift. Dulotic ants are totally dependent on their hosts: they completely rely on their hosts for nest maintenance, nest defence, brood care, foraging and nutrition. Moreover, at least the better examined *Chalepoxenus* species show a specialized pattern of host use and prefer to occupy just one certain host species although other potential hosts may be present and available: mixed slave stocks are extremely rare in *C. muellerianus* and *C. kutteri*, probably due to imprinting effects (Buschinger et al., 1988a). Furthermore, host colonies are a limited resource and shifting to a yet unexploited host species could be advantageous for the parasite because of reduced competition. And finally, the sometimes great diversity of formicoxenine species at certain sites provides the opportunity to find suitable, previously unexploited hosts. Indeed, according to our mtDNA sequence data, speciation by host race formation seems possible for *Chalepoxenus*, although one has to bear in mind that certainly gene trees reflect histories of single traits (Avise, 2000).

In chapter 3, the phylogenetic relationships among several representatives of the genus *Myrmoxenus* are examined. The molecular data confirm that *Myrmoxenus* is a phylogenetically old monophylum with an ancient split into two defined sister groups. *M. gordiagini* represents the most original *Myrmoxenus* type and displays the longest branch lengths without dividing any further. Correspondingly, in contrast to all other *Myrmoxenus* species, *M. gordiagini* females possess 12 antennomeres just like many of the host species of *Myrmoxenus*. The phylogenetic results also support the statements of Buschinger (1989, 2001) and Jessen and Klinkicht (1990) that all *Myrmoxenus* species are monophyletic and closely related. Concluding from the analyses, it seems that workerless parasites evolved from their slave-making ancestors several times. Besides *Myrmoxenus*, this shift from slavery to queen-intolerant, workerless parasitism has also been reported in the genus *Chalepoxenus* (Buschinger et al., 1988a, b). One hypothesis implies that, after different populations of the slavemaker have been isolated for instance by specialization on a certain host, they developed distinct trends in reproductive and raiding behaviour. Indeed, several *Myrmoxenus* species (e.g. *M. adlerzi*, *M. bernardi* and *M. birgitae*) are specialized in exploiting only a small

number of a large set of potential host species. Consequently, these dulotic species are restricted to a much smaller range than the whole genus (Buschinger, 1989). In contrast to the species of *Myrmoxenus* and *Chalepoxenus* that strongly prefer certain hosts, the species-poor, phylogenetically old clades *Harpagoxenus* and *Protomognathus americanus* parasitize a much smaller number of host species over large geographical areas where no other suitable hosts occur. Furthermore, nests of these two slave-making genera often contain slaves of two or three host species simultaneously: in *H. sublaevis*, mixed slave stocks of two or three species have been found in 28% (Foitzik et al., 2003; German colonies) and 41.9% (Fischer-Blass et al., 2006; mature German colonies) up to 57.6% (Buschinger et al., 1988a) of the colonies; in *H. canadensis*, 22.9% of the complete slavemaker colonies contained slaves of both host species (Heinze et al., 1992); in *P. americanus*, mixed slave stocks were found in an average of 10% (Brandt and Foitzik, 2004) up to 19.5% (Buschinger and Alloway, 1977), and genetic analyses showed that no distinct host races exist in this taxon (Brandt and Foitzik, 2004; Brandt et al., 2007). Moreover, various formicoxenine groups seem to differ in their amount of intraspecific variability and species richness. *T. nylanderi* and *T. crassispinus* for example seem to be quite homogenous species with little genetic variation (Pusch et al., 2006), whereas in other species like *T. unifasciatus* or *L. acervorum* the variation is much higher (own observations). To sum up, for these reasons, it is likely that social parasite diversity in the Formicoxenini might, at least partly, reflect the distribution patterns and the diversity of their non-parasitic ancestors or their host species.

Chapter 4 provides evidence that the host species present in *Chalepoxenus muellerianus* nests influences the cuticular hydrocarbons of the slavemaker sexuals. Furthermore, a notable correlation existed between the modified profiles of the *Chalepoxenus* sexuals and their mate preference in mating experiments. Overall, *C. muellerianus* cuticular extracts mainly contained linear and methyl-branched hydrocarbons with 25 to 31 carbon atoms, comparable to the North American slavemaker *Protomognathus americanus* (Brandt et al., 2005). Although the cuticular profile of all *Chalepoxenus* sexuals reared with two different slave species was in general most similar to the profile of *T. unifasciatus* slaves, sexuals reared with *T. recedens* markedly differed from their *T. unifasciatus*-reared counterparts, and their chemical profiles clearly were influenced by their foster parents. In parallel to the host-induced modifications of the hydrocarbon pattern, the interactions among slavemaker sexuals varied. *Chalepoxenus* males reared by *T. unifasciatus* investigated and mounted females reared by the same host significantly more often than females reared by *T. recedens*. Males reared by *T. recedens* did not clearly discriminate and rarely engaged in mating activities with

either type of female sexuals. Our findings indicate that host-derived cuticular hydrocarbons might be important in the mating success of this ant species. In general, an important function of cuticular hydrocarbons in arthropods is to serve as recognition signals between individuals. Besides species- or nestmate-recognition, the recognition of potential mating partners is of prime importance for most insects. In this context, cuticular hydrocarbons play an important role in many species. There are cases in which males and females contain distinctive hydrocarbons (e.g. Sutton and Carlson, 1997; Ginzel et al., 2003), and cases in which the relative abundances of certain hydrocarbons are gender-specific (e.g. Cobb and Ferveur, 1996; Howard, 1998). The use of hydrocarbons for gender recognition in insects was also proven in several bioassays (Carlson et al., 1984; Ginzel et al., 2003; Howard, 1998). Moreover, it was shown that cuticular hydrocarbons influence the mate choice in *Drosophila serrata* (Howard et al., 2003). In many insects, cuticular hydrocarbons also act as sex pheromones (Blomquist et al., 1993, 1998). In bees, behavioural experiments suggest that female sex pheromones are often localized at the surface of the cuticle. Hydrocarbons and additional substances found in female cuticular extracts function as male attractants and elicit copulatory behaviour in males in *Lasioglossum malachurum* (Ayasse et al., 1999), *Osmia rufa* (Ayasse and Dutzler, 1998), and *Andrena nigroaenea* (Schiestl et al., 1999). In ants, it is known that volatile pheromones produced in various glands in many cases attract mating partners from a distance (Ayasse et al., 2001). In addition, sexuals intensively inspect each other by antennation before mating. The importance of cuticular hydrocarbons and hydrocarbon composition for interindividual recognition and their role in the mating process of numerous insects suggests that they might provide signals for short-range communication in ants. Indeed, the results described in chapter 4 seem to confirm the importance of cuticular hydrocarbons in the mating success of *Chalepoxenus* and at the same time demonstrate the influence of the slave species on mate choice. In *Chalepoxenus*, the preference for a certain odour is probably based on imprinting. Schumann and Buschinger (1994, 1995) demonstrated that young mated *C. muellerianus* queens imprint on the odour of their slaves and preferentially invade nests of the host species present in the maternal nest. Similarly, *C. muellerianus* workers raid nests of the host species they are familiar with. If, in parallel, odour components obtained from the rearing slave species are relevant for the mate choice of slavemaker sexuals in such a way that sexuals reared by different host species are less attractive, this might, as mentioned in the previous paragraphs, eventually support the formation of host races with limited gene flow and finally lead to genetic separation and speciation. In fact, our results indicate a potential for the development of isolation among

individuals reared with different host species because sexuals exhibit a preference for mating partners reared with the same host, followed by queens and workers preferentially occupying the familiar slave species with which they then spend their entire life. Consequently, it seems quite plausible that imprinting of a parasite like *Chalepoxenus* on a particular host species, combined with incompatibility of sexuals reared by different hosts, promotes the radiation of a certain slavemaker taxon.

As chemical signals are crucial to ant communication and imprinting, the last chapter of this work finally aims at describing the mutual influences of *Chalepoxenus* slavemakers and their slaves on each other's chemical profile. The results showed that, on the one hand, the hydrocarbon pattern of adult *C. muellerianus* workers changes more or less slowly over time when the tending host species is exchanged. The profile of *Chalepoxenus* workers seems to adjust to their current host species without completely adopting the slaves' profile. On the other hand, the data suggest that eclosing *C. muellerianus* workers also considerably influence the slaves' cuticular hydrocarbons. The impact of the slavemaker on the slaves' profile was also demonstrated in a separate experiment described in chapter 4 with slaves of other species. Some invasive insects managed to exploit their hosts by mimicry or camouflage of cuticular hydrocarbons. Some staphylinid beetles for example live in the nests of termites and produce cuticular hydrocarbons that are similar to those of their respective host (Howard et al., 1980, 1982). The fly larva of *Microdon albicomatus* biosynthesizes cuticular hydrocarbons that are similar to those of its prey, the pupae of *Myrmica incompleta* (Howard et al., 1990). Other parasitoids acquire cuticular hydrocarbons from their hosts, for instance the wasp *Orasma* (Vander Meer et al., 1989a). Furthermore, some social insects manipulate related species and exploit their nests. *Polistes atrimandibularis* for example is able to change its own hydrocarbon profile to match that of its host (Bagnères et al., 1996). In ants, chemical mimicry in the cuticular hydrocarbons has been found in the guest ant genus *Formicoxenus* and its *Myrmica* host (Lenoir et al., 1997). And the inquiline *Leptothorax kutteri* exhibits an exceptional similarity in cuticular hydrocarbons to its *L. acervorum* slaves (Franks et al., 1990). Moreover, it is well known that slavemakers and slaves affect each other's chemical profile. Parasites would benefit from resemblance to their slaves' cuticular profile for example by a more efficient communication with the slaves or lower levels of aggression (Heinze et al., 1994). Kaib et al. (1993) showed that adult *Harpagoxenus sublaevis* adopt the odour of their *Leptothorax acervorum* or *L. muscorum* slaves, and that young isolated slavemakers show weak profiles that are fundamentally distinct from those of their hosts. According to Yamaoka (1990) the slavemaker *Polyergus samurai* similarly displays the same hydrocarbon

pattern as its *Formica* hosts by obtaining cuticular hydrocarbons from its slave workers. In contrast, adult *Polyergus rufescens* workers, a European representative of this genus, actively adjust their cuticular hydrocarbon profiles depending on the slave species to match the host's profile in a reversible way (Bonavita-Cougourdan et al., 1997, 2004). The enslaved *Formica* also tend to adjust the hydrocarbons that are common to themselves and the slavemaker towards the proportions of the latter, but they do not totally adopt the *Polyergus* profile. Thus, each of the two species keeps its own hydrocarbon signature (Bonavita-Cougourdan et al., 1996). Furthermore, *P. rufescens* cocoons from one maternal colony showed cuticular profiles corresponding to the rearing species when being adopted by different *Formica* hosts (D'Ettorre et al., 2002). And although the cuticular profile of *Rossomyrmex minuchae* workers seems to ressemble its host *Proformica longiseta*, both slavemaker and host maintain their own chemical identity (Errard et al., 2006b). Similarly, the slavemaker *Protomognathus americanus* adapts its own chemical signature and furthermore causes a reciprocal adjustment in the cuticular profiles of its slaves (Brandt et al., 2005). These studies suggest that in parasitic ants, several degrees and different ways of hydrocarbon adjustment exist. In *Chalepoxenus muellerianus* colonies, the chemical profile of both slavemakers and slaves also seems to change without totally loosing its own characteristics, but further studies will be needed to confirm these findings.

A fourth hypothesis to explain the remarkable variation in the diversity of socially parasitic Formicoxenini would be that differences in reproductive biology affect the rate of speciation. Mating inside the maternal nest and inbreeding might promote radiation by increasing genetic diversity among previously established host races. Thus, the genetic population structure and genetic divergence is expected to vary between species with intranidal mating and species with nuptial flights. Indeed, pronounced differences in the mating behaviour of different formicoxenine slavemaker taxa exist. Virgin *Harpagoxenus* and *Protomognathus* sexuals for example mate outside the nest. *Harpagoxenus* females are standing on the ground or on low vegetation and attract winged males by releasing sex pheromones (Buschinger, 1968a, b; Buschinger and Alloway, 1979). In comparison, *Protomognathus* females mate during nuptial flights on favourable days (Wesson, 1939), and evidence for inbreeding could not be found (Foitzik and Herbers, 2001a). In contrast, several *Myrmoxenus* species and the degenerate slavemaker *C. brunneus* reproduce by intranidal mating or mating very close to the maternal nest (Buschinger, 1989; Buschinger et al., 1988b). To investigate the influence of mating behaviour, examination of the population structure and gene flow between populations of *Chalepoxenus* or *Myrmoxenus* by micro-

satellite analyses is promising. During this work, the three microsatellite primer pairs L-18 (Foitzik et al., 1997), LXAGT 1 (Bourke et al., 1997), and LXGT 218 (Hamaguchi et al., 1993) were already optimized for *C. muellerianus*.

Studies on slave-making ants and on socially parasitic species in general are difficult due to the rarity of these species. But genetic, chemical and behavioural investigations are extremely challenging and enormously valuable in order to learn more about the distribution and life histories of these parasites. This is the only way to ensure the protection of these rare species, most of which are listed as threatened by the World Conservation Union (IUCN).

SUMMARY

The ecological and physiological relationships between parasites and their hosts constitute some of the most impressive examples of biological adaptation known. Contrary to parasites that exploit the physiology of another individual, social parasites exploit entire societies. A social parasite coexists with another social insect species in the same nest and is the dependent beneficiary. Social parasites are quite common in bees, wasps, and in ants. In the Formicidae, several hundred species are known to be social parasites. Because of their peculiar life style they represent unique model systems to study a number of fundamental problems in evolutionary biology. The more advanced types of social parasitism, slavery or dulosis and inquilinism, are mainly known in the subfamilies Formicinae and Myrmicinae, and they are concentrated in certain genera. The myrmicine tribe Formicoxenini for example is extremely rich in social parasites. Within the Formicoxenini, different degrees of diversification exist. This work is aimed at investigating the causes of variation in species diversity among the six slave-making taxa *Protomognathus* (1 species), *Temnothorax duloticus* and an undescribed *Temnothorax* species, *Harpagoxenus* (3 species), *Chalepoxenus* (8 species) and *Myrmoxenus* (12 species). For this purpose, there is a focus on the age and distribution patterns of the parasitic lineages, as well as on the chemical basis of colony odour and its role in imprinting and host race formation.

First, phylogenetic analyses based on mitochondrial DNA sequences confirmed six convergent origins of slavery within the Formicoxenini, which is one more than previously known. The phylogenetic trees document different degrees of genetic divergence between the slave-making monophyla, suggesting that they evolved from their non-parasitic ancestors at different times. By comparing the genetic data of the socially parasitic lineages, it can be concluded that the differences in species-richness among the parasitic Formicoxenini are not only due to their different age. Furthermore, sequence analyses of the Mediterranean representatives of the genus *Chalepoxenus* and its hosts indicate two lineages, one comprising *C. muellerianus* and *C. tauricus*, and a sister group containing *C. kutteri* and *C. brunneus*. The grouping of the *Chalepoxenus* samples points to the possibility of speciation by host race formation in this genus, as demonstrated especially in *C. muellerianus*. In the genus *Myrmoxenus*, phylogenetic data suggest that a shift from slavery to workerless parasitism took place several times independently. It is known that both *Chalepoxenus* and *Myrmoxenus* species strongly prefer certain hosts and rarely keep mixed slave stocks, in

contrast to *Harpagoxenus* or *Protomognathus*. Therefore, it is likely that parasite diversity reflects at least in part the diversity and distribution patterns of their ancestor or host species.

Cuticular extracts of *C. muellerianus* analysed by gas chromatography and mass spectrometry mainly contained linear and methyl-branched hydrocarbons with chain lengths ranging from C25 to C31. Statistical analyses of chromatograms of *C. muellerianus* sexuals reared with two different suitable host species indicated that the host species present in the nest clearly influenced the slavemakers' chemical profile. Simultaneously, a distinct correlation existed between the modifications of the sexuals' odour components and their mate choice. Especially *Chalepoxenus* males reared by the main host species were much more interested in females that were reared with the same host. Several years ago, imprinting was demonstrated in *C. muellerianus* queens and workers. It seems likely that a preference for mating partners reared by the same host promotes the formation of host races and eventually speciation in *Chalepoxenus*. Finally, the mutual influences of *C. muellerianus* and their slaves on each others' cuticular hydrocarbon pattern were examined. The results suggest that the chemical profiles of both slavemakers and slaves appear to adjust without totally losing their own characteristics.

Additional studies are recommended to ensure the protection of these rare and threatened parasitic ant species.

ZUSAMMENFASSUNG

Die ökologischen und physiologischen Beziehungen zwischen Parasiten und ihren Wirten gehören zu den eindrucksvollsten Beispielen biologischer Anpassung. Im Gegensatz zu Parasiten, die die Physiologie eines anderen Individuums für sich ausnutzen, beuten Sozialparasiten komplette Gemeinschaften aus. Ein Sozialparasit lebt mit einer anderen sozialen Insektenart zusammen in einem Nest und ist der abhängige Nutznießer in dieser Beziehung. Sozialparasiten sind bei Bienen, Wespen und Ameisen ziemlich verbreitet. Bei den Formiciden sind mehrere hundert sozialparasitische Arten bekannt. Wegen ihrer eigentümlichen Lebensweise stellen sie einzigartige Modellsysteme zur Erforschung einer Vielzahl von grundlegenden evolutionsbiologischen Problemen dar. Die fortgeschritteneren Arten des Sozialparasitismus, die Sklavenhaltung oder Dulosis und der Inquilinismus, sind vor allem in den Unterfamilien Formicinae und Myrmicinae bekannt und treten in bestimmten Gattungen gehäuft auf. Die myrmicine Tribus Formicoxenini zum Beispiel ist äußerst reich an Sozialparasiten. Innerhalb der Formicoxenini bestehen verschiedene Grade der Diversifikation. Diese Arbeit zielte darauf ab, die Gründe für die unterschiedliche Artenvielfalt der sechs sklavenhaltenden Taxa *Protomognathus* (1 Art), *Temnothorax duloticus* und einer unbeschriebenen *Temnothorax* Art, *Harpagoxenus* (3 Arten), *Chalepoxenus* (8 Arten) and *Myrmoxenus* (12 Arten) aufzuklären. Zu diesem Zweck lag der Schwerpunkt der Arbeit auf der Untersuchung des Alters und der Verteilungsmuster der parasitischen Lineages, sowie der chemischen Grundlage des Koloniegeruchs und dessen Rolle bei der Prägung und Wirtsrassenbildung.

Phylogenetische Analysen basierend auf mitochondrialen DNA-Sequenzen bestätigten sechs konvergente Ursprünge von Sklavenhaltung innerhalb der Formicoxenini, einen mehr, als bisher bekannt. Die Stammbäume zeigen verschiedene Grade genetischer Divergenz bei den sklavenhaltenden Monophyla, was nahe legt, dass sie sich zu verschiedenen Zeiten aus ihren nicht-parasitären Vorfahren entwickelten. Durch den Vergleich der genetischen Daten der sozialparasitischen Lineages kann man schließen, dass die Unterschiede im Artenreichtum der parasitischen Formicoxenini nicht nur durch unterschiedliches Alter begründet sind. Weiterhin lassen Sequenzanalysen der mediterranen Vertreter der Gattung *Chalepoxenus* und ihrer Wirte zwei Abstammungsgruppen erkennen, von denen eine *C. muellerianus* und *C. tauricus*, die andere *C. kutteri* und *C. brunneus* enthält. Die Gruppierung der *Chalepoxenus*-Proben weist auf die Möglichkeit der Artbildung durch Wirtsrassenbildung in dieser Gattung hin, was besonders bei *C. muellerianus* demonstriert wird. In der Gattung

Myrmoxenus deuten die phylogenetischen Daten darauf hin, dass mehrere Male unabhängig voneinander ein Übergang von Sklavenhaltung zu arbeiterinnenlosem Parasitismus stattfand. Es ist bekannt, dass *Chalepoxenus* und auch *Myrmoxenus* Arten bestimmte Wirte besonders bevorzugen und selten gemischte Sklavenverbände halten, ganz im Gegensatz zu *Harpagoxenus* oder *Protomognathus*. Aus diesen Gründen ist es wahrscheinlich, dass die unterschiedliche Diversität der Parasiten zumindest zum Teil die Diversität und Verteilungsmuster ihrer Ahnen- oder Wirtsarten widerspiegelt.

Cuticuläre Extrakte von *C. muellerianus*, die mittels Gaschromatographie und Massenspektrometrie analysiert wurden, enthielten hauptsächlich lineare und Methyl-verzweigte Kohlenwasserstoffe mit Kettenlängen von C25 bis C31. Statistische Analysen der Chromatogramme von *C. muellerianus* Geschlechtstieren, welche mit zwei verschiedenen, geeigneten Wirtsarten aufgezogen wurden, zeigten, dass die im Nest befindliche Wirtsart das chemische Profil der Sklavenhalter eindeutig beeinflusste. Gleichzeitig bestand eine deutliche Korrelation zwischen den Modifizierungen der Geruchs-Bestandteile der Geschlechtstiere und ihrer Partnerwahl. Insbesondere vom Hauptwirt aufgezogene *Chalepoxenus* Männchen waren viel stärker an Weibchen interessiert, die mit der gleichen Wirtsart aufgezogen worden waren. Vor einigen Jahren wurde Prägung bei *C. muellerianus* Königinnen und Arbeiterinnen nachgewiesen. Es scheint somit durchaus glaubhaft, dass eine Vorliebe für Paarungspartner, die von der gleichen Wirtsart aufgezogen wurden, die Bildung von Wirtsrassen und letzten Endes die Artbildung in *Chalepoxenus* fördert. Schließlich wurden die wechselseitigen Einflüsse von *C. muellerianus* Arbeiterinnen und ihrer Sklaven auf die chemischen Profile des jeweils anderen untersucht. Die Ergebnisse deuten darauf hin, dass sich die chemischen Profile von beiden, Sklavenhaltern und Sklaven, scheinbar anpassen, ohne ihre eigenen charakteristischen Merkmale völlig zu verlieren.

Zusätzliche Studien werden empfohlen, um den Schutz dieser seltenen und bedrohten parasitischen Ameisenarten voranzutreiben.

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APPENDIX

Further publications resulting from this thesis:

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