Distribution, phylogeography and hybridization between two parapatric sibling ant species of the genus *Temnothorax*

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vorgelegt von Katja Pusch aus München

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Die Arbeit wurde angeleitet von: Prof. Dr. Jürgen Heinze
Prüfungsausschuss: Vorsitzender: Prof. Dr. S. Schneuwly

1. Prüfer: Prof. Dr. J. Heinze
2. Prüfer: Prof. Dr. S. Foitzik
3. Prüfer: Prof. Dr. P. Poschlod

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General Introduction

The mechanisms underlying species evolution are of fundamental importance to science and therefore have attracted much attention. According to Darwin (1859), the evolution of species is triggered by natural selection. It forces the modification of species in order to obtain optimal adaptation in a constantly changing environment. Hence, all species represent only temporary stages on the slow, steady and gradual continuum of time. The meanwhile widely accepted Biological Species Concept (BSC) emerged in the middle of the 20th century. This concept regards species as interbreeding units, separated from other species by reproductive isolation (Dobzhansky, 1937; Mayr, 1942). Further, Mayr (1942) and Dobzhansky (1937) argued that species might originate in geographically isolated regions and therefore stressed the importance of environmental factors already stated by Darwin (1859). Since then, numerous species concepts have been developed, like the Recognition Species Concept (RSC), the Cohesion Species Concept (CSC) or the Phylogenetic Species Concept (PSC). According to the first, species represent a population of biparental organisms with a common fertilization system. Adaptation to new habitats leads to the development of specific mate recognition systems and new species emerge as a by-product (Paterson, 1985). The Cohesion Species Concept (CSP) enhanced this definition to asexual organisms and syngameons. All members of a species have to exhibit similar ecological adaptations in order to enable free geographical exchangeability (Templeton, 1989). The Phylogenetic Species Concept (PSC) considers species as a group with identical ancestry and descent (Cracraft, 1989).

Many theoretical ideas on speciation are based on the ‘island’ model developed by Wright (1931). According to this, a population consists of subpopulations, within which individuals are freely exchangeable. Hence, all subpopulations are equally accessible for any individual belonging to this population. He further translated his mathematical work on evolutionary processes into the term ‘shifting balance’. This process includes three phases: first, subpopulations with varying fitness arise within a population due to random genetic drift. In the second phase, the fitness of these subpopulations is enhanced by directed selection. Finally, a raise in fitness of the whole population is accomplished through interdemic selection. The metaphor ‘adaptive landscape’ should illustrate these complicated processes. This landscape is very hilly; the hills illustrate peaks of well-adapted genepools, that are separated by valleys of mal-adaptation. To fullfill optimal conditions, a population should be able to move from one fitness peak to the next in order to reach highest fitness (Wright, 1932). However, this model has been discussed controversely. According to Fisher (1941), the idea of multiple peaks is

flawed, because an increasing net of gene combinations would lead to a decrease of peaks. Fisher's criticism has turned out to be correct accroding to recent simulations (Whitlock et al., 1995). Today, both Wright's multiple peaks and Fisher's single peak theory have been questioned. Because an increasing number of gene interactions leads to incompatible interactions, reproductive isolation would soon arise (Gavrilets, 1997, see below). The migration rate between the adaptive peaks should be in balance, hence being neither too strong nor too weak. Recombination could be a possible inhibitor and the completion of phase three of Wright's shifting balance theory can be accomplished easiest in peripheral demes (Gavrilets, 1996; Coyne et al., 1997).

A species' geographical distribution often exceeds the average migration distance of the individual by far, thus 'isolation by distance' might inhibit complete panmixie in a species (Wright, 1943). The decrease of genetic correlation with distance was later also verified by Kimura & Weiss (1964), based on the stepping stone model. It separates species into units, from which geneflow per generation is resticted to the adjacent unit (Kimura, 1953).

However, the impact of geographical range on speciation has been controversely debated. If fixation of mutations is a neutral process, no correlation between the degree of population subdivision and speciation rate can be found (Orr & Orr, 1996). Under rejection of the neutrality hypotheses, with mutation and random genetic drift as the only factors promoting genetic diversity, population subdivision would positively influence speciation. Under these conditions, neither extreme founder events nor complete geographical isolation are required for reproductive isolation. The geographical range of a species is positively correlated to the number of subpopulations and larger species ranges therefore promote speciation (Gavrilets et al., 1998). An extension to this model confirmed the impact of geographical variation on speciation. According to this, species with smaller range sizes and reduced dispersal rates apparently underlie higher speciation rates (Gavrilets et al., 2000).

Besides the need for geographical isolation, lineage divergence might also occur under sympatric conditions. In theoretical terms, sympatric speciation requires disruptive selection. Thus, an environment has to favour the selection of two extreme traits, while intermediates have to be selected against. However, subsequent mating events apparently counteract this process and therefore, sympatric speciation in nature had been denied for decades. To solve the problem of recombination, recent models required tight linkage of genes for mating preference and ecological traits. Or, the coding of both traits by a single gene, which however is rather unlikely to occur in nature (Turelli et al., 2001, and see references therein). Hence, subsequent

theoretical work tried to adjust to natural conditions, including variable genetic compositions and low linkage disequilibrium. By this, disruptive selection without strong selection against intermediates could be demonstrated and the idea of sympatric speciation was legalized (Dieckmann & Doebeli, 1999; Kondrashov & Kondrashov, 1999). The elimination of intermediate phenotypes by incompatibility selection may also initiate sympatric speciation (Artzy-Randrup & Kondrashov, 2006). Several independent fish lineages exhibiting closely related species pairs within one lake give evidence of sympatric speciation in nature (e.g. Schluter, 1996; Barluenga et al., 2006).

The outcome of sterile or inviable offspring by interbreeding of genetically divergent populations is a paradoxon according to Darwin's theory of natural selection (1859). This phenomenon was explained by the Dobzhansky and Muller model, initiated by Bateson (1909) (BDM model). It assumes the existence of two allopatric populations with identical genotypes at two loci (aa, bb). A single mutation (Aa, bb and AA, bb) becomes fixed in one population, whereas in the other population, the mutation becomes fixed at the second allele (aa, Bb and aa, BB). Apparently, the new 'A' allele is compatible with the 'b' allele and vice versa, but the interaction of the new alleles 'A' and 'B' could lead to incompatibilities (Dobzhansky, 1936; Muller, 1939, 1940). The origin of both substitutions in one population under the maintenance of the ancestral genes in the other population causes the same outcome (Muller, 1942). If the principle of the BDM model is applied to more than two loci, genic incompatibilities arise faster than linearly with time. Apart from that, evolutionary derived alleles cause much more incompatibilities than the ancestral ones. The probability of negative genic interactions increases with the number of substitutions (Orr, 1995). This theoretical framework explains how endogenous selection leads to hybrid inferiority due to negatively interacting genes. Empirical work on a grasshopper hybrid zone demonstrated the evolution of hybrid incompatibilities under neutral expectations (Shuker et al., 2005).

In general, hybridization has often been regarded to lead to an evolutionary dead end. Two models have been developed to explain this. The 'tension zone' model ('dynamic equilibrium' model) predicts stability of hybrid zones due to selection against hybrids together with the continuous migration of parentals into the contact zone (Barton & Hewitt, 1985). One of the best investigated examples of genetic incompatibilities comes from *Drosophila* (Wu & Palopoli, 1994 and see references therein; Hollocher & Wu, 1996). In this genus, at least 40 loci are thought to be involved in male sterility (Palopoli & Wu, 1994). Male-biased inviability or sterility is another frequently observed outcome of hybridization, that causes the

heterogametic sex to 'suffer' more than the homogametic sex (Haldane's rule: Haldane, 1922). Because alleles of decreasing hybrid fitness are partially recessive, the x-chromosome therefore has a disproportionate effect on hybrid inviability or sterility (Turelli & Orr, 1995). Negative effects of hybridization were demonstrated by numerous studies in many taxa (Burke & Arnold, 2001). Endogenous selection could even be directly shown in a bird hybrid zone where breeding success of mated F1 hybrids pairs and those of the parental species was investigated within the natural hybrid zone (Bronson et al., 2003).

According to the second model, the 'mosaic' model, different habitat preferences of the parental species inhibit frequent interspecific mating (Harrison, 1986). Several well-investigated hybrid zones between the toads *Bombina bombina*, that prefers to breed in pondlike habitats and *B. variegata*, adapted to live in puddles, fit this model. Hybrids are viable and fertile, but rare, because of habitat differentiation of the parental species (e.g. Szymura & Barton, 1986, 1991; Vines et al., 2003).

Still, hybridization may also initiate successful speciation in forming new evolutionary lineages (Arnold & Hodges, 1995; Arnold et al., 1999). On the genetic level, this so-called hybrid vigor might be caused by increased heterozygosity, especially, if both parental species are inbred. But heterosis is often only a short-term effect, as in further generations, favourable gene combinations might be destroyed by recombination (Burke & Arnold, 2001). In contrast to hybrid inferiority, which is often caused by endogenous factors (see above), exogenous factors play a considerable role for hybrid superiority. Introgression of one adaptive trait into the other lineage might improve adaptation to new or extreme environments. This mainly accounts for hybrid zones at the edge of a species' range, where parental species are not well adapted to their environments. Sunflower hybrid zones, investigated in detail by genetic mapping, provide a good example for this (Rieseberg et al., 1999; Rieseberg & Buerkle, 2002; Rieseberg et al., 2003).

Hybridization is exhibited more frequently in plants than in animals (Ellstrand et al., 1996). Nevertheless, successful hybridization has also been observed in different animal taxa, e.g. in a frog hybrid zone. In this case, the males' mating behaviour of one parental species apparently does not underlie sexual selection (Lengagne et al., 2006). High introgression rates found in a deer hybrid zone also do not indicate strong selection against hybrids (Goodman et al., 1999). Moreover, speciation by hybridization has been stated recently in *Heliconius* butterflies, where the hybrid phenotype is maintained by strong assortative mating (Mavárez et al., 2006; Kronforst et al., 2006).

Rather early, scientists were aware of the importance of geographical and historical parameters for speciation and the distribution of species (DeCandolle, 1820). Nowadays, the use of molecular methods allows the determination of phylogenetic relationships among groups of animals. A milestone was the discovery of mitochondrial DNA. It is maternally inherited, does not undergo recombination and mutation rates are generally higher than those of nuclear DNA (Ballard & Whitlock, 2004, and see references therein). The use of molecular markers, mainly mt DNA, to investigate the association of different genetic lineages with geographical distribution patterns is described by the term 'phylogeography'. Phylogeography enables the determination of both interspecific and intraspecific lineage divergence (Avise et al., 1987, 1989, 2000). Meanwhile, the apparent relationship between gene trees and species trees is widely accepted (e.g. Pamilo & Nei, 1988; Avise, 1989). Empirical studies on Hawaiian *Drosophila* were one of the first to use genetic markers in order to deduce lineages and build phylogenies (Carson, 1983). It was followed by numerous studies such as the investigation of allopatric speciation in different populations of a Darwin finch species (Grant et al., 2000).

As nucleotide changes accumulate over time, sequence data additionally allow inferences from time. Such estimates required the setting of an independent molecular clock. This had been realised in two major approaches: the nested clade analysis relates phylogenetic patterns to geographical regions (Templeton, 1998); minimum spanning trees and median joining networks of mitochondrial DNA allow the determination of ancient and derived haplotypes (Bandelt et al., 1999). The advances in molecular genetics are joined by advances in the field of paleoclimatology (Hewitt, 1996, 2001). Due to investigations by paleoclimatological methods, vast ice-sheets covered the northern hemisphere in the late Pliocene around 2.4 million years ago. Prior to 700.000 years, the ice was less intense and from this point until now, the climate was dominated by four major glacials, interrupted by relatively short interglacials (Webb & Bartlein, 1992). The last cycle, which is best understood, began around 135.000 years ago, and included considerable climatic oscillations itself (Roy et al., 1996). Pollen remains from Europe and North America during the last 20.000 years allow inferences from the large effects of these climatic oscillations. In Europe, the relatively warm Iberian peninsula in the west and the Balcans in the east harbored deciduous forests during glaciation and thus served as potential refugias for many thermophilic animals (Huntley & Birks, 1983; Huntley & Webb, 1989; Bennett et al., 1991). The effects of re-colonization on species distribution and genetic variability have been investigated in numerous taxa where the existence of northern populations deprived of genetic variability ('bottleneck effect') was evidenced (Hewitt, 1996). The same accounts for postglacial expansion and migration routes from different refugia in

such diverse organisms as freshwater amphipods, grasshoppers, and bears (Hewitt, 1996; Taberlet et al., 1998; Vainio & Väinölä, 2003; Hewitt, 2004a).

The phylogeography of ants, one of the ecologically most important groups of invertebrates, has only recently found attention (Goropashnaya et al., 2004). Ants belong to the eusocial insects, where individuals are organized in colonies of related individuals and thus constitute the final stage of evolutionary transitions (Maynard Smith & Szathmáry, 1995). Colonies usually remain closed entities because all nestmates share a common colony odour consisting of chemical cues. These are produced by and exchanged among colony members, often with considerable contributions from nest material, food or other environmental sources (Hölldobler & Wilson, 1990; Vander Meer & Morel, 1998; Lenoir et al., 1999). Ant colonies often contain one single-mated queen and her offspring: male and female sexuals and sterile workers. The hallmark of eusociality is the sterile helper caste that is explained by kin selection theory (Hamilton, 1963, 1964). However, relatedness asymmetry, combined with conflicts over reproduction and aberrations from high intra-colony relatedness due to multiple mating or polygyny have lead to many parodoxons and various adaptations to them in this system (Hamilton, 1964; Bourke & Franks, 1995).

The special features of eusociality also influence speciation and have lead to the evolution of several outstanding phenomena. In the harvester ant *Pogonomyrmex* for example, distinct lineages have evolved by hybridization between two species. Now, colony foundations are only successful, if the queen is mated to both males from the same and males from the other lineage. Heterozygous individuals develop into sterile workers, whereas homozygous individuals all develop into virgin queens. Meanwhile, this system has lost flexibilty and thus, hybridization finally has culminated in genetic caste determination (Helms Cahan et al., 2002; Julian et al., 2002; Volny & Gordon, 2002; Helms Cahan & Keller, 2003; Helms Cahan et al., 2004). Another example constitutes intraspecific social parasitism, exhibited in different ant species (Buschinger, 1986). It has been shown to trigger sympatric speciation in several *Myrmica* species (Savolainen & Vepsäläinen, 2002). In any case, the social structure of ant colonies is fragile and can be tremendously changed by migration events. For example, the accidental introduction of the Argentine ant *Linepithema humile* to California and Southern Europe has apparently led to a loss of discrimination among members of different colonies and resulted in unicoloniality, which again contributed largely to its ecological dominance (Tsutsui et al., 2000; Giraud et al., 2002). Therefore, phylogenetic studies on ant species remain a vast field to explore.

Aim of the study

The parapatric ant sibling species *Temnothorax nylanderi* and *Temnothorax crassispinus* (Hymenoptera: Formicidae) belong to the most common ant species in Europe. They probably derived from one ancestor species and diverged in different glacial refugia. Hence, they may represent another species pair that originated in allopatry due to climatic oscillations. Only recently, it was recognized, that the morphologically very similar ant species belong to separate species. Despite great morphological similarity, the species can be distinguished by respective private alleles at the enzyme locus GPI and two mitochondrial DNA loci (Seifert, 1995, 1996; Strätz et al., 2002). Presently, *T. nylanderi* is widely distributed throughout deciduous forests in Western Europe, whereas *T. crassispinus* inhabits similar habitats in Eastern Europe (Seifert 1995, 1996; Radchenko et al., 1999; Radchenko, 2000). Both species meet along the line Schwerin-Magdeburg-Halle-Leipzig-Döbeln-Olbernhau, where they occasionally hybridize (Seifert, 1995).

T. nylanderi and *T. crassispinus* are monogynous (single-queened) and monandrous (single-mated) and sexuals mate during nuptial flights (Buschinger, 1968; Plateaux, 1970; Seifert, 1996; Tichá & Štys, 2002). In Central European populations of the well-investigated species *T. nylanderi*, nestmate recognition appears to rely strongly on environmental and not on genetic odour cues (Heinze et al., 1996). Besides, remarkably reduced genetic variability despite outbreeding was observed (Foitzik & Heinze, 2001). This lack of genetic variability is thought to facilitate the occasional fusion of unrelated, neighbouring colonies and the usurpation of established colonies by alien founding queens (Foitzik & Heinze, 1998, 2000, 2001). The same apparently accounts for the lesser intensively studied sibling species *T. crassispinus* (Seifert, 1995; Tichá, 2002; Tichá & Štys, 2002; Strätz & Heinze, 2004).

Due to re-immigration into Central and Northern Europe, both species apparently experienced a genetic bottleneck. Therefore, this study aimed at investigating distribution and genetic variability of populations from both Northern and Southern Europe by inference of phylogenetic approaches. The use of morphometric and allozyme analyses allowed to determine the position of the contact zone and to locate populations with hybrids (Chapter 1). Further, hybridization and its impact on colony level were investigated in detail at a site from the contact zone in Southern Germany (Chapter 2). Observed interspecific colony fusions and the effect of the mainly environmental determined nestmate recognition system were tested in laboratory colony fusion experiments (Chapter 3). Moreover, the extend of genetic variability and its impact on colony structure in South European populations were investigated with

nuclear markers (Chapter 4). By collecting ants in Southern Europe, a new species was discovered (Chapter 5).

Figure 0: Workers of *T. nylanderi* (left) and *T. crassispinus*

Distribution and genetic divergence of two parapatric sibling ant species in Central Europe *

Katja Pusch, Bernhard Seifert, Susanne Foitzik and Jürgen Heinze

Abstract

The two sibling ant species *Temnothorax nylanderi* and *Temnothorax crassispinus* are widely distributed throughout deciduous forests in Europe. Their resemblance in morphology and similar ecological requirements suggest that they evolved from the same ancestral species in different glacial refugia and re-immigrated into Central Europe after the last ice age. Here, we show that the two species are parapatrically distributed in South-Eastern Germany and hybridize along a narrow contact zone close to the continental divide. Phylogeographical data based on the mitochondrial genes cytochrome oxidase subunit I and cytochrome b suggest that the dominant haplotypes for *T. nylanderi* and *T. crassispinus* might have diverged already 1.5-2 Mya. Intraspecific variability is extremely low in both species, which might be explained by severe bottlenecks during rapid post-glacial expansion into Central Europe.

Key words: allozymes, colony odour, glacial refugium, hybridization, morphometry, mt DNA, phylogeography, sibling species, *Temnothorax crassispinus*, *Temnothorax nylanderi*

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Introduction

Geographical regions where pairs of sibling species come into secondary contact after allopatric speciation provide interesting systems for investigation of the early stages of speciation and of competition between closely related taxa (Hewitt, 1989). Postglacial expansion from different refugia has led to the formation of a number of such zones throughout Europe (Gollman et al., 1988; Hewitt, 1990; Szymura & Barton, 1991; Hewitt, 1996; Serrano et al., 1996; Szymura et al., 2000; Hewitt, 2004). The extent of hybridization and the competitiveness of the respective species might either stabilize the contact zone, as in *Chorthippus* and *Bombina* (Gollman et al., 1988; Hewitt, 1990; Szymura & Barton, 1991; Serrano et al., 1996; Szymura et al., 2000), or lead to a shift in their geographical range, as in chickadees (Bronson et al., 2003).

Postglacial migration routes and contact zones have been studied in diverse organisms such as freshwater amphipods, grasshoppers, and bears (Hewitt, 1996; Vainio & Väinölä, 2003; Hewitt, 2004a). However, the phylogeography of ants (one of the ecologically most important groups of invertebrates) has only recently recieved attention (Goropashnaya et al., 2004). This is surprising given that immigration events in social insects may be associated with tremendous changes in the social structure of their colonies. For example, the accidental introduction of the Argentine ant *Linepithema humile* to California and Southern Europe has apparently led to a loss of discrimination among members of different colonies and resulted in unicoloniality, which again contributed largely to its ecological dominance (Tsutsui et al., 2000; Giraud et al., 2002). The introduction of European yellowjacket wasps to New Zealand was associated with similarly dramatic changes in life history (Goodisman et al., 2001).

Approximately ten years ago, Seifert (1995, 1996) recognized that one of the most common ant species in deciduous forests in Central Europe actually comprises two morphologically very similar sibling species, *Temnothorax nylanderi* (formerly *Leptothorax* (*Myrafant) nylanderi* Förster, 1850; Bolton 2003) and *Temnothorax crassispinus* (Karavajev, 1926) (previously *Leptothorax* (*Myrafant*) *crassispinus*; Bolton, 2003). These species probably originated from populations of a common ancestor species in different glacial refugia in Southern Europe and re-immigrated into Central Europe after the retreat of glaciation. Presently, *T. nylanderi* is widely distributed throughout deciduous forests in Western Europe, whereas *T. crassispinus* inhabits similar habitats in Eastern Europe (Seifert, 1995, 1996; Radchenko et al., 1999; Radchenko, 2000).

The biology of *T. nylanderi* has been extensively studied (Buschinger, 1968; Plateaux, 1970, 1972; Foitzik et al., 1997; Foitzik & Heinze, 1998, 2000, 2001; Foitzik et al., 2003). Its small

colonies consist of a few dozen workers and a single queen (monogyny) that inhabit rotten sticks and hollow acorns. Populations may reach extremely high nest densities of up to ten nests per square meter. Nestmate recognition in this species appears to rely strongly on environmental and not on genetic odour cues (Heinze et al., 1996). This is thought to facilitate the occasional fusion of unrelated, neighbouring colonies and the usurpation of established colonies by alien founding queens (Foitzik & Heinze, 1998, 2000, 2001). The biology of *T. crassispinus*, albeit not as intensively investigated, appears to be very similar (Seifert, 1995; Tichá, 2002; Tichá & Štys, 2002; Strätz & Heinze, 2004; Pusch, unpublished data).

In North-Eastern Germany, the two taxa meet along the line Schwerin-Magdeburg-Halle-Leipzig-Döbeln-Olbernhau. Despite their large morphological similarity, the two species can be separated reliably by a discriminant analysis with various morphometric characters, including the length and shape of the propodeal spines (Seifert, 1995). Exact measurements even allowed the distinction of more traits, such as head width and length. Moreover, the allozyme locus glucose-6-phosphate-isomerase (GPI) exhibits a private allele for each species, which supports discrimination (Seifert, 1995). Within this contact zone, morphometric and allozyme analyses indicate occasional hybridization (Seifert, 1995).

In Austria, *T. crassispinus* occurs only east of Vorarlberg (Glaser, 1998, 2000) and this species was recently also recorded from several places in Southern Germany (Strätz et al., 2002). We therefore attempted to determine the position of the contact zone in Southern Germany and Northern Italy. Furthermore, using two mitochondrial DNA (mt DNA) markers we examined the genetic differentiation within and between the two taxa. With these analyses, we also intended to show whether the unusual system of environment-based nestmate recognition might result from a loss of genetic variability during the colonization of Central Europe, analogous to the suggested evolution of unicoloniality in Argentine ants (Tsutsui et al., 2000).

Material and Methods

Collection of colonies and morphometric species determination

Ants were collected by searching potential nest sites in oak – pine – beech – forests in various places in Europe (Figures 1.1 and 1.2). At least five colonies were taken at each collecting site to minimize errors through accidentally missing an equally common second species. In a few areas with very low nest site densities, only less than five colonies could be taken. For further analyses, the ants were frozen at –20°C. Because the most distinct feature differing between the two species is the shape of the propodeal spine (Seifert, 1995), we measured spine length (the distance from the tip of the propodeal spine to the centre of the propodeal spiracle in lateral

view) and spine tip distance (the maximum distance between the tips of the propodeal spines in dorsal view) in three workers from each colony at x 120 magnification using a binocular microscope. The measurement error of spine length, based on 10 measurements of one reference individual of *T. crassispinus*, was 3.3 µm (5.2 % of the largest difference), and that of spine tip distance was 7.0 µm (8.6%). Measurement errors of spine length and spine tip distance were 4.6 µm (6.3%) and 5.4 µm (5.7%), respectively, in *T. nylanderi*. We measured 435 workers from 136 colonies from 37 populations in *T. nylanderi* and 360 individuals from 120 colonies from 31 populations in *T. crassispinus*.

Allozymes

The two taxa differ strongly in the frequencies of electrophoretic variants of the enzyme GPI, but do not show consistent differences in other enzymes (P. Douwes, personal communication) or microsatellites (K. Pusch, unpublished data). We therefore determined the GPI electromorph for two to five ants from each colony. Individual ants were homogenized in 20µl Tris-EDTA pH 7.0 buffer. Proteins were separated by 90min electrophoresis at 10V/cm and 20mA on 10cm x 8cm x 0.75mm 8% polyacrylamide slab gels using a Tris-glycine pH 8.3 buffer. The enzyme was stained using standard histochemical techniques (Murphy et al., 1996). Electromorphs were named according to their migration velocities (fast *f*; medium *m*; slow *s*; very slow *v*).

DNA-isolation, Polymerase Chain Reaction (PCR) and sequencing

DNA was extracted from complete workers using the Puregene kit method (Gentra Systems) as previously described (Foitzik & Herbers, 2001). We analysed a 430bp fragment of the mitochondrial cytochrome b (Cyt b) gene and a 500bp fragment of the mitochondrial cytochrome oxidase (CO I) gene using the primers 5'-TAT GTA CTA CCA TGA GGA CAA ATA TC-3' and 5'-ATT ACA CCT CCT AAT TTA TTA GGA AT-3' for Cyt b; 5'- TTG ATT TTT GGT CAT CCA GAA GT-3'and 5'-CCA CAA ATT TCT GAA CAT TGA CCA-3' for CO I (Simon et al., 1994). The 20µl PCR reaction mixture consisted of 1µl DNA, 2 pmol dNTPs, 0.5 pmol of each primer, 9µl dd H_2O, 2µl 10x PCR buffer (without $MgCl_2$), 2 mM $MgCl_2$, and 0.4µl of 1 unit/µl Taq Polymerase (Q Bio Gene). Both genes were amplified at an annealing temperature of 68°C with 33 and 36 cycles for the Cyt b and CO I genes, respectively. PCR products were separated by electrophoresis on a 1% ethidiumbromide-stained agarose gel (TAE buffer) for 30 min at 100mA and then purified using the NucleoSpin-kit (Macherey-Nagel). The amount of DNA in 5µl of the purified solution was quantified on a

1.5% agarose gel (TBE buffer). The second PCR reaction mixture contained 0.5 pmol primer, 3µl of 5x sequencing buffer, 2µl Big dye terminator version 1.1 (Applied Biosystems), supplemented with dd H_2O and 2 - 12µl amplified ant DNA to a final volume of 20µl. Single-stranded PCR products were sequenced using an ABI PRISM 310 automatic sequencer (Perkin-Elmer, Applied Biosystems). Sequences were read and aligned with GeneScan Analysis Software version 3.1 (Perkin-Elmer, Applied Biosystems).

In *T. nylanderi*, we analysed 430 bp of the Cyt b sequence in 99 workers and colonies from 26 populations and 500 base pairs of the CO I sequence from 71 workers and colonies from 19 populations. In *T. crassispinus*, the Cyt b fragment was sequenced in 32 workers and colonies from 10 populations and CO I in 24 workers and colonies from five populations. Sequences were aligned using the algorithm CLUSTAL W (Thompson et al., 1994) and checked by eye with the program BioEdit (Hall, 1999). Gaps and double peaks were substituted with 'N's. Minimum spanning networks were constructed according to the nucleotide differences calculated with Bioedit. Mismatch distributions revealing characteristic features of the nucleotide differences were calculated and tested against a sudden expansion model (Slatkin & Hudson, 1991; Rogers & Harpending, 1992) for recently expanding populations with DnaSP version 4.0.5 (Rozas et al., 2004). Observed and expected values were compared using the chi-square goodness-of-fit test implemented in Arlequin 2.0 (Schneider et al., 2000). Furthermore, this program was also used to perform analyses of molecular variance (Excoffier et al., 1992) and to calculate Φ_{st} values with 2000 permutations to test for geographical differentiation.

Results

Distribution

In Southern Germany, the two sibling species meet close to the continental divide in the Franconian Alb, following the line Neumarkt - Berching - Beilngries - Ingolstadt -Allershausen - Mering (Figure 1.1). Both species were found syntopically in four collecting sites in this area (Dietfurt, Manching, Eichstätt, Berching) and almost syntopically in Velburg, where the two populations are divided by a field approximately 150 m in width. The ranges of both species therefore appear to overlap for at most 25 km. Specimens collected further west in Germany and other parts of Europe were all *T. nylanderi*, whereas all material collected east and south-east of the contact zone were *T. crassispinus*. In some areas with apparently suitable habitat (light forests with pines, beeches, and oaks) close to the expected contact zone, neither *T. crassispinus* nor *T. nylanderi* could be found despite the presence of other ants, which regularly co-occur with the two sibling species in other sites (e.g. *Leptothorax gredleri*, *Leptothorax*

acervorum, *Temnothorax unifasciatus*). Furthermore, neither *T. crassispinus* nor *T. nylanderi* were found in deciduous forests at the northern border of the Alps (Kempten, Marktoberdorf, Schongau, Weilheim; Figure 1.1, see also Appendix).

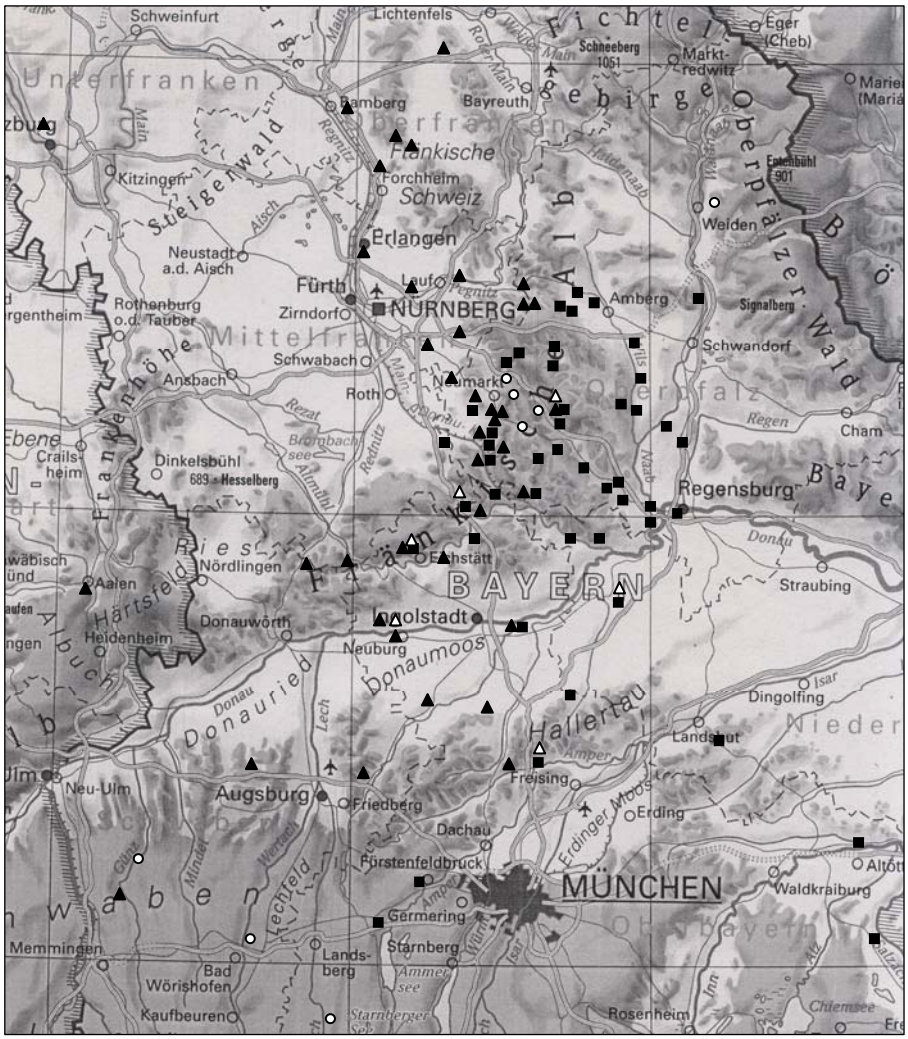


Figure 1.1: Distribution of the two parapatric sibling ant species *Temnothorax nylanderi* (black triangles) and *Temnothorax crassispinus* (black squares) in Southern Germany. Collecting sites where presumed hybrids between the two taxa were found are symbolized by white triangles, sites lacking any of the two species by white circles.

South of the Alps, specimens from Lago Maggiore (Ticino, Switzerland), several sites in Northern Italy, Abruzzo, Campania, and Sila Mountains in Southern Italy, and in Catalonia (Spain) were all *T. nylanderi*, whereas samples from Škocjan (Slovenia), Trebesing (Carinthia, Austria) and Krk (Croatia) were *T. crassispinus* (Figure 1.2, see also Appendix).

Figure 1.2: The distribution of *Temnothorax nylanderi* (hatched) and *Temnothorax crassispinus* in Europe (hatched with broken lines). Collecting sites of samples for this study are indicated by black triangles (*T. nylanderi*) and black squares (*T. crassispinus*). Open symbols represent reports from other studies (Glaser, 2000, 2001; Radchenko et al., 1999; Strätz et al., 2002). The black lines represent the contact zones in north-eastern Germany (Seifert, 1995) and Southern Germany (location of the detailed map in Figure 1.1 indicated by a rectangle).

Morphological species determination

Both spine length and spine tip distance were significantly larger in *T. crassispinus* than in *T. nylanderi*. The values for spine length were not normally distributed in either of the species (Kolmogorov-Smirnov-test: $N = 265$; $p < 0.05$). In *T. nylanderi* spine length was in the range

142 – 200 µm (median = 170 µm; 25 - 75 % quartile = 165 µm –182 µm) and was significantly smaller than in *T. crassispinus* (190 – 250 µm; median = 210 µm; quartile = 202 – 220 µm; Mann-Whitney-U-test: U = 368.5; $p < 0.001$). Spine tip distance in *T. nylanderi* (180 – 250 µm; mean = 220 µm; SD = 18.0 µm) was also significantly smaller than in *T. crassispinus* (227 – 300 µm, mean = 258 µm; SD = 19.0 µm; Kolomogorov-Smirnov-test, not significant; two–tailed t-test: $t = - 16.9$; d.f. = 263; $p < 0.001$).

Measurements allowed a quick determination of most specimens collected at a distance from the contact zone. However, the values overlapped to some extent (Figure 1.3). In such unclear cases, unequivocal classification could be achieved with allozyme investigations. Although Seifert (1995) showed that the discriminant values separating *T. crassispinus* and *T. nylanderi* differ more strongly away from the contact zone, correlations between morphology and site could not be detected. For an exact morphological determination of species, more detailed morphological measurements may be needed, as suggested by Seifert (1995, 2002).

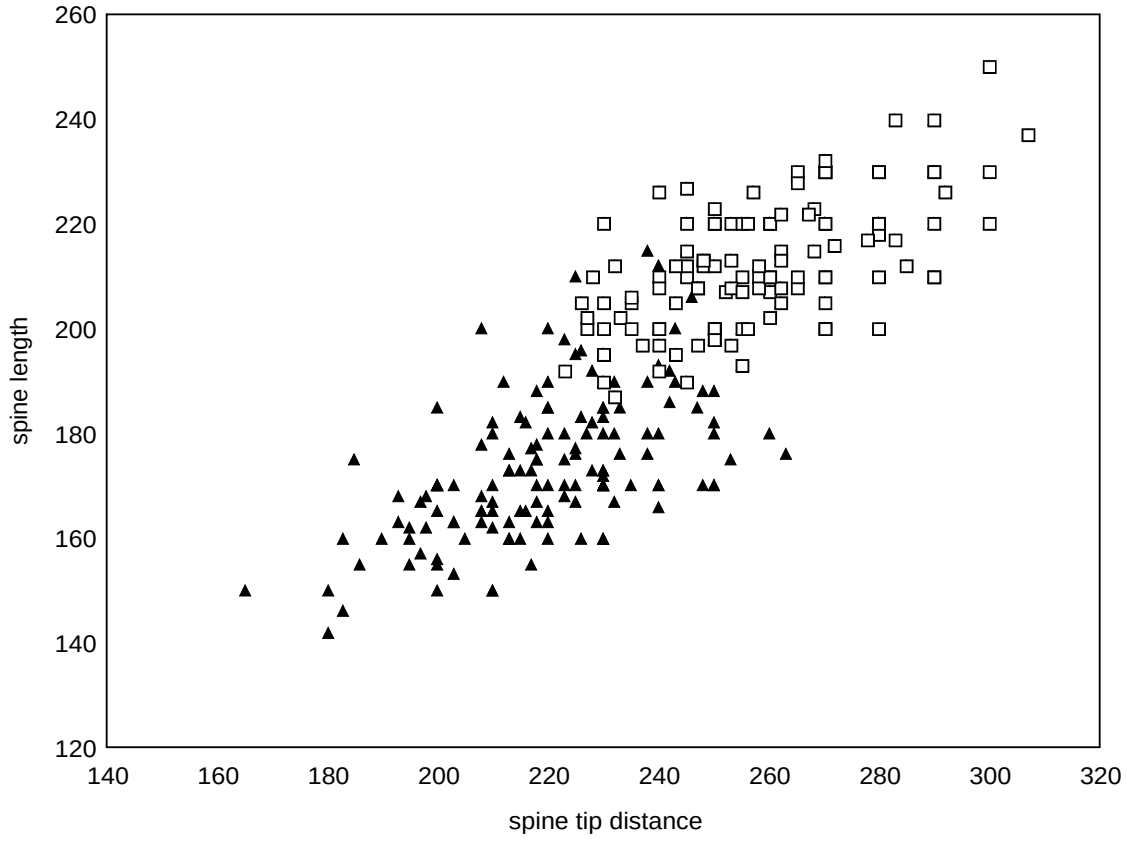


Figure 1.3: Scatterplot of the two measurement parameters including all samples (in µm). The black dots represent data from *Temnothorax nylanderi*, the open squares *Temnothorax crassispinus*.

Allozyme frequencies

Four different electromorphs of GPI were separated by gel electrophoresis. Our allozyme investigations confirmed earlier results (P. Douwes, personal communication) indicating that the two species are almost completely fixed for different electromorphs. In total, we investigated 639 *T. crassispinus* workers from 178 colonies from 55 populations and 677 *T. nylanderi* workers from 183 colonies of 54 populations. Almost all workers (95.1%; 608 of 639 from 176 colonies) of *T. crassispinus* were homozygous for electromorph *m*. Only 31 workers from 11 colonies had aberrant electromorph patterns (*ss*, *ms*, *mf*, *ff)*. In *T. nylanderi*, 605 of 677 individuals from 173 colonies (89.4%) had electromorph *ff* and 72 workers from 31 colonies had other electromorph patterns (*vv*, *ss*, *sf*, *mf*, *ms*, *mm*; Table 1.1).

Table 1.1: Genotypes at the enzyme locus GPI in workers of the two parapatric sibling ant species *Temnothorax nylanderi* and *Temnothorax crassispinus*

	T. nylanderi			***T. crassispinus***		
Genotype	N of individuals	N of colonies	N of populations	N of individuals	N of colonies	N of populations
ff	**605**	**173**	**52**	3	2	2
mm	17	7	4	**608**	**176**	**52**
mf	7	4	4	26	7	5
fs	24	10	7	-	-	-
ss	20	8	5	1	1	1
ms	1	1	1	1	1	1
vv	3	1	1	-	-	-

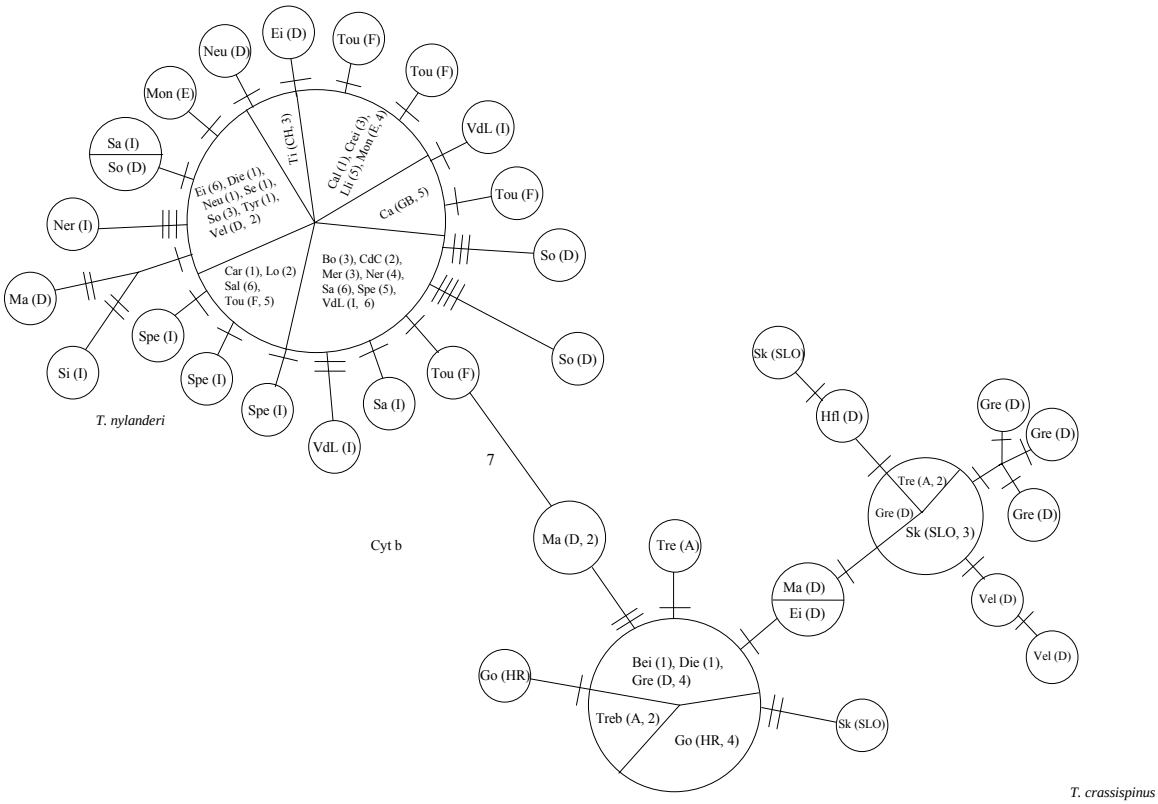
A few individuals that, based on their morphology and mt DNA belonged to one species had only the typical electromorph of the respective other species (i.e. *f* in *T. crassispinus* and *m* in *T. nylanderi*). Most of these individuals were found within 50 km of the contact zone, which might suggest low levels of introgression (Table 1.2). *Temnothorax crassispinus* with a heterozygous genotype, combining the typical electromorphs of both species (*mf*), were found only in the contact zone and might therefore represent hybrids. By contrast, the few *T. nylanderi* individuals with *mf* were found throughout the entire range of this species (Table 1.2). From the low frequency of colonies with *mf* individuals, we conclude that hybridization is a rare event even in the contact zone, although it might be more common in some sites.

Table 1.2: Rare genotypes at locus GPI in workers of the two parapatric sibling ant species *Temnothorax nylanderi* and *Temnothorax crassispinus*

Genotype	Population	N of colonies	N of individuals
	T. nylanderi		
mm	Neuburg	2	7 (2, 5)
	Pfaffenhofen	1	2
	Sengenthal	1	2
	Velburg	3	6 (1, 1, 4)
mf	Montana	1	1
	Locoal	1	1
	Neuburg	1	3
	Mte. Verità	1	2
fs	Eichstätt	2	6 (2, 4)
	Merano	2	4 (1, 3)
	Montseny	1	4
	Schrobenhausen	1	2
	Sengenthal	1	2
	Sperrone	2	5 (1, 4)
	Wäschenbühl	1	1
ss	Merano	2	5 (2, 3)
	Neuburg	2	5 (1, 4)
	Valle d. Lucania	1	1
	Creixelles	1	4
	Llinars	2	5 (1,4)
ms	Velburg	1	1
vv	Neuburg	1	3
	T. crassispinus		
ff	Regensburg	1	2
	Tittmoning	1	1
mf	Abensberg	1	2
	Allershausen	1	4
	Eichstätt	1	3
	Greding	2	15 (7, 8)
	Velburg	2	2
ss	Vilsbiburg	1	1
ms	Velburg	1	1

Species differentiation and phylogeography

Temnothorax nylanderi and *T. crassispinus* differed on average in 14 of 430 sites of the Cyt b gene (3.3%) and in 12 of 500 sites of the CO I (2.4%). Neither *T. nylanderi* nor *T. crassispinus* haplotypes revealed a clear geographical pattern. In *T. crassispinus*, Φ_{st} was 0.02 (p = 0.4) for the CO I fragment and 0.24 (p = 0.001) for the Cyt b gene fragments. The Φ_{st} values in *T. nylanderi* showed the opposite pattern (Cyt b, Φ_{st} = -0.003; p = 0.5; CO I Φ_{st} = 0.41; p < 0.001). The haplotype networks exhibit star-like topologies, with two main Cyt b haplotypes in *T. crassispinus* (Figure 1.4).



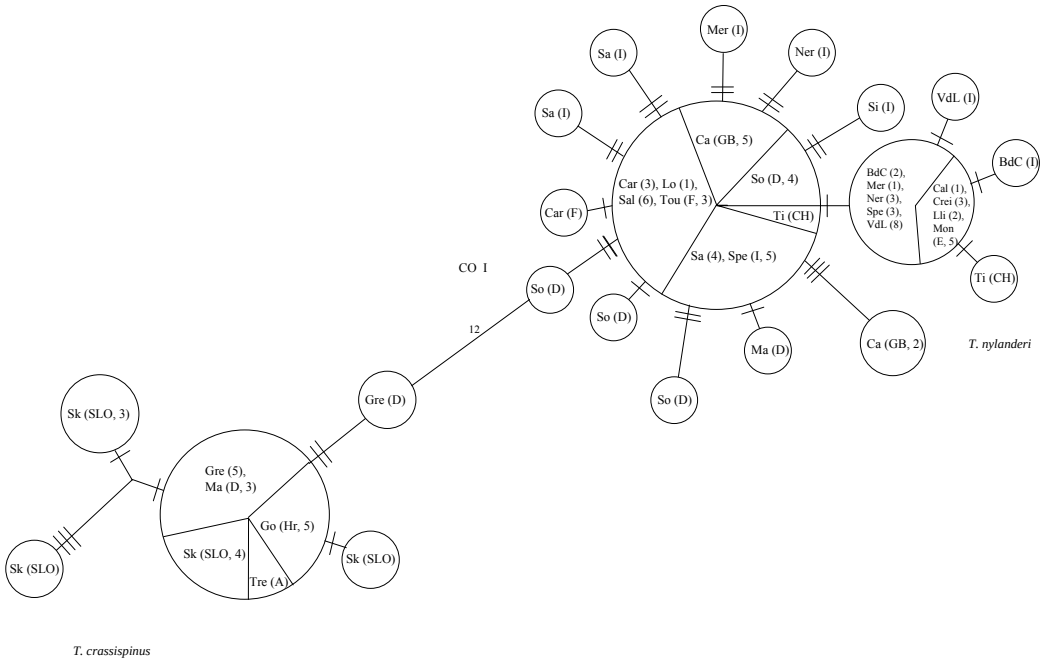


Figure 1.4: Minimum spanning networks based on the cytochrome b (Cyt b) and cytochrome oxidase subunit I (CO I) gene fragments of both *Temnothorax nylanderi* and *Temnothorax crassispinus*. Bars on the lines connecting the haplotypes indicate the number of nucleotide substitutions. The size of the circles corresponds to the number of sequences sharing the same haplotype. Collecting sites: Bo, Montana Bolzano (I); BdC, Bosco del Consiglio (I); Bei, Beilngries (G); Cal, Caldes (ES); Ca, Cambridge (GB); Car, Carnac (F); Crei, Creixelles (ES); Die, Dietfurt (G); Ei, Eichstätt (G); Go, Gostinjac (HR); Gre, Greding (G); Hfl, Höfling (G); Lli, Llinars (ES); Lo, Locoal-Mendon (F); Ma, Manching (G); Mar, Marburg (G); Mer, Merano (I); Mon, Montseny (ES); Ner, Nervesa (I); Neu, Neuburg a.d. Donau (G); Sa, Salò (I); Sal, Sault (F); Se, Sengenthal (G); Si, Sila (I); Sk, Škocjan (SLO); So, Sommerhausen (G); Spe, Sperrone (I); Ti, Mte. Verità (CH); Tou, Tours (F); Tre, Trebesing (A); Tyr, Tyrolsberg (G); Vel, Velburg (G); VdL, Vallo d. Lucania (I). Countries indicated in parentheses: I, Italy; G, Germany; ES, Spain; GB, Great Britain; F, France; HR, Croatia; SLO, Slowenia; CH, Switzerland; A, Austria.

Both species showed surprisingly little intraspecific variation throughout the examined range. The average sequence divergence of CO I was 0.18% in *T. nylanderi* and 0.15% in *T. crassispinus*, and that of Cyt b 0.09% in *T. nylanderi* and 0.44% in *T. crassispinus*. This suggests a recent and fast expansion of both species. In an intraspecific, pairwise comparison, approximately 40% from both species were identical in CO I haplotypes. For the Cyt b sequence, the percentage of identical haplotypes in *T. nylanderi* (64%) was almost twice as high as in *T. crassispinus* (37%), due to some aberrant sequences in workers from Greding, which shared three of the 14 distinguishing nucleotides with *T. nylanderi*. The observed nucleotide distribution mostly matched the expected distribution assuming sudden expansion

for both loci (simulated Ssd ≥ observed Ssd; *T. nylanderi*, Cyt b: p = 0.59; CO I: p = 0.42; *T. crassispinus*, Cyt b: p = 0.58; CO I: p = 0.09; Figure 1.5).

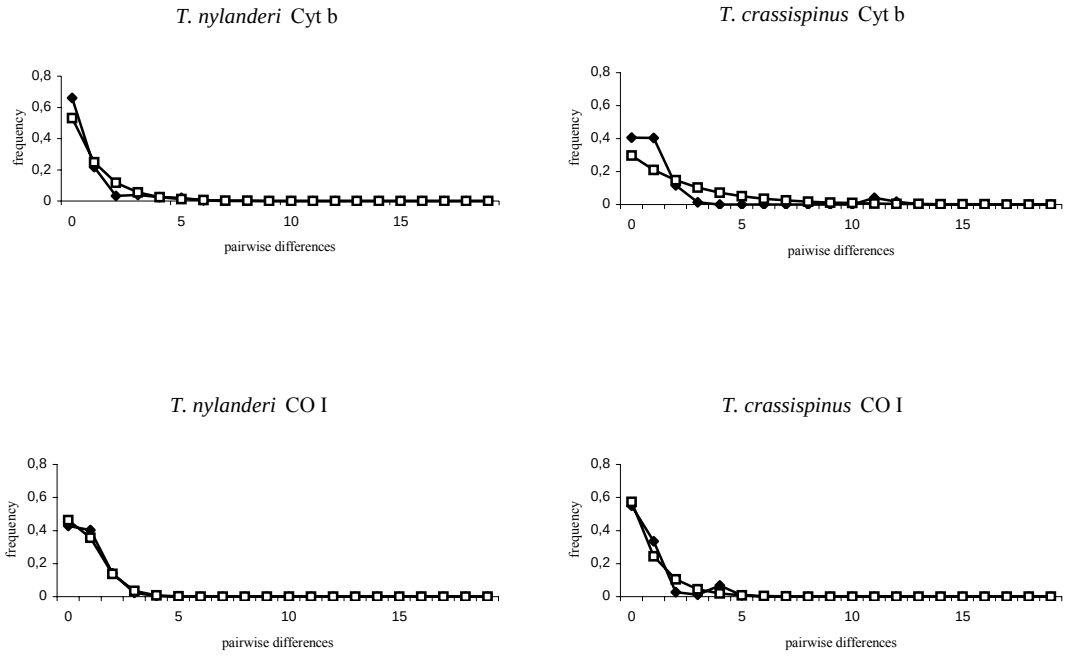


Figure 1.5: Frequency distribution of pairwise distances between sequences of the mitochondrial cytochrome oxidase (CO I) and cytochrome b (Cyt b) genes in the sibling ants *Temnothorax nylanderi* and *Temnothorax crassispinus*. The number of nucleotide differences between any pair of sequences is given on the x-axis, the frequency of differences on the y-axis. Black rhombs indicate observed differences; open squares indicate the expected differences under the expansion model.

Discussion

The two parapatric ant species *T. crassispinus* and *T. nylanderi* meet in a narrow contact zone in Central Bavaria close to the continental divide. This is much farther west than expected from the location of the contact zone in Northern Germany, which turns sout-heast in Saxony (Seifert, 1995), but matches the observation that *T. nylanderi* is restricted to the westernmost parts of Austria (Glaser, 1998).

Because both species preferably nest in hollow acorns and beechnuts, their re-immigration into Central Europe was presumably closely linked to the postglacial advance of oaks and beeches (Seifert, 1995), which spread into Central Europe 10 000 years ago (Brewer et al., 2002). *Temnothorax nylanderi* presumably spread north and northwest from southern and south-western refugia, whereas *T. crassispinus* re-immigrated from the east and south-east without

reaching far into Central Europe (Seifert, 1995, 1996). Presently, the easternmost extension of *T. nylanderi* in Northern Germany follows the Elbe valley and only *T. crassispinus* has been collected farther east (Seifert, 1995; Köbernick, 1999; Radchenko, 2000; Czechowski & Czechowska, 2001; Tichá & Štys, 2002; S. Foitzik, unpublished data). The finding of *T. nylanderi* on Wolin island, Poland, 200 km east of the presumed borderline (Czechowski & Czechowska, 2001), requires further investigation. South of the Alps, the contact zone between the two species presumably lies somewhere in North-Eastern Italy. In Slovenia and Croatia, only *T. crassispinus* has been found (Bračko, 2003). The range of *T. crassispinus* therefore extends comparatively far west in Southern Germany and Austria. This might suggest that its postglacial advance followed the Danube valley.

Interestingly, both species were apparently absent in several suitable oak and pine forests close to the extensively studied contact zone in Bavaria. Instead, many nest sites were inhabited by the oak – preferring, less thermophilic *L. gredleri*, the more boreal *L. acervorum*, and the more thermophilic *T. unifasciatus*, which also regularly co-occur with *T. nylanderi*, *T. crassispinus*, or both, in other sites (Seifert, 1995). At present, it is unknown whether *T. nylanderi* and *T. crassispinus* have not yet reached these areas or have secondarily become extinct. The high inbreeding coefficient at two microsatellite loci in *T. nylanderi* indicates local mating swarms and comparatively limited dispersal capabilities (Foitzik & Heinze, 2001), but the migration distance of queens has not yet been determined.

As in Northern Germany, both species co-occur in a rather narrow contact zone that does not extend over more than 25 km. A few specimens from two sites within this area were presumably hybrids, as demonstrated by an intermediate morphology and heterozygous GPI genotypes with the two 'species-specific' alleles. Occasional hybridization has previously been detected, both morphologically and by allozymes, in the contact zones in Northern Germany (Seifert, 1995). The narrowness of the contact zone probably suggests reduced hybrid fitness (Barton & Hewitt, 1985). Indeed, hybrid colonies produced virgin queens, which weighed significantly less than those of either species (K. Pusch, S. Foitzik, & J. Heinze, unpublished data).

The present parapatric distribution and genetic uniformity of both *T. nylanderi* and *T. crassispinus* indicate a rapid postglacial re-colonization of Central Europe from small, genetically homogeneous populations in southern refugia and repeated and prolonged bottlenecks. Such a scenario is clearly mirrored in the star-like haplotype networks (Avise, 2000; Mardulyn, 2001) and is supported by the mismatch pair histograms, which fit the sudden expansion model (Slatkin & Hudson, 1991; Rogers & Harpending, 1992). Because the

analysed populations south of the Alps, including Southern Italy, France, Spain and Croatia were not more diverse than populations in Central Europe, reduction of genetic variability by recolonization of the north can be excluded. The sequence divergences of 2.4% and 3.5 % in the CO I and Cyt b haplotypes between the species indicate that separation must have taken place much earlier. If ant mitochondrial DNA accumulated a 2 % divergence per million years, as in the honey bee (Arias & Sheppard, 1996), the two dominant species-specific haplotypes would have split already 1.5 - 2 Mya. However, haplotype divergence does not necessarily indicate such an early separation of the two taxa because the typical haplotypes of both species may represent ancestral polymorphisms (Nichols, 2001).

In comparison with related, ecologically similar species, *T. nylanderi* and *T. crassispinus* show unusually low intraspecific genetic variability. For example, CO I sequences of *Temnothorax unifasciatus* from a single location in Southern Germany differed on average by 1.1% at the same CO I gene fragment (H. Sturm, unpublished data). More data from nuclear markers, such as microsatellites, are therefore needed to investigate the causes of genetic homogeneity in the two study species. An alternative explanation for the extremely reduced mitochondrial DNA variability might be selective sweeps due to incompatibility of different strains of the endoparasitic bacterium *Wolbachia*, which has been suggested to cause incompatible matings in *T. nylanderi* (Wenseleers, 1998). The loss of DNA variation due to maternally transmitted parasites (Johnstone & Hurst, 1996) has indeed been repeatedly reported from insects (Jiggins et al., 2003).

Whatever the cause, the uniformity at mitochondrial genes and the nuclear enzyme locus might be indicative of a similarly limited variation at other loci, including those coding for genetic recognition cues. Low genetic variability might therefore explain the frequent occurrence of colony fusion and usurpation in *T. nylanderi* (Foitzik & Heinze, 1998, 2000, 2001) and the ease with which colonies of both species merge in the laboratory when reared in similar nest sites (Heinze et al., 1996; Tichà, 2002). Whereas the homogeny of genetic odour cues in invasive ants was suggested to result from bottlenecks during the man-made introduction into new habitats (Tsutsui et al., 2000), in our study species, natural bottlenecks may have led to an analogous phenomenon.

Acknowledgements

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Appendix

Germany	**other European countries**
Aalen	Bosco del Consiglio BdC (I)
Abensberg	Caldes (Cal), Catalonia (E)
Abensberg	Cambridge (Ca) (GB)
Allershausen	Carnac (Car), Bretagne (F)
Altdorf	Colle di Croce (CdC) (I)
Altötting	Creixelles (Crei), Catalonia (E)
Amberg	Gleno, Bolzano (I)
Babenhausen	Gostinjac (Go), Krk (HR)
Bamberg	Lana, Merano (Mer) (I)
Beilngries (Bei)	Leuven (B)
Berching	Llinars (Lli), Catalonia (E)
Böhming	Locoal (Lo), Bretagne (F)
Breitenbrunn	Magel, Alsatia (F)
Buchberg	Monieux, Provence (F)
Buchenhüll	Mont Ventoux, Provence (F)
Burglengenfeld	Montana, Bolzano (Bo) (I)
Danlohe	Mte. Verità (Ti), Lago Maggiore (CH)
Dasing	Montseny (Mon), Catalonia (E)
Dietfurt	Nervesa (Ner) (I)
Ebermannsdorf	Salò (Sa), Lago di Garda (I)
Eichstätt (Ei)	Salorno, Bolzano
Endorfmühle	Sault (Sal), Provence (F)
Erlangen	Serniga, Lago di Garda (I)
Essing (Schulerloch)	Sila Mts. (Si) (I)
Feucht	Škocjan (Sk) (SL)
Forchheim	Sperrone (Spe) (I)
Fürnried	Subit (I)
Greding (Gre)	Tours (Tou) (F)
Greifenberg	Trebesing (Tre), Carinthia (A)
Greißelbach	Vallo di Lucania (VdL) (I)
Hagenacker	
Haidenbach	
Hartmannshof	
Heldmannsberg	

Germany
Hexenagger
Höfling (Hfl)
Hohenkammer
Hohensolln
Illschwang
Kauerlach
Kinding
Kipfenberg
Lillachtal
Lohhof
Ludwigskanal, Abzw. Deining - Velburg
Mainburg
Manching (Ma)
Marburg (Mar)
Moritzberg
Muggendorf
Mühlberg
Mühlhausen
Nabburg
Neuburg (Neu) (A.d. Donau)
Neunkirchen am Sand
Niederhofen
Oberleinach
Pavelsbach
Pentling
Pfaffenhofen
Pilsach
Ponholz
Prunn
Puch
Pürschläg
Rückersdorf
Schlierferheide
Schmidmühle
Schmidmühlen
Schnufenhofen
Schönhofen
Schrammlhof
Schrobenhausen
Seelach
Sengenthal (Seng)
Siegenhofen

Germany
Solnhofen
Sommerhausen (So)
Stadlhof
Thurnau
Tittmoning
Treidlheim
Tyrolsberg (Tyr)
Uni Regensburg
Unterisling
Utzenhofen
Velburg (Vel)
Vilsbiburg
Wäschenbühl
Zeubelrieder Moor (WÜ)
Zusmarshausen

The influence of hybridization on colony structure in the ant species *Temnothorax nylanderi* and *T. crassispinus**

Katja Pusch, Jürgen Heinze and Susanne Foitzik

Abstract

The parapatric sibling ant species *Temnothorax nylanderi* and *T. crassispinus* hybridize in the contact zone in the Franconian Jura, Southern Germany. Aim of our study was to investigate the impact of hybridization on colony composition and fitness. We classified colonies as either 'pure' or containing hybrids by determining their allozyme pattern at GPI, an enzyme that is fixed for different alleles in the two parental species, and quantified their reproductive output. Most colonies with hybrid workers had a *T. crassispinus* queen. Colonies with heterozygous, hybrid workers produced more young workers than colonies of the parental species but similar numbers of male and female sexuals. Female sexuals from colonies with heterozygous workers had a significantly lower weight than female sexuals from pure colonies. Only a single reproductive queen was found to be heterozygous, suggesting reduced fitness of hybrid queens. As in the parental species, hybrid colonies appear to be frequently taken over by alien queens, which obscures the genetic colony structure.

Key words: contact zone, sibling species, hybridization, reproductive investment

* Insectes Sociaux, in press

Introduction

Hybridization has long been regarded as a phenomenon of minor importance in evolution, which mostly leads to sterility and thus presents an evolutionary dead-end (Mayr, 1963). Hybrids frequently have lower fitness than the parental species, and hybrid zones, where two closely related, parapatric species meet, are stabilized by hybrid inferiority or breakdown (e.g., Barbash et al., 2000; Cianchi et al., 2003). However, research during the last decade has shown that gene introgression may increase the genetic diversity of the hybrid relative to its parental species and can lead to hybrid vigor (Grant & Grant, 1994; Wang et al., 1997). In such cases, new evolutionary lineages may arise, and hybridization is therefore now seen as a process of evolutionary significance (Burke & Arnold, 2001).

In social animals, hybridization does not only affect the phenotype of individuals but can also be expressed on the level of the whole society. Eusocial insects provide a number of interesting examples for such higher-level consequences (Hölldobler & Wilson, 1990). For example, in a hybrid zone of two North American species of harvester ants, *Pogonomyrmex*, female caste is no longer determined by environmental factors, as is normally the case in social insects (Hölldobler & Wilson, 1990), but instead genetically: eggs inseminated with heterospecific sperm develop into sterile workers, whereas eggs fertilized by sperm from the same species develop into female sexuals (Julian et al., 2002; Volny & Gordon, 2002; Helms Cahan & Keller, 2003; Helms Cahan et al., 2004). Similarly, in some fire ant colonies in the hybrid zone of *Solenopsis xyloni* and *S. geminata*, workers are hybrids and female sexuals are of pure *S. xyloni* ancestry (Hung & Vinson, 1977; Helms Cahan & Vinson, 2003).

Morphological and genetic analyses suggest that hybridization is particularly common in *Temnothorax* ants, where up to 44% of all colony founding queens were reported to mate with heterospecific males (Douwes & Stille, 1991; Seifert, 1999). According to Plateaux (1979), the ovaries of *T. parvulus* x *T. lichtensteini* hybrid queens consist of fewer ovarioles and their eggs do not develop, but beyond that, little is known about the consequences of hybridization and how species boundaries are maintained in this genus.

Temnothorax nylanderi (Förster, 1850) and *T. crassispinus* (Karavajev, 1926) (originally referred to as *Leptothorax slavonicus,* Seifert, 1995) are among the most common ant species in deciduous forests throughout Central Europe. *T. nylanderi* occurs in Western Europe; its sibling, which is morphologically very similar, in Eastern Europe. Both species meet along a narrow contact zone close to the river Elbe in North-Eastern Germany (Seifert, 1995) and in the Franconian Jura in Southern Germany (Pusch et al., 2006). Previous morphological and genetic studies suggested occasional hybridization (Seifert, 1995; Pusch et al., 2006). In this study, we

investigated in more detail the frequency of hybridization in the contact zone in the Franconian Jura. Because of the narrowness of the contact zone we aimed at clarifying whether hybrids are selected against and whether interspecific matings are asymmetrical. We therefore genotyped virgin and resident queens to analyse the frequencies of hybrid queens among the female sexuals. Sequencing of a mt DNA fragment allowed us to determine the direction of hybridization. To examine the impact of hybridization on colony productivity we compared the overall productivity of hybrid colonies to that of pure species colonies of *T. nylanderi* and *T. crassispinus.*

Material and methods

Species

Colonies of *T. nylanderi* consist only of a few dozen workers and a single queen (monogyny) (Buschinger, 1968; Plateaux, 1970, 1972; Foitzik et al., 1997; Foitzik & Heinze, 1998, 2000, 2001; Foitzik et al., 2003) and nest in rotting branches and hollow acorns in deciduous forests throughout Central Europe. Populations may reach extremely high nest densities of up to ten nests per square meter. Virgin queens usually mate with a single male (monandry; Foitzik et al., 1997) and thereafter found a new colony solitarily, in a pleometrotic association with other founding queens, or by usurping an already established colony (intraspecific parasitism, Foitzik & Heinze, 1998, 2001). Furthermore, mature colonies may occasionally fuse when their nests decay and empty nest sites are not available (Foitzik & Heinze, 1998, 2000, 2001). Despite of regular monogyny and monandry, colonies may therefore contain workers from several genetic lineages. The biology of *T. crassispinus*, although not as intensively investigated, appears to be very similar, with monogyny, monandry, and occasional colony fusion (Seifert, 1995; Tichá, 2002; Tichá & Štys, 2002; Strätz & Heinze, 2004; Pusch, unpublished data).

T. nylanderi and *T. crassispinus* can only be distinguished by detailed morphometry (Seifert, 1995), sequencing of the mitochondrial genes cyt b (different in 14 of 430bp) and CO I (different in 12 of 500bp), and electrophoresis of the enzyme glucose-6-phosphate isomerase (GPI; P. Douwes, cited in Seifert, 1995, 1999). Whereas almost all investigated *T. crassispinus* workers were homozygous for an electromorph with medium electrophoretic migration velocity (*m*), most *T. nylanderi* were homozygous for a fast electromorph (*f*) (Pusch et al., 2006). Heterozygote *mf* individuals have been found near the contact zone, but are extremely rare in more distant populations (Seifert, 1995; Pusch et al., 2006). Heterozygotes show an intermediate morphology and can therefore be considered as hybrids (Seifert, 1995). Because of their extremely low genetic diversity, the two species do not differ reliably in other enzymes.

Collecting sites

In May 2003 and 2004, we collected 66 and 89 colonies, respectively, in three beech forests with scattered oaks and pines near Schwaighof, 2km west of Velburg, district Neumarkt, in the Franconian Jura (Figure 2.1). Previous morphological, allozyme, and mt DNA data had suggested that both species co-occur and hybridize in this area (Pusch et al., 2006). Site 1 (Schlossberg, N 49°14'14", E 11°38'33") is occupied almost exclusively by *T. nylanderi*, with only two colonies collected in 2003 containing GPI heterozygotes. Site 2 (Eichelberg, N 49°14'09", E 11°39'12") is separated from site 1 by an approximately 300m wide field and inhabited by *T. crassispinus,* colonies containing both *T. crassispinus* and heterozygous individuals, and colonies with only heterozygotes. Site 3 (N 49°14'31", E 11°38"49") is a small forest patch situated in the field between sites 1 and 2 and is solely occupied by *T. crassispinus*.

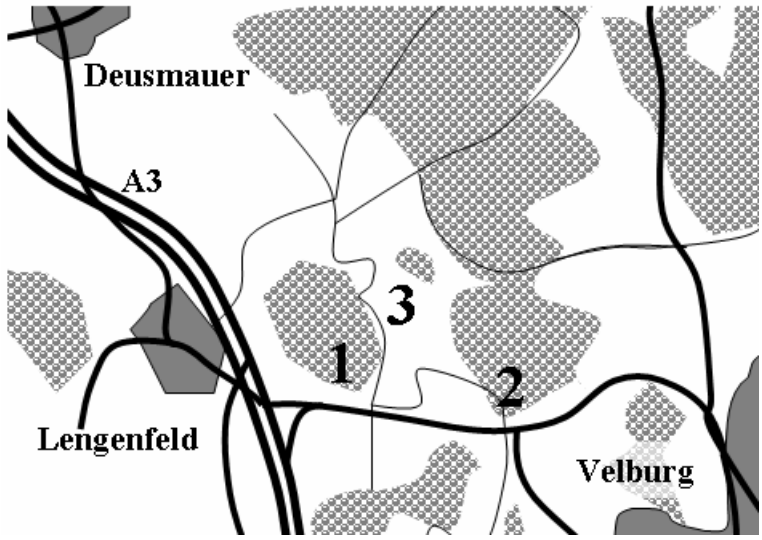

Figure 2.1: Map showing the location of sample sites near Velburg. "A3" stands for highway A3; sample sites are indicated by 1, 2, and 3. Forests are indicated by shaded areas.

Following collection, all colonies were censused and set up in plastic nest boxes with a plaster floor under standard laboratory conditions (Buschinger, 1966; Heinze & Ortius, 1991) for five months. Ants were fed twice a week with diluted honey and pieces of cockroaches. Until the end of May, all colonies were kept in incubators at artificial spring conditions (12h: 20°C light; 12h: 12°C dark) and later at artificial summer conditions (14h: 25°C light; 10h: 20°C dark). Workers, female sexuals and males emerged from late June on.

Genetic analyses

In 2003 and 2004, we genotyped a total of 215 and 732 *T. crassispinus* workers and 104 and 384 *T. nylanderi* workers, respectively (Table 2.1). In addition, we examined the genotypes of colonies with apparent hybrid workers. In 2003, we determined the GPI genotypes of at least eight individuals per colony, except for three colonies, of which only six or seven individuals were available (Table 2.2a). In 2004, we tested at least eight adult workers and, whenever available, freshly eclosed workers and female sexuals from newly collected colonies, except for two colonies, of which only four or five workers were available (Table 2.2b). Electrophoresis conditions are described in Pusch et al. (2006). Electromorphs were named according to their electrophoretic migration velocities (fast *f*, medium *m*, slow *s*, Pusch et al., 2006). Colonies were categorized as *T. crassispinus* or *T. nylanderi*, when all genotyped individuals were homozygous for *m* or *f*, respectively. The electromorph *s* occurs occasionally in both species (Pusch et al., 2006). Colonies with at least one heterozygote *mf* worker were classified as "heterozygous / mixed colonies" (H-colonies). Those colonies consisted exclusively of heterozygous *mf* hybrid workers or of a mixture of heterozygous and homozygous workers. By comparing the genotype frequencies of old and freshly eclosed workers in colonies collected in 2004 we attempted to determine whether a colony had recently been usurped by an alien queen or fused with another colony. In the case of colony usurpation, freshly eclosed workers would occasionally have other genotypes than old workers.

At least one worker from each colony was frozen and bisected. Heads and alitrunks were used for allozyme screening and the gasters for mt DNA analysis. Each worker from colonies from site 2 with the highest frequency of hybrid colonies was treated in this way, and DNA was isolated from GPI heterozygotes. The queens of queenright colonies collected in 2004 were analyzed in the same way after completion of the annual brood production and hatching of all new individuals. DNA was extracted using the Puregene DNA purification kit (Gentra Systems) as described in Foitzik and Herbers (2001), and a 430bp fragment of the cyt b gene was amplified (Simon et al., 1994). For details see Pusch et al. (2006).

Colony productivity

Colonies were frozen in September and all larvae (investment for the following year) were counted. In 2003, five female sexuals and five males per colony were dried for at least 24 hours in an open glass vial at 65°C and their dry weights were determined to the nearest 0.1µg using a Sartorius Microscale. In 2004, we only determined the wet weight of sexuals, which allowed subsequent enzyme staining. To estimate the dry weights of these individuals from their wet

weight, we determined both wet and dry weights of five additional male and five additional female sexuals from three colonies each of *T. crassispinus*, *T. nylanderi*, and H-colonies. As in previous studies on these ants (Foitzik & Heinze, 2000; Strätz & Heinze, 2004), the cost ratio was calculated as (mean dry weight of female sexual / mean dry weight of male sexual)$^{0.7}$ (Boomsma, 1989). For colonies that produced both sexes, we calculated the cost ratio for each colony individually. In the absence of one sex, we used the cost ratio of the respective group, *T. crassispinus*, *T. nylanderi*, and H-colonies. To test whether heterozygosity itself or heterogeneity and / or worker heterozygosity has an impact on brood production, we analysed female sexuals with the genotypes *mm* and *mf* from H-colonies separately. In an additional analysis, we compared the weight of *mm* female sexuals from pure *T. crassispinus* colonies with those of *mm* and those of *mf* female sexuals from H-colonies. All statistical analyses were conducted with Statistica 6.0 (Statsoft, Tulsa, OK, USA).

Results

In 2003, we collected 12 *T. nylanderi* colonies (*Tn)* at site 1 and 12 and 19 *T. crassispinus* colonies (*Tc*) at sites 2 and 3, respectively. Individuals from *T. nylanderi* colonies had mostly the genotype *ff*, individuals from *T. crassispinus* colonies were *mm* (Table 2.1). A total of 23 additional colonies – 21 from site 2 and 2 from site 1 – contained one or several heterozygous *mf* individuals (H-colonies, Table 2.1). Most homozygous workers from H-colonies had the *T. crassispinus* genotype *mm* and the *T. nylanderi* genotype *ff*' was found only in two H-colonies from site 1 (H6 and H14) and one colony (H28) from site 2 (Table 2.2a).

Table 2.1: Glucose-6-phosphate-isomerase (GPI) genotypes of workers and colonies of *Temnothorax crassispinus* (31 and 48 colonies collected in 2003 and 2004, respectively), *T. nylanderi* (12 and 24 colonies collected in 2003 and 2004 respectively) and hybrid colonies (23 collected in 2003 and 17 collected in 2004) from the contact zone in Velburg, Southern Germany. Bold figures indicate the predominant genotypes of the two species.

		T. crassispinus	**hybrids**	***T. nylanderi***
Genotype	Year	Colonies / individuals	Colonies / individuals	Colonies /individuals
ss	2003	1 / 2 site 2	-	1 / 2 site 1
	2004	1 / 1 site 2	-	-
ms	2003	-	-	-
	2004	5 / 58 site 2	1 / 2 site 2	-
mm	2003	**31 / 213 sites 2,3**	17 / 87 site 2	1 / 2 site 1
	2004	**47 / 668 sites 2,3**	14 / 108 site 2	2 / 10 site 1
mf	2003	-	**23 /145 sites 1,2**	-
	2004	-	**17 /224 site 2**	-
ff	2003	-	3 / 18 sites 1,2	**12 / 100 site 1**
	2004	1 / 4 site 2	2 / 4 site 2	**23 / 333 site 1**
fs	2003	-	-	-
	2004	1 / 1 site 2	1 / 1 site 2	5 / 41 site 1

In 2004, we collected 24 *Tn* colonies at site 1 and 27 and 21 *Tc* colonies at sites 2 and 3, respectively. Heterozygous *mf* workers were found in 17 additional colonies from site 2 (Table 2.1). In three H-colonies (H13, H44, H45), all workers were heterozygous, while in the other colonies *mf*-workers co-occurred with homozygous *mm*-workers, *ms*-workers (H40), *fs*-workers (Hx), and *ff* –workers (H11, Hx). By comparison of the genotypes of old and freshly eclosed workers we attempted to determine whether the genotype mixture could be explained by queen replacement through usurpation or colony fusion. Despite the limited sample size, genotype frequencies differed significantly between old and young workers in three colonies. All old workers of colonies H34 and H23 were *mf*- heterozygotes, while young workers had the genotypes *mm* and *mf*, suggesting a recent colony takeover by a *T. crassispinus* queen. In colony H43, heterozygous and homozygous individuals were found among old and young workers in significantly different ratios, also suggesting colony takeover. Two completely different lineages were found in colony Hx (Table 2.2b).

Table 2.2: GPI genotypes and cyt b haplotypes of workers found in presumed hybrid colonies of *T. crassispinus* and *T. nylanderi* from the contact zone near Velburg in 2003 (a) and 2004 (b). In 2004, differences in the genotype distributions between old and young workers were tested by χ^2-tests to reveal possible queen replacement. In colonies H33 and H45, mt DNA sequences were intermediate between the two species.

a) 2003	**GPI genotype**			**Cyt b haplotype**
Colony	***mm***	***mf***	***ff***	
H 1		11		*T. c.*
H 3	4	6		*T. c.*
H 6		1	9	*T. n.*
H 7	4	6		*T. c.*
H 9	1	9		*T. n.*
H 10	1	6		*T. c.*
H 12	9	7		*T. n.*
H 13	1	10		*T. c.*
H 14		2	7	*T. n.*
H 18	8	2		*T. c.*
H 19	10	1		*T. c.*
H 20	8	4		*T. c.*
H 21	2	8		*T. c.*
H 22	6	6		*T. c.*
H 24	8	7		*T. c.*
H 27	9	1		*T. c.*
H 28	1	8	2	*T. c.*
H 31	1	12		*T. c.*
H 35	6	6		*T. n.*
H 51		6		*T. n.*
H 52	8	4		*T. c.*
H 291		14		*T. n.*
H 292		6		*T. c.*

b) 2004	Young workers GPI					Old workers GPI			Old workers haplotype	Queens GPI				Queens haplotype	Female sexuals GPI			Fisher's exact (p)
colony	*mm*	*mf*	*ms*	*ff*	*sf*	*mm*	*mf*	*ms*	cyt b	*mm*	*mf*	*ms*	*sf*	cyt b	*mm*	*mf*	*sf*	
H 34	5	6					8		*T. c.*	1				*T. c.*	1	3		0.04
H 13		10					9		*T. c.*	1				*T. c.*		3		
H 18							5		*T. c.*	1				*T. c.*				
H 11		7		2			10		*T. c.*							2		0.21
H 23	3	7					28		*T. c.*	1				*T. c.*				0.01
H 42						1	3		*T. c.*							3		
H x				2	1	2	6		*T. c.*		1			*T. c.*			5	
H 41	2	9					10		*T. c.*							5		0.48
H 44		10					19		*T. c.*	1				*T. c.*				
H 24		10				1	8		*T. c.*							4		0.47
H 33	6	4				4	3		*T. n./T. c.*	1				*T. c.*				1.00
H 43	6	2				3	24		*T. n.*				1	*T. n.*	3	2		0.001
H 45		10					10		*T. n./T. c.*	1				*T. c.*		5		
H 29	9	1				10			*T. c.*	1				*T. c.*	1			1.00
H 31	9	1				12			*T. c.*	1				*T. c.*	5			0.45
H 53	10					11	1		*T. c.*	1				*T. c.*	5			1.00
H 40	5	2	1			9		1	*T. c.*			1		*T. c.*				χ_2^2=1.03, p > 0.5

All individuals from *Tc* and *Tn* colonies showed their respective typical cyt b haplotypes different in 14 of 430bp. Heterozygous workers from seven H-colonies sampled in 2003, including two from site 1 (H6, H14), and one H-colony from 2004 (H43) had the *T. nylanderi* haplotype, while workers from all remaining H-colonies had the *T. crassispinus* haplotype (Table 2.2a,b). Haplotype distributions did not differ significantly between the years (χ^2-test, Yates correction: $\chi^2 = 1.82$, $p = 0.17$). In two H-colonies (H33, H45), haplotypes were intermediate between the pure species haplotypes (Figure 2.2). To exclude a sequencing artifact, we repeated the analysis with two additional heterozygous individuals, from each of these two colonies, with the same result.

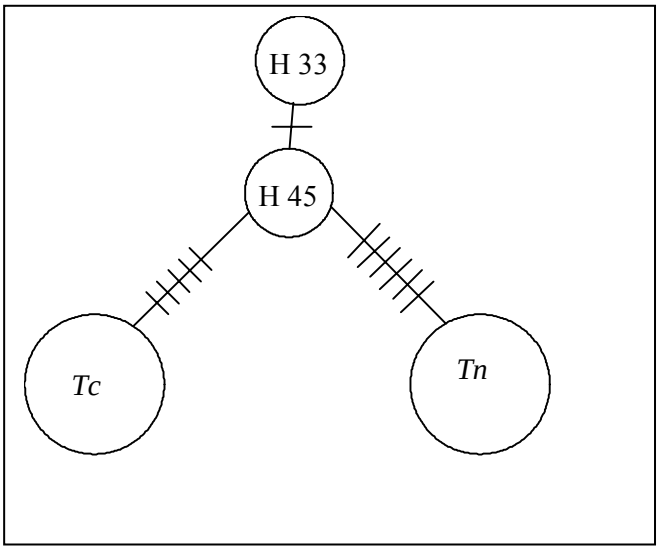

Figure 2.2: Minimum spanning network of the pure species *T. crassispinus* (*Tc*) and *T. nylanderi* (*Tn*) cyt b haplotypes and the two intermediate haplotypes found in colony H45 and H33.

For both years, the proportion of queenright and queenless colonies did not differ between the three groups (χ^2–test: 2003, $\chi_2^2 = 0.26$, $p > 0.5$; 2004, $\chi_2^2 = 1.27$, $p > 0.5$). Furthermore, for both years, no difference was found in the number of colonies producing reproductives of both sexes, only males, and only female sexuals (2003, $\chi_4^2 = 6.53$, $p > 0.1$; 2004, $\chi_4^2 = 2.22$; $p > 0.5$). A significantly larger percentage of *Tc* colonies produced sexuals in 2004 than in 2003 ($\chi_1^2 = 19.69$; $p < 0.001$), while the likelihood to produce sexuals did not vary between years in *Tn* and H colonies (Table 2.3).

Table 2.3: Overview of statistical results from ANCOVA on reproductive and sociometric traits of colonies of *T. crassispinus*, *T. nylanderi*, and colonies containing hybrids collected in the contact zone at Velburg in 2003 and 2004.

Analysis	**parameter over all**	**Effect**	**F**	**p-value**
ANCOVA	n of new workers	Year	3.4 1	0.07
controlled for worker		Group	2.45	0.09
number	n of v. queens	Year	15.91	0.0001
		Group	1.79	0.17
	n of males	year	0.0004	1.00
		group	1.57	0.85
	n of larvae	year	40.78	0.00
		group	1.93	0.15
ANCOVA	annual investment	year	5.93	0.02
controlled for worker		group	0.65	0.52
number	sexual allocation	year	4.95	0.03
		group	0.91	0.41
	male allocation ratio	year	14.66	0.0001
		group	3.33	0.04
	reproductive allocation ratio	year	0.53	0.47
		group	4.15	0.02
	annual investment & larvae	year	6.35	0.01
		group	1.67	0.19
	productivity	year	7.7	0.006
		group	0.93	0.40
ANCOVA	numerical ratio	year	19.87	0.0002
controlled for worker		group	1.92	0.15
number	female ratio	year	1.93	0.17
		group	4.63	0.02
main effect ANOVA	**dry weight v. queens**	year	0.03	0.86
		group	**3.32**	**0.04**
main effect ANOVA	dry weight males	year	8.99	0.006
		group	0.35	0.71

Heterozygous *mf*-female sexuals from H-colonies were significantly lighter than *mm*-and *ff*-female sexuals from pure species colonies (Table 2.3, Fisher's LSD post hoc test: *Tc* vs. *Tn*: p = 0.26; *Tc* vs. H: p = 0.04; *Tn* vs. H: p = 0.01, Figure 2.3). To analyse the cause for this weight difference in more detail, we compared the weights of *mm*-female sexuals from pure *Tc*

colonies, and *mm*- and *mf*-sexuals from H-colonies in a second ANOVA. We found no significant weight difference between *mm*-and *mf*-sexuals from H-colonies, but *mm*-sexuals from *Tc* colonies were heavier than *mm*- and *mf*-sexuals from H-colonies (one-way ANOVA: $F_2 = 4.38$, $p = 0.02$; Fisher's LSD post hoc test: *mf* from H vs. *mm* from H: $p = 0.25$; *mm* from H vs. *mm* from *Tc*: $p = 0.01$; *mf* from H vs. *mm* from *Tc*: $p = 0.09$). The three groups did not differ in the ratio of wet and dry weights (Kruskal-Wallis ANOVA: $H = 2.25$, $p = 0.32$). When controlled for the number of adult workers present in a colony, there was a trend that H-colonies raised more new workers. (GLM ANCOVA, $F_2 = 2.45$, $p = 0.09$, Table 2.3). New workers reared in hybrid nests were not lighter than those from the pure colonies (one-way ANOVA: $F = 1.55$, $p = 0.22$). Based on the dry weights of male and female sexuals we calculated cost ratios of 2.33 for *Tn* colonies, 2.64 for *Tc* colonies, and 2.32 for H-colonies. Differences in brood production (= number of larvae) between the three groups were not significant (Table 2.3).

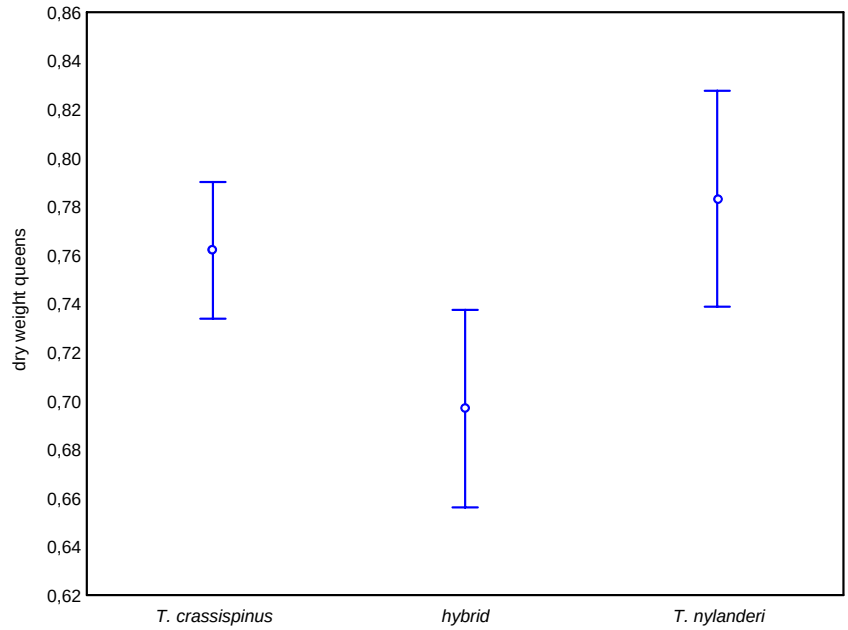

Figure 2.3: Dry weight (mg, mean ± 95% confidence interval) of female sexuals produced in colonies of the ants *Temnothorax crassispinus* and *T. nylanderi* and colonies containing hybrids collected in the contact zone near Velburg. Data from the collecting years 2003 and 2004 were combined.

Male allocation ratio (the investment allocated to male sexuals relative to the investment in both sexes) differed significantly between years and among species which was mainly caused by a difference between *Tn* and the other types of colonies in 2003 (Fisher's LSD post-hoc test: *Tn* vs. *Tc*, $p = 0.01$; *Tn* vs. H, $p = 0.003$, Table 2.3). Moreover, we obtained significant differences in reproductive allocation ratio (the investment allocated to sexuals relative to the

total investment) among species predominantly because *Tc* and H colonies differed in 2003 (post-hoc test, p = 0.009, Table 2.3). Caste bias (the ratio of female sexuals and new workers in the female brood) differed significantly between *Tn* and *Tc* colonies, but not between H-colonies and pure colonies (post hoc test: *Tn* vs. *Tc*: p = 0.03; *Tc* vs. H: p = 0.13; *Tn* vs. H: p = 0.10). Differences in any other parameter were not significant, although we frequently found significant differences between the two years (Table 2.3).

To determine whether the lower weights of female *mf* sexuals from H-colonies affects their recruitment into the breeding population, we compared the number of female sexuals produced in 2004 with the number of inseminated queens found in mature colonies. A significantly higher proportion of young *Tc* and *Tn* queens compared to heterozygote *mf*-queens from H-colonies succeeded in starting new colonies (χ^2–test: $\chi_2^2 = 8.67$, $p < 0.02$).

Discussion

Our investigation of the hybrid zone between *T. nylanderi* and *T. crassispinus* revealed a distinct distribution pattern of colonies of the two species and their hybrids. Workers with heterozygous GPI genotypes were found in 66% (2003) and 39% (2004) of the colonies collected at one of three neighboring study sites, while the other sites were almost exclusively inhabited by *T. nylanderi* or *T. crassispinus*. Hybrid colonies co-occurred mostly with *T. crassispinus* at site 2, where also pure *T. crassispinus* colonies could be found and most heterozygous workers had the *T. crassispinus* mt DNA haplotype. This suggests that interspecific mating is asymmetrical and involves mostly female sexuals of *T. crassispinus* and males of *T. nylanderi*. Asymmetrical or unidirectional mating events are common in hybridizing species (Szymura et al., 2000; Redenbach & Taylor, 2003; Salazar et al., 2005). In our study system, it might result from morphological or behavioral differences between sexuals of the two species or from the prevailing direction of the wind, promoting only a one-way drift of *T. nylanderi* males to the *T. crassispinus* site. The finding of intermediate mt DNA haplotypes in two hybrid colonies is difficult to explain, given that intraspecific variation in mt DNA sequences is extremely low in both parental species (Pusch et al., 2006). It might result from paternal leakage and mt DNA recombination, but both processes appear to be extremely rare in animals (Ladoukakis & Zouros, 2001; Rokas et al., 2003).

Only four of the collected colonies with hybrid workers consisted exclusively of heterozygous *mf*-individuals, while most colonies in addition contained *mm*-homozygotes. Such heterogeneity could be explained either by *mf* queens mated with *T. crassispinus* males, multiple mating (e.g., by an *mm*-queen with both *m*- and *f*-males), or the co-existence of several

matrilines. The first two explanations are rather unlikely. Fertile hybrid queens appear to be uncommon (see below), and though multiple mating has been observed in *T. nylanderi* in behavioral studies in the laboratory (Plateaux, 1970, 1978), all population genetic analyses suggest regular monandry (Foitzik et al., 1997; Foitzik & Heinze, 1998, 2000; Foitzik et al., 2003; Strätz & Heinze, 2004). Nevertheless, occasional multiple mating cannot be excluded. The common co-occurrence of two or more matrilines in the parental species is typically due to colony usurpation by founding queens or colony fusion (Foitzik & Heinze, 1998, 2001; Strätz et al., 2002; Tichá, 2002; Tichá & Štys, 2002; Strätz & Heinze, 2004). Therefore, the differences in genotype frequencies between old and new workers are best explained by the hypotheses that genetic heterogeneity results from colony fusion or take-over by an alien queen. Unpublished microsatellite data on both pure species and hybrid colonies from Velburg (S. Träger, personal communication) indicate that the frequency of colonies with several matrilines is equally high in colonies of the parental species and H-colonies.

Nearly all fertile queens from H-colonies were homozygous with the *T. crassispinus* genotype. However, equally high numbers of virgin queens were produced in the colonies of the three groups. Though we found only a single *mf* queen with both *mm* and *mf* workers and five *mf* queens were collected in 2005 (S. Träger, personal communication). Hence hybrid queens of *T. crassispinus* and *T. nylanderi* appear to be rarely recruited into the breeding population. This clearly suggests reduced hybrid fitness, which is common and supposed to stabilize species boundaries (Arnold, 1997). From the narrowness of the hybrid zone in North-Eastern Germany, Seifert (1995, 1999) concluded that hybrids of *T. nylanderi* and *T. crassispinus* are selected against. The fitness of hybrids appears to be similarly reduced relative to their parental species also in other ants (Plateaux, 1979; Shoemaker et al., 1996). Their reduced success in the field might be due to the lower weight, which, however, is probably not a direct effect of hybridization, as homozygous and heterozygous female sexuals from H-colonies did not differ significantly in their dry weights. At present it is unknown whether homozygous virgin queens reared in an H-colony also have a lower founding success than female sexuals from pure *T. crassispinus* colonies. In any case, heterozygosity of female sexuals alone is apparently not completely responsible for the observed reduction of body weight. Instead, it might be caused by reduced work efficiency of hybrid workers, reduced cooperation between homozygote and heterozygote workers, or a mismatch between larval needs and H-mixed colonies.

That genetic heterogeneity may negatively affect work efficiency in *Temnothorax* ants has previously been demonstrated in *T. longispinosus* where the co-occurrence of different matrilines within a colony results in lower productivity (Trampus, 2001; Foitzik et al., 2003).

The small differences between colonies of the parental species and H-colonies in brood production, sexual allocation, male allocation ratios, and the weight of female sexuals might be similarly explained by colony heterogeneity. Various productivity traits differed strongly between years, again documenting the strong sensitivity of *Temnothorax* ants to environmental changes (Foitzik et al., 2003).

Although our results show reduced hybrid fitness and asymmetric mating, additional long-term investigations in the Velburg population will be necessary to determine the impact of hybridization in more detail.

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Heterospecific colony fusion in two *Temnothorax* (Hymenoptera: Formicidae) sibling ants *

Katja Pusch, Christina Meindl and Jürgen Heinze

Abstract

The two monogynous (single-queened), parapatric ant species *Temnothorax nylanderi* (Förster, 1850) and *T. crassispinus* (Karavajev, 1926) are both characterized by a rather inefficient, environment-based nestmate recognition system, which does not prevent alien colonies from moving in with an unrelated colony when their own nest has decayed. Colony fusion results in a genetically heterogeneous colony, in which later one of the two queens is eliminated. The sporadic occurrence of mixed colonies with workers from both species or from a parental species and a presumed hybrid colony suggested that interspecific fusion may occasionally occur in the narrow contact zone in the Franconian Jura. Colony fusions could be observed after initial aggressions in one third of our laboratory experiments. One of the two queens was usually killed within a few days. Queens of the two species did not differ in their survival rate. Apparently, the close relatedness between the two species and the environment-based recognition system facilitate such heterospecific fusion.

Key words: *Temnothorax nylanderi*, *T. crassispinus*, nestmate recognition, colony odour, queen survival rate, aggressive interactions

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Introduction

The colonies of social insects usually consist of more or less extended family groups that are closed to unrelated freeloaders and parasites through an efficient nestmate recognition system. All nestmates share a common colony odour consisting of chemical cues that are produced by and exchanged among colony members, often with considerable contributions from nest material, food or other environmental sources (Hölldobler & Wilson, 1990; Vander Meer & Morel, 1998; Lenoir et al., 1999). Despite its evolutionary robustness, nestmate recognition may fail under special circumstances, e.g., when social parasites manage to acquire or mimic the colony odour of their hosts (Lenoir et al., 2001) or when unrelated colonies share genetic odour cues due to a loss of genetic diversity and / or selection against heterogeneity (Tsutsui et al., 1999; Giraud et al., 2002). In the latter situation, individual nests are no longer distinct entities but form huge "supercolonies" amongst which individuals are freely exchanged, as in the Argentine ant, *Linepithema humile* (Mayr, 1868).

Previous research has also suggested a lack of variability in genetic odour determinants in *Temnothorax nylanderi* (Förster, 1850), one of the most common ant species of deciduous woodlands throughout Central and Western Europe. *T. nylanderi* nests in cavities in plant material, such as hollow acorns, hazelnuts, and rotting branches. Aggression assays in the laboratory showed that nestmate recognition relies predominantly on transient, environment-derived odour cues, and that unrelated colonies living in the same nesting material easily merge in a single nest site (Heinze et al., 1996).

Sexuals of both sibling species, *T. nylanderi* and *T. crassispinus* (Karavajev, 1926) mate during nuptial flights and colonies are founded by single-mated queens (monandry) either solitarily, by pleometrosis, or by usurpation of alien nests. Though both species are monogynous (single queen per colony; Buschinger, 1968; Plateaux, 1970), their colonies may occasionally contain workers from several matrilines. This heterogeneity results from natural colony fusion and the usurpation of mature colonies by founding queens when populations are dense and suitable nest sites are rare (Seifert, 1995; Foitzik et al., 1997; Foitzik & Heinze, 1998, 2000, 2001; Foitzik et al., 2003; Strätz & Heinze, 2004). Fighting among queens quickly leads to the restoration of monogyny (Strätz et al., 2002).

While *T. nylanderi* and *T. crassispinus* are parapatrically distributed throughout most of their range, they meet and hybridize in a narrow contact zone close to the Elbe River in Northern Germany and near the continental divide in the Franconian Jura in Southern Germany (Seifert, 1995; Pusch et al., 2006). The two species are morphologically very similar and, though they can be discriminated by detailed morphological analysis (Seifert, 1995), are more easily

determined by their different electromorphs of the enzyme glucose-6-phosphate isomerase (GPI; Seifert, 1995; Pusch et al., 2006). In one particularly well-studied population, a few colonies simultaneously contained workers with the GPI genotypes of *T. crassispinus*, *T. nylanderi*, and heterozygous workers, presumably hybrids (Pusch et al., in press).

Since in both species, nestmate discrimination seems to rely mainly on environmental cues and genetic data indicated occasional heterospecific fusion (Pusch et al., in press), we conducted laboratory experiments in arenas with only one nest available for two heterospecific colonies (see Foitzik & Heinze, 1998) to determine in more detail how readily such fusion occurs.

Material and Methods

Complete ant colonies were collected in September 2004. *Temnothorax crassispinus* colonies came from beech sticks and acorns in a beech-oak-pine forest in Pentling near Regensburg (Germany). *Temnothorax nylanderi* colonies were sampled from beech sticks in three beech-oak forests close to Altdorf, east of Nuremberg (Germany). Five additional *T. nylanderi* colonies were collected from beech twigs in a beech – pine forest near Velburg (Germany) and exclusively used in series 1 (see below). All colonies were left in their natural nests until the experiments to avoid a change in their natural colony odour profile. Ants were fed twice a week with undiluted honey and pieces of cockroach. We counted all ants per colony and individually marked their queens with Edding paint marker.

The fusion experiments were conducted in 19x19 cm^2 arenas. In each we provided one standard laboratory nest (Heinze & Ortius, 1991), into which one colony was allowed to move in (series 1: N = 21, resident species = *T. nylanderi*; series 2: N = 20, resident species = *T. crassispinus*). One day after the first colony had successfully moved into the provided nest site, we released the second colony directly in front of the nest entrance. Colonies varied considerably in size and consisted of about 40 to almost 300 workers. We therefore chose pairs of queenright colonies of similar size (= number of workers; Wilcoxon matched pair test; series 1: $Z = 1.25$; $p = 0.21$; series 2: $Z = 0.02$; $p = 0.99$) to avoid any effect from differing colony size. Behaviour was observed for 30 minutes after the second colony had been added. Every ten minutes, all aggressive interactions (= mandible opening, biting, and fighting) among workers, between queens and workers, and between queens were scanned for two minutes. For each experiment, the number of aggressive interactions recorded during the 30 minutes in three two-minute observations was summed and divided through the number of scans. On the following days, the colonies were scanned once per day for two minutes to check for aggression or queens' death. All statistical analyses were conducted with Statistica 6.0

(Statsoft, Tulsa, Ok, USA). Data that were not normally distributed were analysed with non-parametric statistics.

Results

In all experiments, workers of the newly added colony discovered the entrance of the nest site within the first 30 min and entered the nest without being attacked, as the nest entrance was usually left unguarded (see also Foitzik & Heinze, 1998). Six of 21 *T. crassispinus* colonies moved into a nest site occupied by *T. nylanderi* and eight of 20 *T. nylanderi* colonies moved into a nest site inhabited by *T. crassispinus* always resulting in colony fusion (Fisher's exact test: $p = 0.51$). In the other experiments, the introduced colonies did not manage to successfully usurp the inhabited nest within 20 days and in most of the experiments instead settled in one corner of the arena. Which species initially inhabited the nest site did not influence the time until fusion (Gehan's Wilcoxon test, test statistic = 0.826, $p = 0.409$).

Workers of the confronted colonies behaved aggressively towards each other only after direct contact. Aggressions were frequent on the day when the second colony was released into the arena, became much less intense the following two days and in almost all experiments ceased completely after five days. The frequency of worker-worker aggression on the first day of the experiment did not differ between the two series (two-tailed t-test: $t = 1.1$, $p = 0.28$, Figure 3.1).

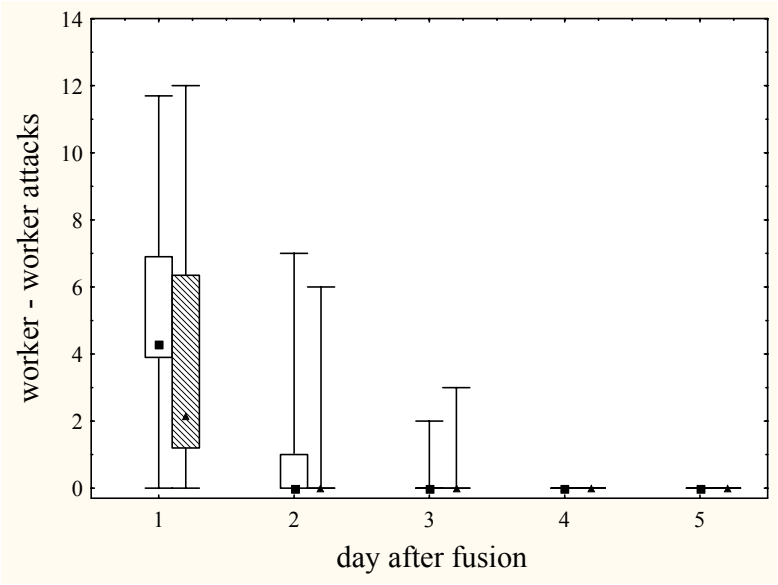

Figure 3.1: Aggressive interactions (median, 25%-75% quartiles, number of all aggressions per scan) among workers of the ant species *Temnothorax nylanderi* and *T. crassispinus* observed in all experimental setups during five days after one colony without nest was added into an arena with a nest containing a colony of the other species.

In the case of successful nest usurpation, both resident and usurping queens were attacked. As workers were not marked and the two species are difficult to distinguish by eye it remains unclear, whether queen-worker aggression always involved pairs of heterospecific individuals. Worker aggression towards queens became less intense after the first day of the encounter and almost completely ceased on day five. Interestingly, aggression among queens and workers was considerably more frequent on day 1 of the experiment when *T. nylanderi* occupied the nest (Mann –Whitney U-test: median, quartiles, series 1: 1, 0.3, 1.9; series 2: 0, 0, 0.3; $U = 92$, $p < 0.02$, Figure 3.2). However, this did not affect the outcome of the fusion experiment.

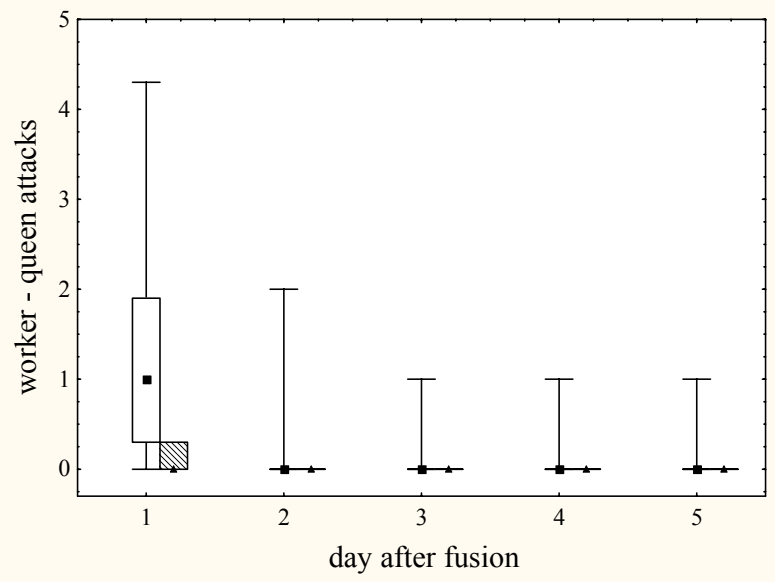

Figure 3.2: Aggressive interactions (median, 25%-75% quartiles, number of all aggressions per scan) between workers and queens observed in the fusion experiments. The left (open) bars indicate experiments with *T. nylanderi* as resident colony and *T. crassispinus* as intruders, the right (hatched) bars experiments with *T. crassispinus* as residents and *T. nylanderi* as intruders.

In nine of 14 colonies, only one queen survived colony fusion, whereas the other was finally killed by the workers. Queen-queen aggression was not observed. In series 1 with *T. nylanderi* as residents, the *T. nylanderi* queen was killed in one colony, the *T. crassispinus* queen was killed in three colonies, both queens died in one colony, and both queens stayed alive in one colony. In test series 2 with *T. crassispinus* as residents, two *T. nylanderi* queens and three *T. crassispinus* were killed, both queens died in one setup and both survived in two experiments. Resident queens did not survive better than intruder queens (Fisher's exact test, $n_1 = n_2 = 14$, p

= 0.500). As we stopped the experiment after 20 days, we could not observe if both queens permanently staid in the nest and laid eggs.

Discussion

Our experiments corroborate field data, which indicate that colonies of the two sibling species *T. crassispinus* and *T. nylanderi*, and presumably also colonies of their hybrids, may occasionally fuse when nest sites are scarce and that they may subsequently form stable, mixed colonies. Heterospecific colonies fused in only one third of the experiments. This contrasts with an earlier observation that colonies fused in 30 of 38 experiments with two *T. nylanderi* colonies (Foitzik & Heinze, 1998), in eight of eight (K. Pusch, unpublished data) and in 24 of 30 (Tichá, 2002; Tichá & Štys, 2002) experiments with paired *T. crassispinus* colonies. The difference may be explained by the higher similarity of conspecific colonies and our usage of colonies from different populations. Consistent with previous experiments with *T. nylanderi* (Foitzik & Heinze, 1998) and *T. crassispinus* (Pusch, unpublished data), in most cases (nine of 14 merged colonies), only one queen survived, independent of whether the queen was nest resident or not. In a study on colony usurpation by founding queens of *T. nylanderi*, Strätz et al. (2002) suggested that workers choose the younger and / or more fertile queen and eliminate the other. At present it is not known how workers in merged colonies chose the queen to be eliminated and whether only heterospecific workers attacked and killed the queen. *T. nylanderi* and *T. crassispinus* are very closely related and queen fertility signals might perhaps be sufficiently conserved to be understood across species borders and lead to maladaptive favouring the more fertile queen even when it belongs to the wrong species. Queen-queen interactions may have preceded worker aggression, as in *T. nylanderi* (Foitzik & Heinze, 1998; Strätz et al., 2002). The take-over of a colony with the subsequent elimination of one queen reminds of interspecific social parasitism, a life history that has convergently evolved in several clades of the ant tribe Formicoxenini, including the genus *Temnothorax* (Buschinger, 1986; Beibl et al., 2005).

Aggression both between workers and queens and among workers was frequent and intense only on the first day of the experiment and later decreased rapidly. This might suggest a change in colony odour. Environmental odour cues appear to play an important role in *T. nylanderi*, where individuals from different colonies reacted more aggressively towards each other when the colonies inhabited nests of different material (Heinze et al., 1996). Chemical analyses of cuticular hydrocarbons suggested that colony odours are not strongly differentiated and also do not reliably differ between the two sibling species: 3.3% of *T. nylanderi* individuals and 6.7%

of *T. crassispinus* individuals were incorrectly classified as belonging to the respective sibling species by discriminant analysis (Foitzik et al., in press). The predominance of environmental odour cues in nestmate recognition might be associated with the surprisingly low genetic variation of both species (Pusch et al., 2006).

To conclude, interspecific fusion of colonies of *T. nylanderi* and *T. crassispinus* appears to be possible where the two species co-occur. The ease of fusion might therefore explain previous data on the genetic composition of colonies in a population near Velburg, Bavaria (Pusch et al., in press), where hybrid colonies frequently also contained non-hybrid workers of one parental species.

Acknowledgements

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Zusammenfassung

Die beiden monogynen, parapatrisch vorkommenden Arten *Temnothorax nylanderi* (Förster, 1850) und *T. crassispinus* (Karavajev, 1926) zeichnen sich durch ein ineffizientes, durch Umweltfaktoren geprägtes Kolonieerkennungssystem aus. Dies ermöglicht Kolonien, in ein bereits von einer fremden Kolonie der gleichen Art bewohntes Nest einzuziehen, wenn ihr eigenes Nest unbewohnbar wird. Dadurch entstehen genetisch heterogene Kolonien, in denen jedoch eine der beiden Königinnen getötet wird. Das Auftreten gemischter Kolonien mit Arbeiterinnen beider Arten bzw. Arbeiterinnen einer Art und Hybriden innerhalb der schmalen Kontaktzone beider Arten im fränkischen Jura zeigt, dass gelegentlich interspezifische Fusionen vorkommen. In einem Drittel der Laborexperimente fusionierten die Kolonien nach anfänglicher Aggression und in den meisten Fällen wurde nach wenigen Tagen eine der beiden Königinnen getötet. Unterschiede in der Überlebensrate der Königinnen beider Arten konnten nicht gefunden werden. Offenbar ermöglicht die nahe Verwandtschaft beider Arten und ihr umweltbedingtes Kolonieerkennungssystem heterospezifische Fusionen.

Inbreeding and genetic structure in European populations of the monogynous ant *T. nylanderi* *

Katja Pusch and Jürgen Heinze

Abstract

The ant *Temnothorax nylanderi*, a very common European species exhibits low mt DNA variability across populations from North-and South-Western Europe suggesting repeated bottlenecks and rapid expansion after the last glaciation. Despite of being monogynous and monandrous, colonies of this species can differ in their genetic structure because of colony fusion due to nest scarcity. Moreover, an extensively studied German population was found to be significantly inbred. Here, we aimed at comparing the genetic structure of *T. nylanderi* populations from Central-and Southern Europe. In the five studied populations from Germany, France and Italy, the ratio of homogenous (one matriline) to heterogenous (two or more matrilines) colonies did not differ significantly. We found spatial structuring between all population pairs that was not correlated with distance. All populations exhibited positive inbreeding. This is remarkable because both male and female sexuals are winged and participate in nuptial flights. However, the fact that only part of the colonies produce sexuals and populations are locally dense but patchily distributed on a wider scale, apparently leads to non-random mating over time in all investigated populations.

Key words: *Temnothorax nylanderi*, *T. crassispinus*, inbreeding, heterogeneity non-random mating

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* Manuscript in preparation

Introduction

Highly variable genetic markers have become an extremely useful tool in detecting intraspecific variation. In numerous studies, species range contractions and bottlenecks due to climatic changes have been revealed by large population genetic studies (e.g. Schmitt et al., 2002; Stone et al., 2002; Brant & Ortí, 2003; Ayoub & Riechert, 2004). Apart from that, genetic markers came out to be an indispensable tool for relatedness studies in group-living animals. In eusocial insects such as ants, nestmate relatedness is considered to be one of the main responsible factors for the evolution of a sterile worker caste (Hamilton, 1964). Here, because of haplo-diploidy, intra-colony worker-worker relatedness is 0.75 under the assumption of monogyny and monandry. However, numerous studies revealed that intra-nest worker relatedness in ant species often is considerably lower than this despite of monogyny due to multiple mating e.g. observed in *Formica* (Keller et al., 1997).

The occasional co-existence of several matrilines in one colony is a typical trait of *Temnothorax nylanderi*, one of the most common ant species in deciduous West European forests. Colonies of this monogynous and single mated ant species are comparably small with a few dozend individuals and inhabit rotten beech, oak or pine twigs, hollow acorns or hazelnuts. Populations often reach very high densities with 10 or more colonies per square meter. Both sexuals are winged and go on nuptial flights in late summer. The inseminated queens either found solitarily, in pleometrosis or by intruding a foreign nest (Buschinger, 1968; Plateaux, 1970; Foitzik et al., 1997). The decay of ephemeral nests like acorns in late summer and autumn may force some colonies to move together. This occasionally leads to the fusion of unrelated colonies under the death of one queen, which happens relatively easy because of the strong environmental influence of nestmate recognition cues in this species. Hence, some colonies contain several, unrelated matrilines (Heinze et al., 1996; Foitzik & Heinze, 1998; Foitzik & Heinze, 2000; Foitzik & Heinze, 2001; Foitzik et al., 2003).

T. nylanderi probably retreated to refugia in South-Western Europe during glaciation (Seifert, 1995). Thus, the apparent weak genetic nestmate discrimination compound, an extremely low allozyme variability and significant inbreeding found for a German *T. nylanderi* population despite of matings outside the nest (Heinze et al., 1996; Foitzik & Heinze, 2001; Pusch et al., 2006), might result from a bottleneck due to re-colonization. However, populations of *T. nylanderi* and of its East European sibling species *T. crassispinus* lacked population–specific mitochondrial DNA haplotypes and higher diversity in southern populations (Pusch et al., 2006).

Therefore, we aimed at gaining further knowledge about the genetic structure in the ant *T. nylanderi* in examining populations from Germany, Southern France, Northern and Southern Italy with two microsatellite markers. We wanted to clarify, whether the high inbreeding found in northern populations is a cause of post glacial re-colonization and therefore might not be found in populations from Italy and France south of the Alps. Furthermore, we compared colony fusion and usurpation rate in all populations. According to the bottleneck theory, one might expect higher variability also at loci determining nestmate recognition cues in southern populations. Therefore colony takeovers would be scarcer because individuals from different colonies would react more aggressively towards each other.

Material and Methods

Populations

Colonies from five populations were included in this study, which were Sommerhausen near Würzburg (Germany), Salorno near Bozen (Italy), Salò at the Lago di Garda (Italy), Malaucène near Mont Ventoux (France) and Sperrone in the National Park of the Abruzzi (Italy). Colonies from the Sommerhausen site were collected in 2002. Here, the colonies were mainly found in acorns, rotten pine and oak sticks. In the same year, we collected colonies in a beech forest near Salorno (N 46°14.331', E 11°13.51'), were they inhabited beech sticks. In 2003, a 15,4 square meter plot was mapped that contained 46 colonies (= three colonies per square meter) in a mixed forest near Salò (N 45°37.232', E 010°32.281'). Colonies were found in acorns, oak and beech sticks. Also in 2003, we collected colonies near Malaucène (N 44°09.935', E 005°09.446') that mainly resided in rotten pine sticks. Colonies near Sperrone (N 41° 55.586', E 013° 43.484') were collected in 2004 and lived in rotten oak sticks and acorns at the edge of an oak forest. Colony density in the Sommerhausen population was about seven colonies per square meter (see also Foitzik et al., 2003), which correlates with colony density from the Malaucène population. In the populations from Salorno and Sperrone, colony density was lower with approximately four colonies per square meter.

Genetics

All individuals were genotyped at the microsatellite loci L-5 (Foitzik et al., 1997) and LX GT 218 (Hamaguchi et al., 1993). From the Sommerhausen (So) population, we genotyped 10 colonies and 10 workers per colony plus the queen (all in all 212 samples due to occasional PCR failure). The same accounts for the Salorno (Sal) population (218 samples on the whole). Nine colonies with 10 workers each were investigated from the Malaucène (Ma) population,

here only five colonies contained a queen (170 samples). From the Salò (Sa) population, seven colonies with nine workers each were investigated. From three colonies also the queen was genotyped (125 samples on the whole). Five colonies with ten workers each and one colony containg a queen were genotyped from the Sperrone (Spe) population (98 samples).

DNA was extracted using the Puregene DNA purification kit (Gentra Systems) as described in Foitzik & Herbers (2001). PCR reaction mixtures consisted of 1µl DNA, 2 pmol dNTPs, 0.5 pmol of each primer, 9µl dd H_2O, 2µl 10x PCR buffer (without $MgCl_2$), 2 mM $MgCl_2$, and 0.4µl of 1 unit/µl Taq Polymerase (Q Bio Gene). Locus L-5 was amplified at 1 min 94°C, 1 min 45°C and 30 sec 72°C, locus LX GT 218 at 1 min 94°C, 1 min 54°C and 30 sec 72°C for 33 cycles each. PCR–products were checked on 1.5% agarose gels stained with ethidiumbromide.

The program Genepop 3.4 (Raymond & Rousset, 1995) via web implementation was used to test for Hardy–Weinberg equilibrium and to calculate expected and observed heterozygosities. With the same program, linkage disequilibrium between the two loci was tested by applying the Fisher's exact test. The inbreeding coefficient (F_{is}), population substructuring (F_{st}), pairwise F_{st} values and the F_{it} (Weir & Cockerham, 1984) values were also calculated with Genepop 3.4. Here permutation exact tests applying the Fisher's method were used to test for departure from the null hypotheses. Relatedness coefficients including the jacknifed standard error (over groups = colonies) were calculated with Relatedness 5.0 (K. F. Goodnight, Rice University). Additionally, as null alleles can also cause heterozygote deficieny, the maximum frequency of potential null alleles was estimated as calculated in Chakraborty et al. (1992) and Brookfield (1996).

Only worker genotypes were considered for the determination of matriline number of the respective colonies, because not all of the genotyped colonies had a queen. Aberrant queen genotypes, probably due to usurpation by a foreign queen, were noted separately.

Results

In all but one population (Salò, where both loci had equal allele numbers) more alleles were found for locus L-5 than LX GT 218. For both loci, the number of alleles did not differ significantly between the populations (chi-square-test: $\chi_4^2 = 8.86$, $p < 0.1$). However there was a trend to significance concerning differences in allele number with 20 alleles for both loci detected in Sommerhausen, 21 in Salorno and 25 in Malaucène against only 12 and 13 alleles found for Salò and Sperrone, respectively (Table 4.1).

Table 4.1: Number of alleles, frequency of most common allele, number of private alleles, expected and observed heterozygosity for both loci. At locus LX GT 218, the most common allele was shared by the populations Sommerhausen, Salorno and Malaucène (underlined numbers). The populations Salò and Sperrone also share the most common allele (bold numbers) at this locus. For abbrevations of populations see Material and Methods.

population	locus	n of alleles	frequency of most common allele	n of private alleles	H_{obs}	H_{exp}
So	L-5	11	0.269	3	0.47	0.88
	LX GT 218	9	<u>0.361</u>	2	0.68	0.77
Sal	L-5	15	0.216	5	0.71	0.98
	LX GT 218	6	<u>0.266</u>	0	0.85	0.81
Sa	L-5	6	0.478	2	0.20	0.48
	LX GT 218	6	**0.326**	0	0.23	0.54
Ma	L-5	14	0.335	6	0.45	0.75
	LX GT 218	11	<u>0.176</u>	4	0.77	0.78
Spe	L-5	8	0.375	1	0.14	0.39
	LX GT 218	5	**0.540**	0	0.33	0.33

A significant difference in the distribution of private alleles (chi-square-test: $\chi_4^2 = 15.72$, $p < 0.01$) was found between the populations with the highest number of private alleles in the french population collected near Malaucène (6 (L-5) and 4 (LX GT 218)) and the lowest number (1 (L-5) and 0 (LX GT 218)) in the population collected near Sperrone, Southern Italy (Table 4.1). Only at locus LX GT 218, the most frequent allele is shared by populations (Table 4.1). As expected, both loci are not linked (test for linkage disequilibrium: $p > 0.001$). In all five populations, significant deviations from Hardy-Weinberg-equilibrium were found for both loci due to heterozygosity deficiency (Hardy-Weinberg exact probability test for all populations and loci: $p > 0.001$, S.E. > 0.0000, test for heterozygote deficiency for all loci and all populations: $p > 0.001$, S. E. > 0.001). The observed heterozygosity was almost always lower than the expected heterozygosity for both loci in all five populations (Table 4.1).

The level of inbreeding (F_{is}, Weir & Cockerham, 1984) was generally high except for locus LX GT 218 in the populations Malaucène and Sperrone (Table 4.2). In the Salò population, where also the smallest number of alleles was found for both loci, the inbreeding coefficient was equally high in both loci. In all other populations, the F_{is} value was higher for locus L-5.

Table 4.2: Level of inbreeding for both loci in all populations (significance values for both loci combined) and average intra-nest worker-worker relatedness in all populations. The p-value indicates significance of deviation from 0.75.

		Fis (W&C)			r +/- SE	
population	n colonies	L-5	LX GT 218	p	both loci	p
So	10	0.47	0.12	$p < 0.001$	0.66 +/- 0.068	> 0.1
Sal	10	0.28	0.05	$p < 0.001$	0.81 +/- 0.092	> 0.5
Sa	7	0.59	0.58	$p < 0.001$	0.91 +/- 0.048	< 0.02
Ma	9	0.40	0.01	$p < 0.001$	0.81 +/- 0.051	> 0.1
Spe	5	0.64	0.01	$p < 0.001$	0.83 +/- 0.030	< 0.1

The highest F_{is} value was found in the South Italian population Sperrone for locus L-5 (F_{is} = 0.64, Table 4.2). The mean inbreeding coefficient considering all populations therefore also deviates significantly from null ($F_{is} = 0.28 \pm 0.0033$, $p < 0.001$; χ_4^2 = infinite, $p < 0.001$). The same accounts for the F_{it} value ($F_{it} = 0.37 \pm 0.00039$, $p < 0.001$; χ_4^2 = infinite, $p < 0.001$). The mean intra-nest relatedness in all populations except Sommerhausen was higher than 0.75 (the expected value), because of relatedness-values of 1 in colonies, where all workers had the same genotype per locus. In heterogenous colonies, the relatedness was lower than 0.75 or 0.5 depending on the number of matrilines. However, only the r-value for Sa derived significantly from the expected value (0.75); here the highest F_{is}- values were found. In the Sommerhausen population, containing the most heterogenous colonies of all populations, the relatedness value was lower than 0.75, but not significantly (Table 4.2). If the decreased heterozygosity was caused by null alleles alone, its frequency would be 28% and 6% for the loci L-5 and LX GT 218, respectively. The overall estimate of the F_{st}-value (0.18 ± 0.00029, $p<0.001$; χ_4^2 = infinite, $p < 0.001$) measuring population substructuring was high. Between all population pairs, significant substructuring could be found with the highest difference between the South Italian population Sperrone and the North Italian population Salò (0.17) and Sperrone and the Southern France population Malaucène (also 0.17, see Table 4.3).

Table 4.3: Matrix of F_{st} values between all population pairs, bold numbers indicate the highest values.

	So	Sal	Ma	Sa
Sal	0.06			
Ma	0.08	0.08		
Sa	0.12	0.14	0.15	
Spe	0.15	0.15	**0.17**	**0.17**

In all populations the number of matrilines ranged from one to three, but no significant difference was found between all five populations (chi-square-test: $\chi_8^2 = 11.61$, $p < 0.2$, Figure 4.1). A foreign queen with a completely different genotype was found in 2 out of 10 colonies in the Sommerhausen and Salorno populations, respectively. The queen genotypes in the remaining three populations suggested all workes to be descendants of one queen.

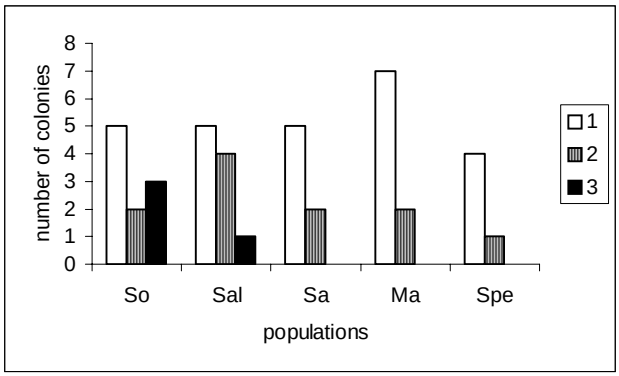

Figure 4: Number of homogenous (one matriline) colonies and heterogenous colonies containing two or three matrilines.

Discussion

Our study reveals similar patterns of colony structure, intra-colony worker-worker relatedness and inbreeding in all investigated populations of the West European ant *T. nylanderi*.

The total number of alleles, including private alleles, was highest for both loci in the Southern France population. In this population, also a slightly higher number of homogenous colonies was found. This is probably due to the abundance of stable pine sticks at this site. These do not decay rapidly and therefore, the frequency of colony movements might be lower in comparison to sites with ephemeral nests. However, the number of both alleles and private alleles for the two loci did not differ significantly among the five populations. These results are in accordance with a previous investigation using two mitochondrial DNA markers (Pusch et al., 2006), but contrast with the clines of allelic diversity observed, e.g., in several well-studied butterfly species (Schmitt & Hewitt, 2004). Thus, the low genetic diversity observed in *T. nylanderi* throughout its distribution range is not a cause of bottlenecks resulting from the postglacial colonization of Central Europe.

The remarkably low allelic diversity in the Salò population probably results from having mistaken polydomous units as separate colonies. Polydomy is common in ants (Herbers, 1986; Herbers & Grieco, 1994; DeHeer et al., 2001; Elias et al., 2005) and was also reported for *T. nylanderi*. Here, large colonies split in spring where empty nest sites are abundant (Heinze et al., 1996; Foitzik & Heinze, 1998). Three of seven colonies from the Salò site had the same

homozygous genotype for locus L-5. For two of these colonies, this also accounts for the genotypes of locus LX GT 218. Additionally, only one of these two colonies contained a queeen. This might be a reason why this population exhibited very high inbreeding values at both loci.

All in all, the positive inbreeding is remarkable. However, it was observed before in a population from Sommerhausen (Foitzik & Heinze, 2001). In general, inbreeding in ants is rare and depends on special life history traits such as intranidal sib-mating e.g. observed in *Cardiocondyla batesii*, where males are wingless and mate with winged females in the nest (Schrempf et al., 2005) or sex-biased gene flow found in *Formica exsecta* (Sundström et al., 2003). In ants where both sexes are winged and participate in mating flights, positive inbreeding is mainly a consequence of population substructuring, leading to the Wahlund effect (Hartl & Clark, 1989). This e.g. accounts for the North American congener *Temnothorax ambiguus*. It also inhabits preformed nest sites, but is very patchily distributed within the closely related species *T. longispinosus* and *T. curvispinosus* (Herbers & Grieco, 1994). The locally dense but on a wider scale patchily distributed populations of *T. nylanderi* found both in Northern and in Southern Europe and the fact that only part of the colonies produce sexuals, apparently leads to non-random mating over time. Thus, a combination of spatial and temporal effects might lead to the positive inbreeding. Non-random mating over time had also been considered to lead to significant inbreeding in the earlier *T. nylanderi* study from Sommerhausen (Foitzik & Heinze, 2001).

At least for locus L-5, null alleles alone can not explain the considerable inbreeding, because the theoretical frequency of 28% is very high. Frequencies of below 15% have been stated for null alleles (Jarne & Lagoda, 1996). The second locus, LX GT 218, exhibited only significant inbreeding for Salò and Sommerhausen. Here, the calculated null allele frequency was 6%, which would explain, at least partly, the heterozygote deficiency. The variation between the two loci is astonishing, as real inbreeding consequently must affect the whole genome. However, different results at different loci for the same population have also been found in other studies (Lampert et al., 2003). Locus L-5 probably exhibited the more reliable result, because in the previous investigation of the *T. nylanderi* population from Sommerhausen, equally high levels of heterozygote deficiencies for the loci L-5 and L-18 were reported. Moreover, even diploid males were detected (Foitzik & Heinze, 2001). Interestingly, the here applied loci L-5 and LX GT 218 did not exhibit significant inbreeding in one population of the sibling species, *T. crassispinus* that is morphologically and biologically very similar (Seifert, 1995; Strätz & Heinze, 2004).

The values of spatial structuring are significant between all population pairs, but not distance-correlated. Because the smallest distance between the sites is approximately 90 km (between Salorno, near Bozen and Salò, near the Lago di Garda), geneflow between any of the populations can be ruled out. Apart from that, the here investigated populations exhibited similar relatedness values, which did not differ significantly from 0.75, except in the Salò population. However, in all other populations except for Sommerhausen, the relatedness-value exceeded 0.75, probably due to the significant inbreeding. The small differences between populations found in this study might depend on unequal sample size and ecological differences in the respective locations; ecological factors seem to play a considerable role in *T. nylanderi* (Foitzik et al., 2003).

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Description of two new *Temnothorax* species (Hymenoptera: Formicidae) from Italy *

Andreas Schulz, Jürgen Heinze and Katja Pusch

Abstract

We describe two species of the ant genus *Temnothorax*: *T. alienus* nov. spec. and *T. saxatilis* nov. spec. Both new species seem to be endemic in Middle and Southern Italy. We characterize the two species, compare them with the next closely related *Temnothorax* from the Western Palaearctic and morphologically similar species from Italy, and present a provisional key for identifying workers and gynes of these and related species from Italy.

Key words: Formicoxenini, species diversity, taxonomy of ants, radiation, European fauna

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* Manuscript in preparation

Introduction

In his recent revision, Bolton (2003) divided the myrmicine genus *Leptothorax* (sensu lato) into three genera: *Leptothorax* (sensu stricto), *Nesomyrmex* and *Temnothorax*. The great majority of former species of *Leptothorax* (s.l.) were transferred to the genus *Temnothorax*, established by Mayr in 1861 for a number of small Palaearctic Formicidae. During the last decades, a wealth of biological information has been accumulated about species of this and other genera in the tribe Formicoxenini. As colonies are generally small, with often less than 200-300 individuals, they can be collected completely, and reared under controlled conditions in the laboratory (Buschinger, 1968, 1974). The large interest in the Formicoxenini stems from the fact that its species serve as model systems for the investigation of population and colony structures of ants with small societies and pronounced kin conflict (Heinze, 2004), and also because numerous species are social parasites – guest ants, slave-makers, and workerless inquilines (Buschinger, 1981, 1986; Hölldobler & Wilson, 1990).

In contrast to the attention, species of *Temnothorax* and other formicoxenine genera have already received, their taxonomy is still unclear. There are nearly 600 described taxa in the genus *Temnothorax*, of which approximately 300 are recognized as valid species (Bolton, 1995a, 1995b). *Temnothorax* is much less diverse in tropical ecosystems of the "Old World" than in the Palaearctic region (Bolton, 1982, 1991). Mediterranean habitats are especially rich in species. *Temnothorax* inhabits nearly all terrestrial environments in the Palaearctic and is found in almost all habitat structures from sea level up to altitudes of 2700 m. It seems that a higher proportion of endemic *Temnothorax* species occurs at higher altitudes, especially on isolated mountains (A. Schulz, unpublished data).

In Italy, the number of described species of *Temnothorax* is high and the diversity of species seems to be similar from north to south (Baroni Urbani, 1971). However, Southern Italy is less well studied than the northern part of the country and additional species can be expected. Here, we report on two new species of *Temnothorax* from Southern Italy, with interesting or geographically far distant relatives. We hope that our study inspires more interest in the remarkable diversity of *Temnothorax* in the Palaearctic, which matches that of some of the “most diverse” ant genera in the tropics.

Material and methods

Material studied

The samples of the described species were collected in 1994 (M. Sanetra) and 2004 (J. Beibl, P. D'Ettorre, K. Pusch, C. Wanke). In addition, we examined material from the private collection of A. Schulz and type material of *T. korbi* (Emery, 1922), *T. lichtensteini* (Bondroit, 1918), *T. parvulus* (Schenck, 1852), and *T. luteus* (Forel, 1874). Collections are referred to by the following acronyms:

EMAU Ernst-Moritz-Arndt-Universität - Zoologisches Institut und Museum, Greifswald, Germany
MCSN Museo Civico di Storia Naturale `Giacomo Doria´, Genova, Italy
MHNG Muséum d'Histoire Naturelle, Genève, Switzerland
MNHN Muséum National d'Histoire Naturelle. Paris, France
PCAS Private Collection Andreas Schulz
SMFM Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt a. M., Germany
SMNG Staatliches Museum für Naturkunde, Görlitz, Germany
SMNK Staatliches Museum für Naturkunde Karlsruhe, Karlsruhe, Germany

Measurements

All measurements were taken using a Zeiss Stemi SR stereomicroscope equipped with an ocular graticule, at a maximum magnification of 250x. The data are presented in mm, as mean ± standard deviation, with minimum and maximum value in parentheses, holotype in brackets.

The following measurements were taken:

HL Head length: measured the maximum real distance from anteriormost to posteriormost margin along median axis. Both anterior and posterior margin of head must be in focus.
HW Head width: maximum head width posterior to eyes.
HS Head size – the arithmetic mean of HL and HW.
SL Maximum chord length of scape. The distal measuring point is the most distal point of the dorsal lamella of the hinge joint capsula, the proximal measuring point is the most proximal point of the scape shaft near the neck of the articular

condyle. To obtain the real maximum, a frontal to dorsal viewing position is necessary.

FCD Frontal carinae distance: Distance between the frontal carinae, measured in full face dorsal view. The measurements were taken at the level of a transverse line where the articulatory bulb of scapes inserts in the antenna socket.

ED Eye diameter: The largest measurable line across the compound eye including all structurally visible ommatidia irrespective of the pigmentation status, measured in lateral view.

ML Mesosoma length measured in lateral view from the frontalmost point of the anterior pronotal slope to the caudalmost portion of the propodeum.

MW Maximum mesosoma width measured in dorsal view at the widest part of mesosoma

PSL Propodeal spine length, measured in workers and gynes. In dorsocaudal view, the tip of the measured spine, its base, and the center of the concavity between the spines must all be in focus (Figure 5.1: points 1, 2 and 3). Using a cross-shaped ocular graticule, point 1 is placed at the median point of the concavity between the spines, point 3 at the tip of a spine. The spine length is measured as the distance from point 2 to point 3. We measured always the right spine (see also Güsten et al., 2006).

PEL Petiole length: The maximum length of the petiolar node is measured in dorsal view from the anterior notch close to the propodeum to the articulation with the postpetiole. Both points must be in focus.

PEW Maximal measured width of petiole in dorsal view.

PEH Petiole height: The maximum height of the petiolar node, measured in lateral view from the highest (median) point of node to the ventral margin. The ventral margin always has a short concave portion, which is the ventral measuring point.

PPW Maximal measured width of postpetiole in dorsal view.

PHD Distance between the bases of the uppermost erect hairs on petiolar node, measured in dorsal view.

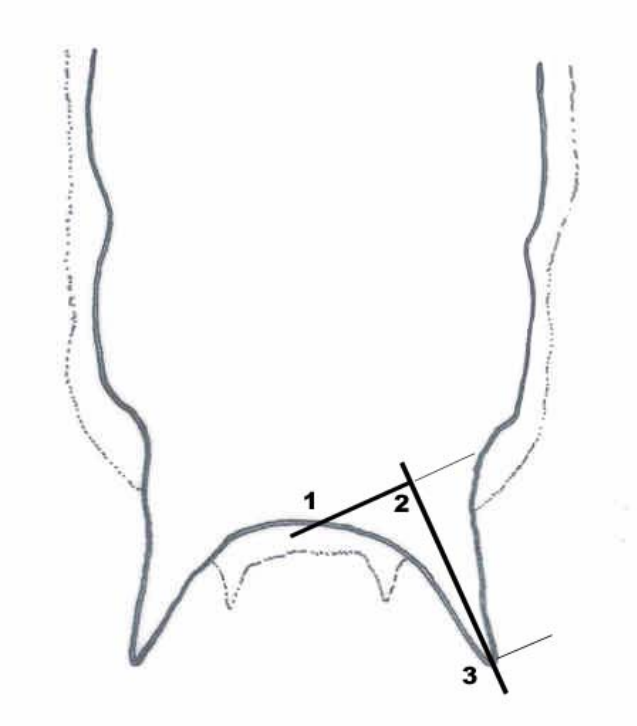


Figure 5.1: Dorsal view of mesosoma, and the instruction of propodeal spine measuring.

Photography

The photographic images (Figures 5.2 – 5.45) were taken using a digital camera (JVC KY-70B) attached to a Leica Z6 APO stereomicroscope. The microscope was equipped with a Z-stepper (Syncroscopy, Synoptics Ltd.) to enable the generation of usually 100 images in different focus layers from which a montage image was computed using AutoMontage Pro 5.02.0096 (Synoptics Ltd.). Montage images were enhanced (Photoshop 7.0, Adobe Systems Inc.) by removing out of focus structures and artefacts caused by the montage process (see also Güsten et al., 2006).

Temnothorax alienus nov. spec.

(Figures 5.2, 5.3, 5.26, 5.30, 5.32, 5.33)

Holotype worker

ITALY, Campania, near Tortora, N. Sapri, Parco Nazionale del Cilento, 39°57.879'N 15°48.809E, 633 m.a.s.l., 28.iv.2004 (Leg. K. Pusch, C. Wanke, J. Beibl, P. D'Ettorre) [SMNK].

Paratypes

12 workers and 4 gynes, same data as holotype [SMNG, MHNG, MCSN, PCAS]; 10 workers, ITALY, Campania, near Carpaccio, N. of Agropoli & Paestum, Mte. Vesole, Parco

Nazionale del Cilento, 730 m.a.s.l., 27.iv.2004 (Leg. K. Pusch, C. Wanke, J. Beibl, P. D'Ettorre) [SMNG, MHNG, MCSN, PCAS].

Etymology

From the Latin word "alienus", meaning “foreigner” or “alien,” referring to the unique combination of characters, which is found only in a small number of other Western Palaearctic *Temnothorax* species.

Description of worker

Measurements and indices (n = 16): HL [0.684] 0.67±0.03 (0.58-0.71), HW [0.580] 0.58±0.03 (0.50-0.63), SL [0.475] 0.48±0.03 (0.43-0.53), FCD [0.220] 0.22±0.02 (0.18-0.24), ML [0.791] 0.77±0.05 (0.67-0.85), MW [0.390] 0.39±0.03 (0.32-0.46), PSL [0.095] 0.09±0.01 (0.07-0.11), PEL [0.238] 0.24±0.02 (0.21-0.27), PEW [0.171] 0.18±0.02 (0.15-0.21), PEH [0.238] 0.23±0.02 (0.21-0.27), PHD [0.090] 0.09±0.01 (0.07-0.11), PPL [0.162] 0.16±0.02 (0.13-0.18), PPW [0.238] 0.23±0.02 (0.20-0.26), HS 0.63±0.03 (0.54-0.67), HW/HL 0.86±0.03 (0.83-0.97), SL/HS 0.77±0.03 (0.70-0.82), FCD/HS 0.34±0.02 (0.31-0.36), MW/ML 0.50±0.03 (0.48-0.63), PSL/ML 0.11±0.01 (0.09-0.13), PEH/PEL 0.98±0.05 (0.91-1.08), PEW/PEL 0.74±0.07 (0.68-0.90), PHD/PEW 0.48±0.07 (0.35-0.56), PPL/PPW 0.72±0.06 (0.64-0.79), PEW/PPW 0.76±0.08 (0.71-0.97).

Head narrower anterior to eyes than posteriorly. Margins of head posterior to eyes weakly convex, vertexal corners evenly rounded, posterior margin of vertex linear. Frontal triangle somewhat impressed but not clearly demarcated. Frontal carinae narrow and short, strongly divergent posteriorly. Mesosoma with dorsal profile evenly and weakly convex, without metanotal groove. Propodeal spines broadly attached, nearly triangular, acute, slightly pointed upward and slightly divergent. Petiole subsessile, its anterior face straight or only slightly concave, node triangular with rounded apex. Posterior face weakly convex or straight, sloping downwards nearly at the same angle as the anterior face. Anterior subpetiolar process large, slightly longer than broad at the base. In dorsal view, petiole with weakly convex to straight sides at midlength, strongly converging anteriorly. In dorsocaudal view the node apex is relatively narrow with a straight dorsal margin. Postpetiole in lateral profile more or less evenly rounded. In dorsal view the postpetiole is subrectangular with weakly rounded corners, slightly broader anteriorly, sides are straight and nearly parallel.
Mandibles very finely irregularly longitudinally striate, sublucid. Frontal triangle smooth

with 1-2 shallow rugulae. Clypeus medially lucid, without a coarse median carina, but with some paramedian striae running half way from anterior to posterior clypeal border. Scapes faintly striate to very finely granulate. Frons medially with a narrow medial unsculptured and lucid part, other surfaces irregularly and divergently rugose with many anastomoses. Interspaces between rugae densely reticulate. Posterior frons reticulate with isolated superficial rugae. Genae, surface around the eyes and vertex irregularly rugose to striate, with densely reticulate interstices. Surface posterior of eyes with semicircular rugulae. Ventral surface of head laterally striate, medially smooth. Entire mesosoma irregularly and densely rugose to rugoreticulate, dorsal median surface of mesonotum alveolate. Space between the propodeal spines and entire petiole and postpetiole reticulate. Petiolar node with some fine rugae superimposed on the reticulum. Gaster lucid. Colour entirely yellowish-orange, appendages with same colour, without darker antennal club. Up to 2/3 of posterior portion of first gastral tergite dull orange-brown. Standing pilosity of head, mesosoma and gaster of medium size, transparent, with blunt tips.

Some specimens have stronger medial carinae on the clypeus, the head may be slightly darker than the mesosoma, the distal antennal club may be slightly darker than the rest of the funiculus. The femur may be darker, the gaster may be mainly brownish only with a more or less extended spot of the most anterior first gaster tergite is yellowish or orange. The sculpture may be coarser in general, the frons may be entirely reticulate.

Description of gyne

Measurements and indices (n = 4): HL 0.74+-0.03 (0.73-0.79), HW 0.69+-0.03 (0.66-0.73), SL 0.52+-0.02 (0.50-0.54), ED 0.21+-0.01 (0.19-0.22), MW 0.77+-0.02 (0.75-0.80), PSL 0.10+-0.01 (0.09-0.11), PEL 0.30+-0.01 (0.29-0.32), PEW 0.23+-0.01 (0.22-0.25), PHD 0.11+-0.01 (0.10-0.11), PPL 0.23+-0.01 (0.22-0.24), PPW 0.31+-0.01 (0.30-0.31), ML 1.19+-0.04 (1.15-1.24), PEH 0.30+-0.01 (0.28-0.31), HS 0.72+-0.03 (0.69-0.76), SL/HS 0.72.0+-0.01 (0.71-0.73), ED/HS 0.29+-0.01 (0.26-0.30), HW/HL 0.93+-0.01 (0.92-0.94), MW/ML 0.65+-0.01 (0.63-0.66), PSL/ML 0.08+-0.01 (0.07-0.09), PEH/PEL 0.97+-0.06 (0.87-1.03), PEW/PEL 0.77+-0.04 (0.72-0.82), PHD/PEW 0.34+-0.02 (0.32-0.36), PPL/PPW 0.74+-0.03 (0.70-0.76), PEW/PPW 0.76+-0.02 (0.75-0.79), PEL/ML 0.28+-0.04 (0.25-0.34).

Head relatively large with weakly convex and convergent genae, rounded vertexal corners and slightly convex anterior clypeus margin. Compounded eyes relatively small. Mesosoma

short, relatively high and robust, with straight dorsal margin, and well visible pronotal corners. Scutellum broader than long, posterior margin of it semicircular. Propodeal spines short, broadly attached and triangular, with pointed tips, caudad oriented. In dorsal view the spines are linear and parallel-sided. Petiole subsessile, average high and long, but relatively broad, with general shaping as described for workers. Postpetiole shaped as in workers.

Mandibles faintly longitudinally striate, sublucid. Frontal triangle unsculptured and lucid. Clypeus medially lucid, without a coarse median carina, but with some paramedian carinae, with lucid interstices. Scapes faintly striate, or faintly granulate. Frons striate to carinate, with unsculptured and lucid interstices. Other parts of head dorsum stronger longitudinally carinate to irregularly rugose, with lucid interstices. Anterior surface of pronotum reticulate, other parts broad-mashed rugose with shining interstices. Mesonotal dorsum with few longitudinal, nearly invisible carinae, mainly shining. Scutellum lucid, laterad with 2-3 very fine striae on each side. Dorsum of propodeum roughly, transversely and diffusely carinate, between and below the spines transversely reticulo-striate. Anepisternit and other lateral parts of mesosoma irregularly and shallowly rugostriate, with shining and lucid interstices. Petiole and postpetiole dorsally rugoreticulate, ventrally reticulate, subopaque. Bicoloured species, mainly orange with equal coloured or paler appendages, without darker antennal clubs. Genae, dorsum of head, two lateral small spots of mesonotum, 50% of scutellum, and 2/3 of first gaster tergite darker coloured, testaceus to brownish. Standing pilosity as described in workers.

The male is unknown

Differential diagnosis

Workers of *T. alienus* show the typical *nylanderi/parvulus*-like petiolar shape, but differ from latter in lacking of any metanotal impression or groove. The colour of *T. alienus* is uniformly light yellow-orange, with only a slightly darker broad band on the first gaster tergite. Other Italian *Temnothorax* are dark brown to black, have distinctly darker antennal clubs, or a shining surface. In the following, we separate morphologically near related and other Italian species by presence/absence of a metanotal groove, and by various morphometric characters (see Table 5.1).

Species without metanotal groove: The central Asian *T. tianshanicus* (Tarbinsky, 1976) has longer scapes, broader head, and shorter propodeal spines than *T. alienus*. The head dorsum of *T. tianshanicus* is more shining. In addition, *T. tianshanicus* has a shallow and broad, just

visible metanotal depression. Other characters are similar, thus *T. tianshanicus* seems morphologically the closest to *T. alienus*.

T. satunini (Ruzsky, 1902) (Figures 5.4 & 5.5), a species from Southern to Eastern Turkey and Caucasus, is also morphologically similar, but differs in the following characters: narrower, and shining, nearly unsculptured head, yellow colour without darker gaster, and often distinctly shorter propodeal spines (Caucasian & East Turkey: PSL/ML < 0.07). An unidentified species from Morocco (PCAS sp. 27 "Morocco") has distinctly longer scapes, a lower, narrower petiole, and a shallow, just visible metanotal depression. Additionally, the petiole is truncated and pedunculate. Sculpture and colour are similar to *T. alienus*.

Another lighter coloured species with slightly convex or straight mesosomal dorsum is *T. luteus* (Forel, 1874) s.l., which is distinguishable from *T. alienus* by longer propodeal spines, broader head, longer scapes, and the distinctly lower, pedunculate and narrower petiole. Sculpture characters are similar to *T. alienus*, but the whole gaster is yellowish. The taxonomic situation of *T. luteus* and related species is not clear yet.

The two arboreal species *T. rabaudi* (Bondroit, 1918) and *T. italicus* (Consani & Zangheri, 1952) (Figures 5.12 & 5.13) are similar in the shape of petiole and mesosoma, and might be confused with *T. alienus*. However, *T. rabaudi* and *T. italicus* differ partially in the length of propodeal spines, and the petioles of both species are distinctly lower and generally more triangular, with a more rounded apex. Especially the petiolar node apex is more rounded in lateral view, with a straight anterior declivitous face. The sculpture of head is more heavily reticulate in both species.

Species with metanotal groove: *T. lichtensteini* (Bondroit, 1918) (Figures 5.10 & 5.11) workers can easily be distinguished from *T. alienus* by their very long and curved propodeal spines, the pedunculate petiole with a more rounded or truncated node, the well visible metanotal groove, and the denser sculpture of head and other parts of the body, but *T. lichtensteini* is smaller, sometimes the colour can be identical with *T. alienus,* but it is mainly darker. *T. nylanderi* (Foerster, 1850) can be distinguished from *T. alienus* by its longer propodeal spines and more apart frontal carinae. *T. nylanderi* (Figures 5.8 & 5.9) and *T. crassispinus* (Karavajev, 1926) workers are darker, with brownish head and mainly dark brown gaster. They can be distinguished from *T. alienus* by their well visible metanotal grooves, more truncated nodes apex, and evident fine and parallel-running, dense striae on the frons. Sometimes the metanotal groove is less visible or rarely absent in *T. lichtensteini, T. nylanderi,* and *T. crassispinus*.

Other yellowish species that might be confused with *T. alienus* are *T. parvulus* and *T. flavicornis* (Emery, 1870). *T. parvulus* has distinctly longer propodeal spines and a deep metanotal groove, a smaller head and gradually narrower frontal carinae. In addition, *T. parvulus* has a less coarse, mainly reticulate sculpture, and a uniform pale yellow colour. *T. parvulus* is rarely found in South Italy, *T. flavicornis* is more common, but differs easily by its 11-jointed antenna, coarser head sculpture, longer propodeal spines and lower petiole.

Table 5.1: Morphometric characters of *T. alienus* nov. spec. workers and related species.

	HS	**SL/HS**	**HW/HL**	**PSL/ML**	**PEH/PEL**	**PEW/PEL**	**FCD/HS**	**N=**
T. alienus **nov. spec.**	0.63±0.03 (0.54-0.67)	0.77±0.03 (0.70-0.82)	0.86±0.03 (0.83-0.97)	0.11±0.01 (0.09-0.13)	0.98±0.05 (0.91-1.08)	0.74±0.07 (0.68-0.90)	0.34±0.02 (0.30-0.36)	18
T. italicus (Consani & Zangheri, 1952)				0,13±0,02 (0,09-0,18)	0,83±0,03 (0,78-0,92)			24
T. lichtensteini (Bondroit, 1918)	0.55±0.02 (0.48-0.58)		0.82±0.02 (0.78-0.85)	0.21±0.01 (0.18-0.24)				39
T. luteus (Forel, 1874)		0.86±0.02 (0.80-0.85)	0.82±0.01 (0.80-0.84)	0.15±0.01 (0.12-0.17)	0.84±0.03 (0.80-0.90)	0.61±0.03 (0.57-0.67)		8
T. nylanderi (Foerster, 1850)				0.15±0.01 (0.12-0.18)			0.37±0.01 (0.37-0.41)	18
T. parvulus (Schenck, 1852)	0.54±0.03 (0.48-0.60)			0.20±0.02 (0.17-0.24)			0.38±0.02 (0.34-0.41)	30
T. satunini (Ruzsky, 1902)			0.81±0.02 (0.77-0.87)					41
T. crassispinus (Karavajev, 1926)				0.19±0.02 (0.13-0.24)				70
T. sp 27 „Morocco“	0.65±0.01 (0.64-0.66)	0.84±0.01 (0.84-0.84)			0.86±0.03 (0.83-0.86)	0.64±0.05 (0.59-0.69)		3
T. tianshanicus (Tarbinsky, 1976)		0.85±0.02 (0.82-0.88)	0.82±0.02 (0.80-0.85)	0.08±0.02 (0.06-0.10)				9

The gynes of *T. alienus* are morphologically similar to *T. parvulus* or look like a pale *T. nylanderi* (Figures 5.34 & 5.35). To distinguish gynes of *T. alienus* from other *Temnothorax* species is more difficult than in workers.

Gynes of *T. tianshanicus* are distinctly smaller, have longer scapes, and a narrower head and petiole, than those of *T. alienus.* The shape of the petiole is different and the colour of the former species is darker. *T. satunini* are morphologically very similar to *T. alienus* and differ in the shorter propodeal spines, in shorter and narrower head, and narrower mesosoma, but sculpture and colour are equal. In *T. luteus* the scapes and propodeal spines are longer, and the petiole is pedunculate and distinctly lower than in *T. alienus* gynes. Nearly the whole

mesonotum and scutellum are strongly longitudinally rugose, whereas in *T. alienus* the surface is mainly unsculptured, shiny. In Southern Italy, *T. luteus* s.l. is brownish and strongly sculptured. Gynes of *T. rabaudi* and *T. italicus* are morphologically more different than workers. *T. rabaudi* and *T. italicus* have tooth-like propodeal spines and a distinctly lower petiole. In both species the head is heavily reticulate. Gynes of *T. lichtensteini* are dark ferrugineous to brown, distinctly smaller, with larger eyes, and longer propodeal spines, than in *T. alienus*. *T. nylanderi* is similar to *T. alienus*, but have a narrower petiole in comparison with the postpetiole, have darker heads, with the same striation of frons as described in workers. *T. crassispinus* has always distinctly longer propodeal spines. Sculpture and colour are similar to those of *T. nylanderi*, but *T. crassispinus* is generally darker than *T. alienus* and *T. nylanderi*. The gyne of *T. parvulus* has a smaller head, larger eyes, longer propodeal spines, and a narrower petiole in comparison with the postpetiole. The colour of *T. parvulus* is sometimes uniformly yellowish-testaceous (including the gaster) in contrast to the more ferrugineous gynes of *T. alienus*, but usually *T. parvulus* has brownish coloured gynes. *T. parvulus* gynes have also a more pedunculate petiole than those of *T. alienus*; yet they are very similar and most safely distinguishable by morphometric characters.

Comments

One part of the *T. alienus* nests were collected in a forest with *Quercus* and *Laurus* trees, at the base of a hill. The ground was covered with rocks and ivy; nests were located in dead sticks on the ground. At the second locality the surface was covered with scattered *Castanea* and *Corylus* trees, and sparse vegetation.

An unpublished cytochrome oxidase (CO I) analysis (K. Pusch and J. Heinze) supports the hypothesis that *T. alienus* is phylogenetically not related with the *T. nylanderi/parvulus* complex, but in this character more similar with species like *T. unifasciatus* or *T. luteus*.

Table 5.2a,b: Morphometric characters of *T. alienus* nov. spec. gynes and related species.

a.

species	***HS***	***SL/HS***	***ED/HS***	***HW/HL***	***PSL***	***PSL/ML***	***MW/ML***
T. alienus **nov. spec.**	0.72±0.03 (0.69-0.76)	0.72±0.01 (0.7.-0.73)	0.27±0.01 (0.26-0.30)	0.93±0.01 (0.92-0.94)	0.10±0.01 (0.09-0.11)	0.08±0.01 (0.07-0.09)	0.65±0.01 (0.63-0.66)
T. italicus (Consani & Zangheri, 1952)		0,67±0,02 (0,67-0,71)			0.03±0.01 (0.01-0.05)	0,02±0,01 (0,01-0,04)	
T. lichtensteini (Bondroit, 1918)	0.65±0.01 (0.63-0.67)	0.75±0.02 (0.73-0.80)	0.32±0.01 (0.31-0.34)		0.14±0.01 (0.13-0.16)	0.12±0.01 (0.10-0.13)	0.58±0.01 (0.56-0.60)
T. luteus (Forel, 1874)		>0.73			>0.11	>0.09	
T. nylanderi (Foerster, 1850)							
T. parvulus (Schenck, 1852)	0.66±0.01 (0.63-0.68)		0.32±0.01 (0.31-0.35)		0.14±0.02 (0.12-0.17)	0.12±0.012 (0.10-0.15)	0.60±0.01 (0.58-0.63)
T. satunini (Ruzsky, 1902)	0.67±0.02 (0.65-0.69)	0.76±0.02 (0.74-0.78)		0.89±0.01 (0.88-0.90)	0.08±0.01 (0.07-0.08)		0.59±0.01 (0.59-0.60)
T. crassispinus (Karavajev, 1926)					0.15±0.02 (0.11-0.18)	0.12±0.02 (0.09-0.15)	
T. tianshanicus (Tarbinsky, 1976)	0.63±0.01 (0.62-0.64)	0.81±0.02 (0.78-0.84)		0.85±0.02 (0.82-0.87)	0.08±0.01 (0.08-0.08)		

b.

species	**PEH/PEL**	**PEW/PEL**	**PEW/PPW**	**N=**
T. alienus **nov. spec.**	0.97±0.06 (0.90-1.03)	0.77±0.04 (0.72-0.82)	0.76±0.02 (0.75-0.79)	4
T. italicus (Consani & Zangheri, 1952)	0,77±0.06 (0.69-0.88)			6
T. lichtensteini (Bondroit, 1918)		0.66±0.04 (0.57-0.70)		14
T. luteus (Forel, 1874)	<0.90	<0.72		1
T. nylanderi (Foerster, 1850)			0.70±0.02 (0.67-0.73)	12
T. parvulus (Schenck, 1852)			0.67±0.03 (0.61-0.72)	13
T. satunini (Ruzsky, 1902)				4
T. crassispinus (Karawajev, 1926)				33
T. tianshanicus (Tarbinsky, 1976)		0.69±0.03 (0.65-0.72)	0.68±0.01 (0.66-0.70)	7

Temnothorax saxatilis nov. spec.
(Figures 5.14, 5.15, 5.27, 5.31, 5.38, 5.39)

Holotype worker

ITALY, Abruzzi, Prov. L'Aquila, Gran Sasso, 6 km NE. Castel del Monte, 1600mH [SMNK].

Paratypes

8 workers and 1 gyne, same data as holotype [SMNG, MHNG, MCSN, PCAS].

Etymology

The Latin word means "between the rocks", a tribute to the name of the type locality, Gran Sasso.

Description of worker

Measurements and indices [holotype] (n=10): HL [0.637] 0.62±0.02 (0.59-0.66), HW [0.532] 0.51±0.02 (0.48-0.54), SL [0.428] 0.42±0.02 (0.38-0.43), FCD [0.214] 0.21±0.01 (0.20-0.22), ML [0.822] 0.77±0.03 (0.72-0.82), MW [0.399] 0.37±0.02 (0.35-0.40), PSL [0.081] 0.08±0.01 (0.07-0.10), PEL [0.261] 0.25±0.02 (0.23-0.28), PEW [0.176] 0.16±0.01 (0.14-0.18), PEH [0.228] 0.21±0.01 (0.19-0.23), PPW [0.228] 0.21±0.01 (0.20-0.23), HS 0.56±0.02 (0.53-0.59), HW/HL 0.82±0.02 (0.78-0.84), SL/HS 0.74±0.02 (0.67-0.77), FCD/HS 0.37±0.01 (0.35-0.39), MW/ML 0.46±0.01 (0.48-0.50), PSL/ML 0.11±0.01 (0.09-0.12), PEH/PEL 0.84±0.05 (0.76-0.90), PEW/PEL 0.64±0.04 (0.59-0.68), PEW/PPW 0.75±0.03 (0.71-0.79).

More slender head, narrower anterior to eyes than posteriorly, vertexal corners evenly rounded, posterior vertexal margin medially sligthly concave. Anterior margin of clypeus slightly convex, medially with a shallow depression or straight. Frontal carinae short, nearly parallel-sided. Scapes short. Mesosoma relatively narrow, in lateral view moderately high, with the dorsal margin mainly straight, or slightly convex. Propodeal spines short, broadly attached, nearly triangular, in dorsal view nearly linear and less divergent distally, with rounded tips. Petiole pedunculate, average high and broad, with broadly truncated posteriorly sloping, and rounded apex, anterior uppermost part with a shallowly visible angle, anterior petiolar face mainly concave. In dorsal view the node is evenly rounded, without angles or a crest. In dorsal view the postpetiole is subrectangular, anteriorly slightly

broader than posterior.

Mandibles partially rugoreticulate, lucid. Frontal triangle faintly granulate, lateral parts of clypeus irregularly striate to rugoreticulate, median faintly granulate, with one superimposed shallow carina, subopaque. Frons reticulate with some striae**,** sublucid. Genae rugoreticulate, around the eyes, and whole vertex mainly reticulate, larger specimens more rugoreticulate. Head ventrum faintly reticulate, medially lucid. Entire mesosoma densely reticulate, with a few superimposed rugulae on dorsum and pronotum. Between the propodeal spines and postpetiole reticulate, petiolar dorsum rugoreticulate, basal face reticulate. Gaster lucid. Colour dark ferrugineous to brown, with more dark brown head, and lighter gaster and appendages. Antennal clubs dark brown. Standing pilosity of head, mesosoma and gaster sparse, transparent, with blunt tips.

Description of gyne

Measurements and indices (n = 1): HL 0.74, HW 0.67, SL 0.50, FCD 0.26, ED 0.20, ML 1.33, MW 0.76, PSL 0.05, PEL 0.31, PEW 0.23, PEH 0.28, PPL 0.20, PPW 0.30, HS 0.72, HW/HL 0.94, SL/HS 0.70, FCD/HS 0.36, ED/HS 0.28, MW/ML 0.57, PSL/ML 0.04, PEH/PEL 0.89, PEW/PEL 0.73, PPL/PPW 0.68, PEW/PPW 0.76.

Head in relation to the mesosoma large and broad, especially behind the eyes. Genae weakly convex and convergent. Behind the eyes the margins are convex, vertexal margin broadly rounded, medially nearly linear. Anterior margin of clypeus slightly convex, medially with a shallow depression. Frontal triangle negligibly impressed. Eyes very small. Frontal carinae short and widely separated. Scapes short. Mesosoma in lateral view flat. Scutellum distinctly broader than long, posterior margin straight. Propodeal spines very short, dentiform.

Petiole pedunculate, average high, but broad, node with a truncated and rounded apex. Anterior-dorsal margin is slightly concave, in profile. Subpetiolar process inconspicuous, nearly triangular. In dorsal view with narrow peduncle, strongly divergent, from midlength the sides are nearly parallel. The node apex has rounded nearly invisible lateral corners, in dorsocaudal view, postpetiole of same shape as in workers.

Mandibles longitudinally striate, lucid. Frontal triangle lucid, clypeus carinate. A small strip of frons nearly unsculptured, lucid, bordered by longitudinal carinae, which connected by shallow transverse strigae. In prolongation of frontal carinae stronger carinae occur. Posterior part of frons only reticulostriate. Genae, around the eyes and vertex stronger rugate, with reticulate interstices. Head ventrum reticulostriate. Lateral parts of mesosoma +/- rugose to carinate with scattered reticulate interstices. Pronotum rugose, mesonotum

irregularly and densely carinate with some anastomoses, anterior surface unsculptured medially. Scutellum lucid medially, lateral surface striate. Dorsum of propodeum transversally carinate, also between the spines. Petiole rugoreticulate, with transverse strigae on dorsum, basal petiolar face and entire postpetiole irregularly reticulate. Mainly unicoloured species, dark brown, gaster somewhat lighter testaceus to brown. Appendages orange-brown, darker scapes, antennal clubs, and femurae.

Table 5.3: Morphometric characters of *T. saxatilis* nov. spec. workers and related species.

	HS	**SL/HS**	**PEW/PPW**	**FCD/HS**	**FCD/HS**	**N=**
T. saxatilis **nov. spec.**	0.56±0.02 (0.53-0.59)	0.74±0.02 (0.70-0.77)	0.75±0.03 (0.71-0.79)	0.37±0.01 (0.35-0.39)	0.37±0.01 (0.35-0.39)	9
T. cf. *anodontoides* (Dlussky & Zabelin, 1985)	0.63±0.03 (0.57-0.70)	0.79±0.02 (0.76-0.82)				35
T. nigriceps (Mayr, 1855)		0.77±0.02 (0.72-0.81)				20

Table 5.4: Morphometric characters of *T. saxatilis* nov. spec. gyne and related species.

Species	**HS**	**PSL/ML**	**PEH/PEL**	**PEW/PEL**	**N=**
T. saxatilis **nov. spec.**	0.72	0.04	0.89	0.73	1
T. affinis (Mayr, 1855)			0.77±0.03 (0.70-0.82)		17
T. nigriceps (Mayr, 1855)		0.08±0.02 (0.05-0.10)			10
T. cf. *anodontoides* (Dlussky & Zabelin, 1985)		0.09±0.02 (0.07-0.12)	0.75±0.04 (0.68-0.84)	0.64±0.05 (0.56-0.71)	1
T. tuberum (Fabricius, 1775)	0.69±0.02 (0.63-72)	0,07±0,01 (0,04-0,09)			36

Differential diagnosis

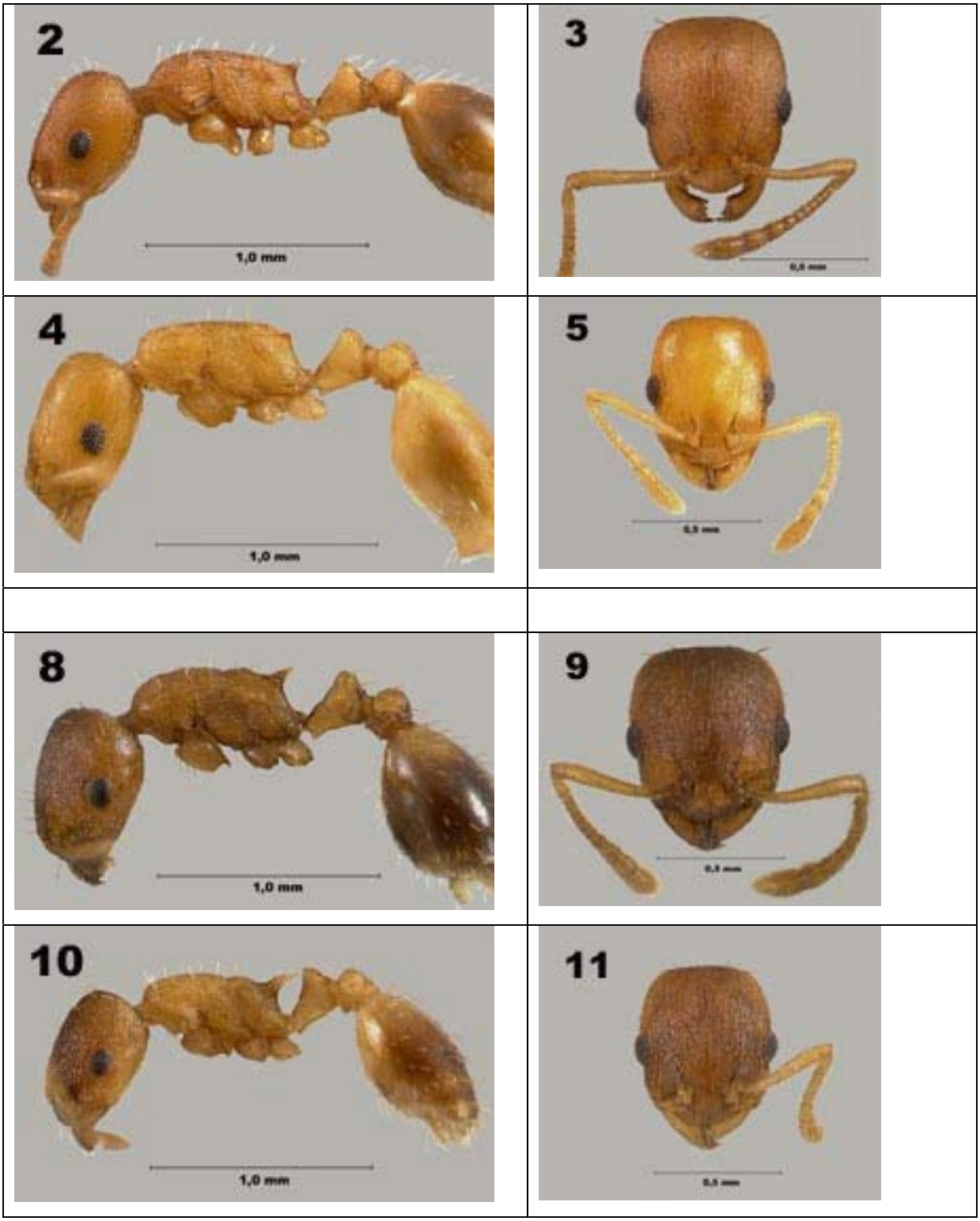
The workers of *T. saxatilis* are distinguishable from most Italian *Temnothorax* by the brown colour in combination with a conspicuous truncated robust petiole. The following species are similar in these characters. *T. saxatilis* has shorter scapes than *T. nigriceps* (Mayr, 1855) (Figures 5.16, 5.17, 5.28), also typical *T. nigriceps* are clearly bicoloured with ferruginous

mesosoma and waist, and contrasting darker head and gaster. Additionally, the sculpture is rougher, better visible on head and mesosoma, with in general stronger rugae. Workers of *T. tuberum* (Fabricius, 1775) (Figures 5.18, 5.19, 5.29) have always a distinctly lighter mesosoma and less dark head than in *T. saxatilis*. Other Mediterranean darker coloured *Temnothorax* species are: *T. laestrygon* (Santschi, 1931), *T. niger* (Forel, 1894) and the usually lighter, but sometimes equally dark coloured *T. exilis* (Emery, 1869). The petiole of all three species is lower, triangular, and on apex with a more or less well visible crest in lateral view. Furthermore, these species occur only at lower elevations with Mediterranean climate. The arboreal species *T. affinis* (Mayr, 1855) is similar, when specimens are darker than usual, than they differ by distinctly longer propodeal spines. Very rarely specimens of *T. affinis* may have shorter spines in combination with darker reddish brown colour. In such a case, these specimens have a more triangular node apex and evenly reticulate head, without superimposed rugae. A morphologically similar species outside Italy is *T. anodontoides* (Dlussky & Zabelin, 1985) (Figures 5.20 & 5.21) from Transcaucasia and probably high mountains of Southern Greece. *T. saxatilis* and *T.* cf. *anodontoides* from Greece can be distinguished by morphometric characters (see Table 5.3), such as distinctly longer scapes, distinctly shorter propodeal spines and lower waist in *T.* cf. *anodontoides,* which is also different in its dark brown, nearly black colour, the coarser rugose sculpture, and the straighter and less rounded truncated petiolar node apex.

For comparison, only one gyne of *T. saxatilis* is available. The gyne of *T. nigriceps* has longer propodeal spines, more triangular, and edged petiolar apex in lateral view, and rougher sculptured head and mesosoma. *T. tuberum* is a morphologically variable species, but has a smaller head, and somewhat shorter propodeal spines than *T. saxatilis*. Furthermore, in *T. tuberum*, the mesosoma and especially the scutellum are more densely rugose, and they have evenly yellowish legs, whereas legs are somewhat bicoloured, mostly brown with ferrugineous patches in *T. saxatilis*. The gyne of T. *affinis* (Figures 5.42 & 5.43) is normally lighter coloured than in *T. saxatilis*, but darker specimens occur, then the propodeal spines are usually longer and the petiole is lower in profile. Head sculpture of *T. affinis* (Figure 5.43) is more reticulate and less rugulose than in *T. saxatilis*. The brownish to black gynes of *T. exilis*, *T. laestrygon* (Figures 5.44 & 5.45) and *T. niger* are distinctive in their low petiole with a triangular and acute node apex in profile. The morphometric differentiation of gynes of *T. saxatilis* and *T.* cf. *anodontoides* from Greece is difficult, only one gyne each is available for comparison. *T.* cf. *anodontoides* has a distinctly lower and

truncated node apex in profile. In addition, in this species the sculpture is mainly rugose and coarser. More material is needed to separate this different species.

Figures 5.2-5.5, 5.8-5.21, 5.26-5.29

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15


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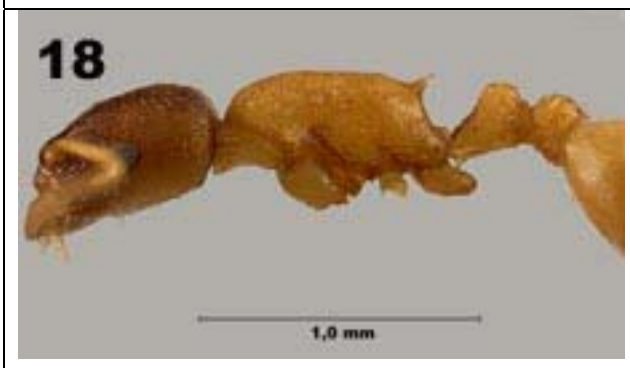
17


18
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19


20
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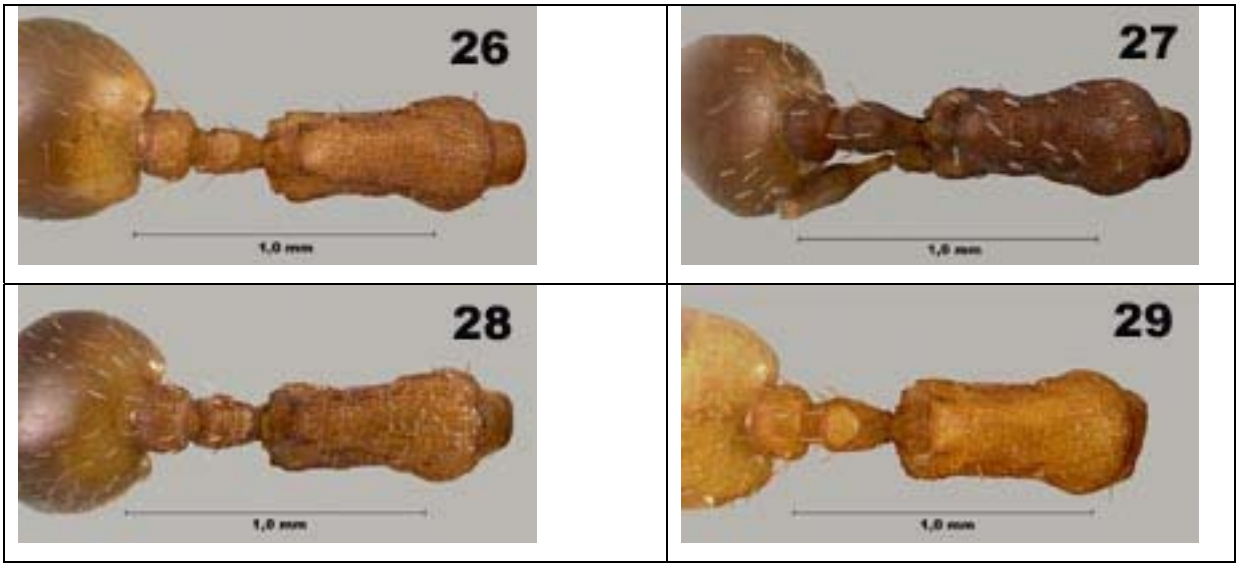
21



Temnothorax workers, left: lateral view of body, right: full-face view of head. Figures 5.2 & 5.3: *T. alienus* Holotype; 5.4 & 5.5: *T. satunini* Prov. Artvin, East Turkey.

Temnothorax workers, left: lateral view of body, right: full-face view of head. Figures 5.8 & 5.9: *T. lichtensteini* Southern Tyrol, Italy; 5.10 & 5.11: *T. italicus* Island Krk, Kroatia; 5.12 & 5.13: *T. saxatilis* Holotype*;* 5.14 & 5.15: *T. nigriceps* Arcadia, Peloponnesos, Greece.

Temnothorax workers, left: lateral view of body, right: full-face view of head. Figures 5.16 & 5.17: *T. tuberum* Southern Tyrol, Italy; 5.18 & 5.19: *T. cf. anodontoides* Arkadia, Peloponnesos, Greece. 5.20 & 5.21: *T. affinis* near Spitz, Wachau, Austria.

Temnothorax gynes, lateral views of body. Figures 5.26: *T. alienus* Holotype; 5.27: *T. saxatilis* Holotype.

Temnothorax gynes, left: dorsal view of body. right: full-face view of head. Figures 5.28 & 5.29: *T. alienus* Holotype.

Figures 5.30-5.35, 5.38-5.45


32
1,0 mm


33
1,0 mm


34
1,0 mm


35
1,0 mm


38
1,0 mm


39
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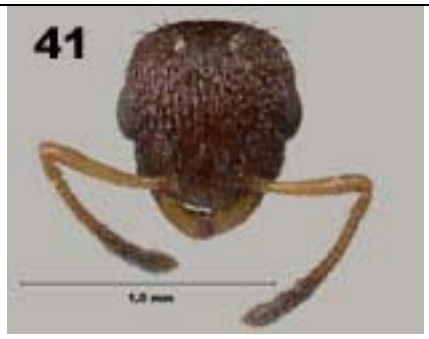
40
1,0 mm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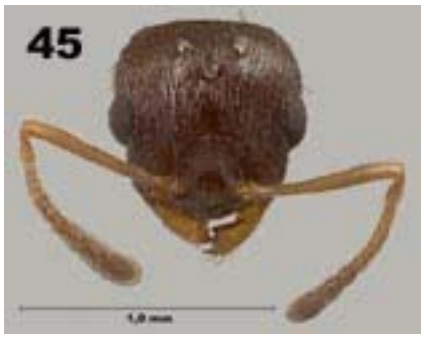

Figures 5.30 & 5.3: *T. nylanderi* Beira Alta, Sierra de Montemuro, Portugal; 5.32 & 5.33: *T. saxatilis* Holotyp. *Temnothorax* gynes, left: dorsal view of body. right: full-face view of head. Figures 5.34 & 5.35: *T. nigriceps* Arcadia, Peloponnesos, Greece.
Figures 5.44 & 5.45: *T. laestrygon* near St Giovanni, Calabria, Italy.

Acknowledgements

We wish to express our gratitude to the Staatliches Museum für Naturkunde, Karlsruhe, and specifically Christiana Klingenberg and Manfred Verhaagh, for providing the photographic equipment and for their gracious and very helpful advice. We also sincerely thank the following persons who made available relevant type material from different museums and were all very helpful: B. Merz (MHNG), V. Raineri (MCSN) R. Schultz (EMAU), J. Casewitz-Weulersse (MNHN) and W. Dorow (SMFM). We are grateful to Jack Longino and Fabricio Rigato for their comments of an early draft of this paper. We are also grateful to Maria-Helene Faulhaber, who most kindly improved the English. The fieldwork was supported by DFG (He 1623/15).

Key for Italian *Temnothorax* species

This key shall help to determine *T. alienus* nov. spec. and *T. saxatilis* nov. spec. The differentiation of *Temnothorax* species is often difficult. More material is needed to create a final key to all species. The gynes of *T. minozzii* and *T. lagrecai* are unknown to us.

SPECULARIS

Workers:

Smaller species HS < 670 µm, ML < 940 µm (rarely single specimens of diverse species have HS between 670 µm and 710 µm, than ML < 910 µm) **2**

-. Larger species HS > 680 µm, ML > 940 µm ..... Gaster without microsculpture ……... …….. (*T. algiricus* partim, *T. clypeatus, T. corticalis* partim, *T. nadigi*)

-. Gaster with microsculpture (*T. rottenbergi*)

Scapus shorter than HS. Mesosoma margin straight, convex or with a delimited metanotal groove (Fig. 2, 6, 8, 12, 14, 16) ……..... **3**

-. Scapus longer than HS. Mesosoma profile very unique with a deep and wave-like metanotal groove or depression ..... (*T. recedens*)

3. Lighter and +/- unicoloured yellow to orange species, with only partially darker gaster (Fig. 2, 3, 6, 7, 10, 11, 12, 13) **4**

-a. Dark coloured, reddish-brown, brown or black (Fig. 8, 9, 14, 15, 24, 25) …..... **7**

-b. Clearly bicoloured species, head and gaster darker than mesosoma and waist, if not bicoloured, than with brownish antennal clubs, which are darker than the remaining parts of antennae (Fig. 16, 17, 18, 19) ..... (*T. affinis p*artim, *T. albipennis, T. corticalis, T. exilis* partim, *T. interruptus, T. jailensis, T.*

lagrecai, T. menozzii, T. nigriceps, T. nylanderi partim, *T. crassispinus, T. tuberum, T. unifasciatus*)

4. Compound eyes smaller ED/HS: < 0.26 ..... **5**

-. Compound eyes larger ED/HS: > 0.26 ..... (*T. exilis* partim, *T. finzii, T. flavicornis*) [*T. flavicornis* mainly have 11 antennal segments (Fig. 6, 7)]

5. Mesosoma margin in lateral view lacking any metanotal groove or depression. Profile straight or slightly convex (Fig. 2, 12) ….....**6**

-. Mesosoma margin in lateral view with a +/- distinct metanotal groove or depression (please note, in single specimens rarely this groove is only a shallow depression, confusion is possible) (Fig. 8, 10) …… ……(*T. lichtensteini, T. nylanderi, T. parvulus*)

6. Propodeal spines shorter, PSL/ML: < 0.14 (mean 0.113), petiole high and sessil PEH/PEL: > 0.90 (mean 0.984), and broader PEW/PEL: > 0.68, anterior declivitous face of petiole margin in lateral view concave. Colour more orange than yellowish, with often partially brownish gaster. (Living on the ground in small sticks) (Fig. 2, 3) .... ***T. alienus* nov. spec.**

-. Petiole lower, and sessil PEH/PEL: < 0.92 (mean 0.833), anterior declivitous face in lateral view straight. (Arboreal species, in small dead sticks of trees, or in the bark, never nesting on the ground) (Fig. 12, 13)..... (*T. italicus, T. rabaudi*)

-. Propodeal spines longer, PSL/ML: > 0.12 (mean 0.146), petiole low and pedunculate PEH/PEL: < 0.90 (mean 0.839), and narrower PEW/PEL: < 0.68, colour lighter, more yellowish than orange, with evenly yellowish gaster. (Living on the ground often under small stones, or in stone cavities, but also in sticks) ..... (*T. luteus* and related species)

7. Without any metanotal groove or impression (Fig. 14, 16, 20) **8**

-. With a well visible metanotal groove or impression, antennal clubs in the same colour as the remaining antennae, or only gradually darker .... (*T. algiricus, T. angustulus, T. kraussei, T. saxonicus, T. sordidulus*)

8. Petiole short and broad, more subsessil. In lateral view with broadly truncated and with a slightly convex node PEH/PEL nest mean > 0.78, PEW/PEL nest mean > 0.61, sculpture of head evenly reticulate with median +/- unsculptured part (Fig. 14, 22) ……..... **9**

-. Petiole long and narrow, more pedunculate, in lateral view with slightly lower rounded or triangular node PEH/PEL nest mean < 0.82, PEW/PEL nest mean < 0.63, sculpture of head mainly missing, surface lucid, if sculptured then with fine longitudinal striae (Fig. 24, 25) ..... (*T. exilis, T. laestrygon, T. niger*)

9. Petiole subsessil, in lateral view broadly truncated, with rounded margin **10**

-. Petiole sessil, in lateral view clearly triangular with an edge on the top (arboreal species) .....*T. angustulus*

10. Propodeal spines longer than proximal antennae diameter or PSL/ML > 0.10 (mean 0.158) (please note, normally *T. affinis* (Mayr) is bicoloured, but sometimes darker specimens occur) (arboreal species) (Fig. 22, 23) ..... *T. affinis*

-. Propodeal spines shorter, rarely as broad as proximal antennae diameter or PSL/ML < 0.13 (mean 0.106) (living on the ground) (Fig. 14, 15) ***T. saxatilis* nov. spec.**

Gynes

1. Smaller species HS: < 900 µm + ML < 1500µm, petiole never globular **2**

-. Larger species HS: > 900 µm + ML: > 1500µm,..... ..... (*T. rottenbergi, T. clypeatus, T. nadigi*)

2. Scapes distinctly shorter than HS (SL/HS < 0.80) **3**

-. Scapes slightly shorter, or as long as HS (SL/HS > 0.90) ..... *T. recedens*

3. Species with larger gynes HL + ML > 1780 µm, MW/ML > 0.54 .....**4**

-. Species with very small gynes, HL + ML <1750 µm, (please note that sometimes microgynes occur in some species *e.g. T. unifasciatus* and sometimes *T. jaliensis* gynes are minimally larger, but than MW/ML is < 0.54 .... (*T. interruptus, T. finzii, T. jaliensis, T. flavicornis*)

4. Species with longer propodeal spines (PSL/ML > 0.04) **5**

-. Species with very short propodeal spines, only angles (PSL/ML < 0.04), if PSL/ML is minimally larger, than ML < 1250 µm .... (*T. albipennis, T. italicus, T. rabaudi, T. unifasciatus* partim)

5. Dark mainly brownish or blackish coloured species in combination with higher, sessil and +/- triangular petiole PEH/PEL > 0.90 (Fig. 40) ..... (*T. algiricus, T. angustulus, T. corticalis*, *T. kraussei,, T. nigriceps* partim, *T. sordidulus* partim)

-. Dark coloured with lower and more pedunculate petiole PEH/PEL < 0.95, not triangular, or lighter coloured with various petiole height and shape (Fig. 30, 31, 32, 34, 36, 38, 42, 44) ……..... **6**

6. Bicoloured with mesosoma and waist always lighter than head and gaster (orange to reddish brown), or evenly yellowish to orange coloured with minimal darker gaster (Fig. 30, 32, 34. 36) …..... **7**

-. Clearly darker, evenly brown to black, only with slightly lighter brown or reddish brown mesosoma and waist (31, 38, 42, 44) **12**

7. With evenly coloured antennae **8**

-. Clearly darker antennal clubs ..... (*T. unifasciatus*)

8. Darker coloured, with contrasting darker head and gaster in relation to lighter mesosoma and waist. Head distinctly denser sculptured with coarse rugae or carinae on frons, and mesosoma, in combination with smaller head HS: < 675 µm and long propodeal spines PSL/ML: > 0.10 ..... (*T. lichtensteini*)

-. Other character combinations HS: > 640 µm **9**

9. Petiole clearly pedunculate, and lower PEH/PEL < 0.86 colour variable .... (*T. luteus, T. crassispinus* partim)

-. Petiole subsessil or sessil and higher PEH/PEL > 0.83 ..... **10**

10. Sculpture of frons dense, but not rough, mainly fine striate, colour variable, propodeal spines longer PSL/ML: > 0.088, petiole narrower PEW/PEL: < 0.74 (Fig. 35) .... (*T. nylanderi, T. crassispinus*)

-. Sculpture of frons variable, never densely striate, median surface often unsculptured (Fig. 33) **11**

11. Propodeal spines longer PSL/ML: > 0.095, mesosoma narrower MW/ML: < 0.64 .... (*T. saxonicus* partim, *T. parvulus*)

-. Eyes shorter ED/HS < 0.30, scapes shorter SL/HS < 0.74, mesosoma broader MW/ML > 0.62, propodeal spines shorter PSL/ML: < 0.095, petiole very short PEL/ML < 0.35 (occur in Southern Italy) (Fig. 33) ..... ***T. alienus* nov. spec.**

-. Eyes larger ED/HS > 0.30, scapes longer SL/HS > 0.72, mesosoma broader MW/ML < 0.63, petiole longer PEL/ML > 0.35 (occur in Northern Italy) ..... *T. saxonicus*

12. With clearly darker antennal clubs **13**

-. With evenly coloured antennae, without darker clubs, or the antennae are generally brownish coloured ..... (*T. affinis*, *T. exilis, T. niger T. parvulus, T. sordidulus*)

13. Propodeal spines shorter PSL/ML: < 0.05, only angles or small tips **14**

-. Propodeal spines longer PSL/ML: > 0.05, well visible spines (Fig. 40, 42, 44) .... (*T. affinis (Mayr), T. exilis T. laestrygon, T. nigriceps, T. tuberum* partim)

14. Only gradually darker coloured femurae in relation to the other parts of the legs .....
[A.] Head size variable HS: 668-762, petiole lower PEH/PEL: < 0.83, node in lateral view +/- triangular. Sculpture dense but not rough (arboreal species) ..... *T. affinis*
[B.] Head size relatively small HS: < 730 µm, petiole height variable PEH/PEL: 0.73-1.00, node in lateral view with a +/- sloping plateau, sculpture deeper and rougher, mainly with dense carinae on head and mesonotum, and less dense developed reticulate sculpture elements (mainly alpine species) ..... *T. tuberum*

-. Clearly darker coloured femurae, in relation to the other parts of the legs. Petiole relatively high PEH/PEL: 0.885, in lateral view with a rounded truncated plateau. Sculpture dense, on head and on waist mainly reticulate to rugo-reticulate. General appearance with only a few carinae on mesosoma (Fig. 31, 38, 39) ..... ……***T. saxatilis* nov. spec.**

General discussion

In diverse taxa, geographical differences in genetic diversity have been interpreted as a consequence of postglacial expansion processes (Hewitt, 1996, 1999, 2000). By retreat of populations into small, separated refugia and their subsequent spreading, distinct lineages and reproductively isolated species were produced. Nowadays, species ranges often overlap to some extend and species sometimes hybridize along their contact zones (Remington, 1968; Taberlet et al., 1998; Swenson & Howard, 2004, 2005). The allopatric distribution of the sibling ant species *Temnothorax nylanderi* and *T. crassispinus* also fits into this scenario. The work presented in this thesis aimed at investigating distribution and genetic variability of North and South European populations of both species. Further, width and position of the contact zone were determined and the hybridization pattern between the species examined in detail. In the following, all results from this study are discussed in regard to speciation and hybridization patterns observed in other species.

Distribution and genetic diversity

In the South European regions, both *T. nylanderi* and *T. crassispinus* seem to be as abundant as in Central Europe. The sites were often densely populated, with few or no congenerics and even no other ant species present. Habitat preference ranged from dry pine forests (Southern France) to small humid ivy-covered woods consisting of beech and hazelnut trees in South Tyrol. Hence, both species seem to be very common over their entire range in Europe. Collection and classification confirmed previous findings that *T. nylanderi* exclusively occurs in Western and *T. crassispinus* in Eastern Europe, separated by a small zone of overlap. However, the contact zone runs far more west in Southern Germany than the previously located demarcation line in Northern Germany (Seifert, 1995) (see below). The two mitochondrial DNA markers did not reveal any phylogeographic structuring between populations in both species. Species division is therefore constant with 2.4 % and 3.5 % divergence in the CO I and Cyt b haplotypes, respectively (Chapter 1). Whereas morphological species determination did often not produce clear-cut results (Chapter 1) and sophisticated morphometric analyses are generally required (Seifert, 1995), DNA barcoding allowed unequivocal species distinction. Thus, this study constitutes yet another example for the more and more popular barcoding that enables species classification and the detection of cryptic species (e.g. Hebert et al., 2004a,b; Hajibabaei et al., 2006; Smith et al., 2006). The existence of two distinct genetic lineages together with the fixation of private alleles at the GPI locus and morphological differences (Seifert, 1995, Chapter 1) supports the idea of differentiation in isolated glacial refugia. Indeed,

an eastern and western lineage with a small zone of overlap can be found in many different taxa, e.g. in the brown bear *Ursus arctos* (Kohn et al., 1995), the grey partridge *Perdix perdix* (Luikkonen-Anttila et al., 2002), the butterfly *Maniola jurtina* (Schmitt et al., 2005) or the grasshopper *Chorthippus parallelus* (Cooper et al., 1995; Lunt et al., 1998).

A refugium in the Balkan region could also explain, why *T. crassispinus* reaches relatively far west in Southern Germany (Chapter 1). The somewhat lower Carpathian Mountains do not form such an invincible barrier as the Alps for populations derived from the western refugia, as also indicated by the distribution pattern of *C. parallelus* subspecies (Lunt et al., 1998). However, all ants from North-Eastern Italy could be unequivocally determined as *T. nylanderi* (Chapter 1). Therefore, it is more likely that *T. crassispinus* might have followed the Danube valley. In general, valleys appear to play an important role as glacial refugia during the last cold period (Lister, 1984). High altitude and east-western range of the Alps suggest the existence of a third genetic lineage in Southern Italy, as found in *C. parallelus* (Lunt et al., 1998). A distinct *T. nylanderi* population could still exist in the very south of Italy. This however is rather unlikely, because the Sila Mountains specimen from the southern tip of the peninsula fitted into the star-like haplotype network for both mt DNA loci (Chapter 1). Apart from that, the occurrence of *T. nylanderi* in the south is restricted to regions of higher altitude. Lower sites are occupied by more thermophilic Mediterranean species, or, potentially suitable *T. nylanderi* sites were inhabited by other congenerics (Chapter 5). In Spain, too, ancestral founder populations could have been overlooked as colonies were only collected in Catalonia.

Due to the absence of any phylogeographic structure, expansion events from cryptic forested refugia in the North (Stewart & Lister, 2001) are unlikely. This would require different centres of higher genetic diversity both in Central and Southern Europe, which has been shown in the forest-living North American chipmunk *Tamias striatus* (Rowe et al., 2004). The mismatch pair histograms fit the sudden expansion model (Slatkin & Hudson, 1991; Rogers & Harpending, 1992) and therefore suggest rapid re-colonization. Populations in the refugia must have already been homogenous, because the central haplotypes in the diagram represent ancestral haplotypes (Avise, 2000) and do not reflect any geographical origin. In the leaf beetle *Gonioctena pallida* that is restricted to only a few plant species, several star-like haplotype networks were geographically related to sites in the Vosges Mountains. This clearly demonstrates rapid expansion from several founder populations (Mardulyn, 2001). Habitat requirements of *T. nylanderi* and its sibling species are much more less specific and both male and female sexuals are winged. Therefore, founder populations and re-colonization routes might be currently undetectable.

The molecular clock is estimated to be rather similar among animals, with approximately 2% divergence per million years in mammals and *Drosophila* (Brown et al., 1979; DeSalle et al., 1987), and 2.3% in arthropods in general (Brower, 1994). Due to the relatively high divergence between the species (2.4% in the CO I and 3.5 % in the Cyt b haplotypes (Chapter 1)), lineage divergence might have started at the end of the Pliocene or in the early Pleistocene. However, gene and species divergence can evolve differently and thus ignored rate variation among lineages might overestimate the divergence time (Nichols, 2001; Aris-Brosou & Yang, 2003; Cranston & Rannala, 2005). The neutrality of mitochondrial DNA markers is meanwhile questioned in several studies (Ballard & Whitlock, 2004). According to a very recent study, mt DNA apparently does not reflect species abundance due to recurrent adaptive evolution (Bazin et al., 2006). Thus, divergence time and size of ancestral populations cannot be clearly deduced from the results obtained by the two mt DNA markers (Chapter 1).

Generally, in contrast to biologically similar congeners, both sibling species exhibit low mt DNA variability. In the North American ant *T. longispinosus*, COI / COII sequences from five populations less than 1000 km apart diverged by up to 12%, despite a lack of genetic differentiation at nuclear loci (Brandt et al., submitted). The European, more thermophilic congener *T. unifasciatus* also seems to exhibit a higher level of mt DNA variability (H. Sturm, unpublished data). However, detailed phylogeographical analyses do not exist. Genetic variability of nuclear markers is also low in comparison to other ants, e.g. *Camponotus* (Burgman et al., 1980) or other *Temnothorax*/*Leptothorax* species (Heinze et al., 1995, 1997). Hence, the maternally transmitted endoparasitic bacterium *Wolbachia*, observed in *T. nylanderi* (Wenseleers, 1998), is probably not the only factor responsible for its genetic impoverishment (Chapter 1). Indeed, *Wolbachia* has not been recorded yet for *T. crassispinus*. Whether the equally low level of mt DNA variability in *T. crassispinus* could also be the consequence of an infection, remains to be investigated. The occurrence of two groups of mitotypes and corresponding different *Wolbachia*-strains in subspecies of the *Porcellionides pruinosus* group (Marcadé et al., 1999) might also apply for the sibling ant species. In any case, the use of nuclear markers is required if populations are infected by *Wolbachia* (Hurst & Jiggins, 2005). Nuclear DNA sequence markers are needed to clarify, whether selective sweeps inflicted by endosymbionts or bottlenecks are responsible for the low variability.

According to the accumulating evidence questioning the exactness of mt DNA molecular clocks (see above), the role of the glacial-interglacial periods during the Pleistocene for the origin of the sibling species remains unclear. Models have given evidence that the Pleistocene has had a positive effect on divergence in grasshoppers (Knowles et al., 2001). Apart from that,

the influence of repeated glaciation on speciation during the Pleistocene is controversially debated (Avise et al., 1998; Avise & Walker, 1998; Klicka & Zink, 1997, 1999). A recent study on Pleistocene mammals, including three extinct species, revealed a complete lack of phylogeographical structuring before the last glaciation (Hofreiter et al., 2004). In the here investigated ants, the distinctive mt DNA lineages might have evolved in small isolated refugia very early, which would fit the molecular clock scenario. Because of the equally low mt DNA variability, both species/lineages apparently have had experienced a similar demographic history. Scientific interest and work on ant phylogeography has just started. The phylogeographical study of two closely related, sympatrically distributed boreal *Formica* species across Eurasia also implies different vicariant histories and similar signs of subsequent expansion (Goropashnaya et al., 2004). However, in one of the species a phylogeographical division was found, suggesting re-colonization from different refugia, which was neither found for *T. nylanderi* nor *T. crassispinus*.

Contact zone and hybridization

The contact zone between *T. nylanderi* and *T. crassispinus* does not exceed 25 km (Chapter 1) and therefore is rather narrow like in many hybrid zones (Barton & Hewitt, 1985). Maintenance of the pure lineages without hybrid inviability is often guaranted by differences in morphology and ecology. This e.g. has been observed in the contact zone between *Heliconius* species (Jiggins et al., 1997). Differences exhibited in courtship behaviour apparently inhibit mating even on the subspecies level in *Chorthippus* (Tregenza et al., 2000). Moreover, species-specific habitat preferences also lead to low levels of introgression despite successful hybridization in the contact zone between *Bombina bombina* and *B. variegata* (Kruuk & Gilchrist, 1997; Vines et al., 2003; Nürnberger et al., 2004). In the here investigated sibling species, rare occurrence of hybrid individuals (Chapter 1) and almost complete absence of heterozygote adult queens despite equal numbers of heterozygote and homozygote virgin queens (Chapter 2) also clearly demonstrate reduced hybrid fitness. However, morphological differences are rather small, no habitat patchiness could be observed and sexuals of both species attend nuptial flights around the same time of the year (Seifert, 1996). Therefore, shifts in mating time have not been observed yet. Apparently, reproductive barriers are not very effective, as many colonies with heterozygous individuals can be found at sympatric sites (Chapter 2). Reviews of hybrid zones in many taxa have claimed endogenous selection to be the most important factor for the stability of hybrid zones (Barton & Hewitt, 1981, 1985). Probably, also here, negatively interacting genes of hybrid individuals are the main cause for hybrid inferiority. Nevertheless,

the evolution of pre-mating isolation mechanisms, like shifts in mating time can not be completely ruled out. They might even exist to some extent in the ant species pair. The described isolation mechanism evolved in the hybrid zone between *Chorthippus brunneus* and *C. jacobsi* and keeps it stable (Bailey et al., 2004).

All in all, hybridization has been reported for several *Temnothorax* species (Douwes & Stille, 1991), for *Lasius* (Pearson, 1983), *Acanthomyops* (Umphrey & Danzmann, 1998), *Formica* (Czechowski, 1993; Seifert, 1999) or *Solenopsis* (Ross et al., 1987; Shoemaker et al., 1996). The majority of these studies suggested hybrid inferiority, as it was found in this study. This is clearly evidenced not only by the lack of heterozygous adult queens, but also by the lower weight of gynes reared in colonies containing hybrid individuals. The latter does not seem to be a direct effect of hybridization, because homozygous and heterozygous female sexuals from hybrid-colonies did not differ significantly in their dry weights. The same accounts for worker weight (Chapter 2). Reduced hybrid worker fitness affecting brood caring and foraging abilities might play an additional role.

The detailed analysis of the Velburg hybrid site revealed an interesting pattern in the distribution of colonies containing hybrids. Almost all heterozygous individuals were collected in colonies from a forest inhabited almost exclusively by *T. crassispinus*, despite the close proximity of both another *T. crassispinus* and a *T. nylanderi* site (Chapter 2). As habitat requirements appear to be identical in both species, an asymmetrical introgression from one species of one habitat type into the other, like in *Bombina* (Vines et al., 2003) can be ruled out. The side-restricted distribution of heterozygous individuals is connected to asymmetric mating. Due to the mt DNA analyses, the hybrid individuals collected within the *T. crassispinus* site were the offspring of *T. crassispinus* queens mated to *T. nylanderi* males. Hybrid individuals from the *T. nylanderi* sites with the respective other haplotype were very rarely found. Unidirectional mating events are common in hybridizing species (Szymura et al., 2000; Redenbach & Taylor, 2003; Salazar et al., 2005). In this study system, it might result from morphological or behavioural differences between sexuals of the two species. The endosymbiont *Wolbachia*, until now only documented for *T. nylanderi* (Wenseleers, 1998), might also affect the direction of hybridization, as it has been observed in hybrid zones of the *Diabrotica* beetles and the *Gryllus* crickets (Giordano et al., 1997). Furthermore, as both species mate in swarms, the prevailing direction of the wind could be another factor leading to the observed asymmetry. This would mean annual fluctuations in the distribution of hybrids at the three sites. Indeed, analyses from 2006 revealed a considerable number of heterozygote workers from the *T. nylanderi* site (J. Trettin, personal communication). The astonishing, yet

rare finding of the intermediate haplotypes (Chapter 2) remains an enigma. Because intraspecific variation in mt DNA sequences is extremely low in both parental species (Pusch et al., 2006) and paternal leakage enabling mitochondrial DNA recombination is an extremely rare event in animals (Ladoukakis & Zouros, 2001; Rokas et al., 2003; Gantenbein et al., 2005). Besides that, the existence of mitochondrial DNA recombination is controversially debated in humans (Awadalla et al., 1999; Eyre-Walker et al., 1999; Kraytsberg et al., 2004; but see Piganeau & Eyre-Walker, 2004).

Pure hybrid colonies with solely heterozygous workers, headed by a homozygous *T. crassispinus* queen have been rarely found in this two-year study. The pre-dominating mixed colonies consisting of both *mm* and *mf* workers could have originated by multiple mating, or the co-existence of several matrilines. Although multiple mating has been observed in *T. nylanderi* in behavioural studies in the laboratory (Plateaux, 1970, 1978), all population genetic analyses suggest regular monandry (Foitzik et al., 1997; Foitzik & Heinze, 1998, 2000; Foitzik et al., 2003; Strätz & Heinze, 2004). Nevertheless, multiple mating, which has been documented in other ants (e.g. Cole, 1983) might sometimes occur. However, colonies with more matrilines are rather common in parental species due to colony usurpation by founding queens or colony fusion (Foitzik & Heinze, 1998, 2001; Strätz et al., 2002; Strätz & Heinze, 2004; Tichá, 2002; Tichá & Štys, 2002). In these species, colony fusions are enabled by the strong influence of nest material on colony odour, which has been shown for *T. nylanderi* (Heinze et al., 1996). Hence, the weak genetically based nestmate recognition system and the fact that most of the colonies were found in the same nest material at Velburg, may explain, why colonies founded by an interspecifically mated queen probably also easily fuse with pure species colonies. Microsatellite data on both pure species and hybrid colonies (S. Träger, personal communication) additionally showed that pure species colonies exhibited two or more matrilines to the same extent as colonies containing hybrids. However, genetic heterogeneity per se, caused by the co-existence of more matrilines, may negatively affect work efficiency as has been previously demonstrated in *T. longispinosus* (Trampus, 2001; Foitzik et al., 2003).

Despite the high similarity between the sibling species, endogenous and probably also exogenous factors seem to keep species boundaries stable. All in all, the influence of hybridization on colony level remains relatively small (Chapter 2) and hybridization apparently leads to an evolutionary dead-end. This stands into stark contrast to well-investigated *Pogonomyrmex* lineages, or a *Solenospis* hybrid zone, where genetic caste determination arouse through hybridization (Helms Cahan et al., 2002; Julian et al., 2002; Volny & Gordon, 2002; Helms Cahan & Keller, 2003; Helms Cahan & Vinson, 2003; Helms Cahan et al., 2004).

More extensive studies with variable nuclear markers and the investigation of several hybrid sites within the contact zone would help to determine the steepness of clines. Another approach would be to test for chromosomal differences between *T. nylanderi* and *T. crassispinus*. Differences on the cytogenetic level have helped to investigate e.g. two hybrid zones of *Sorex* species (Brünner et al., 2002).

Colony structure and inbreeding

Both *T. nylanderi* and *T. crassispinus* are monogynous and certainly also monandrous according to previous population genetic analyses. However, populations exhibit a complex structure, due to polydomy, occasional colony fusions and founder queen usurpations. The latter two explain the unexpected genetic heterogeneity of some nests (Foitzik et al., 1997; Foitzik & Heinze, 2000, 2001; Strätz & Heinze, 2004; Chapter 4). Apparently, the weak endogenous nest-mate discrimination system is characteristic not only for *T. nylanderi* (Heinze et al., 1996), but also for *T. crassispinus*. This is demonstrated by successful interspecific fusions in one third of laboratory experiments under natural conditions, where one colony lacked a nest site (Chapter 3). Normally, endogenous nestmate recognition cues are considered the primary factors for nest-mate recognition (e.g. Smith & Breed, 1995). This also accounts for the North American congener *T. longispinosus*. In this species, genetic cues enable colony-mate recognition despite of spatial subdivision (Stuart & Herbers, 2000). On the other hand, in the also polydomous, very close relative *T. curvispinosus*, colony segregation within multicolonial populations is largely maintained by environmentally-based nestmate recognition cues (Stuart, 1987). In *T. nylanderi*, the environmentally-based nestmate discrimination is explained by loss of genetic variation and microhabitat uniformity (Heinze et al., 1996). This probably also accounts for its sibling species *T. crassispinus*. The high relatedness between the sibling species, supported by chemical analyses of cuticular hydrocarbons (Foitzik et al., in press) explains, why also in interspecific fusions, heterospecific workers remain in the same colony. In most cases, even one queen was killed in order to maintain monogyny (Chapter 3), which reminds of interspecific social parasitism (Buschinger, 1986).

Apart from revealing colony heterogeneity and thus reduced intra-nest relatedness, the population genetic analyses of several populations from both Central and South Europe confirmed the occurrence of the astonishingly high inbreeding found in a well-studied German population (Foitzik & Heinze, 2001). In general, inbreeding effects are normally detrimental and are avoided in natural populations (e.g. Charlesworth & Charlesworth, 1987). However, in species inhabiting patchy, unpredictable microhabitats, where only closely related males are

available, inbreeding without negative effects can be found, e.g. in the rat *Parotomys littledalei* (Pillay, 2002). Inbreeding is quite rare in ants with their large scale mating flights, but resulting inbreeding depressions are less common, probably because of extinction of lethal recessive alleles in the haploid males (Crozier, 1970; Keller & Passera, 1993; Keller & Fournier, 2002). In the polygynous ant *Plagiolepis pygmaea*, inbreeding by philopatry of both sexes and re-adoption of mated queens into their natal nests, enhances intra-colony relatedness that is rather lowered by polygyny. Significant inbreeding exhibited in the ant *Formica exsecta* is caused by sex-biased dispersal (Sundström et al., 2003). Both cases however can not be applied to *T. nylanderi* due to mating swarms, where both sexes take part. Here, the locally dense, but on a wider scale patchily distributed populations and the fact that only part of the colonies produce sexuals, might affect the rate of inbreeding. This was suggested for the lek-mating ant *Pogonomyrmex occidentalis* (Cole & Wiernasz, 1997). Interestingly, inbreeding does not seem to have a negative effect in this species, taking both its high nesting density and wide range into consideration. Low genetic variability might lead to unicoloniality, a trait exhibited in the introduced, very successful invasive ant *Linepithema humile* (Krieger & Keller, 2000; Tsutsui et al., 2000). However, despite occasional colony fusions, the here examined sibling species still exhibit clear colony structures. Apart from that, other characteristics, typical for invasive ants like polygyny or colony reproduction by budding (Holway et al., 2002) lack in *T. nylanderi* and *T. crassispinus*. Thus, the success of both species despite low genetic variation and inbreeding remains unclear.

Figure 6: Virgin queen of *T. nylanderi*

Summary

The present-day range distribution of many species has been influenced by re-occuring glacials of the past. By the retreat into different refugia situated in more southerly regions, species were geographically separated and sometimes diverged into separate lineages. After having re-occupied their formerly ice-covered habitats, these species pairs nowadays often exhibit parapatric distribution and some hybridize along their contact zones. Such a scenario probably also accounts for the origin of the very common monogynous and monandrous ant sibling species *Temnothorax nylanderi* and *T. crassispinus*. While the latter inhabits Eastern Europe and the Caucasus, *T. nylanderi* can be found in Western Europe. They occasionally hybridize along a narrow contact zone in North-Western Germany. In this study, using morphometry and genetic markers, the position of the contact zone in Southern Germany and Northern Italy was determined. The differentiation within and between the two species was analysed and the genetic structure of Central and South European *T. nylanderi* populations was compared. Further, the impact of hybridization on colony level was studied in detail.

Previous findings were confirmed, that *T. nylanderi* can be exclusively found in Western and *T. crassispinus* in Eastern Europe, separated by a small zone of overlap. In Bavaria, the contact zone is situated along the Franconian Jura. South of the Alps, it probably lies somewhere in North-Eastern Italy, because in Slovenia and Croatia, only *T. crassispinus* has been found. The contact zone therefore runs far more west than expected from previous results. The genetic division between the species was constant with 2.4% and 3.5 % divergence in the CO I and Cyt b haplotypes, suggesting that the species might have split 1.5-2 Myr ago. Both species exhibited genetic uniformity on the nuclear and mitochondrial level. Their haplotypes revealed a lack of geographical origin, indicating rapid post-glacial recolonization.

Further population genetic studies using microsatellite markers on several Central and South European *T. nylanderi* populations revealed equal rates of heterogenous (two or more matrilines) colonies due to colony fusion, a typical characteristic of this species. Thus, also in southern populations, colony odour appears to be mainly environmentally determined. Besides that, no bottleneck-induced north-south differences in genetic variability could be proofed. The significant inbreeding in both South and North European populations is probably a species-specific trait. It might result from random mating over time and habitat patchiness.

As the sibling species are morphologically very similar, hybrids were classified by heterozygosity at the diagnostic allozyme locus GPI. Like in Northern Germany, both species co-occured in a rather narrow contact zone, which does not extend over more than 25 km. The narrowness of the contact zone apparently demonstrates hybrid inferiority. This was further confirmed by the detailed analysis of the hybrid zone, were almost no fertile hybrid queens were found. Pure hybrid colonies, consisting of exclusively heterozgous workers were rare. Instead most colonies contained both hybrid and *T. crassispinus* workers and co-occurred with pure *T. crassispinus* colonies. Besides that, the weight of hybrid virgin queens, which were as abundant as pure species' gynes (mostly *T. crassispinus*), is apparently negatively influenced by hybridization. However not directly, because co-occuring homozygous queens in hybrid colonies were also lighter. Mating was found to be almost exclusively unidirectional according to mitochondrial DNA data.

Mixed hybrid colonies apparently originate by colony fusions between pure and hybrid lineages, as other reasons can be ruled out. The existence of interspecific colony fusions in nature has been additionally supported by laboratory experiments, where heterospecific fusions could be documented. This is probably facilitated by the high relatedness between the species and the environmentally influenced nestmate discrimination.

Hybridization between the sibling species *T. crassispinus* and *T. nylanderi* apparently leads to an evolutionary dead-end. However it does not seem to be too costly, as hybrid colonies produced slightly more workers than pure species colonies.

Zusammenfassung

In der Vergangenheit wiederholt auftretende Eiszeiten haben die derzeitige Verbreitung vieler Arten geprägt. Während dieser Eiszeiten wurden manche Arten durch den Rückzug in unterschiedliche Refugien in südlichere Regionen getrennt und entwickelten sich dort zu eigenen Arten. Nach der Wiederbesiedelung ihrer einstmals eisbedeckten Habitate sind diese Artenpaare nun häufig parapatrisch verbreitet. Einige hybridisieren innerhalb ihrer Kontaktzone. Die häufig vorkommenden, monogynen und monandrischen Zwillingsarten *Temnothorax nylanderi* und *T. crassispinus* entstanden wahrscheinlich auch auf diesem Weg. Während letztere Osteuropa und die Kaukasusregion besiedelt, ist *T. nylanderi* in Westeuropa zu finden. Innerhalb einer schmalen Kontaktzone in Nord-Ostdeutschland wurden gelegentlich auch Hybride gefunden. In dieser Arbeit wurde die Position der Kontaktzone in Süddeutschland und Norditalien mit Hilfe von Morphometrie und genetischen Markern bestimmt. Es wurde die Artdifferenzierung untersucht und die Struktur von zentral- und südeuropäischen Populationen miteinander verglichen. Des weiteren wurde untersucht, wie sich die Hybridisierung zwischen den beiden Arten auf Kolonieebene auswirkt.
Die Verbreitung der Arten in den untersuchten Gebieten entspricht vorangegangenen Untersuchungen; *T. nylanderi* konnte also nur in Westeuropa, *T. crassispinus* nur in Osteuropa gefunden werden. Beide Arten sind durch eine schmale Kontaktzone, in der die Verbreitungsgebiete beider Arten überlappen, getrennt. In Bayern befindet sich die Grenze innerhalb des fränkischen Juragebietes. Südlich der Alpen liegt das Überlappungsgebiet wahrscheinlich in Nordwestitalien, da in Slowenien und Kroatien ausschließlich *T. crassispinus* Kolonien gefunden wurden. Im Hinblick auf die vorherigen Untersuchungen befindet sich die Kontaktzone im Süden also weiter westlich als erwartet. Auf genetischer Ebene unterschieden sich die Arten konstant durch 2.4% and 3.5 % Sequenzdivergenz der CO I und Cyt b Haplotypen, was den Beginn der Artentstehung auf 1.5-2 Millionen Jahre datiert. Beide Arten sind sowohl auf nuklearer als auch auf mitochondrialer Ebene äußerst uniform. Die Haplotypen lassen kein geographisches Muster erkennen, was auf eine sehr rasche, nacheiszeitliche Wiederbesiedelung schließen lässt.
Die populationsgenetischen Studien mit Mikrosatellitenmarkern zeigten keinen Unterschied in der Anzahl homogener (eine Matrilinie) und heterogener (zwei oder mehrere Matrilinien) Kolonien von *T. nylanderi* Populationen aus Zentraleuropa und Südeuropa. Heterogene Kolonien wurden in zentraleuropäischen Populationen beobacht und werden durch das hauptsächlich durch Umweltweltfaktoren geprägte Kolonieerkennungssystem erklärt - dasselbe gilt demnach für Populationen aus Südeuropa. In dieser Studie wurden des weiteren keine durch einen ‚Flaschenhals'-Effekt hervorgerufenen Unterschiede an genetischer Variabilität zwischen nordeuropäischen und südeuropäischen Populationen gefunden. Die hohe Inzucht, die in allen europäischen Populationen gefunden wurde, ist demnach ein artspezifisches Merkmal. Sie könnte über einen längeren Zeitraum durch nicht zufällige Paarungen und auf sehr kleine Flächen begrenzte Habitate zustande kommen.
Aufgrund der hohen morphologischen Ähnlichkeit wurden Hybride durch Heterozygosität des artspezifischen Allozymmarkers GPI bestimmt. Ähnlich wie in Norddeutschland kamen beide Arten ausschließlich innerhalb einer maximal 25 km breiten Kontaktzone vor. Die schmale Überlappungszone lässt auf eine Selektion gegen Hybride schließen. Dies legt auch die detaillierte Untersuchung einer Population mit Hybriden nahe, bei der nahezu keine Hybridköniginnen gefunden wurden. Ausschließlich aus Hybridtieren bestehende Kolonien waren sehr selten, statt dessen kamen die meisten Hybridtiere in Kolonien mit *T. crassispinus* Arbeiterinnen vor. In dieser Population kamen neben den gemischten Kolonien auch reine *T. crassispinus* Kolonien vor. Das Gewicht der Hybridjungköniginnen, die genauso zahlreich vorkamen wie die der Elterarten, wird negativ durch die Hybridisierung beeinflusst. Dies scheint jedoch keine direkte Folge ihrer Hybridherkunft zu sein, da Jungköniginnen mit dem

Genotyp einer Elternart (meistens *T. crassispinus*) aus der selben Kolonie auch leichter waren. Gemäß den Untersuchungen mit mitochondrialer DNA kamen Paarungen fast ausschließlich unidirektional zustande.
Die gemischten Hybridkolonien entstehen höchstwahrscheinlich durch Koloniefusionen zwischen Kolonien der Elternart und Hybridkolonien, da andere Gründe hierfür ausgeschlossen werden können. Das Zustandekommen heterospezifischer Koloniefusionen wurde zusätzlich in einem Laborexperiment gezeigt. Dies wird wahrscheinlich durch die hohe Verwandtschaft der Schwesternarten und das hauptsächlich durch Umweltfaktoren beeinflusste Kolonieerkennungssystem begünstigt.
Die Hybridisierung zwischen den beiden Arten führt offensichtlich in eine evolutionäre Sackgasse. Dieser Verlust wird jedoch dadurch ausgeglichen, dass in den Hybridkolonien eine etwas höhere Anzahl an Arbeiterinnen produziert wird.

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