Mitochondrial markers in the ant *Leptothorax rugatulus* reveal the population genetic consequences of female philopatry at different hierarchical levels

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Abstract:

Leptothorax rugatulus, an abundant North American ant, displays a conspicuous queen size polymorphism that is related to alternative reproductive tactics. Large queens participate mainly in mating flights and found new colonies independent of their mother colony. In contrast, small queens do not found new colonies independently, but seek readoption into their natal nest which results in multiple-queen colonies (polygyny). Populations differ strongly in the ratio of small to large queens, the prevalent reproductive tactic and colony social structure, according to ecological parameters such as nest site stability and population density. This study compares the genetic structure of two strongly differing populations within the same mountain range. Data from microsatellites and mitochondrial DNA give no evidence for alien reproductives in polygynous colonies. The incidence of alien workers in colonies (as determined by mitochondrial haplotype) was low and did not differ between monogynous and polygynous colonies. We found significant population viscosity (isolation-by-distance) at the mitochondrial level in only the predominantly polygynous population, which supports the theoretical prediction that female philopatry leads to mtDNA-specific population structure. Nuclear and mitochondrial genetic diversity was similar in both populations. The genetic differentiation between the two investigated populations was moderate at the mitochondrial level, but not significantly different from zero when measured with microsatellites, which corroborates limited dispersal of females (but not males) at a larger scale.

KEYWORDS
alternative tactics • colony structure • local adaptation • nestmate recognition • reproductive strategy • social insects

Article:

INTRODUCTION

The population structure of any species is the outcome of mating, dispersal and mortality patterns of individuals, and many theoretical and empirical studies have demonstrated the effects of inbreeding (e.g. Storz *et al*. 2001), restricted dispersal (e.g. Hansson *et al*. 2002) and local extinction (e.g. Wade & McCauley 1988). The current ease to generate powerful genetic markers and advances in analysis procedures (Sunnucks 2000) has led to studies in a great variety of

plants and animals. Overall, it has become clear that differentiation between populations depends not only on their geographical distance, but also on physical barriers (Harrison & Hastings 1996), the dispersal capabilities of organisms under consideration (Slatkin 1985) and the degree of local adaptation (Cooper 2000).

Commonly, population differentiation is more pronounced in sessile organisms, although many plants and sessile animals have a dispersing life history stage. Social insects in general, and ants in particular, build permanent, sedentary nests and hence can be considered to follow a sessile life history strategy (Bourke & Franks 1995). During mating and dispersal flights the sexual castes function as dispersing propagules, while colonies remain reasonably stationary after their foundation. Furthermore, ants resemble plants in their prevalence of alternative reproductive tactics (Hölldobler & Wilson 1977; Heinze & Tsuji 1995; Rüppell & Heinze 1999). The most common, and presumably ancestral, mode of reproduction is the emission of sexuals, which found a new colony independently after mating and dispersal flights (Hölldobler & Wilson 1990). However, alternative reproductive tactics, dependent on existing colonies, have been demonstrated in some species and suggested in many others (Hölldobler & Wilson 1990; Bourke & Franks 1995; Heinze & Tsuji 1995; Crozier & Pamilo 1996).

The most common alternative reproductive tactic for ant queens is readoption into their natal colonies, where they may remain as additional reproductives or disperse with part of the colony (budding). The readoption of queens leads to colonies with multiple reproductive queens (polygyny), in contrast to colonies with only one queen (monogyny) in independently founding ant species (Bourke & Franks 1995). Budding is considered as a mechanism for short-range dispersal due to the lower mobility of the participating, nonflying workers. Consequently, clusters of related colonies and a significant population viscosity are expected when readoption followed by budding is the predominant reproductive tactic. This has been demonstrated in a few species (e.g. *L. acervorum*: Stille & Stille 1993; *M. ruginodis* and *M. rubra*: Seppä & Pamilo 1995; *Solenopsis invicta*: Ross & Shoemaker 1997) but intraspecific comparative data sets supporting the prediction of a positive association of dependent colony founding and population viscosity are needed.

The genetic colony structure of ants becomes more complex when additional reproductives are (re)adopted. The higher genetic diversity resulting from several reproducing individuals in colonies leads presumably to a more diverse olfactory colony recognition template (Hölldobler & Michener 1980). Thus, polygyny commonly leads to a decrease in nestmate recognition ability (Bourke & Franks 1995) and a reduced discrimination against non-nestmates. This may result in integration of unrelated individuals into the colony and facilitates intraspecific parasitism. However, intraspecific data supporting the link between polygyny and nestmate recognition are rare (Bourke & Franks 1995).

The North American ant *L. rugatulus* exhibits a pronounced queen size dimorphism (Rüppell & Heinze 1998) that is related to alternative reproductive tactics. Several lines of independent evidence suggest that large queens (macrogynes) mainly found their colony independently, while small queens (microgynes) specialize in dependent colony foundation by readoption into the natal colony (Rüppell *et al*. 2001a). The ratio of small to large queens, and consequently the predominant reproductive tactic, differs strongly among populations. Microgynes are prevailing

in populations that are characterized by stable, rocky outcrops as nesting habitat that limit colony growth little in time and space and support high population densities. Macrogynes mainly occur in less dense populations with less stable, smaller nesting sites, presumably because opportunities for independent colony founding are more abundant and readoption is less favourable (Rüppell *et al*. 2001a). In the Chiricahua Mountains (Arizona), these habitat characteristics follow an altitudinal cline: the mountain tops, characterized by rocky outcrops with abundant crevices, are predominated by microgynes, while the downhill slopes, with small stones as *L. rugatulus* nest sites, are predominated by macrogynes.

With nuclear markers (microsatellites) no genetic structure was found in different *L. rugatulus* populations, regardless of the predominant reproductive tactic (Rüppell *et al*. 2001a). To investigate whether population homogeneity is due to extensive male dispersal or long-range budding, our current study combined the mitochondrial and nuclear population structure of two adjacent populations from Chiricahua Mountains, that strongly differed in their predominant queen morphology, reproductive tactic and colony social structure. The selection of these two nearby populations also allowed for a comparison of nuclear and mitochondrial differentiation between them. Furthermore, multiple workers from single colonies were tested for their mitochondrial haplotype to investigate the presence of unrelated individuals in the nest, indicating nestmate recognition errors.

MATERIALS AND METHODS

Colony collection

Complete colonies of *L. rugatulus* (Emery) were collected by aspiration from two populations in the Chiricahua Mountains in Southeast Arizona (32° N, 109 W) close to the southern distribution limit (Creighton 1950; Rüppell *et al*. 1998, 2001a). We compared a typical low-altitude population 'West Turkey Creek' (WTC: 1880 m) with a typical high altitude population 'North Barfoot Lookout' (NBL: 2540 m), which differ strongly in queen morphology and social structure (Rüppell *et al*. 2001a) but are only 7 km apart.

The location of a subset of colonies relative to each other was determined within both populations from direct measures of intercolony distances. After censusing most colonies were stored in 95% ethanol for subsequent DNA extraction, but some were transported back to the laboratory alive and killed by freezing prior to DNA extraction. The different storage conditions did not noticeably affect the quality of the subsequent analyses.

Laboratory procedures

DNA was extracted from whole animals in frozen state or dried at room temperature after ethanol storage. Total DNA extractions for microsatellite genotyping were performed using Chelex® resin (Altschmied *et al*. 1997), but the template quality proved insufficient for RFLP-analyses of the mtDNA. Consequently, DNA for mtDNA analyses was extracted using the PureGene kit (Gentra Systems) as described by Foitzik & Herbers (2001), omitting the ProteinaseK digestion step.

Polymerase chain reaction (PCR)-amplification of four (di-nucleotide) microsatellite loci (LXGT104, LXGT218 and LXGT228 adapted from *L. spinosior* (Hamaguchi *et al*. 1993) and L18 adapted from *L. nylanderi* (Foitzik *et al*. 1997) was performed, using 1/200 of the genomic

DNA as template in a total reaction volume of 20 µL and for each locus slightly differing PCR conditions (see Appendix I). PCR products were labelled internally with P^{33}, separated on standard sequencing gels, which were exposed to Kodak Biomax® film. Fragment sizes were determined by comparison to the Sequamark® size standard.

For mitochondrial genotyping, two fragments were amplified by PCR: approximately 1500 base pairs (bp) between cytochrome oxidase I and II (primers C1-J-2195 and C2-N-3661: Roehrdanz 1993) and 450 bp of cytochrome b (primers CB-J-10933 and CV-N-11367: Simon *et al*. 1994). In a total reaction volume of 25 µL we used 1/25 of the extracted DNA and 1 µ*Taq*-polymerase (MBI-Fermentas) per reaction. $MgCl_2$ concentration was 2.5 mm, primer concentration 25 µm and dNTP concentration 200 µm. Thirty-five cycles of denaturation at 94 °C (1 min), annealing at 44 °C (cyt-b) or 46 °C (cyt-ox) for 1 min, and extension 68 °C (5 min) were performed. The restriction patterns of 17 restriction enzymes were screened for polymorphisms in 10 randomly chosen individuals from 10 different colonies on 0.8–1.5% horizontal agarose gels (size marker: 123 bp DNA ladder, Invitrogen). Five polymorphic restriction sites were detected in the cytochrome-oxidase fragment: Two *Hin*fI, one *Bam*HI and one *Msp*I site were analysed, yielding five distinguishable mitochondrial haplotypes. The fifth polymorphism, in a restriction site of *Dde*I, proved unscorable and was omitted from the analysis. Additional sequencing of the two fragments in eight individuals from different colonies revealed six further putative polymorphic sites in the cyt-b fragment. However, these were not included in the study due to the absence of suitable restriction enzymes.

Data analysis

For microsatellite and mitochondrial haplotype data, the genetic structure of two populations was assessed using multiple statistics. From microsatellite genotypes, we calculated hierarchical *F*-statistics using the computer program Genetic Data Analysis 1.1 (Lewis & Zaykin 1999) with colonies nested in populations. We also performed a four-level analysis to test whether colonies of different social type (monogynous/polygynous) were differentiated genetically. Furthermore, we computed the coancestry coefficient (Reynolds *et al*. 1983) between the two populations without taking substructure into account. The computer program arlequin 2.0 (Schneider *et al*. 2000) was used to compute molecular and standard genetic diversity in each population (see arlequin 2.0 manual) and Fisher's exact test of population differentiation.

Population differentiation at the mitochondrial level was assessed by hierarchical analysis of molecular variance (amova, Euclidian distances), calculation of genetic distance (as F_{ST}-value without colony level structure) and Fisher's exact test, using arlequin 2.0. Genetic differentiation according to social colony type was evaluated with a hierarchical amova, nesting social type in populations. We also calculated molecular diversity for each population.
In order to assess population viscosity at the mitochondrial level we determined the correlation between spatial distance between colonies and their haplotype concordance. Significance was assessed by Mantel tests (Manly 1997) using the computer program Tools for Population Genetic Analysis (Miller 1998). The number of known spatial distances between colonies genotyped with microsatellites was too small to permit a meaningful, comparable analysis at the nuclear level.

RESULTS

Microsatellites

Altogether, 120 queens from 59 colonies were genotyped using microsatellites. In colonies without a present queen, the most likely genotype of the former queen was inferred from worker genotypes. All loci were polymorphic in both populations, with an average of 15 alleles in population NBL and 12 in WTC. The average expected heterozygosity was H_E= 0.73 and H_E= 0.70, respectively. Over both populations, the allele number ranged from 5 to 29 and H_E from 0.52 to 0.92. The molecular diversity estimates (Tajima 1983) were π= 0.60 ± 0.36 for NBL and π= 0.42 ± 0.27 for WTC, the standard diversity indices (Nei 1987) were ˙= 1.00 ± 0.00 for both populations. Overall, the populations were genetically similar: Reynold's coancestry coefficient = 0.006 (NS). Fisher's exact test confirmed that the populations were not significantly different (P = 0.123 ± 0.028).

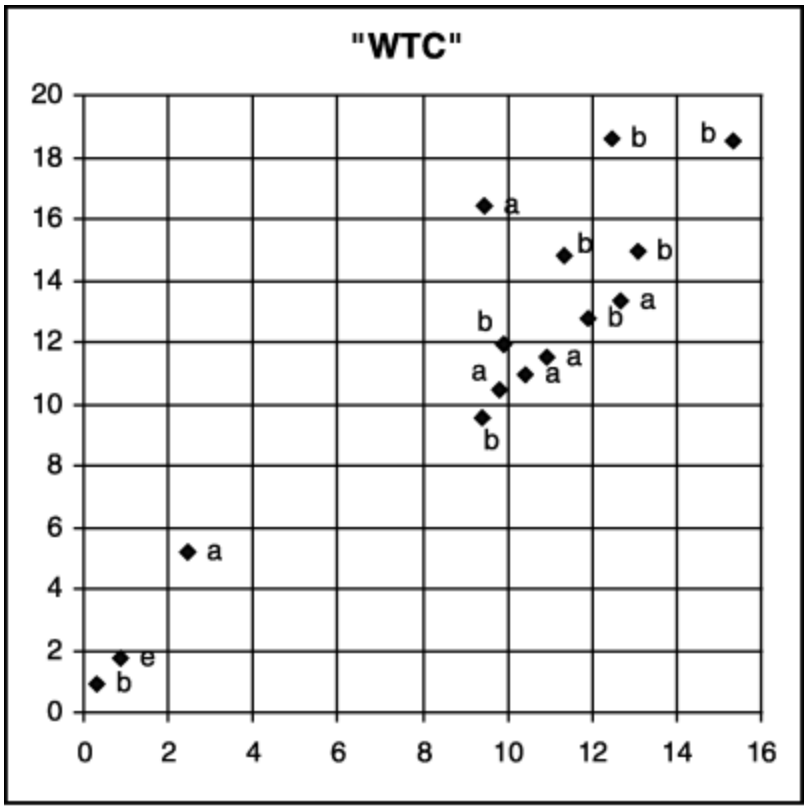
The hierarchical analysis of variance, involving 59 colonies nested in two populations resulted in F_{IS} = −0.157 (– 0.226 to – 0.116). Structuring was significant at the colony level (F_{colony}= 0.178 (0.163–0.191)) but negligible at the population level ($F_{population}$= 0.000 (−0.014–0.007)). These parameters did not change significantly when social type of colony was taken into account as an additional hierarchical level: F_{IS} = −0.157 (−0.226 to –0.116) (F_{colony}= 0.178 (0.162–0.193) ($F_{colonytype}$= 0.002 (−0.018–0.012)) and ($F_{population}$= 0.012 (− 0.023–0.012).

Mitochondrial DNA

Consensus sequences for *L. rugatulus* of the two amplified mtDNA sequences were deposited in GenBank (accession nos AY158899 and AY158900). Overall, 286 workers and 106 queens were scored from the two sampled populations for their mitochondrial haplotype: 15 queens and 154 workers from 15 colonies were studied from population WTC and 91 queens and 132 workers from 22 colonies from population NBL. In population WTC, six colonies contained haplotype a, eight haplotype b and one haplotype e. In population NBL, haplotype a was found in nine colonies, b in one, c in nine and d in three. The molecular diversity was 0.29 ± 0.22 in WTC and 0.28 ± 0.21 in NBL, standard diversity 0.59 ± 0.08 and 0.68 ± 0.06, respectively. Reynold's coancestry distance between the two populations without substructure based on our mitochondrial data was 0.120 (P = 0.001) and population differentiation was highly significant (Fisher's exact test: P < 0.001). The hierarchical analysis of molecular variance indicated significant structure at both the population ($F_{population}$= 0.253, P < 0.001) and at the colony level (F_{colony}= 0.961, P < 0.001). However, no structuring according to colony social type was found ($F_{socialstructure}$=−0.016, P > 0.5).

Nestmate queens always shared one haplotype, indicating only one matriline per colony. Based on our queen data, the probability that 10% or more queens in colonies are unrelated can be rejected with P = 0.006. This is also reflected in the worker's haplotypes, with three (of 154) and one (of 132) exceptions in population 'WTC' and 'NBL', respectively. Unrelated workers were found twice in monogynous and twice in polygynous colonies. Thus, the proportion of unrelated workers in colonies was low, and it did not differ between polygynous and monogynous colonies (Yates" corrected χ^2 = 0.03, P = 0.856).

No significant spatial clustering of colonies with identical mitochondrial haplotype could be detected in the predominantly monogynous population WTC (r = 0.08, P = 0.219). In contrast, a weak but significant correlation (r = 0.19, P = 0.026) between haplotype and spatial distance was found in the predominantly polygynous population NBL (Fig. 1).


"WTC"
20
18
16
14
12
10
8
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6
8
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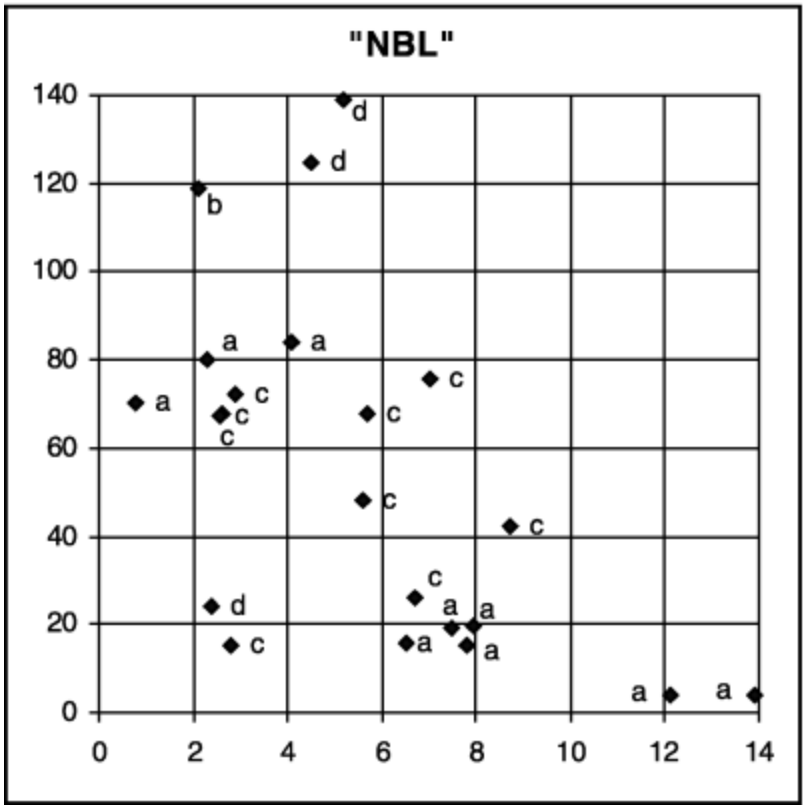
"NBL"
140
120
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Fig. 1 Spatial arrangement of investigated colonies (black dots) and their mitochondrial haplotypes, indicated by letters a–e, in the two compared populations of *Leptothorax rugatulus*. Units are in metres.

DISCUSSION

The results of our study demonstrate the value of employing multiple classes of genetic markers (Ross *et al*. 1999), particularly for social insects, in which mating and dispersal are difficult to monitor, and males and females play very different roles in reproduction. Particularly in social Hymenoptera, males often do not survive the mating period, and females play the major role in reproduction and consequently have developed the majority of alternative reproductive tactics (Hölldobler & Wilson 1990; Heinze & Tsuji 1995; Rüppell & Heinze 1999; Heinze & Keller 2000). There are three major conclusions from our mitochondrial data: (i) successful female dispersal is limited between the two investigated populations in spite of their close proximity; (ii) measurable population viscosity exists in the population that exhibits predominantly dependent colony foundation; and (iii) most colonies are matrilineal, adopt only offspring queens and display few errors in nestmate recognition (resulting in the incorporation of alien workers).

The population differentiation at the mitochondrial level is not reflected in the microsatellite data. Thus, we conclude that significant male gene flow between the two populations exists, in contrast to limited female dispersal between the populations. In contrast to males, *L. rugatulus* queens are adapted strongly to the local environment. Although queen phenotype is plastic to a certain degree (Rüppell *et al*. 2001b), queens leave the colony for mating and colony founding either with or without sufficient nutritional body reserves for independent colony foundation (Keller & Passera 1989).

As in many ant species, *L. rugatulus* males are smaller and have a lower wing load than either queen morph (Rüppell & Heinze 1998), which increases their chances of (active or passive) dispersal. Although it has never been observed directly, *L. rugatulus* queens that seek readoption in their natal colonies presumably do not join mating flights, and male dispersal may be much higher than female dispersal, as it is the case in most polygynous social insect species (Pamilo *et al*. 1997; Ross 2001). The negative inbreeding coefficients in our hierarchical analyses of microsatellite genotypes indicate that queens mate with males that are significantly less related to them then their nestmate queens. At the population level, this amounts to random mating, which is corroborated by the nonsignificant $F_{IS} = 0.039$ (−0.008–0.061), when the colony level is removed from the analysis.

Despite random mating, we found significant population viscosity at the mitochondrial level as evidence for female philopatry within the polygynous population NBL, demonstrating the effect of reproductive tactics and social system on population genetic structure. This expected correspondence of social colony structure and genetic population structure (Pamilo *et al*. 1997; Ross 2001) was not detected employing microsatellites in a previous study that compared monogynous and polygynous populations of *L. rugatulus* (Rüppell *et al*. 2001a). Retrospectively, this previous failure to detect population viscosity with nuclear markers is not surprising, because our present results indicate no nuclear differentiation even between populations 7 km apart. Due to female philopatry in (facultatively) polygynous ant species stronger mitochondrial than nuclear population structuring should be common and the studies conducted so far have

provided supportive evidence (e.g. Stille & Stille 1993; Ross & Shoemaker 1997; Foitzik & Herbers 2001).

Within colonies of *L. rugatulus* we have found no evidence for the existence of multiple established matrilines: queens and workers shared one mitochondrial haplotype in any given colony, which substantiates earlier findings of high intracolonial queen relatedness (Rüppell *et al*. 2001a). Based on our current findings we are confident that adoption of unrelated queens, as in *L. acervorum* (Stille & Stille 1992), or colony take-over by unrelated queens, as in *L. nylanderi* (Foitzik & Heinze 2001), occurs very rarely in *L. rugatulus*. Thus, alternative reproductive tactics in *L. rugatulus* comprise independent colony founding and readoption, but not intraspecific social parasitism. Moreover, in more than 1200 colonies investigated, we have failed to find any kind of interspecific social parasitism (Rüppell & Heinze, unpublished). The common reason for the absence of inter- and intraspecific social parasitism may be the protected nest structure of *L. rugatulus*. This might also be the reason why we found very few workers with alien mitochondrial haplotypes, although empirical and theoretical work suggests that nestmate recognition should be decreased by polygyny (Hölldobler & Michener 1980; Vander Meer & Morel 1998). Our present data indicate that in *L. rugatulus* non-nestmate acceptance occurs rarely, independent of colony social type. However, we have to caution that the small size of our data set allows only for this preliminary conclusion.

In summary, our study demonstrates at three hierarchical levels (among populations, within populations and within colonies) the importance of multiple genetic marker systems to understand a species' reproductive and dispersal behaviour. This is particularly true for a comprehensive understanding of species which cannot be directly observed and mark–recapture techniques yield only fragmentary information, such as most social insects.

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This study resulted as a collaboration from Olav Rueppell's ongoing interest in ant reproductive tactics and Michaela Straetz's work on mtDNA in the ant genus Leptothorax. It was carried out in the laboratory of Juergen Heinze with assistance from Bernd Baier.

APPENDIX 1

PCR conditions used to amplify the four microsatellite loci, which were adopted from different *leptothorax* species

Locus	Concentrations of PCR reactants	Cycling conditions (initial denaturation: 92 °C/120 s)
L18	200 µm dNTP	Denaturations: 92 °C/60 s
	2.5 mm $MgCl_2$	Annealing: 51 °C/60 s
	0.63 µm Primers	Elongation: 70 °C/90 s
	0.04 µ/µL *Taq*-polymerase	No. of cycles: 36
LXGT104	311 µm dNTP	Denaturations: 92 °C/60 s
	2.5 mm $MgCl_2$	Annealing: 51 °C/60 s
	0.63 µm Primers	Elongation: 70 °C/90 s
	0.03 µ/µL *Taq*-polymerase	No. of cycles: 36
LXGT218	200 µm dNTP	Denaturations: 92 °C/60 s
	1.25 mm $MgCl_2$	Annealing: 57–47 °C/90 s
	0.56 µm Primers	(touchdown)
	0.03 µ/µL *Taq*-polymerase	Elongation: 70 °C/90 s
		No. of cycles: 10 × 2 + 20
LXGT228	200 µm dNTP	Denaturations: 92 °C/60 s
	1.84 mm $MgCl_2$	Annealing: 57–47 °C/90 s
	0.41 µm Primers	(touchdown)
	0.03 µ/µL *Taq*-polymerase	Elongation: 70 °C/120 s
		No. of cycles: 10 × 2 + 20