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POPULATION AND COLONY GENETIC STRUCTURE OF THE PRIMITIVE TERMITE *MASTOTERMES DARWINIENSIS*

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Abstract.—The termite *Mastotermes darwiniensis* is the sole extant member of its family and occupies the basal position in the phylogeny of the eusocial order Isoptera. In this study, we investigated the micro- and macrogeographic genetic structure of *M. darwiniensis* in its native range in Australia. A total of 1591 workers were sampled from 136 infested trees in 24 locales. Each locale was separated by 2–350 km, and these locales were found within two broader geographic regions approximately 1500 km apart. The multilocus genotypes of all termites were assayed at six polymorphic microsatellite loci. The genetic data indicated that colonies typically fed on multiple trees within locales and extended over linear distances of up to 320 m. Single colonies were frequently headed by multiple reproductives. Workers were highly related ($r = 0.40$) and substantially inbred ($f = 0.10$). Thus, *M. darwiniensis* colonies are characterized by the input of alleles from multiple reproductives, which sometimes engage in consanguineous matings. Our analyses of population genetic structure above the level of the colony indicated that locales and regions were significantly differentiated ($\theta_{locale} = 0.50$, $\theta_{region} = 0.37$). Moreover, locales showed a pattern of genetic isolation by distance within regions. Thus, *M. darwiniensis* populations display restricted gene flow over moderate geographic distances. We suggest that the genetic patterns displayed by *M. darwiniensis* result primarily from selective pressures acting to maintain high relatedness among colonymates while allowing colonies to grow rapidly and dominate local habitats.

Key words.—gene flow, inbreeding, Isoptera, microsatellite, polygyny, relatedness, social insect.

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Eusocial insects exhibit a variety of remarkable behaviors associated with group living, such as cooperation in rearing offspring and partitioning of reproduction among colony members (Wilson 1971). The development of many of these traits arose via kin selection, the effectiveness of which depended on patterns of genetic structure in populations (Hamilton 1964). Consequently, many studies have investigated the population genetic structure of eusocial insects to better understand the factors affecting the evolution of sociality (reviewed by Crozier and Pamilo 1996; Pamilo et al. 1997; Ross 2001).

However, the vast majority of these studies have focused on eusocial members of the Hymenoptera (all ants, some bees, and some wasps). The emphasis on this order may bias our understanding of the circumstances under which social behaviors evolve and are maintained, because the Hymenoptera possess distinct characteristics that may have played a role in the evolution of sociality in this group. For example, all Hymenoptera are haplodiploid, a condition that alters the genetic relationships within families and may facilitate the evolution of eusociality (Hamilton 1964; Trivers and Hare 1976). Thus, investigations of the genetic structure in distantly related eusocial taxa are critical.

Relatively few population genetic studies in the eusocial order Isoptera (termites) have been undertaken. This is surprising given the distant phylogenetic divergence between the Hymenoptera and the Isoptera (Kristensen 1991). Indeed, despite the overt similarity of their social systems, the Isoptera differ from the Hymenoptera in many important ways. For example, termites are diploid and hemimetabolous (Krishna and Weesner 1970). They also mate throughout their lifetime (Nalepa and Jones 1991) and may possess greater plasticity in their developmental and reproductive programs than the Hymenoptera (Noirot and Pasteels 1987).

A particularly important isopteran candidate for study is the giant termite *Mastotermes darwiniensis*, the sole extant representative of the family Mastotermitidae. Fossil evidence indicates that the family was once distributed worldwide, but it is now confined to Australia and New Guinea (Watson and Gay 1991). *Mastotermes darwiniensis* occupies the phylogenetically basal position in the Isoptera and is distantly related to all other termite families (Thompson et al. 2000). The species possesses morphologically primitive characteristics, such as the structure of its hind wings (Watson and Gay 1991) and its mechanism of egg-laying (Nalepa and Lenz 2000), yet its social system is considered to be derived (Watson and Gay 1991).

Newly hatched *M. darwiniensis* larvae develop into one of several distinct castes after entering one of two developmental pathways (Watson et al. 1977; Watson and Sewell 1981). Larvae entering the alate (winged) pathway pass through a series of nymphal stages and eventually develop into sclerotized adults that participate in dispersal flights (Watson et al. 1975; Watson and Abbey 1989). In contrast, larvae entering the worker pathway molt into workers and cannot develop into alates, although they retain several developmental options. They may go through stationary molts and remain as workers. Alternatively, they may develop into soldiers through a presoldier stage or take on reproductive roles by molting into neotenics (Watson et al. 1977). Neotenics are common in old *M. darwiniensis* colonies (Hill 1942). They are believed to mate within the nest and replace the primary alate-derived reproductives (Watson and Abbey 1985, 1989). Most mature colonies are headed by hundreds of neotenic reproductives, and alate-derived primary reproductives are virtually never found in natural populations (Watson and Abbey 1989).

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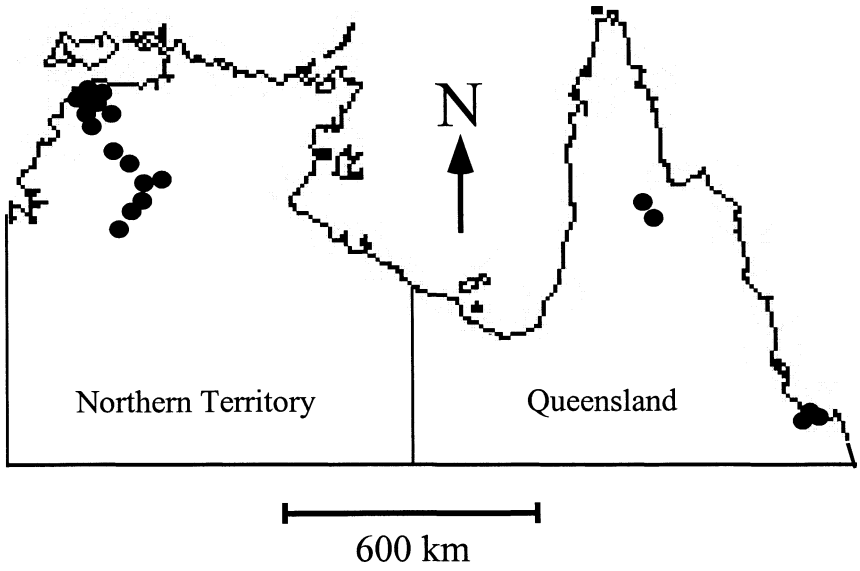

FIG. 1. Map of northeastern Australia showing approximate locations of 20 locales from which *Mastotermes darwiniensis* termites were sampled. The species is found throughout the area depicted.

New *M. darwiniensis* colonies are founded by alate reproductives following dispersal flights (Watson and Gay 1991). In addition, it has been hypothesized that new colonies may arise by budding off from older established colonies (Hill 1942). *Mastotermes darwiniensis* colonies do not inhabit conspicuous nests. Rather, they live underground or within the food (typically living or dead trees) they consume. Mature colonies may nest in many such feeding sites and grow to contain millions of individuals, particularly in disturbed environments where they seem to flourish (Hill 1942).

The goal of this study was to obtain a complete understanding of the population genetic structure of *M. darwiniensis* and interpret the results in the broader framework of sociality in termites. We investigated the sociogenetic structure of *M. darwiniensis* colonies to identify the systems of reproduction and recruitment within colonies. We also tried to distinguish colony boundaries and determine the extent to which individual colonies dominated habitats. Finally, we studied the macrogeographic patterns of genetic structure and attempted to discern the importance of gene flow on larger, regional scales.

MATERIALS AND METHODS

We collected *M. darwiniensis* workers from 20 locales separated by a minimum of 2 km in the two larger regions of the Northern Territory and Queensland during the austral winters of 1999 and 2000 (Fig. 1, Table 1). Termites were

TABLE 1. Locations (latitude south, longitude east), number of trees sampled (*N*), and number of *Mastotermes darwiniensis* workers analyzed (*n*) from 20 locales in two regions of northern Australia. Locales within regions are numbered in order of increasing south latitude.

Region	Locale	Location	*N*	*n*
Northern Territory	N-1	12°23.800′, 130°55.247′	21	299
	N-2	12°24.689′, 130°55.366′	4	19
	N-3	12°24.846′, 131°9.769′	1	2
	N-4	12°28.141′, 131°3.365′	27	437
	N-5	12°39.381′, 131°4.601′	24	341
	N-6	12°42.299′, 131°24.977′	4	17
	N-7	13°1.141′, 130°56.941′	3	32
	N-8	13°6.301′, 131°6.381′	1	4
	N-9	13°31.921′, 131°20.960′	3	13
	N-10	13°37.924′, 131°37.959′	4	19
	N-11	14°11.033′, 132°2.379′	20	302
	N-12	14°21.064′, 132°26.065′	3	14
	N-13	14°35.434′, 132°10.920′	4	19
	N-14	14°38.353′, 132°7.184′	4	14
	N-15	15°28.684′, 131°23.609′	2	9
Queensland	Q-1	14°47.265′, 143°30.218′	2	8
	Q-2	14°56.882′, 143°33.427′	2	10
	Q-3	19°14.148′, 146°46.248′	2	10
	Q-4	19°19.651′, 146°45.585′	3	5
	Q-5	19°19.780′, 146°43.950′	2	7
Total			136	1591

collected from each of several infested trees in each locale, and the position of trees within locales was noted. In four locales (N-1, N-4, N-5, and N-11, hereafter referred to as ''major locales''), we sampled particularly large numbers of workers from many trees. All termites were placed in 95% ethanol after collection for subsequent microsatellite analysis.

The genotypes of two to 20 termites from each tree were assayed at six microsatellite loci, Mdar2, Mdar3, Mdar4, Mdar5, Mdar8, and Mdar13, as described by Goodisman et al. (2001a). To visualize the polymerase chain reaction (PCR) products, the forward primer for amplifying each locus was labeled with a fluorescent dye: Mdar2 and Mdar5 were TET-labeled; Mdar3 and Mdar8, HEX-labeled; and Mdar4 and Mdar13, 6-FAM-labeled. PCRs were conducted in a final volume of 15 μl containing 3 μl genomic DNA and 0.75 U *Taq* DNA polymerase (Promega, Madison, WI) and a final concentration of 200 μM dNTPs, 0.5 μM of each of the forward and reverse PCR primers, and 1X Promega Buffer (with 1.5 mM $MgCl_2$). PCR products from all loci were then combined in a 20-μl cocktail containing 0.5 μl, 8.0 μl, 0.3 μl, 1.2 μl, 6.0 μl, and 4.0 μl of Mdar2, Mdar3, Mdar4, Mdar5, Mdar8, and Mdar13 PCR products, respectively. Two microliters of this cocktail were combined with a labeled size standard and electrophoresed on an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA). Alleles were scored using the program GENOTYPER (Applied Biosystems).

We combined workers from all trees within each locale to obtain locale-level estimates of allele frequencies at the six microsatellite loci. Nei's (1987) unbiased estimate of gene diversity, $h = 2n(1 - \Sigma_i p_i^2)/(2n - 1)$, where n is the number of workers sampled and p_i is the frequency of allele i, was used as a measure of variability for each marker in each locale. We tested whether microsatellite variability differed between the two regions by comparing the locale-level gene diversities for each locus using a Mann-Whitney *U*-test (Sokal and Rohlf 1995).

The relationships of workers sampled from different trees within the major locales were examined to determine colony boundaries. The genotypic frequencies of workers from all pairs of trees were compared by means of an exact test using the program GENEPOP 3.2 (Raymond and Rousset 1995). Significant differentiation suggested that workers sampled from distinct trees did not belong to the same colony, and nonsignificant differentiation was taken as evidence that workers were derived from the same colony. Locale-level significance values were adjusted using the sequential Bonferroni procedure to account for the multiple tests performed (Rice 1989).

We directly examined the genotypes of workers from single colonies in the major locales to determine if workers could have been produced by a single pair of reproductives, by multiple reproductives derived from a single pair, or by multiple unrelated reproductives. The genetic data were considered to agree with the presence of only two reproductives if the genotypes of workers within trees conformed to those expected under Mendelian segregation of alleles from two diploid parents. The genotypes of termites within trees were consistent with having been produced by multiple reproductives descended from a single pair if no more than four alleles were present at any locus. The presence of more than four alleles at any locus signaled that more than two unrelated reproductives had produced the sampled workers.

We next determined if the workers from single colonies were clumped within the major locales by examining the patterns of genetic isolation by distance of workers from distinct trees. We first calculated pairwise estimates of F_{TL} for workers inhabiting different trees within individual locales. Spearman's rank-order correlation coefficient (r_S) was used to measure the correlation between genetic and geographic distance of trees. The significance of the correlation was assessed by a Mantel test with 10,000 permutations using GENEPOP 3.2. A significant association suggested that colonies foraged in discrete areas and were separated from other colonies.

Similar tests were conducted to detect evidence that new colonies originated from old colonies through budding. In this case, we examined patterns of genetic isolation by distance of *colonies* in the major locales. Pairwise estimates of F_{CL} were obtained for all pairs of colonies within locales, and the geographic distances between colonies were estimated as the distance between the midpoints of colonies. Spearman's correlation coefficient was again used to measure the association between the genetic and geographic distance, and the significance of the correlation was determined with a Mantel test as described above. A significant relationship would be consistent with the hypothesis that budding of new colonies occurred.

The program RELATEDNESS 4.2 (Queller and Goodnight 1989) was used to calculate the relatedness (r) of workers belonging to trees or colonies. The deme function was used to account for differences in allele frequencies across locales, and standard errors for estimates were obtained by jackknifing over trees or colonies. We used *t*-tests to determine if estimates differed statistically from each other, from 0.0, or from 0.5.

We then obtained estimates for the level of inbreeding (f) of workers within the major locales using RELATEDNESS 4.2. Score tests executed by GENEPOP 3.2 were used to determine the significance of the levels of inbreeding. To obtain significance values unbiased by the genetic structure of workers cohabiting within the same colony, we randomly selected a single worker from each colony and conducted the test on this reduced dataset. When conducting these tests, we specified the alternate hypothesis to be a deficiency of heterozygotes (Rousset and Raymond 1995).

Inbreeding can affect measures of genetic relatedness (Pamilo 1985). To adjust the estimates of relatedness obtained in this study for inbreeding, we applied Pamilo's (1985) correction to obtain the adjusted estimate $r^* = [r - 2f/(1 + f)]/[1 - 2f/(1 + f)]$, where r^* is the inbreeding adjusted relatedness estimate, r is the unadjusted relatedness estimate, and f is the inbreeding coefficient. This correction appears applicable to cases of true inbreeding (i.e., mating between relatives), although it was derived under models of isolation by distance of social groups (Pamilo 1984, 1985).

Genetic differentiation above the level of the colony was measured by Wright's hierarchical *F*-statistics, which were estimated using Weir and Cockerham's (1996) method as

TABLE 2. Variability of six microsatellite markers in *Mastotermes darwiniensis* within each of 20 locales, as given by the number of alleles (*A*) and Nei's unbiased estimate of gene diversity (*h*).

	Mdar2		Mdar3		Mdar4		Mdar5		Mdar8		Mdar13	
Locale	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*
N-1	6	0.71	3	0.61	2	0.03	7	0.77	3	0.58	4	0.54
N-2	7	0.83	4	0.71	2	0.11	7	0.84	4	0.59	6	0.79
N-3	2	0.48	3	0.67	2	0.30	4	0.80	2	0.48	3	0.73
N-4	2	0.22	3	0.65	3	0.41	2	0.50	4	0.75	1	0.00
N-5	7	0.67	5	0.57	4	0.08	9	0.78	5	0.61	5	0.56
N-6	3	0.59	2	0.40	2	0.35	4	0.64	3	0.60	2	0.48
N-7	4	0.75	3	0.62	2	0.39	4	0.76	4	0.75	3	0.61
N-8	2	0.23	1	0.00	1	0.00	3	0.68	3	0.54	3	0.63
N-9	6	0.75	2	0.10	3	0.27	3	0.65	3	0.41	4	0.57
N-10	3	0.63	6	0.78	2	0.29	4	0.59	4	0.76	2	0.06
N-11	6	0.76	5	0.66	2	0.45	8	0.79	5	0.58	4	0.40
N-12	1	0.00	5	0.66	3	0.50	3	0.65	3	0.55	2	0.33
N-13	5	0.65	5	0.75	3	0.55	5	0.74	4	0.68	2	0.51
N-14	4	0.73	3	0.64	4	0.37	4	0.75	2	0.40	4	0.65
N-15	4	0.76	3	0.56	2	0.52	3	0.63	2	0.29	3	0.68
Q-1	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00
Q-2	1	0.00	2	0.42	2	0.10	2	0.10	1	0.00	1	0.00
Q-3	2	0.48	1	0.00	1	0.00	2	0.11	1	0.00	1	0.00
Q-4	2	0.47	1	0.00	1	0.00	2	0.20	1	0.00	1	0.00
Q-5	4	0.60	2	0.52	1	0.00	4	0.65	1	0.00	2	0.16
All	17	0.71	14	0.73	8	0.36	23	0.82	8	0.71	11	0.54

implemented by the program GDA (Lewis and Zaykin 2000). The resulting nonrandom associations of alleles due to structure of locales within regions and between regions were quantified with the statistics θ_L and θ_R, respectively. The 95% confidence intervals for the statistics were determined by bootstrapping over loci 1000 times. A given statistic was deemed to be significantly different from zero if its 95% confidence interval did not overlap zero.

To analyze patterns of isolation by distance among locales, we obtained estimates of F_{LP} for all pairs of locales within the total population using GENEPOP 3.2. The strength of the association between the pairwise values of F_{LP} and the metric distances between locales was quantified with Spearman's rank-order correlation coefficient, and the significance of the correlation was determined via a Mantel test with 10,000 permutations. The association between the transformed values of $F_{LP}/(1 - F_{LP})$ and log-transformed metric distances was also considered, as the relationship between these latter variables is expected to follow a linear pattern under some models of migration and drift in a two-dimensional sampling scheme (Rousset 1997).

Finally, we constructed an unrooted dendrogram to investigate the genetic relationships among the 20 locales. Nei's (1987) genetic distance was calculated between all pairs of locales from the allele frequency estimates of all workers sampled within locales. The neighbor-joining algorithm (Saitou and Nei 1987), as implemented by PHYLIP 3.572c (Felsenstein 1989), was then used to reconstruct the relationships among locales. The data were bootstrapped over loci 1000 times, yielding confidence levels for the nodes on the final tree.

RESULTS

All six microsatellite markers were polymorphic (Table 2). The number of alleles per locus ranged from eight to 23 when samples from all locales were combined. The gene diversities (*h*) within locales were generally high, suggesting that the markers would provide substantial power in dissecting the social structure of *M. darwiniensis* colonies and in determining levels of gene flow within the population.

The Northern Territory displayed greater locale-level gene diversities than Queensland for all markers ($U = 62.5$, $P = 0.029$ for Mdar2; $U = 67.5$, $P = 0.009$ for Mdar3; $U = 70.0$, $P = 0.004$ for Mdar4; $U = 70.0$, $P = 0.004$ for Mdar5; $U = 75.0$, $P = 0.001$ for Mdar8; $U = 71.0$, $P = 0.003$ for Mdar13). The microsatellite markers used in this study were specifically selected because they were variable in a sample of termites collected from the Northern Territory (Goodisman et al. 2001a). Consequently, the difference in variability between the two regions may have resulted from this sampling bias.

Distinct colonies comprising genetically related individuals were detected in three of the four major locales (Appendices 1–4, Fig. 2). In most cases, the results of exact tests allowed us to sort workers from separate trees into colonies, and anomalous patterns could be attributed to low sample sizes (e.g., only two workers were collected from tree 10 in locale N-11). Consequently, we grouped workers from the 21 trees in locale N-1 into the following three colonies: (1–8), (9–18, 20, 21), and (19); workers from the 24 trees in locale N-5 were grouped into the following nine colonies: (1), (2, 3, 10), (4), (5), (6–8), (9), (11–14), (15, 20), (16–19, 21–24); and workers from the 20 trees in locale N-11 were grouped into the following six colonies: (1, 2), (3), (4–6, 8, 9, 11, 12, 16–20), (7), (10), (13–15). The genotype frequencies of workers from no two trees in locale N-4 were significantly different. Therefore, all workers sampled from locale N-4 were deemed as belonging to a single colony.

Examination of the worker genotypes from the major locales revealed that more than two reproductives frequently

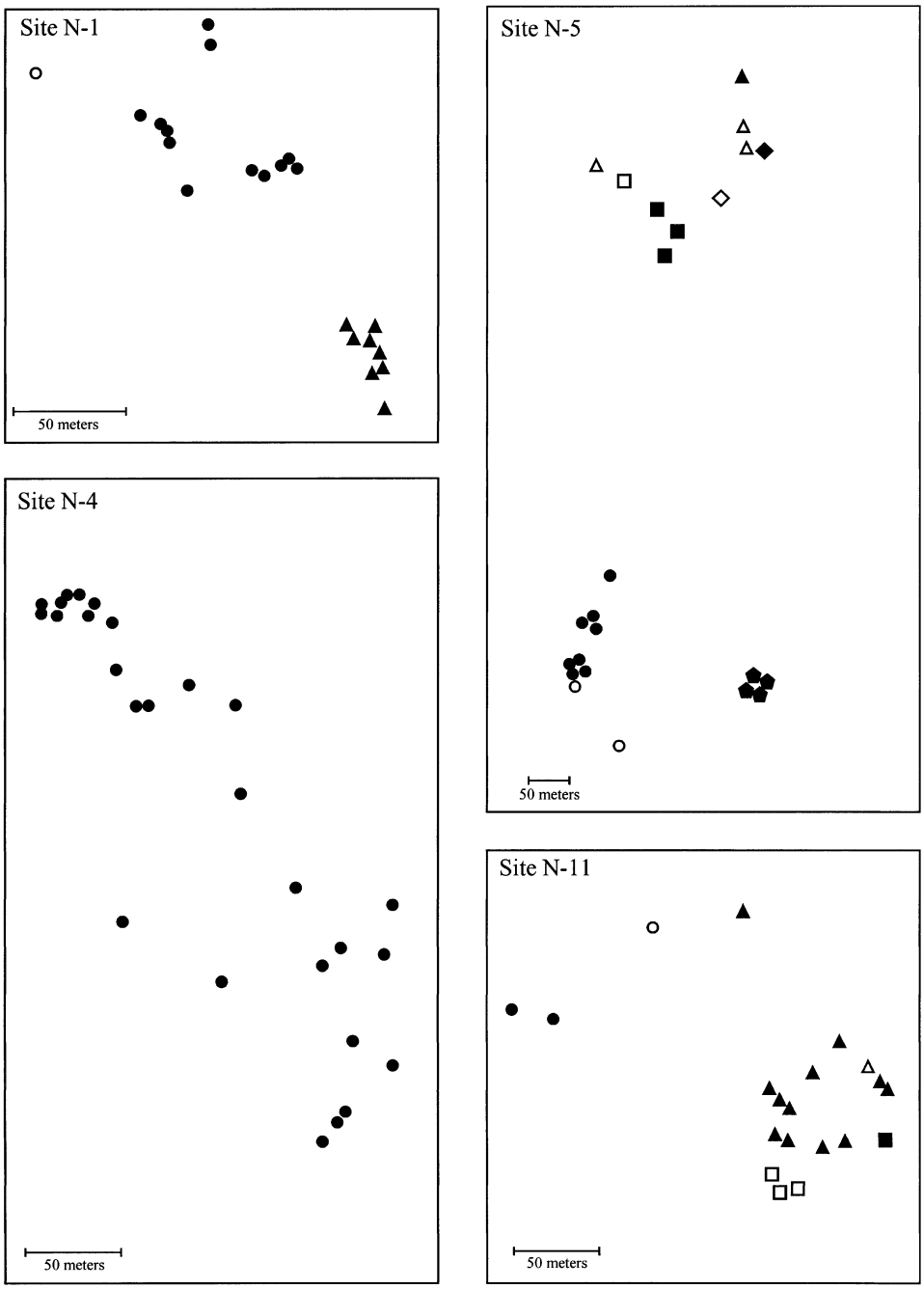

FIG. 2. Locations of trees in major locales from which *Mastotermes darwiniensis* termites were sampled. Trees containing workers belonging to the same colony are indicated by the same symbols within locales.

were active within colonies. In all, workers from only five of the 19 colonies could have been produced by two reproductives, whereas workers in the remaining 14 colonies required contributions from more than two parents. However, clear direct evidence that the multiple reproductives were recently derived from more than two unrelated reproductives was rare, as workers from only five of these 14 colonies possessed more than four alleles. We note that the frequencies of colonies headed by more than two reproductives represent underestimates of the actual proportions because of the limited variation of our markers and the finite number of workers sampled.

We next investigated whether termites belonging to the same colony were clustered within the major locales by examining the patterns of genetic isolation by distance of termites sampled from distinct trees. Not surprisingly, no significant correlation between genetic and geographic distance was discerned in locale N-4 ($r_S = 0.020$, $P = 0.35$); the lack of significant genetic differentiation between trees precluded finding the effects of isolation by distance (Goodisman and

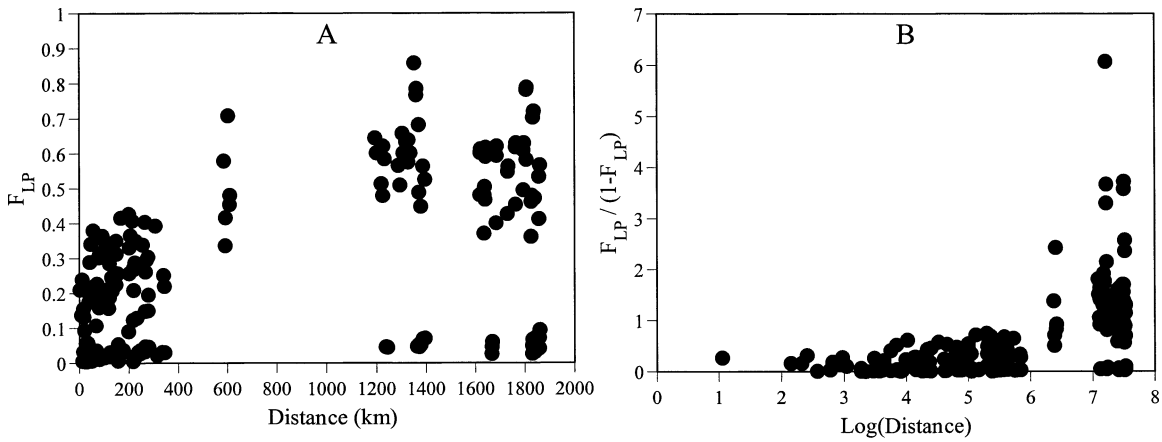

FIG. 3. Patterns of isolation by distance for *Mastotermes darwiniensis* sampling locales. (A) Relationship between pairwise estimates of F_{LP} and geographic distance between locales. (B) Relationship between transformed values of $F_{LP}/(1 - F_{LP})$ and log-transformed geographic distances between locales.

Ross 1998). However, significant associations between genetic and geographic distance were apparent in the other three locales ($r_S = 0.70$, $P < 0.0001$ in locale N-1; $r_S = 0.29$, $P = 0.00040$ in locale N-5; $r_S = 0.23$, $P = 0.039$ in locale N-11), supporting the hypothesis that colonies were geographically contiguous within locales. In contrast, we found no significant correlation between the genetic and geographic distances of *colonies* in major locales where more than two colonies were detected ($r_S = 0.5$, $P = 0.51$ in locale N-1; $r_S = 0.27$, $P = 0.092$ in locale N-5; $r_S = -0.31$, $P = 0.83$ in locale N-11), suggesting that budding was not necessarily a common form of new colony formation.

The mean relatedness (±SE) of workers inhabiting the same tree ($r = 0.21 \pm 0.024$) was significantly greater than 0.0 ($t_{133} = 8.91$, $P < 0.0001$) but significantly less than 0.5 ($t_{133} = 12.14$, $P < 0.0001$). The relatedness of workers from the same *colony*, obtained from the three major locales N-1, N-5, and N-11 was $r = 0.40 \pm 0.037$. This estimate was significantly greater than 0.0 ($t_{17} = 10.71$, $P < 0.0001$), significantly less than 0.5 ($t_{17} = 2.75$, $P = 0.014$), and significantly greater than the relatedness estimate obtained from workers sampled from the same tree ($t_{43} = 4.22$, $P = 0.00012$). The estimate for the level of inbreeding based on colonies from major sites was $f = 0.10$. A score test based on a reduced dataset indicated that this estimate was highly significant ($P = 0.0080$). The substantial levels of inbreeding led us to correct our relatedness estimate using the values $r = 0.40$ and $f = 0.10$ to $r^* = 0.26$.

We next moved to an analysis of genetic structure above the level of the colony. The results of the hierarchical analysis revealed that *M. darwiniensis* displayed substantial differentiation among locales and between regions. Estimates of the relatedness of alleles in locales within regions and between regions were both significantly positive (95% confidence intervals, given in parentheses, did not overlap zero), with $\theta_L = 0.51$ (0.42–0.62) and $\theta_R = 0.37$ (0.23–0.52). In addition, we uncovered a strong ($r_S = 0.51$) and highly significant ($P < 0.0001$) association between pairwise estimates of F_{LP} and distance, indicating an isolation-by-distance effect at the level of locales (Fig. 3A). This relationship was not caused by the large genetic differences between regions alone, because the correlations remained significant even when locales from each region were considered independently (Northern Territory, $r_S = 0.24$, $P = 0.0028$; Queensland, $r_S = 0.73$, $P = 0.040$). We then examined the association between the transformed values, $F_{LP}/(1 - F_{LP})$, and the log of the metric distances. This relationship, however, failed to follow the predicted linear trend (Fig. 3B).

The structure among locales uncovered by analysis of isolation by distance was largely supported by the unrooted dendrogram depicting the genetic relationships among locales (Fig. 4). Neighboring locales typically were genetically similar. Moreover, the node separating the two regions of the Northern Territory and Queensland possessed strong bootstrap support, which indicated substantial genetic differentiation between regions.

DISCUSSION

We investigated the population genetic structure of the primitive termite *M. darwiniensis* in its native range in Australia. Our results revealed that workers within colonies were significantly related and inbred. Colonies reached large sizes and fed over extensive areas. Moreover, *M. darwiniensis* displayed genetic differentiation among locales and between regions. Thus, *M. darwiniensis* populations exhibit substantial micro- and macrogeographic genetic structure.

Relationships of Colonymates

Our estimate for the relatedness of workers within colonies, 0.40, was obtained only after *M. darwiniensis* workers sampled from different trees were grouped into true colonies consisting of related and presumably interacting individuals. Relatedness estimates obtained when workers were not grouped into colonies fell well below 0.40. The difference in the estimates resulted from mistakenly considering workers from the same colony as belonging to distinct colonies. This potential error would have remained undetected if we had not sampled large numbers of workers from many trees within locales, thereby allowing us to detect genetically differentiated colonies. It also highlights the importance of large-scale sampling when studying the structure of social

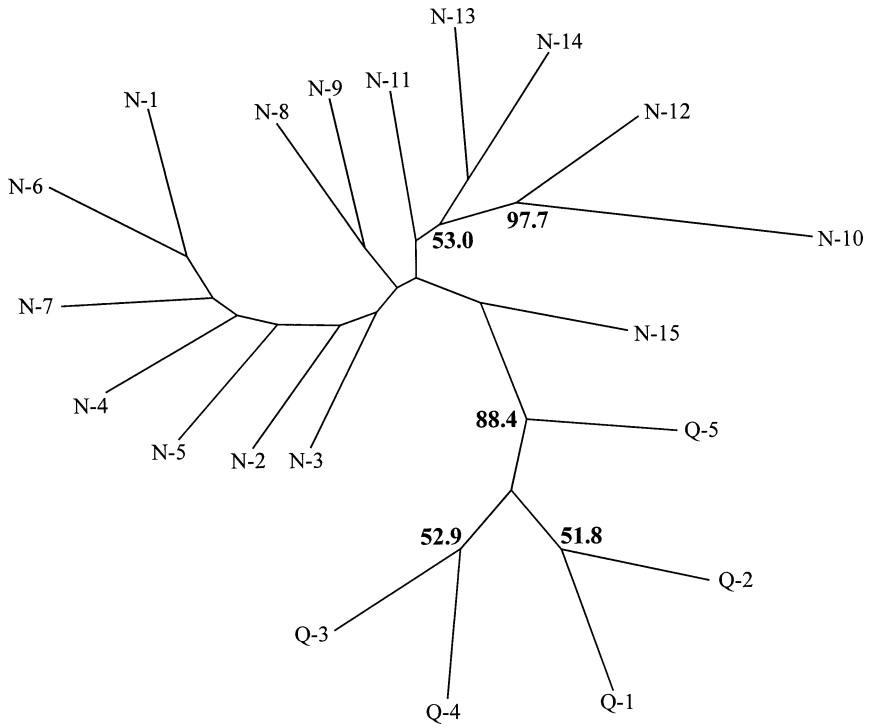

FIG. 4. Genetic relationships among *Mastotermes darwiniensis* locales in Australia obtained using the neighbor-joining algorithm. The percentage of bootstraps out of 1000 replicates is given in bold for nodes with greater than 50.0% support. Note that locales within regions are numbered in order of increasing south latitude (see Table 1).

insects that inhabit multiple feeding sites or nests (Crozier and Pamilo 1996; Chapuisat et al. 1997).

The relatedness estimate of colonymates adjusted for inbreeding ($r^* = 0.26$) fell below the value of 0.5 expected if workers within colonies were full-siblings. Indeed, worker genotypes frequently revealed genetic input into colonies from more than two reproductives. This result agrees with some aspects of the known social structure of *M. darwiniensis*. Colonies are typically headed by many neotenic reproductives (Hill 1942; Watson and Abbey 1985, 1989). However, the finding that these neotenics may have originated from more than two genetic lineages (i.e., were not recently descended from a single reproductive pair) was unexpected. We note, however, that worker relatedness was relatively high and significantly greater than zero. Therefore, *M. darwiniensis* nonreproductives presumably obtain substantial inclusive fitness benefits because they aid reasonably close relatives.

The recruitment of multiple reproductives within eusocial Hymenoptera and Isoptera is fairly common (Keller 1993; Crozier and Pamilo 1996; Myles 1999). It is possible that more than two unrelated primary reproductives are sometimes responsible for founding *M. darwiniensis* colonies. Associations of multiple primary reproductives during colony founding (pleometrosis) have been suggested in other termites and may permit rapid colony growth during the critical stage of colony initiation (Nutting 1969; Thorne 1985; Roisin 1993). Moreover, multiple alate-derived reproductives do head colonies of other termite species (Thorne 1985; Atkinson and Adams 1997). However, strong evidence for pleometrosis is still lacking in termites. Alternatively, foreign reproductives may be accepted into colonies after the colony has been founded. Until recently this possibility seemed unlikely, because the boundaries of most termite colonies typically are vigorously defended against foreign conspecifics once the colony has been established (Thorne and Haverty 1991; Shelton and Grace 1996). However, recent genetic data suggest that colony boundaries may be less rigid in some taxa (Clément 1986; Broughton 1995; Jenkins et al. 1999a; Bulmer et al. 2001).

The relatedness of *M. darwiniensis* colonymates was lower than that estimated in other Isoptera. For example, Husseneder et al. (1999) found that *Schedorhinotermes lamanianus* nestmates were related as siblings and suggested that a single pair of outbred primary reproductives typically founded colonies. Similar conclusions arose from genotypic analysis of *Nasutitermes nigriceps* colonies (Thompson and Hebert 1998). Reilly (1987) also obtained a relatedness estimate for *Reticulitermes flavipes* that was not significantly different from the value expected if workers were full-siblings. However, Bulmer et al. (2001) uncovered highly variable genetic structure in *R. flavipes* colonies and suggested that nestmate relatedness could fall to relatively low levels in older colonies.

Mastotermes darwiniensis workers were significantly inbred, a finding that supported expectations based on the reproductive and dispersal biology of the species. Neotenic reproductives are incapable of flight and mate with nestmates in the laboratory (Watson et al. 1975; Watson and Abbey 1985), strongly suggesting that they mate with relatives in natural populations. Evidence of inbreeding arising from the consanguineous mating of neotenics has been uncovered in

other Isoptera (Reilly 1987; Husseneder et al. 1999; Bulmer et al. 2001), although this has not always been the case (Luykx 1985; Thompson and Hebert 1998). Inbreeding had been suggested as a possible factor affecting the evolution of social behavior, because it may increase the relatedness of interacting individuals (Hamilton 1972; Michod 1993). Indeed, the frequent observation of significant inbreeding in empirical studies suggests that it may play an important role in termite social evolution.

Bulmer et al. (2001) simulated the effects of putative termite reproductive and dispersal strategies (e.g., recruitment of inbred neotenics or founding of colonies by multiple unrelated reproductives) on Wright's *F*-statistics (F_{IL}, F_{CL}, and F_{IC}, where I, L, and C stand for individual, locale, and colony, respectively). We calculated these statistics and their 95% confidence intervals for the three major locales in the *M. darwiniensis* population where colonies could be distinguished. We found that in locale N-1, $F_{IL} = 0.19$ (0.11 to 0.24), $F_{CL} = 0.19$ (0.12 to 0.26), and $F_{IC} = -0.02$ (-0.04 to 0.01). In locale N-5, $F_{IL} = 0.16$ (0.08 to 0.22), $F_{CL} = 0.24$ (0.19 to 0.29), and $F_{IC} = -0.11$ (-0.15 to -0.07). In locale N-11, $F_{IL} = -0.02$ (-0.15 to 0.12), $F_{CL} = 0.28$ (0.17 to 0.38), and $F_{IC} = -0.42$ (-0.59 to -0.27). Comparison of these values to those obtained by Bulmer et al. (2001) reinforces previous analyses suggesting that *M. darwiniensis* colonies are frequently headed by more than two reproductives. In addition, considerable variation exists in these statistics among locales, suggesting that colonies in distinct areas may be in various stages of ontogeny (Bulmer et al. 2001) or may be responding in different ways to environmental variation (Clément 1986). Indeed, direct examination of *M. darwiniensis* worker genotypes revealed variation in colony types within the population. The genotypes of workers in 26.3% of colonies could have been explained by the presence of only a single reproductive pair. In addition, 47.4% of sampled colonies may have been headed by multiple inbred neotenics derived from a single pair. However, the other colonies within the population were apparently headed by more than two unrelated reproductives.

Colony Boundaries

By grouping workers from different trees into colonies we were able to reveal the territories that *M. darwiniensis* colonies occupied. Statistical analyses of genetic isolation by distance within locales, as well as direct assessment of colony boundaries (Fig. 2), revealed that workers belonging to the same colony lived in proximity and that the boundaries of distinct colonies typically did not overlap. In fact, workers from distinct colonies were always separated by at least 10 m. Thus, our data did not support the hypothesis that *M. darwiniensis* formed unicolonial groups in which colony boundaries were absent.

We also discovered that the territory size of colonies varied from distances of only a few meters to linear ranges extending over 100 m (Fig. 2). An extraordinary case was discovered in locale N-4, where all workers apparently belonged to a single colony. The distance between the most widely separated trees sampled in this locale was 320 m. This colony probably contained millions of termites and the total area occupied by the colony extended at least 15,000 m^2 (the edges of the colony were not detected). The predominant vegetation in this locale was a large stand of *Pinus caribaea* planted about 30 years ago (G. Young, pers. comm.). As the stand matured, it became infested with *M. darwiniensis* and is now in advanced stages of decay. Previous studies using mark-recapture techniques have also reported *M. darwiniensis* colonies containing millions of termites (Hill 1942; French 1986), which foraged linear distances of about 100 m (Paton and Miller 1980; Miller 1993). Thus, single *M. darwiniensis* colonies may come to dominate exceptionally large territories.

Hill (1942) suggested that *M. darwiniensis* colonies could originate as buds of existing colonies. Yet our test for the presumed consequences of colony budding (i.e., genetic isolation by distance of colonies within locales) yielded nonsignificant results. However, the negative finding arising from this test does not represent convincing evidence against colony budding, because our test would only return a significant correlation under certain conditions. Specifically, new buds would have to be more similar to their parental colony than to other unrelated colonies within the locale, but sufficiently different from the parental colony so as to be distinguishable. This scenario would be likely only after new buds have had the opportunity to differentiate for some time.

Genetic Structure among Locales and Regions

In addition to documenting the genetic structure of colonies, we investigated patterns of higher-level genetic differentiation. The highly polymorphic microsatellite markers used in this study possessed substantial power in detecting population genetic structure. Indeed, we found that locales separated by tens to hundreds of kilometers differed genetically, as did more widely isolated geographic regions. We also found significant patterns of genetic isolation by distance among locales within regions. Overall, these patterns indicated that gene flow was restricted over relatively short distances.

There are at least two nonexclusive explanations that may account for these patterns of differentiation. First, primary *M. darwiniensis* reproductives may disperse poorly, thereby allowing genetic differentiation to build up among locales over time. However, a recent study suggests that *M. darwiniensis* winged reproductives are relatively strong flyers (Nalepa et al. 2001). Alternatively, winged reproductives may disperse widely but account for only a small fraction of new colonies formed. Although primary founding pairs are frequently observed after nuptial flights (Watson and Abbey 1989), they may suffer high mortality and incipient colonies may fail to mature. In this case, the primary mechanism of new colony formation may be budding, which would lead to strong patterns of genetic isolation among locales separated by relatively short distances (Crozier and Pamilo 1996).

The patterns of genetic isolation by distance observed in this study as described by the relationship between pairwise estimates of $F_{LP}/(1 - F_{LP})$ and log-distance (Fig. 3B) failed to follow the predicted linear trend expected under models of migration and drift (Rousset 1997). One explanation for this lack of fit is that the number of genetically independent

samples obtained from each locale was low, because many of the sampled workers belonged to the same colony. Consequently, our estimates of pairwise genetic differentiation between locales probably lacked accuracy and did not represent true estimates of population (i.e., groups of interbreeding organisms) differentiation. If this is the case, then the linear trend predicted may not apply.

A second factor contributing to the lack of fit of the model to the data is that the dispersal patterns of *M. darwiniensis* may differ from those assumed under simple models of isolation by distance. *Mastotermes darwiniensis* is particularly successful at invading and occupying environments disturbed by human activity. Previous studies in other taxa have noted that long-distance dispersal mediated by human transportation may be an important mode of movement for social insects under these conditions, leading to complex patterns of genetic structure (Vinson 1986; Goodisman et al. 2001b; Suarez et al. 2001).

Genetic structure above the level of the colony has been investigated in other isopteran taxa. Husseneder et al. (1998) observed genetic isolation by distance over areas of less than 1 km in *S. lamanianus*, a result that was interpreted as evidence for colony budding. However, they failed to find evidence for genetic structure among sites separated by 2–10 km. Similarly, little evidence for strong genetic differentiation among sites separated by about 1 km was discovered in the termite *R. flavipes* (Reilly 1987; Jenkins et al. 1999b, 2000; Bulmer et al. 2001). Differentiation of areas separated by larger distances has been frequently observed in other termite taxa. *Nasutitermes nigriceps* populations separated by about 100 km displayed significant differences in allele frequencies (Thompson and Hebert 1998), and *Coptotermes acinaciformis* and *C. lacteus* populations separated by hundreds to thousands of kilometers also differed genetically (Wang and Grace 2000a,b). Overall, data on the genetic structure of termite populations indicate that differentiation occurs over moderately large distances (10–100 km).

Conclusions

The genetic structure of *M. darwiniensis* populations is complex. The patterns observed within locales may result from combinations of selection pressures including the need to maintain high relatedness among colonymates and the necessity of rapid colony growth and dominance of local habitats. Dispersal of individuals is restricted over larger areas, suggesting that colonization of new sites may be difficult. Consequently, the importance of local competition may dictate the life-history strategies displayed by *M. darwiniensis*.

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APPENDIX 1

Genotypic differentiation of *Mastotermes darwiniensis* workers from pairs of trees (1–21) sampled from local N-1 (above diagonal) and distances in meters between trees (below diagonal).

Tree	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1									*	*	*	*	*	*	*	*	*	*	*	*	*
2	18								*	*	*	*	*	*	*	*	*	*	*	*	*
3	18	5							*	*	*	*	*	*	*	*	*	*	*	*	*
4	25	7	9						*	*	*	*	*	*	*	*	*	*	*	*	*
5	35	19	18	14					*	*	*	*	*	*	*	*	*	*	*	*	*
6	33	15	16	8	7				*	*	*	*	*	*	*	*	*	*	*	*	*
7	41	24	24	19	6	11			*	*	*	*	*	*	*	*	*	*	*	*	*
8	38	20	21	13	11	5	12		*	*	*	*	*	*	*	*	*	*	*	*	*
9	133	118	116	113	100	105	94	103											*		
10	119	103	102	96	84	88	78	84	34										*		
11	120	103	102	96	85	88	79	83	44	10									*		
12	116	99	99	92	81	84	75	80	49	15	6								*		
13	119	107	106	101	88	92	82	88	30	5	14	20							*		
14	120	103	103	96	85	88	79	83	45	11	1	5	15						*		
15	161	146	139	139	127	132	121	129	29	49	56	61	14	56					*		
16	155	141	144	134	121	127	116	124	24	45	53	58	40	54	6				*		
17	163	148	147	142	129	134	124	131	31	51	57	63	45	58	3	8			*		
18	172	157	155	151	139	144	133	141	39	61	68	73	56	68	12	17	10		*		
19	219	206	203	200	186	193	182	190	87	113	119	126	107	120	64	68	62	52		*	*
20	189	165	165	159	147	150	142	146	66	64	63	67	60	63	44	48	42	45	80		
21	183	172	172	166	155	158	149	152	74	71	70	74	67	70	51	56	49	51	82	8	

* Significant genotypic differentiation ($P < 0.05$) after Bonferroni corrections.

APPENDIX 2

Genotypic differentiation of *Mastotermes darwiniensis* workers from pairs of trees (1–27) sampled from locale N-4 (above diagonal) and distances in meters between trees (below diagonal). Note: Termites from no two pairs of trees were significantly differentiated.

Tree	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1																											
2	9																										
3	24	16																									
4	2	8	24																								
5	9	7	14	11																							
6	14	11	14	16	4																						
7	2	16	12	21	11	6																					
8	26	2	7	27	18	13	8																				
9	37	29	14	37	29	27	23	13																			
10	5	41	32	47	44	44	44	36	25																		
11	72	64	54	7	67	68	67	58	46	22																	
12	76	69	59	74	71	71	7	62	48	27	5																
13	88	79	66	87	8	8	76	67	53	42	29	24															
14	116	17	94	115	19	18	12	94	8	7	54	48	27														
15	144	135	124	141	139	138	136	126	114	94	72	66	63	46													
16	21	192	183	198	195	196	195	185	171	152	129	124	121	12	58												
17	239	232	221	239	235	236	231	225	29	191	169	165	16	14	98	38											
18	242	235	225	238	238	239	235	228	213	193	17	165	164	145	12	44	12										
19	32	312	35	317	317	320	317	311	298	275	252	25	251	235	19	135	13	94									
20	314	35	297	39	311	311	311	31	29	264	244	241	243	225	18	125	91	83	12								
21	311	31	293	36	38	37	39	297	286	262	241	238	238	223	176	119	86	78	18	5							
22	284	274	264	28	28	279	279	27	257	235	213	28	26	189	144	85	5	44	56	44	38						
23	37	298	289	33	33	33	31	291	277	257	235	23	227	29	165	18	69	64	54	42	38	24					
24	257	248	237	255	252	253	249	239	226	27	183	18	174	152	113	57	2	29	13	89	85	46	59				
25	244	236	222	241	239	239	235	225	29	194	173	167	158	133	99	5	35	48	129	117	112	74	84	27			
26	175	168	164	171	173	177	175	17	161	135	117	116	13	131	91	96	117	11	157	154	153	138	162	137	142		
27	221	216	26	219	219	22	219	211	2	175	154	153	159	148	11	64	65	56	1	95	92	77	11	85	99	61	

APPENDIX 3

Genotypic differentiation of *Mastotermes darwiniensis* workers from pairs of trees (1–24) sampled from locale N-5 (above diagonal) and distances in meters between trees (below diagonal).

Tree	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
2	60			*	*	*	*	*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*
3	86	26		*	*	*	*	*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*
4	94	36	19		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
5	155	99	73	80		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
6	212	163	141	148	72				*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
7	241	189	166	173	94	28			*	*	*	*	*	*	*	*	*	*		*	*	*		*
8	196	155	136	148	81	38	59		*	*	*	*	*	*	*	*	*	*		*	*	*	*	*
9	200	174	163	179	127	93	110	56		*	*	*	*	*	*	*	*	*	*	*	*	*		*
10	218	203	195	212	166	132	146	96	40		*	*	*	*	*	*	*	*	*	*	*	*	*	*
11	772	718	695	686	623	588	563	628	668	696					*	*	*	*	*	*	*	*	*	*
12	769	718	695	686	623	588	563	628	668	696	3				*	*	*	*	*	*	*	*	*	*
13	770	718	695	686	623	588	563	628	668	696	3	3			*	*	*	*	*	*	*	*	*	*
14	773	707	689	687	619	583	554	616	659	687	14	15	14		*	*	*	*	*	*	*	*	*	*
15	854	797	772	772	700	651	626	681	711	736	194	196	192	179		*	*	*	*		*	*	*	*
16	773	717	691	693	620	569	540	592	622	638	224	224	224	208	102					*				
17	770	716	689	691	615	563	533	588	615	630	233	233	233	217	112	11				*				
18	761	709	681	684	609	558	525	587	608	625	234	234	234	220	117	16	6			*				
19	777	720	695	697	623	571	541	597	623	639	234	234	234	221	107	13	6	14		*				
20	792	734	710	712	639	582	553	613	635	653	235	235	235	219	95	19	20	28	14		*	*	*	*
21	715	660	633	636	562	508	482	536	564	583	218	218	218	205	152	59	57	50	62	77				
22	703	651	621	624	550	498	469	522	550	569	232	232	232	217	164	70	65	59	71	86	16			
23	714	661	635	636	559	509	479	535	559	578	236	236	236	220	158	61	56	49	62	77	17	11		
24	647	591	565	569	494	441	411	466	498	516	236	236	236	223	215	126	124	116	130	143	67	57	69	

* Significant genotypic differentiation ($P < 0.05$) after Bonferroni corrections.

APPENDIX 4

Genotypic differentiation of *Mastotermes darwiniensis* workers from pairs of trees (1–20) sampled from locale N–11 (above diagonal) and distances in meters between trees (below diagonal).

Tree	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1			*	*	*	*	*	*	*		*	*	*	*	*	*	*	*	*	*
2	27		*	*	*	*	*	*	*		*	*	*	*	*	*	*	*	*	*
3	97	80		*	*	*	*	*	*		*	*	*	*	*	*	*	*	*	*
4	150	129	54				*						*	*	*					
5	197	171	131	95			*						*	*	*					
6	185	157	131	105	24		*			*			*	*	*					
7	217	190	154	118	22	33		*	*		*	*	*	*	*	*	*	*	*	*
8	228	201	167	131	36	43	12						*	*	*					
9	230	203	171	135	39	46	17	3		*			*	*	*					*
10	237	211	188	161	64	58	45	35	30		*					*	*	*		*
11	215	189	172	148	58	45	46	42	40	23			*	*	*					
12	205	179	169	150	66	48	57	56*	55	38	15		*	*	*					
13	203	178	180	168	91	71	85	83	82	61	42	27				*	*	*	*	*
14	194	169	173	165	94	72	90	90	89	70	49	34	10			*	*	*	*	*
15	187	162	166	157	88	66	86	86	86	70	48	32	16	8		*	*	*	*	*
16	176	150	146	135	68	45	74	74	74	66	43	30	34	30	22					
17	185	158	151	138	65	44	65	67	67	58	34	21	30	29	22	8				
18	176	149	135	119	50	26	55	61	61	62	40	34	49	48	41	19	19			
19	170	144	129	114	49	25	56	64	64	67	45	40	53	53	45	23	26	5		
20	163	137	121	108	49	24	59	69	70	74	53	48	62	60	51	30	32	15	8	

* Significant genotypic differentiation ($P < 0.05$) after Bonferroni corrections.