

Evaluation of DNA Fingerprinting, Aggression Tests, and Morphometry as Tools for Colony Delineation of the Formosan Subterranean Termite

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*Multilocus DNA fingerprinting, aggression tests, and morphometry were compared to evaluate their potential for the delineation of colonies of *Coptotermes formosanus* Shiraki (Isoptera; Rhinotermitidae) in Hawaii. DNA fingerprinting segregates the termites from all collection sites and allows the assignment of all individuals to their original collection site. The genetic similarity of termites from different collection sites approaches the population's genetic background similarity, consequently collection sites represent independent colonies. Aggression between colonies is comparatively low and does not provide reliable colony delineation. Morphometry allows a 79% classification rate of termites to their colony of origin. No correlation among genetic similarities, aggression levels, and morphometric distances is found. Of the three investigated methods, we conclude that the genetic approach is the most useful tool for colony delineation in *C. formosanus*.*

KEY WORDS: genetic similarity; Mahalanobis distances; population structure; *Coptotermes*; Isoptera.

INTRODUCTION

Colony delineation in termites, i.e., the assignment of collection sites (infested structures, aggregation traps) to specific colonies, has become essential for the study of termite biology and in pest management for the effective

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evaluation and application of control techniques such as toxic baits. The Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Rhinotermitidae, Isoptera), is a severe pest species in Hawaii and in numerous locations on the U.S. mainland. Colony delineation in this species remains a challenge for several reasons. Cryptic nesting and feeding habits prevent colony delineation by direct observations of behavior (Shellman-Reeve, 1997; Thorne *et al.*, 1999). Foraging areas are dynamic and the use of feeding sites by more than one colony is probable (Jenkins *et al.*, 1999b). Mature colonies contain up to 10 million individuals and their foraging areas can extend 100 m and more (Tamashiro *et al.*, 1987). Multiple reproductives and subsidiary nests (Bess, 1970; Su and Tamashiro, 1987) may lead to a complicated colony structure with separate breeding units.

Behavioral bioassays and epicuticular hydrocarbons provide somewhat ambiguous information for the differentiation of *C. formosanus* from different collection sites (e.g., Su and Haverty, 1991; Shelton and Grace, 1996; Haverty *et al.*, 1996). For example, *C. formosanus* from Florida sites are not aggressive, but some Hawaiian colonies fight. These observations require further interpretation on a population genetic basis to assess genetic differentiation and define colony membership, as accomplished in other termite species [*Reticulitermes* (Clément, 1986; Reilly, 1987; Jenkins, 1999a,b), *Schedorhinotermes* (Husseneder *et al.*, 1998)].

Aggression levels in *C. formosanus* have not yet been linked to the genetic structure of populations because genetic markers such as alloenzymes and mitochondrial DNA did not provide sufficient polymorphism to reveal differences between colonies (e.g., Korman and Pashley, 1991; Strong and Grace, 1993; Broughton and Grace, 1994; Wang and Grace, 1995). Yet colonies of an African rhinotermitid species could be identified even on a small geographic scale by congruent results from multilocus DNA fingerprinting, observation of aggressive behavior, and morphometric measurements (Husseneder *et al.*, 1998). The study presented in this paper was initiated to determine if *C. formosanus* colonies in Hawaii could be delineated with similar DNA fingerprinting techniques, aggression tests, and morphometric methods. We compared genetic similarities, aggression levels, and morphometric distances to determine if aggressive behavior and phenotypic features are correlated with genetic factors and can therefore be used as indirect markers for describing the population structure.

MATERIALS AND METHODS

Collection of Termites

In November 1998, *C. formosanus* were collected from five sites on the Manoa campus of the University of Hawaii (aggregation traps, 120- to 540 m

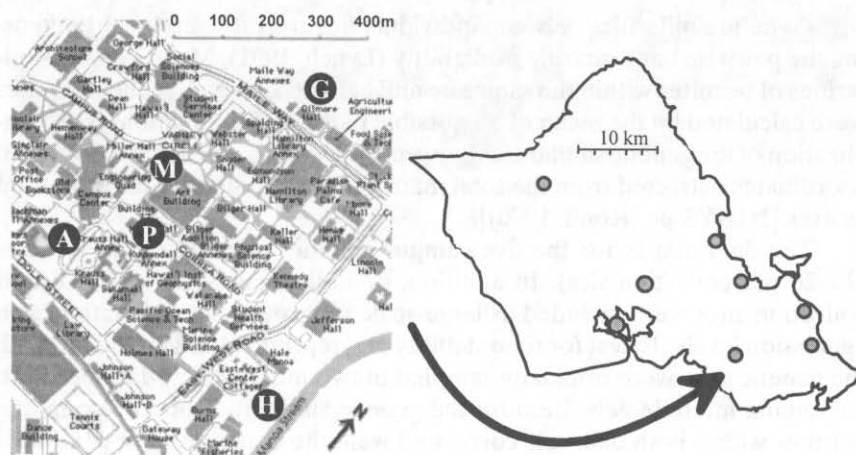


Fig. 1. Collection sites (naturally infested wood, aggregation traps containing cut wood) for *C. formosanus* on the Manoa campus of the University of Hawaii and at additional collection sites across the island of Oahu.

distance; Fig. 1) for quantifying genetic similarities, aggressive behavior, and morphometric distances among individuals from these sites. Termites from distant sites on the island of Oahu (naturally infested wood, 4- to 39-km distance; Fig. 1) were used as outgroups for determining aggression levels and correlating aggression levels with genetic similarities and morphometric data. All colonies had been observed for several years and were considered to be mature. For DNA analysis, workers were frozen in liquid nitrogen or preserved in 95% ethanol (Quantum Anaheim, CA). For aggression tests, living workers were kept in petri dishes (diameter, 10 cm) with moist filter paper for 24 h prior to the tests. For morphometric measurements, soldiers were placed in 70% ethanol (Fisher Scientific Fair Lawn, NJ).

Multilocus DNA Fingerprinting

Multilocus probes target simple repetitive sequences in the genome. They generate individual-specific banding patterns that follow Mendelian rules of inheritance (Jeffreys *et al.*, 1991). For visualization of DNA fingerprint patterns, DNA was extracted from individual workers and digested with *Hae*III (Roche Molecular Biochemicals, Indianapolis, IN). The DNA fragments were separated through electrophoresis on 20 × 25-cm 0.8% agarose gels at 45 V for 72 h, blotted onto nylon membranes (Roche), hybridized with the digoxigenated oligonucleotide probe (GTG)₅ (MWG-Biotech AG, Ebersberg, Germany) and visualized by a chemiluminescence reaction (Roche).

Genetic similarities between individual fingerprints were quantified using the pairwise band-sharing probability (Lynch, 1991). Mean genetic similarities of termites within the same site and between different collection sites were calculated by the mean of all possible pairwise combinations. For visualization of the genetic similarities between individuals, the first two principal coordinates extracted from the total matrix of genetic similarities were used as axes [NTSYS-pc (Rohlf, 1990)].

The data matrix for the five campus colonies is based on 83 workers (14–20 per collection site). In addition, 67 individuals from seven distant collection sites were included as large-scale outgroups for correlation with aggression levels. To test for time stability and reproducibility of the method, the genetic data were originally sampled in two independent datasets, both combining multiple gels. Because the genetic similarities of corresponding pairings within both data sets correlated well, the data sets were combined ($r = 0.97$, $P < 0.001$, one-tailed permutation test).

Aggression Tests

The experimental setup to determine the aggression level between termite workers was derived from Kaib and Brandl (1992). For each experiment, two groups of five workers, each from the same or from different collection sites, were paired in petri dishes (diameter, 10 cm) with moist filter paper. The number of termites involved in aggressive behavior (biting) was scored at 1-min intervals for as long as 15 min. The mean of these 15 scores, the aggression index, ranges theoretically from 0 (none of the termites showed aggression) to 10 (in each interval all termites were involved in aggressive behavior). Each experimental pairing was repeated up to six times, with a new set of termites each time, and the mean aggression index (AI) was calculated. To check for temporal bias in the aggression levels, we repeated the first pairings at the end of the study. There was no significant difference in the repeated measures, so we combined all data for the entire study period ($Z = -0.58$, $P > 0.20$, two-tailed Wilcoxon test).

Morphometry

Nine linear parameters of 15 soldiers from each collection site on the campus and from the site most distant from the campus (39 km) were measured according to Kaib and Brandl (1992): maximum head capsule width and length, distance between ocelli, distance between antennal bases, pronotum width and length, mesonotum width, metanotum width, and distance between cerci. Log transformation and size correction of the parameters prior

to analysis did not improve the resolution. Therefore the original data set was subjected to canonical variate analysis using collection sites as group variables. The first two canonical variates were used to visualize morphometric distances (Mahalanobis distances) between termites from different collection sites.

Statistical Evaluation

Statistical evaluations, canonical variate analysis, principal coordinate analysis, and graphic visualization were performed using NTSYS-pc 1.70 (Exeter Software) (Rohlf, 1990), Sigma Plot 5.0, and SPSS 9.0 for Windows (SPSS Inc.). In general, nonparametric tests were applied because data were not normally distributed. Aggression levels were compared by two-tailed Kruskal-Wallis one-way ANOVA (note that, because of the pairwise interdependencies, this test is considered approximative). Due to the nonindependence of data points, correlations between data sets and one-tailed error probabilities were calculated via permutations (1000 times).

The classification rate of individuals was estimated by the Mahalanobis distance method [*U*-method (Lachenbruch, 1967; Sharma, 1996)]. Each case was classified to the nearest group by the discriminant functions derived from all cases other than that case (cross validation). Prior probabilities of group membership were assumed to be equal.

RESULTS AND DISCUSSION

Multilocus DNA Fingerprinting

Banding patterns of 83 individuals from the five campus collection sites were scored between 4- and 23-kb fragment size, which included an average of 10.2 bands ($SD = 2.3$). Pairwise genetic similarities between individual termites range from 0.20 to 0.72 on a scale from 0 (no common bands) to 1 (identical banding patterns). Principal coordinate analysis of these similarities visualizes the genetic relationship of the termites from the five collection sites (Fig. 2a): individuals of the same collection site consistently group together. Termites from different sites form separate clusters. Three of the five sites (G, H, P) overlap in the two-dimensional projection of the multivariate space; however, they can be separated in the third dimension. The mean genetic similarity within collection sites is 0.66 ($SD = 0.12$; $N = 659$). The mean genetic similarity between collection sites, 0.29 ($SD = 0.12$; $N = 2247$), is significantly lower than that within collection sites ($P = 0.002$, two-tailed *U* test). Genetic similarity between collection sites

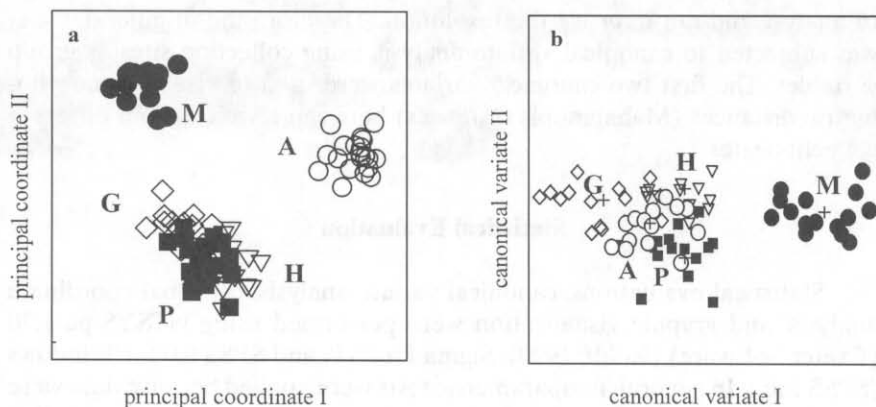


Fig. 2. Genetic and morphometric differentiation of termites from the five campus colonies. (a) Principal coordinate analysis of genetic similarities between workers. Axes combine 34% of the total variance. Termites from Miller Hall and Amphitheater form distinct groups. Gilmore, Hale Halawai, and Publication segregate in higher dimensions. (b) Canonical variate analysis of morphometric distances of individual soldiers. + indicates group centroids. Axes combine 86% of the total variance.

approximates the population's background similarity of 0.21, i.e., the average genetic similarity between nonrelated individuals (Husseneder and Grace, 2001, in press). Because termites from disparate collection sites are no more related than the population average, we conclude that collection sites in this study represent independent colonies. To determine how these findings translate into successful classification of individuals to colonies, the predicted group membership of each individual (based on the nearest distance to one of the five group centroids) was compared to their actual site of origin. Due to the considerable genetic differentiation between the colonies, individual termites can be assigned to their actual colony of origin in 100% of all cases.

Accumulating genetic studies on termites increasingly shed light on the structure of termite colonies, e.g., degrees of inbreeding [*Incisitermes* (Luykx, 1985), *Reticulitermes* (Reilly, 1987), *Nasutitermes* (Atkinson and Adams, 1997; Thompson and Hebert, 1998), *Schedorhinotermes* (Husseneder *et al.*, 1999)], kin-biased foraging (Kaib *et al.*, 1996), evidence of budding, and genetic substructure within colonies (Atkinson and Adams, 1997; Husseneder *et al.*, 1998; reviewed by Thorne *et al.*, 1999). The complex patterns require highly sensitive methods to identify colonies unequivocally. We believe that DNA fingerprinting is a valid tool for delineation of colonies and colony membership, because it shows clear genetic differentiation even on a small spatial scale (<500 m). In a natural population of the African termite species *Schedorhinotermes lamanianus* Sjöstedt, nine collection sites could

be grouped to three colonies, one of which exhibited an intracolony genetic substructure (Husseneder *et al.*, 1998). In the present study on an introduced species, termites could be assigned to collection sites that, in this case, represented independent colonies. Further studies with *C. formosanus* are using this successful delineation of colonies to provide detailed analyses of the relationships between adjacent colonies and between different collection sites within the same colony. In synopsis with genetic studies in other termite species (Thorne *et al.*, 1999), we hope to describe processes and sources of colonization into new areas as well as reinvasion of areas where termite activity has previously been eliminated.

Aggression

Mean aggression levels from 31 different pairings (112 single aggression tests) of workers from the same colony, different colonies at the University of Hawaii campus, and geographically isolated colonies from across Oahu are shown in Table I. No aggression was recorded in intracolony pairings of workers. Termites from colonies that showed aggressive behavior in a different bioassay several years ago (Shelton and Grace, 1997a) still demonstrate aggression.

Compared to another rhinotermitid species, *S. lamanianus*, the level of aggression in *C. formosanus* is low (Husseneder *et al.*, 1998). Aggression in single tests ranges only between 0.0 and 2.1 on a scale from 0 to 10. Due to the low aggression levels and the variation between pairings, no statistically significant differences were found among intracolony pairings, intercolony pairings on a small spatial scale, and intercolony pairings on a large spatial scale ($\chi^2 = 3.66$, $df = 2$, $P = 0.16$, Kruskal-Wallis ANOVA; Table I). Even aggression tests on a large scale between geographically isolated colonies

Table I. Mean Aggression Levels Between Groups of Workers from the Same and from Different *C. formosanus* Colonies^a

Pairings of workers	AI	SD	N
Intracolony	0.00	0.00	8
Intercolony			
Small scale	0.15	0.32	10
Large scale	0.31	0.61	13

^aAI, mean aggression index of all intra- and intercolony pairings of *C. formosanus* workers (small scale, University campus; large scale, island of Oahu); N, number of pairings (each consists of three to five repeated tests). No statistically significant difference was found between intracolony pairings and intercolony pairings ($\chi^2 = 3.66$, $df = 2$, $P = 0.16$, Kruskal-Wallis ANOVA).

across Oahu do not consistently demonstrate more aggression than within colonies. Distances up to 39 km were measured between nonaggressive termites. Tolerance was confirmed through additional mortality counts after 24, 48, 72, and 96 h following the bioassay of Shelton and Grace (1997a). Even after 4 days the mortality of pairings between termites found nonaggressive in previous aggression tests was not significantly different from that of intracolony pairings ($P = 0.15$, two-tailed U test). Obviously, tolerance does not necessarily mean membership in the same physical colony. Contrary to the results in most termite species, which show consistently aggressive discrimination of noncolony mates (e.g., Thorne and Haverty, 1991; Shelton and Grace, 1996; Husseneder *et al.*, 1998; Jmhasly and Leuthold, 1999), aggression tests cannot be used unequivocally for the determination of colony membership in Hawaiian *C. formosanus* (Delaplane, 1991; Su and Haverty, 1991).

Aggression between colonies does not occur in all termite species or under all conditions. Considering the sum of the studied termite species, passive encounters between disparate colonies are rather uncommon (reviewed by Thorne and Haverty, 1991; Shelton and Grace, 1996) and may be triggered by a high genetic relatedness between colonies or an environment where monopolizing and defending resources is of little importance (e.g., Clément, 1986; Grace, 1996). The possible influence of genetic factors is discussed below.

Morphometry

The data set combines nine morphometric parameters measured in 15 soldiers from each of the five campus colonies. All nine parameters show significant differentiations of group means between the colonies ($df = 4, 70$, $P < 0.01$, Wilks' λ U statistics). The best discrimination according to Wilks' λ (differentiation of group means) and the structure coefficients (contribution of each parameter to the formation of the discriminant functions) is provided by pronotum length (Wilks' $\lambda = 0.23$, $F = 60.3$), followed by head length (Wilks' $\lambda = 0.24$, $F = 57.3$) and head width (Wilks' $\lambda = 0.33$, $F = 36.3$, $df = 4, 70$, Wilks' λ U statistics). Pronotum length is only moderately correlated with head length and width ($r < 0.45$). The latter two, however, are not independent ($r = 0.68$). The first two of the four canonical discriminant functions combine 86% of the total variance and are used to visualize the morphometric distances between individual termites from the five colonies (Fig. 2b).

The group means of the colonies are differentiated. This corroborates that the average size of certain morphometric parameters (e.g., pronotum

length, head capsule length, and head capsule width) can be used to search for differentiation between large termite samples from known locations. However, single individuals or small samples cannot be reliably assigned to colonies by morphometry alone, as shown in Fig. 2b. The plot of the morphometric distances between individuals shows considerable scatter. Patterns of colony A, G, H, and P members overlap, while termites from colony M are separated. For individuals, 93% can be correctly classified as M members, 87% as G members, and 80% as H and P members, but only 53% as A members. On the average, in 79% of all cases the predicted group membership coincides with the actual colony of origin. Compared to the 100% successful classification based on genetic similarities, morphometry is a less accurate predictor of colony membership in *C. formosanus*.

Morphometry was used previously for determining colony structure in other termite species. Kaib and Brandl (1992) inferred spatial dynamics in the colony structure of *S. lamanianus* using morphometric measurements and aggression tests. In a later study on the same species, congruent results of morphometry and DNA fingerprinting allowed grouping of individuals from different collection sites to colonies (Husseneder *et al.*, 1998). Due to the considerable variance of morphometric distances within groups, however, no attempt was made to assign single individuals to collection sites based on morphometric data alone (see also Hostettler *et al.*, 1995).

Correlation of Genetic Similarities, Aggression Levels, and Morphometric Distances?

Aggression between *C. formosanus* colonies is low or absent and does not correlate with the genetic similarities (Fig. 3a). In theory, this can be explained by three hypotheses: (1) the population is genetically homogeneous, i.e., there is no difference in the genetic similarities within and between colonies; (2) the studied colonies are highly related; and (3) aggression is not entirely a function of genetic relatedness.

Each hypothesis is considered in turn.

(1) Studies in certain European *Reticulitermes* show that termite societies can be "open," i.e., low aggression permits exchange of individuals between neighboring sites, leading to genetic uniformity, which in turn may promote tolerance (Clément, 1986). This phenomenon depends on the species and environmental conditions (Kaib and Brandl, 1992). However, in the Hawaiian *C. formosanus* genetic similarities within colonies (0.66) are significantly higher than between colonies (0.29). This did not change over time (Husseneder and Grace, 2001, in press). Genetic uniformity is therefore low and colonies clearly are genetically distinct entities.

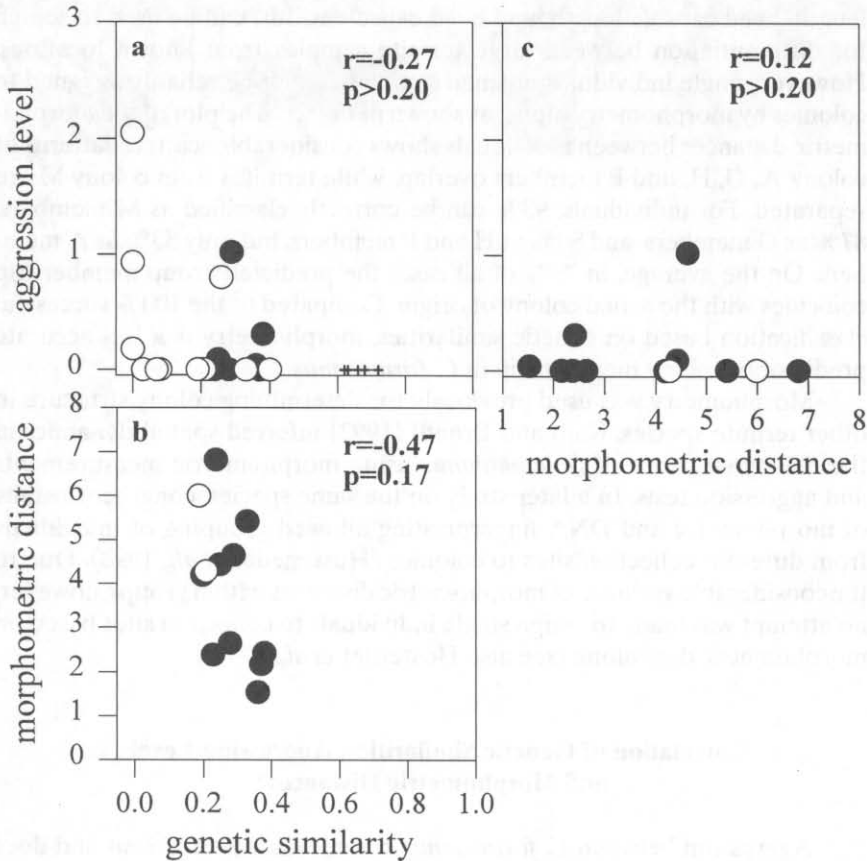


Fig. 3. Lack of correlation among genetic similarities, aggression levels, and morphometric distances. (a) Relationship of aggression levels and genetic similarities between the five campus colonies (●) and seven distant colonies across Oahu (○). In addition, the control pairings of workers from the same colony are shown (+) but excluded from the calculation of Pearson's correlation coefficient. (b) Relationship of morphometric distances and genetic similarities between all five campus colonies (●) as well as between two campus colonies (G, H) and the colony of Oahu with the greatest geographical distance from the campus (○). (c) Relationship of aggression levels and morphometric distances between campus colonies (●) as well as between two campus colonies and a distant colony (○). The correlation coefficients were not significant (one-tailed permutation test; due to missing pairs in the matrix, the values are considered approximate).

(2) *C. formosanus* is an introduced species in Hawaii. A limited number of introductions and genetic bottlenecks are suggested to have resulted in a purported lack of genetic variation in the population. High genetic background similarity in the population together with limited dispersal may have

led to extensive local inbreeding (as discussed by Haverty *et al.*, 1996; Strong and Grace, 1993; Broughton and Grace, 1994). In contrast to these assumptions, however, the present study does not corroborate an ample lack of genetic variability in the population. The population's genetic background of 0.21 (Husseneder and Grace, 2001, in press) lies in the same magnitude of natural populations [0.25 for an African population of *Schedorhinotermes* (Husseneder, 1998); for other species including mammals, see Lynch (1991)]. Local inbreeding appears to be moderate. Even on a small spatial scale the genetic similarities between colonies are not considerably higher than the genetic background (0.29 compared to 0.21 background similarity).

(3) After exclusion of alternate explanations we suggest that, contrary to similar studies in other termite species (e.g., Adams, 1991; Husseneder *et al.*, 1997), aggression in *C. formosanus* is not necessarily evoked through cues that would be affected by low genetic relatedness. Variation in aggressive responses may be triggered by environmental factors. According to previous studies in social insects, aggression is dependent on the size, health, and maturity of colonies, as well as on the availability of nutrients [termites (Thorne and Haverty, 1991; Clément, 1986; Polizzi and Forschler, 1998), bees (Ribbands, 1954), ants (Wallis, 1962)]. Furthermore, aggression can be experimentally modulated by temperature, diet, or nest material [termites (Shelton and Grace, 1997a,b), ants (e.g., Le Moli and Mori, 1990)].

Morphometric measurements show differentiation between campus colonies, but they are not significantly correlated with the genetic similarities (Fig. 3b). Size parameters averaged over a large pool of individuals may show qualitative differentiations between colonies, but they do not measure the relative degree of difference between colonies that is necessary for the analysis of population structures.

To demonstrate that the lack of correlation is not due to the limited spatial scale of the campus area, we included genetic similarities and morphometric distances between two campus colonies and the colony most distant from the campus in our correlation analysis (Fig. 3b; ○). Although the genetic similarities of the campus colonies to the distant colony descend as low as the population's background similarity, the morphometric distances still vary within the range defined by the morphometric distances between the campus colonies on a small spatial scale. Thus, the correlation is not improved by including geographically isolated colonies.

These results are in contrast with other termite studies, where morphometric distances proved to be a good predictor of genetic similarity even on a small spatial scale (Husseneder *et al.*, 1998). As suggested for aggression levels, phenotypic features in *C. formosanus* appear to be more influenced by environmental factors than genetic cues. Behavioral and phenotypic patterns,

though, are not determined by the same factors, at least not in the same way. The aggression levels are low in spite of the considerable range of morphometric distances, including the data from a colony outside the campus (Fig. 3c; ○). No correlation could be found between aggression and morphometric distances (Fig. 3c).

In summary, classical approaches to colony delineation, such as aggression tests and morphometry, can be applied with only limited success in *C. formosanus* and are not clearly correlated to the genetic structure. DNA fingerprinting, however, is able to elucidate a pattern of differentiation between colonies and to segregate individuals to specific colonies even on a small spatial scale. The genetic approach appears to be, therefore, the most useful tool available for the analysis of population structure in *C. formosanus*.

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