

Characterization of Microsatellites for Population Genetic Analyses of the Fungus-Growing Termite *Odontotermes formosanus* (Isoptera: Termitidae)

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ABSTRACT The fungus-growing subterranean termite *Odontotermes formosanus* Shiraki (Isoptera: Termitidae) is a destructive pest in Southeast Asia. To facilitate studies on the biology, ecology, and control of *O. formosanus*, we isolated and characterized nine novel microsatellite loci from a mixed partial genomic library of *O. formosanus* and the sympatric *Macrotermes barneyi* Light enriched for di-, tri-, and tetranucleotide repeats. We screened these loci in three populations of *O. formosanus* from China. All loci were polymorphic. Three loci showed heterozygote deficit possibly because of the presence of null alleles. The remaining six loci with 4–15 alleles per locus and an average observed heterozygosity of 0.15–0.60 across populations were used for population genetic analysis. Populations from different provinces (Guangdong, Jiangxi, and Hubei) were genetically differentiated, but the genetic distance between populations was surprisingly small (F_{ST} : 0.03–0.08) and the gene flow was considerable (N_m : 3–8), despite the geographical distance being >300 km. Genetic diversity within populations was low (allelic richness: 5.1–6.3) compared with other subterranean dwelling termites, but consistent with the diversity in species of the family Termitidae. Microsatellite markers developed for *O. formosanus* will allow further studies to examine the phylogeography, population genetic and colony breeding structure, dispersal ranges, and size of foraging territories in this and closely related species, as well as aid in assessing treatment success.

KEY WORDS social insect, genetic marker, genotyping, allele, gene flow

Fungus-growing termites (Macrotermitinae) are found throughout the tropics and subtropics of Africa and Asia and have attracted widespread interest among researchers because of their obligatory symbiosis with basidiomycete fungi (*Termitomyces* spp., reviewed in the article by Bignell 2000). The majority of research has focused on the evolution and transmission of the fungal (Aanen et al. 2002) and bacterial (Shinzato et al. 2007) symbionts and the function of their enzymes (Pan et al. 2009, Zhou et al. 2010). As the center of diversity of Macrotermitinae is Africa, the general biology and ecology of macrotermitine species in Asia are less known compared with their counterparts in Africa (Cheng et al. 2011 and references therein).

The black-winged subterranean fungus-growing termite *Odontotermes formosanus* Shiraki (Termitoidea: Termitidae) is an important decomposer (Bignell and Eggleton 2000), but also a major pest in forestry and agriculture in Southeast Asian countries, including China, Myanmar, Thailand, Vietnam, India, and Japan (Huang et al. 2000, 2006, Cheng et al. 2007). In addition to damaging trees and crops, this species is also known to attack buildings and destroy earthen dams and dykes (Cai et al. 1965, Huang et al. 2008).

Similar to other subterranean dwelling termites, the cryptic lifestyle of *Odontotermes* has hindered direct observation and the execution of field experiments with appropriate replications. Current knowledge comprises mainly descriptive studies of flight phenology, colony development, and physical nest structure published in the Chinese literature (reviewed in the article by Cheng et al. 2007). Molecular markers have proven especially useful to overcome these limitations and shed light on many aspects of subterranean termite biology (reviewed in the article by Vargo and Hussener 2009, 2011). The goal of this study was to characterize genetic markers that would aid in providing comprehensive knowledge about *O. formosanus* biology comparable with that already gained in other termites with subterranean habits, such as *Re-*

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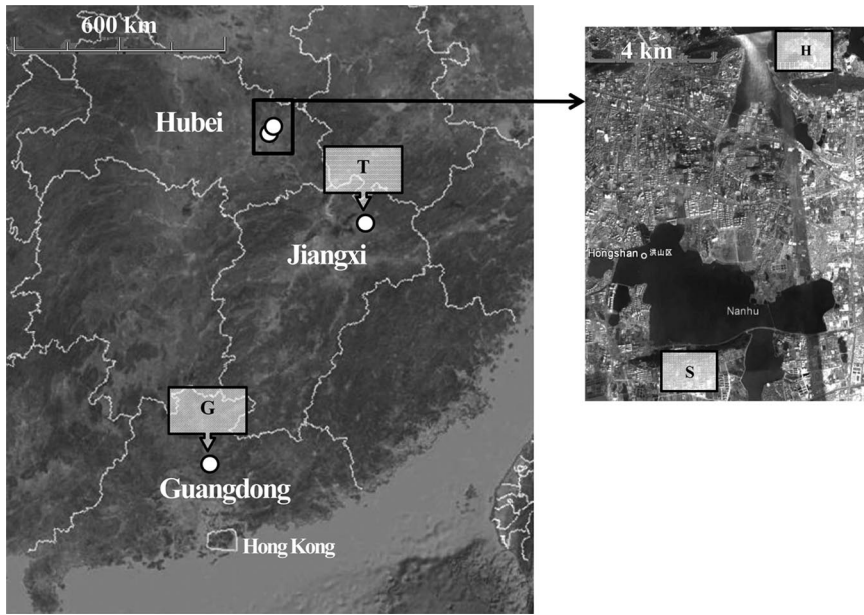


Fig. 1. Map of the four sample sites of *O. formosanus* (T, G, H, S) in three provinces of China (Hubei, Jiangxi, and Guangdong).

ticulitermes and *Coptotermes* spp (Vargo and Hussener 2009, 2011).

Microsatellites are the most popular genetic markers because of their broad scope of applicability ranging from the study of colony breeding structure to global population genetics. Microsatellites are simple sequence motifs of one to six nucleotides that are repeated typically between 5 and 40 times (Selkoe and Toonen 2006). Repeat units have a considerable mutation rate (10^{-2} – 10^{-6} per locus and generation) because of slip strand mispairing and proofreading errors during replication (Eisen 1999). The high allelic diversity and Mendelian co-dominant mode of inheritance of microsatellite loci make them especially useful genetic markers to study the hierarchical population genetic structure in social insects from large-scale gene flow and migration rates to the degree of inbreeding within and among colonies and individual assignment to colonies and populations (Goldstein and Schlötterer 1999).

In this study, we developed microsatellite markers from a selectively enriched clone library of *O. formosanus* and *Macrotermes barneyi* Light by using the SNX linker and subtractive hybridization method (Hamilton et al. 1999) and subjected them to quality control screening, including tests for linkage disequilibrium, large allele dropout, and presence of null alleles. The final set of six loci that passed the screening process was used to provide the first insights into the population genetics of *O. formosanus*.

Materials and Methods

Termite Collections and DNA Extraction. To develop microsatellites for Macrotermitinae, we col-

lected separate samples of ≈ 150 *O. formosanus* and *M. barneyi* individuals. Each sample represented a colony located in a wood stump in Changsha, China, in spring 2008. For population genetic studies of *O. formosanus*, workers of this species were collected in August 2008 from four sample groups (Fig. 1): 1) Huolu Hill and nearby South China Botanical Garden in Guangzhou City, Guangdong Province (G); 2) Baihe Lake and nearby Zhiguang Town in Yingtang City, Jiangxi Province (T); and 3) Houshan Hill (H), which is located 8 km from 4) Shizishan Hill in Wuhan City, Hubei Province (S). Within each sample group, termites were obtained from 25 different sites (putative colonies) within a radius of 5 km, with a minimum distance of 10 m between sites to minimize the possibility of collecting individuals from the same colony (personal observation from excavating *O. formosanus* colonies by Q.H.). The distances between sample groups ranged from 8 km within the same province to 300–800 km between different provinces (Fig. 1).

Specimens were stored in 95% ethyl alcohol until extraction of DNA. For the creation of a microsatellite-enriched partial genomic library, ≈ 12 μ g of high-quality genomic DNA was extracted from legs and antennae (to avoid contamination by gut contents) of ≈ 100 *O. formosanus* and *M. barneyi* workers from Changsha by using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). DNA was also extracted from whole bodies of three workers each from 25 putative colonies from each of the four sample sites of *O. formosanus* (Fig. 1). DNA concentrations were determined with a ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE) and ranged from 58 to 190 ng/ μ l (1.45–4.75 μ g per individual).

Table 1. Characteristics of nine microsatellite loci developed for *Odontotermes formosanus*

Locus	Repeat motif	Sequence length (bp)	GenBank accession number	Primers	T _m °C	<i>O. formosanus</i>				<i>M. barneyi</i> allele number and size
						Allele number	Allele size	Ho	He	
T5-5F	(agt) ₄ agc (agt) ₁₃	186	JN980106	tgtatccctaggacatgg gttctgagccgagtggtagc	59.6 60.0	9	317-341	0.58	0.71	203, 233
T7-10E	(actt) ₅	484	JN980109	ctcgttaaccaggccacattt gggaaaccaggaggaaagag	59.9 59.9	5	388-408	0.56	0.54	404, 408
13-10D	(ca) ₁₀	146	JN980111	ccgtctctgttggtaggttt gaagcctgatacacagttca	60.1 59.8	15	151-185	0.56	0.76	167
14-2A	(tac) ₁₁	170	JN980112	ccttctcgatttaccacacag accctctatcccaaaagt	58.8 60.0	10	157-202	0.60	0.75	175
15-11C	(gt) ₂₀	146	JN980113	ttgtgcgtggtttgttttt tactgttgaccaccgatcac	60.0 59.6	5	133-149	0.43	0.54	135
15-12B	(ac) ₈	178	JN980114	ggttttcagctggtccagaa gcagaagcctgtgggataag	60.2 59.8	4	188-196	0.15 0.17	0.14	190
6-1A ^a	(actt) ₇	222	JN980107	cacgaaccatatttctctg gttgctcaggatagggaaca	59.3 60.1	11	216-328	0.21	0.77	344
T6-1C ^a	(gt) ₂₀	165	JN980108	agccttcttctctcctggt aatgcagtcgcttaccagcag	60.1 60.1	18	162-204	0.52	0.87	368
13-3B ^a	(gt) ₁₈	284	JN980110	tcgctgtttgttctcaggtg gtcgactgtaggcctctgc	60.0 60.0	19	184-332	0.29	0.67	284

T_m = melting temperature; Ho = observed heterozygosity, He = expected heterozygosity.

^a Loci significantly deviated from Hardy-Weinberg equilibrium because of heterozygote deficit in most *O. formosanus* populations and were thus not included in population genetic analysis.

Note that allele number and size in *M. barneyi* are only based on a single colony and thus preliminary.

Microsatellite Development. Microsatellite development essentially followed the protocol described by Booth et al. (2008), with some minor modifications (Husseneder et al. 2010) by using the SNX linker and subtractive hybridization method (Hamilton et al. 1999) for enrichment, followed by polymerase chain reaction (PCR) amplification of the enriched fragments and cloning them into *Escherichia coli* by using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA).

In total, 192 clones containing inserts with a size range of 400–1,150 bp were sequenced at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) by using the universal M13 forward primer. Microsatellites with at least five repeats of di-, tri-, or tetramers were detected in 66 sequences by using MSATCOMMANDER (Faircloth 2008). After removal of vector and SNX linker sequences, primer pairs were designed for 36 different loci that contained microsatellites with at least five tandem repeats (Primer3Plus software available at SourceForge.net). The primer pairs were synthesized by Eurofins MWG Operon (Huntsville, AL) with the forward primer end-labeled with a M13 forward tail.

Initially, gradient PCR reactions (with annealing temperatures ranging from 55 to 60°C and varying MgCl₂ concentrations from 2 to 3 mM) were run for each primer pair with DNA from at least three individual termite workers of *M. barneyi* and *O. formosanus*. After optimization, the PCR reaction mixes contained ≈20 ng DNA template, 1× PCR buffer, 0.2 μg/μl BSA, 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 U *Taq* polymerase, 0.32 pmol M13 F-29-IRD700 or 800, 2.0 pmol of each primer, and Millipore water to a total volume of 10 μl.

Microsatellite loci were amplified with a PTC-200 thermal cycler (MJ Research Inc., Littleton, MA) by

using a touch-down program: initial denaturation step at 94°C (30 s), followed by 3 cycles at 94°C (30 s), 60–1°C per cycle (30 s), and 72°C (30 s), and then 30 cycles at 94°C (30 s), 58°C (30 s), and 72°C (30 s), with a final extension step at 72°C (3 min). After adding 5 μl Blue Stop dye solution (Li-Cor Inc., Lincoln, NE), the amplified DNA fragments were denatured at 95°C for 5 min, loaded onto a 6.5% 1× TBE polyacrylamide gel, and separated according to their size by running the gel in a Li-Cor 4300 automated DNA analyzer with size standard IRDyes of 50–350 and 50–700 bp (Li-Cor Inc.). Microsatellite allele sizes were scored by using the program GeneProfiler software (Scanalytics, Inc., Fairfax, VA). Of the 36 primer pairs, 13 yielded scorable products that consistently amplified in *O. formosanus*. Four loci, however, were monomorphic in all samples. The original sequences and primers of the nine polymorphic loci (Table 1) were submitted to GenBank under the accession numbers JN980106–JN980114. We tested the microsatellites primarily for their applicability to analyze population genetics of *O. formosanus* because multiple colonies and populations were only sampled for this species. Data of cross-amplification in *M. barneyi* were included in Table 1, but have to be considered preliminary because they only represent one colony. Population genetic studies of *M. barneyi* will follow in the future.

Statistics. For each locus, the genotype of only one *O. formosanus* worker per colony was included in the database to avoid bias because of relatedness among workers from the same colony. Numbers of alleles per locus and observed and expected heterozygosity were calculated for each locus and population by using the program GDA (Lewis and Zaykin 2000). Genetic diversity at each locus was calculated by using the sample-size independent rarefaction analysis of

allelic richness as implemented in the FSTAT software package (Goudet 2001). Log-likelihood ratio G -statistics for randomized data sets were used to test for gametic disequilibrium and population differentiation (FSTAT).

Monte Carlo simulations implemented in the software Micro-Checker (Van Oosterhout et al. 2004) were used to test all loci for null alleles and possible scoring errors derived from large allele dropout and the presence of microsatellite stutter bands. Population genetic analyses were performed by using tests for population differentiation (see previously) and F -statistics (Weir and Cockerham 1984) as implemented in FSTAT. Heterozygote deficit in individuals relative to the population (F_{IS}) was used as a measure of inbreeding, and heterozygote deficiency among populations (F_{ST}) was used as a measure of pairwise genetic distance among populations; 95% CIs were estimated by bootstrapping over loci. Finally, individuals were probabilistically assigned to genetic clusters based on their multilocus genotypes by using Bayesian clustering implemented in *STRUCTURE* 2.3 (Pritchard et al. 2000). Simulations used 100,000 MCMC iterations in the burn-in phase and data collection phase and the default settings of the program. Separate simulations were run three times for models with different numbers of assumed genetic clusters ($K = 1-6$). The number of genetic clusters was determined following the Delta K method of Evanno et al. (2005) by using the Web-based *STRUCTURE HARVESTER* v.0.6 software (Earl and vonHoldt 2012). Then, the estimated membership coefficients of each individual multilocus genotype were plotted to test if the genetic cluster it was assigned to was congruent with actual population of origin.

Results and Discussion

Microsatellite Characteristics. Nine microsatellite loci amplified consistently in *O. formosanus* and results were repeatable when the same individuals were run on different gels. Of these loci, two had tetra-nucleotide repeat motifs, two had tri-nucleotide, and five had di-nucleotide repeats (Table 1). Eight loci contained perfect repeats; locus T5-5 F was interrupted by a nucleotide transition from T to C (Table 1).

All of the nine microsatellite loci were polymorphic, with 4–19 alleles observed across all four *O. formosanus* sample groups, that is, putative populations and an allelic richness ranging from 2.9 to 13.8 per sample group. At two loci (T5-5 F and T7-10E), the allele size of the PCR product differed from the expected size based on the original sequence. This discrepancy may be because of cross-species amplification. Locus T5-5 F was probably of *M. barneyi* origin because allele sizes derived from *M. barneyi* were much closer to the original sequence length than those of *O. formosanus* (Table 1). In case of T7-10E, allele sizes derived from both species were at least 76 bp smaller than the original sequence, which might be because of mutation. Short microsatellites typically are expected to follow the step-wise-mutation model, where one or

more repeat units are added or subtracted at a constant rate leading to a Gaussian-shaped allele frequency distribution (Eisen 1999, Ellegren 2004). However, once microsatellites increase in size, multiple repeats can be added or lost, and microsatellites then follow the infinite allele or two-phase models of mutation. Unequal crossing over, gene conversion, and transposon excision can lead to considerable expansion or contraction in allele length (Richard and Paques 2000). None of the 36 pairs of loci showed significant gametic disequilibrium ($P > 0.0013$, 720 permutations). Thus, the genotypes at all nine microsatellite loci assorted independently.

Mean observed heterozygosity for all loci across all sample groups was 0.43 ($SD = 0.17$; Table 1). We tested each locus for heterozygote excess or deficit, that is, deviations from Hardy-Weinberg equilibrium. None of the loci showed significant heterozygote excess. Loci 6-1A and 13-3B showed heterozygote deficiency in all four sample groups, and locus T6-1C showed heterozygote deficit in three sample groups ($P = 0.0013$, 720 randomizations). Heterozygote deficit is the typical direction of deviation from HWE and can be because of biological reasons (high inbreeding levels, selection for or against certain alleles), or population substructure within samples (Wahlund effect, Wahlund 1928). However, if these were the causes of heterozygote deficit, all loci should be affected. Because only three of nine loci showed heterozygosity deficit, this was probably caused by amplification failure of “null alleles.” Micro-Checker software (Van Oosterhout et al. 2004) was used to examine the patterns of heterozygote deficit as a statistical approach to identify null alleles. Results obtained by Micro-Checker software confirmed deviations from HWE because of heterozygote deficit at these loci and suggested the occurrence of a null allele with a frequency of $<10\%$ in most sample groups at each of these loci. None of the loci showed evidence for large allele dropout (Wattier et al. 1998). However, an excess of large homozygote classes at locus 6-1A in three sample groups indicated stutter. Consequently, loci 6-1A, T6-1C, and 13-3B may not be suitable for population genetic studies in *O. formosanus* for the primers that we designed here. At least one of the loci in question (13-3B) was probably of *M. barneyi* origin, as indicated by the allele size in this species being identical to the original sequence (Table 1). If additional loci are needed in the future, better primers may be re-designed from the sequences submitted to GenBank (Table 1) that do not yield null alleles.

Population Genetic Analysis. The remaining six loci (T5-5 F, T7-10E, 13-10D, 14-2A, 15-11c, and 15-12B; Table 1) with 4–15 alleles per locus and observed heterozygosity of 0.15–0.60 (mean = 0.48, $SD = 0.17$) did not show significant heterozygote deficit and were in HWE within sample groups and were thus used for population genetic analyses. Permutation tests of the distribution of genotypes between pairs of *O. formosanus* sample groups showed significant differentiation between the sample groups collected from different provinces in China, that is, between the groups from

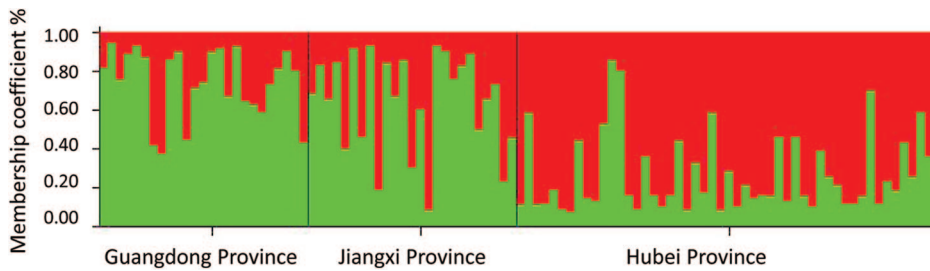


Fig. 2. Assignment of individual multilocus genotypes to two genetic clusters inferred from STRUCTURE simulations. Samples originated from three *Odontotermes* populations located in three provinces of China. Number of genetic clusters ($K = 2$) was inferred based on the model with the highest posterior probability (Pritchard et al. 2000). Each bar represents the membership coefficient, Q , divided into parts proportional to proposed ancestry of an individual genotype in each genetic cluster. (Online figure in color.)

Guangdong, Jiangxi, and Hubei Province, which were separated by 300–800 km. They were thus considered as different populations. However, no significant differentiation was detected between samples from two sample groups located only 8 km apart within Wuhan City in Hubei Province (H, S; Fig. 1). Therefore, the sample groups from the two sites in Wuhan City were combined and considered as the same population for the following analyses. Both Nei's (1978) unbiased identity index (0.87–0.91) and F_{ST} (0.03–0.08) indicated surprisingly small genetic distances among populations from different provinces. Populations were separated by 10 private alleles at loci 13–10D (four alleles), 15–11C (1), 15–12B (1), and 14–2A (4), but only three private alleles at locus 14–2A occurred in frequencies >5% in each of the populations. STRUCTURE HARVESTER analyses suggested the existence of two genetic clusters (peak distribution of Delta $K = 22.680$ observed at $K = 2$), with the populations from Guangdong and Jiangxi being more similar to each other than to the population of Hubei (Fig. 2). This separation is possibly caused by mountain ranges, for example, the Mufu mountains between Hubei and Jiangxi and the Wuling mountain chain, which limits access to Hubei from Guangdong via Hunan. Weak differentiation among populations and genetic clusters was confirmed by the fact that only 52, 40, and 60% of samples from the populations of Guangdong, Jiangxi, and Hubei, respectively, could be assigned with >80% to a particular genetic cluster (Fig. 2).

Consistent with the weak differentiation, genetic distances between populations were small. The F_{ST} values between populations of *O. formosanus* ranged from 0.03 to 0.08 and were at the lower end of genetic differentiation reported between termite populations (Vargo and Husseneder 2011). The mean number of migrants exchanged between populations ($N_e m$) can be estimated according to the formula $N_e m = (1 - F_{ST}) / 4 F_{ST}$ and ranged from three to eight individuals. Kimura and Weiss (1964) suggested that an $N_e m$ more than four signifies sufficient gene flow to create effectively panmictic populations. Although surprisingly high, gene flow among *O. formosanus* populations lies in the same range as reported for a species from a closely related genus of fungus-growing ter-

mites, *Macrotermes michaelseni* Sjöstedt (Brandl et al. 2005). Similar to *O. formosanus* in this study, the African fungus-growing termite *M. michaelseni* showed panmictic populations on spatial scales of up to 50 km and a mean F_{ST} value of 0.08 with genetic differentiation leveling off at a spatial distance of 100 km. Other termite species for which population differentiation in the range of >100 km was low include the neotropical *Nasutitermes nigricaps* Haldeman ($F_{ST} = 0.11$; Thompson and Hebert 1998), *Coptotermes formosanus* Shiraki in Japan ($F_{ST} = 0.12$; Vargo et al. 2003), and between certain populations from South China, Hawaii, and New Orleans ($F_{ST} = 0.04$ –0.09; Husseneder et al. 2011) and *Coptotermes lacteus* Froggatt in Australia ($F_{ST} = 0.04$; Thompson et al. 2007). This extensive gene flow across large scales could be attributed to sufficient habitat connectivity combined with considerable dispersal ability and/or transport by humans. A colony produces thousands of alates during its lifetime, and a population consists of hundreds if not thousands of colonies. Although the likelihood for a single individual is low, a small fraction of the large number of alates might disperse over extreme distances, forming the tail of a leptokurtic distribution (Brandl et al. 2005). Anecdotal reports of termites found 1 km high in the atmosphere have been cited (Müller 1981). In a more down-to-earth fashion, movement of infested material may counteract genetic differentiation and mitigate genetic drift, in particular in termites associated with wood (e.g., *Mastotermes darwiniensis* Froggatt, Goodisman and Crozier 2002). Human transport is most likely the reason for gene flow across surprisingly large distances in several of the invasive termites species, such as in *Reticulitermes santonensis* Feytaud (= *Reticulitermes flavipes* Kollar) between North America and France (Dronnet et al. 2005); in *Reticulitermes urbis* Bagnères, Uva et Clement between the Balkans and Western Europe (Luchetti et al. 2007, Leniaud et al. 2010); and in *C. formosanus* between China, Hawaii, and the U.S. mainland (Husseneder et al. 2011).

Low F_{ST} values between populations could be caused by the use of highly variable microsatellite loci (>25 alleles or 85% heterozygosity), because of increased large allele dropout, stutter, and homoplasy

associated with high mutation rates, which can bias allele frequency estimates and diminish F_{ST} values (Selkoe and Toonen 2006 and references therein). However, none of the six loci included in the population genetic analysis showed excessively high variability (≤ 10 alleles, 60% heterozygosity; Table 1). Micro-Checker software (Van Oosterhout et al. 2004) did not detect large allele dropout, and loci with potential stutter were not included in the final six (see previously).

Genetic diversity within populations measured by allele numbers (2–11 per locus and population) and allelic richness across six loci was rather low, considering *O. formosanus* is a native species in the range sampled (Guangdong: 6.31 [SD = 3.20], Jiangxi: 5.96 [SD = 3.25]; Hubei: 5.11 [SD = 2.02]). The genetic diversity of *O. formosanus* was comparable with that of *C. formosanus* populations from the introduced range and clearly lower than that of native *C. formosanus* populations (Husseneder et al. 2011). Genetic diversity of *M. michaelseni* from seven colonies in eastern Africa (6–11 alleles per locus) and two *Odontotermes* species from the Malaysian Peninsula, *Odontotermes denticulatus* Holmgren and *Odontotermes srinakariensis* sp. nov., with an average allelic richness per locus and population of 5.5–7.4 and 4.5, respectively (Cheng et al. 2013), was similar to that of *O. formosanus*. However, the genetic variability in a population of *Macrotermes gilvus* Hagen in Malaysia with only 2–4 alleles per locus (Singham et al. 2012) was even lower than those aforementioned. All these results warrant further study to determine whether low genetic diversity is a biological trait of fungus-growing termites, or reflects the history of certain populations, for example, a bottleneck because of a recent introduction (founder effect) or treatment efforts.

The mean levels of inbreeding of individuals versus populations (F_{IS}) across all six loci were 0.07 (SD = 0.15), 0.18 (SD = 0.17), and 0.15 (SD = 0.18) for the populations of Guangdong, Hubei, and Jiangxi, respectively. The standard inbreeding coefficient F_{IS} value is analogous to F_{IT} in other studies that considered colonies as an additional level of genetic variation. Compared with most other subterranean termites, the level of inbreeding in individuals relative to the population was low to moderate in *O. formosanus* (reviewed in the article by Vargo and Husseneder 2011). For comparison, *C. formosanus* in its native range of China showed higher levels of inbreeding (0.18–0.29; Husseneder et al. 2011).

Whether the comparatively low level of inbreeding is because of the majority of colonies in *O. formosanus* populations being headed by unrelated pairs of reproductives needs to be tested in future studies of colony breeding systems in *O. formosanus*, which also will confirm whether *O. formosanus* have just one pair of primary reproductives, as generally assumed (Cheng et al. 2007), or multiple queens, as shown to occur in an African Macrotermitinae (Hacker et al. 2005).

The microsatellite markers developed in this study may also make it possible to assign foragers and alates to colonies of origin. This information is important for

determining numbers and density of colonies infesting an area, as well as sources of reinfestation after treatment (Vargo 2003; Husseneder et al. 2005, 2007; Vargo and Parman 2012). Assignment to colonies is also necessary to confirm foraging areas in *O. formosanus* (which few observations indicate to be as large as 368 m²; Hu et al. 2006) and to calculate dispersal distances. All of these are important parameters to consider when attempting area-wide control (Simms and Husseneder 2009, Husseneder and Guillot 2010).

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