Polymorphic Microsatellite Loci From an Indigenous Asian Fungus-Growing Termite, *Macrotermes gilvus* (Blattodea: Termitidae) and Cross Amplification in Related Taxa

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ABSTRACT The fungus- growing termite, *Macrotermes gilvus* (Hagen), an indigenous species from Southeast Asia distributed from Myanmar to Indonesia and the Philippines, offers great potential as an ecological model system to elucidate the effects of geography on gene flow within this region. We used next generation sequencing (Roche 454 pyrosequencing) to identify microsatellite markers from the genomic DNA of *M. gilvus*. A modest sequencing volume generated 34,122 reads, with 1,212 (3.6%) reads contains microsatellites with di-, tri-, tetra-, penta-, and hexa-nucleotide repeat motifs. Thirty-seven loci were selected for primer development and tested for polymorphism across 22 colonies of *M. gilvus*. Eleven loci were found to be polymorphic with 2–4 alleles per locus. Observed and expected heterozygosities ranged between 0.091–0.727 and 0.090–0.540, respectively. Cross taxa amplification was successful across a panel of four related termite species and four multiplex groups were designed for future population genetic studies. These markers will open new avenues for the study of phylogeography and population genetics of this fungus-growing termite. This study also has effectively demonstrated the use of 454 pyrosequencing for the rapid development of informative microsatellite markers from a termite genome.

KEY WORDS microsatellite, 454 pyrosequencing, next generation sequencing, termite, *Macrotermes gilvus*

Macrotermes gilvus (Hagen) is a common fungus-growing termite that is indigenous to Southeast Asia (SEA) from Myanmar, Thailand, Vietnam, and Peninsular Malaysia to Indonesia, Borneo, and the Philippines (Snyder 1949, Roonwal 1970, Thapa 1982, Tho 1992). It is regarded as a pest of economic importance as it damages wooden structures and often is responsible for secondary reinfestation in premises previously infested by other termite species (Roonwal 1970, Lee 2002, Lee et al. 2007). Despite the fact that it is widely distributed in this region, few studies have been made on this species. Most of the previous studies on *M. gilvus* focused on biological and fundamental ecological aspects such as mound structures, role of fungal symbionts in lignin degradation, foraging territories, flight activity, and caste developmental pathway (Inoue et al. 1997; Hyodo et al. 2000; Acda 2004; Neoh and Lee 2009a,b). Thus, there has been a lack of information on the molecular ecology and population biology of this species. The use of genetic and molecular markers will aid and enable more accurate determination of its population dynamics and genetic structure. Large spatial distribution, encompassing mainland SEA and geographically fragmented island populations together with its limited natural dispersal ability, make M. *gilvus* a good subject to investigate the effects of vicariance events on the genetic structure, population biology, and biogeography of terrestrial fauna in this region.

Microsatellite DNA, widely distributed in the genome of prokaryotes and eukaryotes (Field and Wills 1998, Schlotterer 2000), has been used repeatedly in studies of genetic diversity, population genetic structure, genomic mapping, and phylogeography (Sunnucks 2000, Zhang and Hewitt 2003). High levels of polymorphism, reproducibility, and codominance make microsatellites one of the most powerful molecular markers for fine scale studies of the population genetic structure, and thus are likely to be ideal for studies of genetic structure of *M. gilvus*.

Next generation sequencing, such as the Roche 454 GS-FLX Titanium pyrosequencing platform, holds a great promise for the isolation of microsatellite markers from the genome of model and nonmodel species with no prior reference genome available (Margulies et al. 2005, Malausa et al. 2011). There are many advantages to using 454 pyrosequencing over traditional enrichment procedures in isolating microsatellite

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markers because of high throughput, relatively low cost, quick time, and low labor requirements (Rothberg and Leamon 2008). There is a growing list of publications describing the isolation of microsatellite markers from insect genomic DNA through the use of 454 pyrosequencing, including Hemiptera (Perry and Rowe 2011); Hymenoptera (Santana et al. 2009); Diptera (Rasmussen and Noor 2009, Copeland et al. 2011, in press; and Coleoptera (Smith et al. 2011). However, this technique has not been used to isolate microsatellite markers from the Blattodea (including termites). Thus, the objectives of this study were to isolate and characterize polymorphic microsatellite loci from *M. gilvus*, conduct a preliminary assessment of genetic diversity, and to test the utility of the developed loci across a panel of related termite species via cross amplification. Our results provide valuable new microsatellite markers for a number of higher subterranean termites in SEA.

Materials and Methods

Sample Preparation and 454 Pyrosequencing. *M. gilvus* worker termites were collected from 22 colonies in suburban environments within the state of Sarawak, East Malaysia and stored in 95% ethanol. Voucher specimens have been deposited in Vector Control Research Unit, Universiti Sains Malaysia (Penang, Malaysia). Total genomic DNA was extracted from the whole body of one worker termite from each colony by using CTB Tissue Extraction Kit protocol (Intron, Seongnam-Si, Gyeonggi-do, Korea) after being pulverized in liquid nitrogen. DNA quality was assessed by spectrophotometric absorbance (BioSpec-nano, Shimadzu, Columbia, MD) and electrophoresis on 0.8% agarose gel.

In total, ≈2 μg of gDNA extracted from a single individual termite was used for $1/16^{th}$ of a 70-mm by 75-mm Titanium PicoTiter plate of sequencing on a Roche 454 GS FLX sequencer with titanium chemistry at the Genome Sequencing and Analysis Core Facility, Duke University (Durham, NC). Sample preparation and analytical processing of sequence reads were performed according to manufacturer's protocol for the Titanium series. The techniques we used are described in more detail in Margulies et al. (2005).

Microsatellite Detection and Primer Design. The program Msatcommander version 0.8.2 (Faircloth 2008) was used to detect reads containing microsatellite repeats. The search criteria were set to a minimum of six repeats of di- to hexa-nucleotides with perfect repeat motifs. Of the repeats identified, subsets of tri- and tetra-nucleotides with at least eight and six perfect repeat motifs respectively were selected. The selection was made on the basis that tri- and tetra-nucleotides generally are easier to score than dinucleotide, because of a reduced amount of strand slippage (Weber et al. 1993), reducing the likelihood of stutter bands and scoring error.

Primer3 version 0.4.0 (Rozen and Skaletsky 2000), incorporated within the Msatcommander software, was used to design a subset of primer pairs that fit the following stringent criteria: 1) amplification product size range of 100−350 bp, 2) maximum of four bases homopolymer stretch at flanking region, 3) optimal GC content of 50%, 4) annealing temperature of primer pairs ranging between 57°C and 61°C, 5) primer length ranging between 18 and 23bp, and 6) low levels of primer complementarity fixed at maximum local alignment score of 5.0 and maximum allowable 3′ anchored global alignment score of 3.0.

Testing Markers. Thirty-seven primer pairs were selected for preliminary tests of polymorphism across seven individual termites from different localities (Malaysia, Indonesia, Thailand, the Philippines). The M13 forward sequencing primer (CACGACGTTGTAAAACGAC) was incorporated into the 5′end of each forward primer by using the methods of Oetting et al. (1995). Infrared dye-labeled primers (IRDye 800CW/IRDye 700CW) were included in the polymerase chain reaction (PCR), yielding a product detectable on a LI-COR 4300 dual laser DNA Analyzer (model 4300, LI-COR Bioscience, Lincoln, NE). Primer pair optimization and sample preparation for analysis on LI-COR 4300 was carried out by using PCR conditions and methodologies described by Booth et al. (2008) with annealing temperature ranging between 57 and 59°C. Primer pairs that produced scorable, unambiguous PCR products of the expected size, and that demonstrated allelic variation were selected for further testing.

The resulting polymorphic loci were evaluated across a total of 22 individual termites by using forward or reverse primers labeled with the fluorescent dyes 6-FAM, HEX, TAMRA-S, and ROX performed in a multiplex PCR volume of 12.5 μl containing ≈50–100 ng of genomic DNA; 1 X Master Mix (Type-It Microsatellite PCR kit, Qiagen, Venlo, Netherlands); 0.10 μM of forward and reverse primers; and sdH_20 added up to final volume. Amplification was carried out on a thermal cycler (model PTC200, MJ Research, Inc., Waltham, MA) with initial denaturing at 95°C for 5 min, followed by 35 cycles of denaturation step at 95°C for 30 s, annealing ranging between 57 and 59°C for 90 s, and extension at 72°C for 1 min, followed by a final extension of 72°C for 30 min. PCR amplicons were electrophoresed on a 3130 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA), with allelic size determined using 500LIZ as an internal size standard and PeakScanner software version1.0 (Applied Biosystems, Inc., Foster City, CA).

Data Analysis. We used the software Arlequin version 3.5.1.2 (Excoffier and Lischer 2010) to determine departures from Hardy–Weinberg equilibrium, observed and expected heterozygosity (H_O and H_E), allelic diversity, gene diversity, number of alleles per locus, number of polymorphic loci and linkage disequilibrium among all pairs of loci. Evidence for large allelic drop out, scoring error because of stutter, and null alleles was determined using Microchecker version 2.2.3 (van Oosterhout et al. 2004).

Cross Taxa Amplification. To test the transferability of the developed microsatellite loci across the genomes of related taxa, a panel of four higher termite

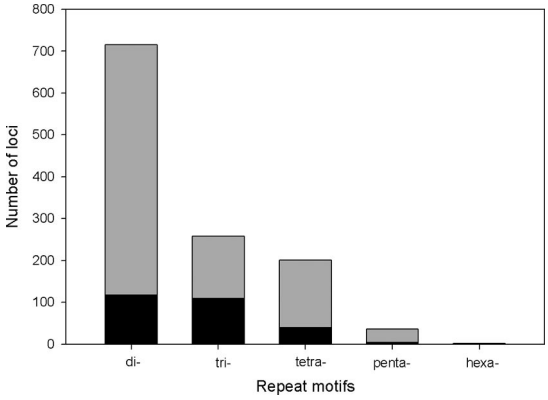

Fig. 1. Number of loci with microsatellite repeats (gray and black), and the number of these loci with suitable flanking regions for primer design (black) isolated from *M. gilvus*.

species, *M. carbonarius* (Hagen), *M. barneyi* Light, *M. malaccensis* (Haviland), and *Globitermes sulphureus* (Haviland), were tested for cross amplification by using the PCR conditions described in the testing markers. PCR amplicons then were electrophoresed on a 2% agarose gel and bands were detected under UV illumination.

Results and Discussion

454 Pyrosequencing. In total, 34,122 sequence reads were generated by the 454 pyrosequencing with a mean length of 357 bp and median length of 392 bp. The search using Msatcommander yielded 1,212 sequences containing microsatellite repeat motifs with 715 di- (59.0%), 258 tri- (21.3%), 201 tetra- (16.6%), 36 penta- (3.0%), and two hexa-nucleotide repeats (0.2%), a trend similar to that found in various other invertebrates for whom microsatellite loci were identified using 454 pyrosequencing (see Gardner et al. 2011). Of the 151 primer pairs suitable for primer design (tri- and tetra- repeats only with sufficient flanking region), 37 primer pairs successfully were developed (20 tri- and 17 tetra-nucleotides) by using the stringent criteria set in PRIMER 3. The proportion of loci with perfect repeat motifs and the number of loci with suitable flanking region for primer design are displayed in Fig. 1.

The frequency of finding microsatellites within the *M. gilvus* genome by using high throughput 454 technology is 3.6% of which 274 sequences (22.6%) contained suitable flanking sites for primer design. Although the most commonly found repeat motif was dinucleotide, only 16.5% of the 715 sequences had suitable flanking regions for primer design in contrast to trinucleotide repeats with nearly triple the percentage (42.6% of 258 sequences) with suitable flanking regions, followed by tetranucleotide motifs (20.4% of 201 sequences) (Fig. 1). This result is in accordance with the findings of Gardner et al. 2011 and Copeland et al. 2011, who reported that trinucleotide repeats have the highest proportion of all microsatellite motifs suitable for primer design among the invertebrates.

Genetic Analysis. Of the 37 developed primer pairs, 20 loci produced reliable, unambiguous amplifications within expected allele size, of which 15 loci showed allelic variation, whereas five loci were monomorphic when tested across individuals from multiple populations (preliminary test result by using the Li-Cor 4300). When evaluated within a single population (Sarawak), 11 loci were found to be polymorphic with 2–4 alleles per locus (mean equals 2.9 alleles per locus) (Table 1). Observed heterozygosities ranged from 0.09 to 0.64 and expected heterozygosities from 0.09 to 0.54. We did not detect any evidence of scoring error because of stutter or large allelic drop out. There were no significant departures from Hardy–Weinberg equilibrium across all loci ($P > 0.05$) after Bonferroni correction for multiple comparisons (Table 1). We did not find any significant linkage disequilibrium between any pairs of loci. There was no evidence of null alleles for any of the loci tested. For future use in population genetic studies, we designed four multiplex groups for these 11 polymorphic primer pairs as follows: G1 (*Mg*1, *Mg*2, *Mg*30); G2 (*Mg*3, *Mg*7, *Mg*5); G3 (*Mg*8, *Mg*11); and G4 (*Mg*34, *Mg*37, *Mg*18).

The single population studied displayed a fairly low level of genetic diversity (allelic diversity, $A = 2.9$ alleles per locus and gene diversity, h [Nei 1973] = 0.30) compared with other studies of insect populations using microsatellite data: $h = 0.58$, $A = 6.1$ in a water strider (Perry and Rowe 2011); $h = 0.68$, $A = 7.2$ in an ambrosia beetle (Smith et al. 2011); $h = 0.60$, $A = 4.7$ in a drywood termite (Booth et al. 2008); $h = 0.36$, $A = 5.5$ in the cat flea (Husseneder et al. 2010); and $h = 0.65$, $A = 6.1$ in a lower subterranean termite (Vargo 2000). It remains unclear why the population studied here showed such low genetic diversity; however, a genetic bottleneck because of either historical events or contemporary processes may explain this result (Freeland 2005). It is worth noting that the population studied was sampled from the island of Borneo, which separated from continental Asia ≈20,000 yr ago after the Sunda shelf (land bridge) was flooded with rising sea water at the end of Last Glacial Maximum. This event created a physical barrier that likely restricted gene flow between the island and the mainland. Testing this hypothesis will require knowledge of the genetic diversity of *M. gilvus* populations from mainland Asia. Hence, this preliminary study warrants further investigation of these microsatellite loci across different populations in the region.

SEA is one of the most intriguing areas of the earth from the geological and biological viewpoint. It includes areas with the highest global rate of plate convergence and separation involving subduction of tectonic plates and collisions between island arcs, between island arcs and continents, and between continental fragments (Hall 1998). These intense geological processes coupled with other more subtle geologically related forces such as links between tectonics and sea levels, the rise of mountains and global or local climate, and closure of seaways and oceanic circulation has influenced the biogeographic patterns of flora and fauna in the region in unique ways (Hall 2001),

Table 1. Characteristics of 11 polymorphic microsatellite loci in the fungus-growing termite, *Macrotermes gilvus*, isolated using 454 pyrosequencing

Locus	Repeat motif	Primer sequence (5′–3′)	Ta (°C)	N	N_A	Expected allele size (bp)	H_O	H_E	P_{HWE}	GenBank accession no.
Mg 18	$(GAT)_8$	F:GGAAGAGGCTGTGGACCTG R:GTGCGTGCGTGATATTTAAACG	58.5	22	4	184	0.363	0.325	1.000	
Mg 34	$(AACT)_6$	F:TACCCGTAGCCAGAAACGC R:CCTGGTTCTGGGTGAGGAC	58.5	22	3	328	0.091	0.090	1.000	
Mg 2	$(ATC)_8$	F:GCATCATCAGTGAGAAAGGTTTG R:TGAGACAGCAGGTTCCTTTG	58.5	22	4	185	0.636	0.538	0.017	
Mg 1	$(AAT)_9$	F:TTCCGCTTACTTCTGCACAC R:TTGTGACCCCGTAAGTCTGA	58.5	22	4	272	0.318	0.285	1.000	
Mg 30	$(AAGT)_{13}$	F:GCTGCAAAACCAGTTAGCTTC R:TTGACTCGTTACACAATCCGTA	58.5	22	2	227	0.091	0.089	1.000	
Mg 37	$(ACTT)_{11}$	F:TTTGGCACCTGTTCGTACC R:AGGACTACTGCCACAGAGC	58.5	22	3	196	0.363	0.540	0.088	
Mg 3	$(AAT)_8$	F:TAGATAGGGGTGGGTGGTCA R:CCGAAAGGCGACTTATGAAA	58.5	22	3	158	0.091	0.090	1.000	
Mg 7	$(ATT)_8$	F:CGAATATTTGCATCCTGGAA R:AGCCCCGTAGCAGGTCTCTAT	58.5	22	2	226	0.545	0.444	0.360	
Mg 5	$(AAT)_9$	F:AAGCGATCTTGAACGTCTCTC R:GCACATTTCTTAGCCCTGGT	58.5	22	2	154	0.227	0.206	1.000	
Mg 11	$(ATT)_9$	F:CCTCCCATTGGTATAAAGGCAC R:GGATAGGGACAGGTGGCG	57.0	22	2	201	0.227	0.206	1.000	
Mg 8	$(AAT)_{11}$	F:CGTTGCCTCGTTCCACTAAC R:ACGTCGTAGAACTCAGCGG	57.0	22	3	193	0.727	0.489	0.017	

T_a, Primer annealing temperature ; N, sample size for genetic analysis; N_A, number of alleles; H_O, observed heterozygosity, H_E, expected heterozygosity; P_{HWE}, Hardy–Weinberg equilibrium value.

Primer sequence and GenBank accession numbers of the four other monomorphic loci can be obtained from Supplemental Material (Table S1).

making this region one of the most biologically diverse areas of the world. Thus, the development of sensitive molecular marker such as microsatellites for *M. gilvus* is essentially useful in understanding patterns of speciation and genetic differentiation of fauna in this region.

The microsatellites developed here should provide important tools for the study of the molecular ecology of Asian termites. To date, microsatellites have been developed for 15 termite species belonging to five families, of which there are only five termitids, and none are from Asia (Yeap et al. 2009, Vargo and Husseneder 2011). There are only three species from Asia for which microsatellites have been developed, and they are all rhinotermitids; *Coptotermes formosanus* Shiraki (Vargo and Henderson 2000), *C. gestroi* (Wasmann) (Yeap et al. 2009), and *Reticultermes speratus* (Kolbe) (Hayashi et al. 2002). Furthermore, we attempted to use microsatellites developed for the congeneric African species, *M. michaelseni* (Sjöstedt) (Kaib et al. 2000), but found these to be monomorphic in *M. gilvus* and thus unsuitable for population genetic analysis of this species.

Species Cross Amplification of Loci. Thus, this study not only provides valuable informative microsatellite loci for *M. gilvus* but also a subset of usable microsatellite loci that cross amplifies in other termitids of Asian origin. Cross amplification generally was successful on all four of the termite species tested (see Table 2). Only one of the 11 loci tested showed no positive PCR amplification of expected allele size across all taxa. Six loci displayed successful amplification with a single product of appropriate size across the whole panel of termites. Loci that produced multiple bands, with at least one band within the expected allele sizes require further investigation to determine if they represent the sequenced microsatellites and whether are variable in each species.

The developed microsatellite loci also should prove useful in clarifying the taxonomic status of *M. gilvus* in the region. To date, there are eight forms or variants of *M. gilvus* have been described from SEA, of which a few have been designated as subspecies based on very brief morphological characters and localities (Snyder 1949). A recent biometric analysis based on 13 morphological characters poorly discriminated the various forms of *M. gilvus* into distinctive groups (G.V.S. et al., unpublished data). The sensitivity of these microsatellite markers thus should be helpful in revealing the taxonomic status of this species and to further understand its phylogeography in SEA.

Table 2. Cross amplification of 11 polymorphic microsatellite loci in four species related to *M. gilvus*

Locus	*M. carbonarius*	*M. malaccensis*	*M. barneyi*	*G. sulphureus*
*Mg*18	+	+	+	+
Mg 34	−	−	−	−
Mg 2	+	+	+	+
Mg 1	+	+	+	+
Mg 30	+	#	+	+
Mg 37	−	#	+	+
Mg 3	+	+	+	+
Mg 7	+	+	+	+
Mg 5	#	+	#	−
Mg 11	+	+	+	#
Mg 8	+	+	+	+

+, positive amplification with a single band within the expected allele size; #, positive amplification with multiple bands, with at least one band within expected allele size; −, no amplification of the expected allele size.

In conclusion, 454 pyrosequencing technology proved to be a powerful tool for isolating microsatellite markers from the *M. gilvus* genome, in which a previous attempt by using a traditional enrichment procedure failed to produce functional polymorphic microsatellite loci (G.V.S. et al., unpublished data). Fifteen polymorphic loci were developed successfully, of which 11 loci were polymorphic within a single population. We found low levels of genetic diversity in the studied population, but the reasons for this are not clear and require further study. We were successful in obtaining cross species amplification, suggesting the loci may be of use in population genetic studies of related taxa. Our results also provide a valuable database with over 200 sequences containing suitable flanking regions for development of additional microsatellite loci which are available upon request from the authors. The successful isolation of polymorphic loci from *M. gilvus* opens new avenues for future studies of the phylogeography and genetic structure of this species in SEA.

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