Gene 518 (2013) 412–418

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Microsatellite markers uncover cryptic species of *Odontotermes* (Termitoidae: Termitidae) from Peninsular Malaysia

S. Cheng [a,*], C.T. Lee [a], M.N. Wan [b], S.G. Tan [c,**]

[a] *Genetics Laboratory, Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor, Malaysia*
[b] *Recreational Impact and Management Branch, FRIM, 52109 Kepong, Selangor, Malaysia*
[c] *Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ARTICLE INFO

Article history:
Accepted 19 December 2012
Available online 15 January 2013

Keywords:
Odontotermes
Microsatellites
Macrotermitinae
Cryptic species
Taxonomy

ABSTRACT

Termites from the genus *Odontotermes* are known to contain numerous species complexes that are difficult to tell apart morphologically or with mitochondrial DNA sequences. We developed markers for one such cryptic species complex, that is, *Odontotermes srinakarinensis* sp. nov. from Maxwell Hill Forest Reserve (Perak, Malaysia), and characterised them using a sample of 41 termite workers from three voucher samples from the same area. We then genotyped 150 termite individuals from 23 voucher samples/colonies of this species complex from several sites in Peninsular Malaysia. We analysed their population by constructing dendograms from the proportion of shared-alleles between individuals and genetic distances between colonies; additionally, we examined the Bayesian clustering pattern of their genotype data. All methods of analysis indicated that there were two distinct clusters within our data set. After the morphologies of specimens from each cluster were reexamined, we were able to separate the two species morphologically and found that a single diagnostic character found on the mandibles of its soldiers could be used to separate the two species quite accurately. The additional species in the clade was identified as *Odontotermes denticulatus* after it was matched to type specimens at the NHM London and Cambridge Museum of Zoology.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

The *Odontotermes* belong to the Macrotermitinae subfamily of termites, well-known for their symbiosis with basidiomycete fungi from the genus *Termitomyces* which they cultivate in order to digest plant materials into food for their colonies. The *Odontotermes* is also the most species-rich and, incidentally, better studied compared to its other relatives within the Macrotermitinae. Their taxonomy however is poorly resolved because of the limited number of morphological characters available to separate them. Species within the *Odontotermes* are usually separated based on differences in the size and shape of the head capsules of its soldiers and the variable position of an apical tooth that is located along the inner margin of the left mandible of these forms (Ahmad, 1958; Holmgren, 1913).

Several closely related and morphologically similar species are found within the genus and to add to this complexity, many species have been described based on small differences that could be attributed to intraspecific variation (Tho, 1991). Additionally, many of these characters constantly overlap between species. Although data on behaviour, nest architecture and mitochondrial DNA sequences have been used to illuminate species boundaries in the *Odontotermes*, they have not been able to differentiate between the numerous cryptic species found within the genus (see Cheng et al., 2011; Darlington, 1997; Darlington et al., 2008; Davison et al., 2001).

Microsatellite markers or short tandem repeats (STRs) are increasingly being applied to study cryptic speciation in birds (Förschler et al., 2009), frogs (Elmer et al., 2007), giraffes (Brown et al., 2007), termites (Roy et al., 2006) and tsetse flies (Dyer et al., 2011). Because STR regions are also among the most variable in the genome, primer-binding sites are generally not well conserved among distantly related species. There are, however, instances of successful cross-species microsatellite amplification in birds (Pinheiro et al., 2009), chameleons (Feldheim et al., 2012), and flowering plants (Primmer et al., 2005) to name a few. It is often the case that microsatellite markers need to be isolated *de novo* for each species or group of closely related species. But methods where DNA libraries of the focal species are enriched for the microsatellite motif of interest can increase the efficiency and lower the cost of microsatellite isolation. Once a microsatellite-containing fragment of interest is isolated and its nucleotide sequence

Abbreviations: bp, base pair(s); CD-Hit, Cluster Database at High Identity with Tolerance; F.R., Forest Reserve; F_{IS}, inbreeding coefficient of an individual relative to the subpopulation; F_{IT}, inbreeding coefficient of an individual relative to the total population; FRIM, Forest Research Institute Malaysia; F_{ST}, fixation index; H_e, expected heterozygosity; H_o, observed heterozygosity; HWE, Hardy–Weinberg equilibrium; LB, Luria–Bertani (medium); MISA, Microsatellite Identification tool; mtDNA, mitochondrial DNA; NHM, Natural History Museum; NJ, neighbour-joining; PCR, polymerase chain reaction; STR, short tandem repeat; UPGMA, unweighted paired group method with arithmetic mean; X-gal, 5-Bromo-4-chloro-3-indolyl-β-D-galactoside.

* Corresponding author. Tel.: +60 3 6279 7110.
** Corresponding author. Tel.: +60 3 8946 8098.
E-mail addresses: shawn@frim.gov.my (S. Cheng), sgtan_98@yahoo.com (S.G. Tan).

0378-1119/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2012.12.084

determined, primers can be designed to produce a sequence-tagged microsatellite marker.

Here we report on the isolation and characterisation of microsatellites for *Odontotermes srinakarinensis* sp. nov (Takematsu, submitted), a species complex thought to contain more than one species unit (Cheng et al., 2011). We also report its subsequent use in teasing apart cryptic species within this clade of *Odontotermes* from Peninsular Malaysia which was assigned the morphospecies rank of *Odontotermes* sp. 1 in Cheng et al. (2011). Initially, we tested primers developed for *Amitermes meridionalis* (Schmidt et al., 2007) and *Macrotermes michaelseni* (Kaib et al., 2000) on this focal group. However, these markers proved to be unspecific as polymerase chain reaction (PCR) amplicons showed multiple banding patterns when visualised on agarose gel even after repeated optimisation experiments.

We utilised synthetic oligonucleotide probes bound to magnetic beads in a hybridisation solution to obtain a DNA library enriched for microsatellite core sequences (Kijas et al., 1994) and adopted a microsatellite isolation protocol detailed in Lee et al. (2004) for *Shorea leprosula* (Dipterocarpaceae) to isolate microsatellite markers for *O. srinakarinensis*. Once these markers have been isolated, they may also be used to solve the problem of cryptic species complexes in other species of *Odontotermes*. It is also to date, the first attempt at developing microsatellite markers for the genus.

2. Materials and methods

2.1. Isolation of microsatellites

Briefly, DNA from five *O. srinakarinensis* soldiers from Maxwell Hill Forest Reserve (F.R.), Maxwell Hill (Fig. 1) was pooled and digested with *Nde*II. Fragments between 300 and 1000 bp were then isolated and ligated to *Sau*3A1 linkers. DNA fragments were hybridised with $(CT)_{15}$ and $(GT)_{15}$ biotinylated repeat oligonucleotides and bound to Streptavidin MagnaSphere® Paramagnetic Particles (Promega). The streptavidin beads were then rinsed repeatedly to remove unwanted DNA fragments. Repeat-enriched DNA fragments were recovered and amplified with the C1 cassette primer using PCR conditions described in Lee et al. (2004). Sau3A1 linkers were removed before the DNA fragments were ligated into the plasmid vector pUC118 Bam HI/BAP (Takara, Tokyo). DNA fragments were then amplified in a PCR reaction using the M13 forward and reverse primers. The ligated plasmids were subsequently cloned into TOP10 *Escherichia coli* (Invitrogen) competent cells and positive clones were identified using blue/white screening on LB-agar plates containing ampicillin and X-gal. Randomly selected positive clones were then subcultured and the plasmids were sequenced in Big-Dye® Terminator ver 3.1. cycle-sequencing reactions on an ABI PRISM *3130xl* genetic analyzer.

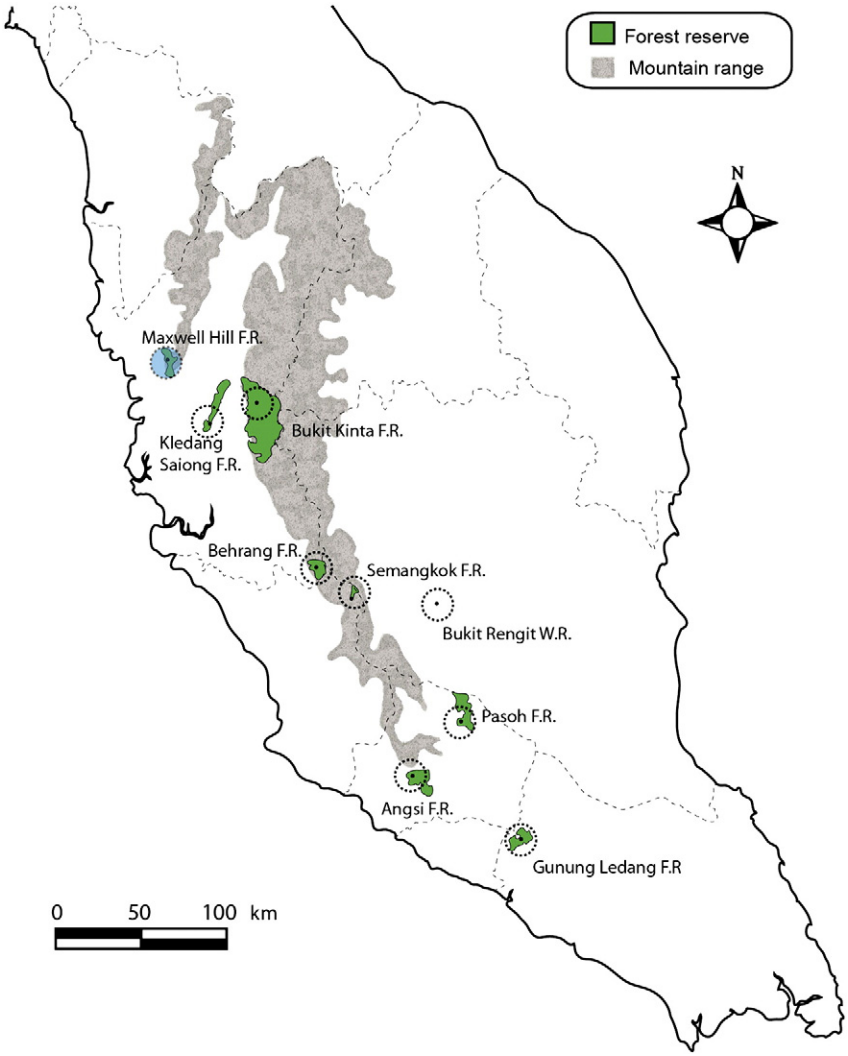

Fig. 1. Sampling sites in Peninsular Malaysia (broken circles). Microsatellites were isolated and characterised using voucher samples of *O. srinakarinensis* from the area highlighted in blue.

After correcting for ambiguous nucleotide base calls in Sequencher ver. 4.9 (Gene Codes Corp., Ann Arbor, MI), redundant clone sequences were removed using CD-Hit (Li and Godzik, 2006). Microsatellite sequences were then identified using Micro-FamilyWIN ver 1.2 (Meglécz, 2007) and MISA (Thiel et al., 2003) before PCR primers were designed using OLIGO6 (Molecular Biology Insights Inc.). An initial screen was performed on four termite individuals to determine if the primers amplified fragments within the expected size ranges. Optimisation experiments were performed to determine the appropriate annealing temperatures for some of the primer sets, where it was necessary. Primer pairs that could amplify the targeted fragments and which could be interpreted robustly were then labeled fluorescently.

2.2. Microsatellite genotyping

The markers were characterised using 41 termite individuals from three collection sources in Maxwell Hill F.R. Following this, 150 termite individuals from 23 collection sources from Behrang F.R., Bukit Rengit Wildlife Reserve, Pasoh F.R. and Semangkok F.R. (see Fig. 1) were genotyped with an ABI Prism *3130xl* Genetic Analyzer using ROX400 (GeneScan) as an internal size standard. Samples from the *O. srinakarinensis* species complex from four of the other study sites, that is, Kledang Saiong F.R., Bukit Kinta F.R., Angsi F.R. and Mount Ledang F.R. were not genotyped because only a limited amount of morphological samples preserved in 70% ethanol could be obtained from these sites (see Fig. 1). Sites shown in Fig. 1 were systematically sampled for *Odontotermes* between 2006 and 2009 as part of a larger study to understand their diversity, abundance and ecology in Peninsular Malaysia.

Allele sizes were assigned based on the internal size standard of ROX400 and individuals were genotyped using GeneMapper ver. 3.7 (Applied Biosystems). Microsatellite loci were amplified in multiplex reactions using the Type-It® Microsatellite PCR Kit (Qiagen) or individually in singleplex reactions. Multiplex reactions were prepared according to the protocol detailed in the Qiagen Type-It® Microsatellite PCR handbook. However, annealing temperatures were adjusted to temperatures lower than the one recommended by the optimised Qiagen cycling protocol to enable the amplification of the microsatellite loci.

2.3. Genetic data analysis

The Excel Microsatellite Toolkit (Park, 2001) was used to check the data set for errors and to create input files for subsequent analyses. The programme Micro-Checker (Van Oosterhout et al. 2004) was then used to identify and correct genotyping errors in the data set caused either by large allele dropout, short allele dominance or stutter products resulting from slippage during PCR amplification. We also identified the presence of null alleles in the multi-locus data set using Micro-Checker. The programme PowerMarker ver 3.0 (Liu and Muse 2005) was used to draw dendograms from the proportion of shared-alleles in the total data set. Population trees using Nei et al.'s (1983) D_A distance and Goldstein et al.'s (1995) $(\delta\mu)^2$ distance were also constructed using PowerMarker ver 3.0. We used Structure (Pritchard et al., 2000) to infer the actual number of populations in the microsatellite genotype data. Lastly, F-Stat ver. 2.9.3 (Goudet, 2001) was used to obtain diversity statistics such as allelic richness and differentiation statistics such as F_{IS}, F_{ST} and F_{IT}.

3. Results

3.1. Microsatellite identification and characterisation

Starting initially with 263 clone sequences, 184 were found to be unique and non-redundant. From these clone sequences, 73 were found to contain microsatellites. Primers could only be designed for 46 of these clones because in the rest of the clones, the microsatellite sequences were either too close to the 5′- or the 3′-end. Out of the 46 primer pairs, 29 showed potential to be used for a panel of microsatellite markers for *O. srinakarinensis*. Details on the microsatellite loci that were isolated, primer pairs and GenBank accession numbers of the sequences are in Table 1.

Close to 90% of the 29 microsatellite loci that were isolated contained dinucleotide repeats motifs with 14 of the loci containing CT/TC/AG/GA repeats motifs and the other 11 containing GT/TG/CA/AC repeat motifs (Table 1). The dinucleotide repeats were either simple or compound repeat motifs (uninterrupted and interrupted). A trinucleotide repeat $(GAA)_7$ was found at locus Oskar 37-3 while a compound repeat of $(AGTA)_4$ $(AAGTA)_4$ was found at locus Oskar 59-2 (Table 1).

The results of the marker characterisation experiment are shown in Table 2. A single locus, Oskar 33-4, was monomorphic while the rest of the loci had between 2 and 8 alleles or an average of 4.28 alleles per locus (Table 2). Three of the loci, Oskar 22-5, Oskar 34-5 and Oskar 59-2, showed signs of null alleles as there were significant departures from the Hardy–Weinberg equilibrium or HWE ($p<0.05$), indicated by an excess of homozygotes at these loci (see Table 2). After removing these loci, the population was found to be in Hardy–Weinberg equilibrium. Three loci, Oskar 9-5, Oskar 10-5 and Oskar 67-5, particularly, were the most variable with 7 to 8 alleles found at these loci (Table 2). Observed heterozygosity (H_o) was also found to be equal or higher than expected heterozygosity (H_e) at 20 of the 29 loci that were analysed.

3.2. Population analysis

For the population genotyping experiments, primers that could be multiplexed with the Qiagen Type-It® Microsatellite PCR are listed in Supplementary Table 1 together with the annealing temperature for each primer combination. The neighbour-joining (NJ) tree calculated from the proportion of shared-alleles among 189 termite individuals is shown in Fig. 2. The individuals that were genotyped clustered according to their collection sources and their sampling locations (Fig. 2). Two major clusters were evident in the NJ tree. The first cluster consisted solely of samples from Maxwell Hill (Perak) while the second cluster consisted of samples from Semangkok, Behrang, Pasoh and Bukit Rengit (Fig. 2). Within the second cluster, the Semangkok and Behrang samples formed one subgroup while the Pasoh and Bukit Rengit colonies formed another subgroup. The unweighted pair-group method with arithmetic mean (UPGMA) tree which was also calculated from the proportion of shared-alleles produced a similar tree (Supplementary Fig. S1).

The NJ tree calculated from Nei et al.'s (1983) D_A for 20 of the *O. srinakarinensis* colonies also grouped the Semangkok and Behrang in one cluster and the Pasoh and Bukit Rengit colonies in another cluster; the Maxwell Hill colonies formed a distinct cluster that had a high bootstrap support (Fig. 3). The bar plot outputs from structure ver 2.3 (Pritchard et al., 2000) together with the corresponding delta *K* versus *K* plots (Earl and vonHoldt 2012; Evanno et al., 2005) are shown in Fig. 4. When admixture was assumed and allele frequencies were set to be independent or correlated, two clusters were recovered (Fig. 4). The Maxwell Hill samples formed a single cluster while the Pasoh, Bukit Rengit, Semangkok and Behrang samples formed another cluster (Fig. 4). When allele frequencies were correlated, the likelihood of $K=2$ was about 30 times higher than the corresponding value when *K* was equal to 3 (Fig. 4).

3.3. Relationship between populations assuming the presence of two species

The various methods of analysis indicate the presence of two major population clusters in the data set. The Maxwell Hill population appeared to be highly distinct based on the shared-alleles tree, NJ tree based on the genetic distances between colonies and the Bayesian

Table 1
Characteristics of 29 microsatellite loci and primers developed for *Odontotermes srinakarinensis* sp. nov.

Locus	Repeat motif	Primer sequence (5′ to 3′)	Size (bp)	GenBank accession no.
Oskar 2-5	$(TC)_{16}$	F:CAACTATGTACCGCCGTGCTA R:ATTTCCCGCAAGACGCATTC	327	JQ665975
Oskar 8-5	$(CT)_{14}TTT\ (TC)_7$	F:GGGCAGATTAAGGAATGAATA R:ACATAGCCCTGAATGAGCA	418	JQ665976
Oskar 9-5	$(AG)_{15}C\ (GA)_7$	F:CGTACACCGCACAACT R:TACCTGCATTGTTTTAACTCC	618	JQ665977
Oskar 10-5	$(TG)_{27}(AG)_{12}$	F:CCAACAACAGCGCCTACAGAC R:CCCGTTATTATTGTCAGATTT	313	JQ665978
Oskar 14-5	$(TC)_{14}$	F:TTCGACTGCACACTGCCATTC R:GACGCATTTGCACTCCATCA	342	JQ665979
Oskar 19-5	$(AG)_{14}(GA)_8$	F:ATGGGTGAGGTGGCAGA R:GTCCATTGCATTTGATTGTCC	275	JQ665980
Oskar 20-5	$(AG)_{24}$	F:AATGGACCGAGTTTCGAGATG R:TGCGTGCCAATATGCGTGTA	281	JQ665981
Oskar 21-5	$(GA)_{10}GG\ (GA)_7$	F:TTACAGCCCTCTGGATGTCTT R:CAGCCCATGCAGTACAGTTT	302	JQ665982
Oskar 22-5	$(TC)_{25}$	F:GTTAGTTTCCCAAGGTCTTGT R:CGCAGTAAGAAAGCAGAA	345	JQ665983
Oskar 24-1	$(AG)_{15}$	F:AGGGTTTCACAGCAGCGTGAC R:AGCCGTGGAACAATTATCTC	317	JQ665984
Oskar 27-5	$(AC)_9$	F:AAGGGCCATCGTGCATTC R:CATCCAGTTGGGCTCCGACAT	461	JQ665985
Oskar 31-5	$(GT)_{16}$	F:TAATCAGTCATGAAGCAGCAG R:CGGGAAGCGATTGAGA	209	JQ665986
Oskar 33-4	$(CA)_6$	F: GTTGCACGTGTGATGGTATAG R: GAACGCTTGGAGAGGA	325	JQ665987
Oskar 34-5	$(TG)_6$	F:CGTATCGACTGTGGATTGAGT R:TGCGTACACGTCACAATG	339	JQ665988
Oskar 37-3	$(GAA)_7$	F:TAGCGGTGAGAGGTGGAACGTCTA R:GAATGTGGCCTCATCCCTAGTTCA	340	JQ665989
Oskar 40-3	$(AGTA)_{19}$	F:CGCTTGTGTGTACGGTAA R:GATTCTAACCGACAAATG	318	JQ665990
Oskar 49-2	$(GT)_4$	F:GCGCTGTAAACACT R:CAGGTAGCAGGAATTAAC	456	JQ665991
Oskar 59-2	$(AGTA)_4$ $(AAGTA)_4$	F:TGGATGGAGTTGGCTCAGGATAGG R:CCTATCCTGAGCCCACTCCATCCA	454	JQ665992
Oskar 60-5	$(AC)_{11}$	F:GACACAAAGCCTCGGTTAGTA R:TTGGTGCAAACGGAAAGTA	305	JQ665993
Oskar 62-5	$(GT)_6n(TG)_8$	F:TCCTACCGGGTTAGTG R:TATCCCGAATACCTAGCATAG	446	JQ665994
Oskar 66b-5	$(AG)_{14}$	F:AGTGAGTGAGTGGCCTCT R:GTTCCATCAGCGATAAGTCAG	316	JQ665995
Oskar 67-5	$(GA)_{15}$	F:CATGGCACGGATGAGTCAGTC R:TGGCGTCAGTACACTCGTA	369	JQ665996
Oskar 68-5	$(TC)_{13}$	F:TAGTAGGCATTCCCTGA R:AGGCAAAAACATACACTCTAT	335	JQ665997
Oskar 69-5	$(AG)_{19}$	F:GAACCCACGCATTCTCCTAGT R:GTTGTAACAAGTGCCCAGTA	366	JQ665998
Oskar 77-5	$(AC)_9$	F:GCAACGAATGAGCGCAGTTAT R:CATGGGGAGTACGCAGTGGA	345	JQ665999
Oskar 80-1	$(GT)_{29}$	F:CCGCTACACAAAGTGCCTGATACT R:GGCCACCACTGACTGTCGAAACAT	326	JQ666000
Oskar 83-5	$(TG)_9$	F:TGAAAAACAGTGCGTGCGTAT R:CGCTTTGCTTCAGGGTATCAC	183	JQ666001
Oskar 84-2	$(AG)_{16}$	F:CGGTTCTGTGAACGTTTTATGTCC R:GCCAAACAGATGACTAACCCAC	415	JQ666002
Oskar 93-5	$(CA)_7$	F:AAATTTAAGTTAGGGCAGTGA R:AACTGTGCGAAACACCATTCC	456	JQ666003

clustering analysis (Figs. 2–4). When the genotype data of the Pasoh population were reanalysed with Micro-Checker, null alleles were found at six loci, that is, Oskar 8-5, Oskar 9-5, Oskar 27-5, Oskar 31-5, Oskar 40-3 and Oskar 68-5. Nevertheless, the population was still in Hardy–Weinberg equilibrium after the Pasoh population data set was reanalysed with these loci removed.

The NJ and UPGMA trees based on the proportion of shared alleles, rebuilt after the additional 6 loci were excluded, showed that two clusters were still evident, one comprising the Maxwell Hill samples and the other comprising the Pasoh, Bukit Rengit, Semangkok and

Table 2
Characterisation of loci using 41 *O. srinakarinensis* individuals from three collecting sources in Maxwell Hill (Perak). Annealing temperatures T_A, number of alleles N_A. When more than 3 alleles were present in the population, N_A are shown in parenthesis.

No.	Locus	T_A	Allele size (bp) and N_A	H_e	H_o
1.	Oskar 2-5	47.0	232–242 (4)	0.68	0.78
2.	Oskar 8-5	47.0	289–307 (6)	0.82	0.93
3.	Oskar 9-5	47.0	214–223 (7)	0.85	0.98
4.	Oskar 10-5	52.0	261–298 (8)	0.85	1.00
5.	Oskar 14-5	47.0	216–237 (4)	0.56	0.54
6.	Oskar 19-5	47.0	178–184 (4)	0.69	0.93
7.	Oskar 20-5	47.0	115–131 (5)	0.73	0.88
8.	Oskar 21-5	47.0	173–188 (6)	0.70	1.00
9.	Oskar 22-5*	47.0	236–258 (4)	0.42	0.00
10.	Oskar 24-1	48.9	219–232 (6)	0.81	0.83
11.	Oskar 27-5	55.9	371, 372	0.05	0.05
12.	Oskar 31-5	47.0	156, 178	0.03	0.03
13.	Oskar 33-4	47.0	115	–	–
14.	Oskar 34-5*	47.0	244, 245, 247	0.27	0.00
15.	Oskar 37-3	47.0	250, 253, 256	0.56	0.73
16.	Oskar 40-3	48.4	191–222 (5)	0.65	0.63
17.	Oskar 49-2	47.9	322, 353, 364	0.55	1.00
18.	Oskar 59-2*	47.0	215, 216, 225	0.65	0.47
19.	Oskar 60-5	47.0	276, 278, 280	0.59	0.47
20.	Oskar 62-5	47.0	145, 155	0.27	0.32
21.	Oskar 66b-5	47.0	187–205 (6)	0.68	0.61
22.	Oskar 67-5	47.0	75–100 (7)	0.84	1.00
23.	Oskar 68-5	47.0	264–289 (5)	0.72	0.90
24.	Oskar 69-5	47.0	197–207 (4)	0.67	0.80
25.	Oskar 77-5	47.0	275–295 (6)	0.72	0.70
26.	Oskar 80-1	50.2	142,153	0.39	0.53
27.	Oskar 83-5	47.0	80–96 (6)	0.72	0.76
28.	Oskar 84-2	47.0	210–221 (4)	0.75	0.98
29.	Oskar 93-5	47.0	169, 171, 173	0.48	0.55

Behrang samples (Supplementary Fig. S2). UPGMA and NJ trees constructed from D_A and $(\delta\mu)^2$ distances using the reduced loci data set also showed that the Maxwell Hill samples had the highest genetic distance from the rest of the population pairs (not shown). This was still consistent with the findings made earlier when the loci with null alleles were included in the analysis.

Following this, we reexamined the morphology of the specimens in light of the genetic data and found morphological differences between samples from the two clusters. Comparisons made with identified materials at the NHM London and Cambridge Museum of Zoology (United

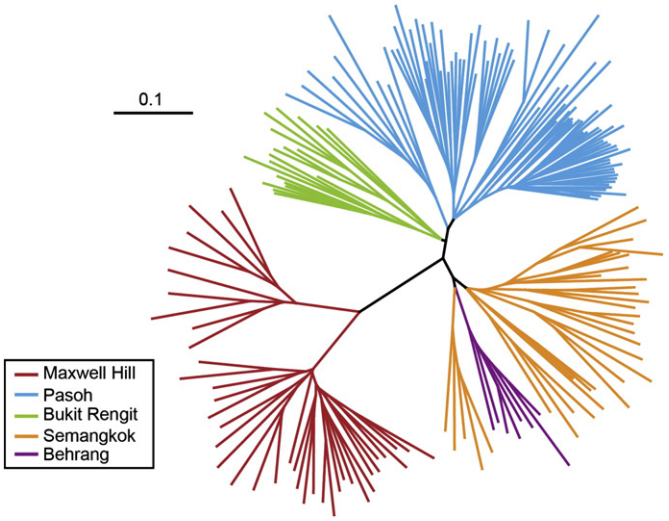

Fig. 2. NJ tree constructed from the proportion of shared-alleles from 26 loci amplified in 189 termite individuals. (Note: each branch represents a single termite individual. Two samples were removed from the data set as they were suspected to have been labelled wrongly during DNA extraction.)

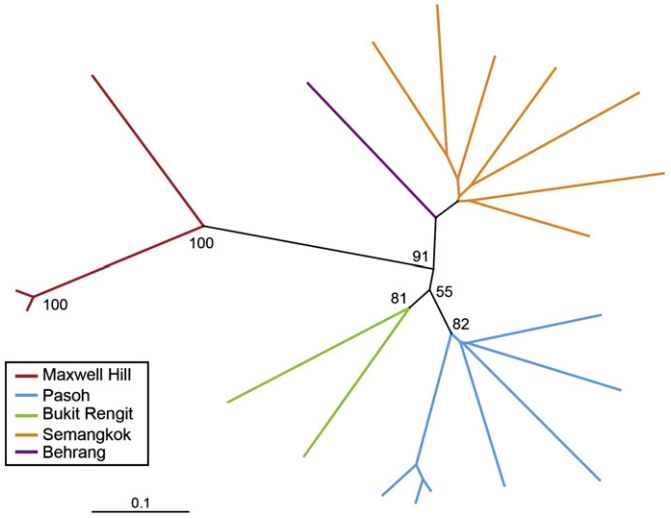

Fig. 3. NJ tree calculated from D_A (Nei et al., 1983). (Note: branch tips represent a single termite colony. Only colonies with four or more termite individuals genotyped were used to construct the tree.)

Kingdom) matched the soldier samples from Pasoh, Bukit Rengit, Semangkok and Behrang F.R.'s to *Odontotermes denticulatus* Holmgren while soldier samples from Maxwell Hill were matched to *O. srinakarinensis* sp. nov (Takematsu, submitted). A single character, that is, the shape of the internal margin of the left mandible of its soldiers could be used quite accurately to differentiate *O. denticulatus* from *O. srinakarinensis*.

Examination of our records show that the actual *O. srinakarinensis* occurred in three of the nine study sites in Peninsular Malaysia, that is, Maxwell Hill F.R., Bukit Kinta F.R. and Angsi F.R (Fig. 1). The species appears to primarily inhabit hill forests which is, similarly, where the type specimen *O. srinakarinensis* was found. Srinakarin Dam (Thailand), the type locality of this species is a high elevation site and the only site where Takematsu has found *O. srinakarinensis* (Takematsu, submitted). *O. denticulatus* on the other hand was found in both lowland and hill forests. However, our collection records also show that *O. srinakarinensis* and *O. denticulatus* occur sympatrically in Bukit Kinta F.R. and Angsi F.R.; samples from Bukit Kinta F.R. and Angsi F.R. could not be included in the microsatellite analysis because insufficient samples for DNA analysis were obtained from these sites.

Table 3 shows the allelic richness, gene diversity and F_{IS} values for populations of *O. denticulatus* and *O. srinakarinensis* that were analysed in this study. The Pasoh population of *O. denticulatus* appeared to have the highest allelic richness followed by the Semangkok and Bukit Rengit colonies. Although gene diversity values were comparable in populations of both species, the inbreeding coefficient (F_{IS}) in *O. denticulatus* was larger compared to *O. srinakarinensis* indicating that the former species had a tendency to inbreed.

4. Discussion

Microsatellites were developed for *O. srinakarinensis*, a species-group or clade that was previously found to consist of samples with highly divergent DNA sequences (Cheng et al., 2011). The basis of the application of microsatellite markers to study this clade was that it would allow us to determine the genetic structure of the population, that is, whether it consisted of a population of randomly mating individuals or if the genetic variation seen in the mtDNA data was due to significant genetic structure at the population level of the species. Alternately, it could provide us with evidence of the existence of cryptic species within this clade. The shared alleles trees, population trees and Bayesian clustering of the genotype data showed that the *O. srinakarinensis* species complex was actually composed of two distinct clusters (Figs. 2–4). Samples from the first cluster belonged to *O. srinakarinensis* while samples from the second cluster belonged to *O. denticulatus*. The finding of a diagnostic morphological character that could be used to differentiate between the species provided further support that they were indeed distinct.

There was, as well, an indication of a difference in breeding behaviour in that *O. denticulatus* tended to inbreed to a larger extent compared to *O. srinakarinensis*. This was based on the strongly negative F_{IS} value in the single *O. srinakarinensis* population in Maxwell Hill compared to the F_{IS} values in the three populations of *O. denticulatus* from Pasoh, Semangkok and Behrang F.R.s (Table 3). A higher rate of inbreeding in organisms such as termites indicates that the species has a shorter mating flight range and hence are more likely to pair with relatives during colony-founding. However, additional samples or colonies of *O. srinakarinensis* need to be analysed in order to confirm this postulation.

O. srinakarinensis primarily inhabited hill forests whereas *O. denticulatus* was found in lowland and hill forests. Both species were found occurring in sympatry in two sites, that is, Bukit Kinta F.R. and Angsi F.R.; however, insufficient samples were obtained from these sites for genetic analysis. If additional samples are obtained from these sites in the future, we will be able to examine if *O. srinakarinensis* and *O. denticulatus* remain reproductively isolated here, or if these areas

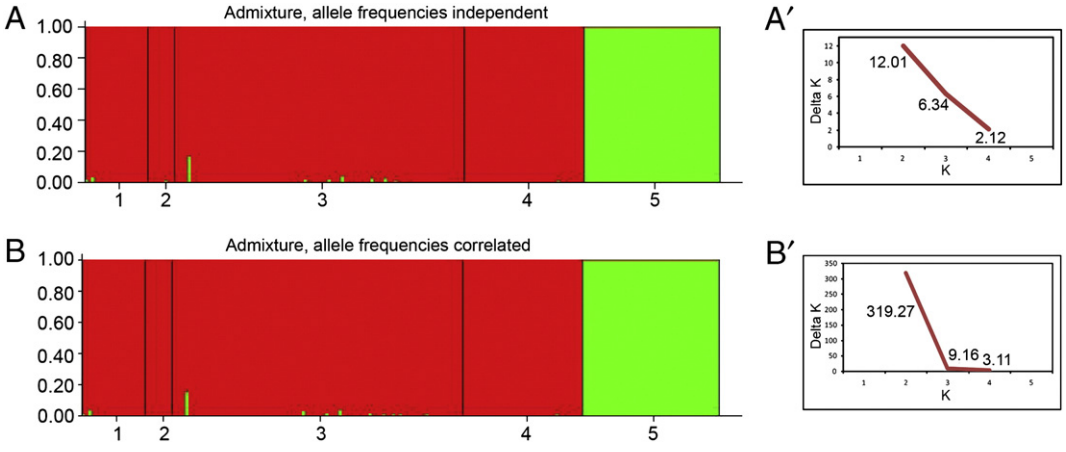

Fig. 4. Structure bar plots showing Bayesian clustering patterns of the genotype data assuming admixture under two different conditions of allele frequencies (populations 1–4: Bukit Rengit, Behrang, Pasoh and Semangkok; population 5: Maxwell Hill).

Table 3
Allelic richness, gene diversity and F_{IS} values for populations of *O. denticulatus* and *O. srinakarinensis*, abbrev. as *O. sri* ($p = 0.05$).

No	Locus	Allelic richness, R_S				Gene diversity				F_{IS}*			
		O. denticulatus			*O. sri*	*O. denticulatus*			*O. sri*	*O. denticulatus*			*O. sri*
		Psh.	Br.	Smk.	Tpg.	Psh.	Br.	Smk.	Tpg.	Psh.	Br.	Smk.	Tpg.
1	*Oskar* 49-2	3.00	2.00	2.00	3.00	0.50	0.50	0.39	0.55	−0.44	−0.67	−0.35	−0.83
2	*Oskar* 67-5	3.00	2.90	3.00	6.88	0.49	0.32	0.29	0.84	−0.45	−0.17	−0.14	−0.19
3	*Oskar* 24-1	7.00	4.00	3.78	5.99	0.69	0.63	0.27	0.81	0.03	−0.09	−0.13	−0.02
4	*Oskar* 80-1	1.00	1.00	1.00	2.00	0.00	0.00	0.00	0.39	NA	NA	NA	−0.35
5	*Oskar* 14-5	7.00	3.83	3.00	3.95	0.15	0.16	0.50	0.56	−0.04	−0.03	−0.12	0.04
6	*Oskar* 10-5	5.95	4.00	5.94	8.00	0.54	0.54	0.66	0.85	−0.39	−0.18	−0.30	−0.18
7	*Oskar* 19-5	8.93	6.88	6.94	4.00	0.77	0.69	0.63	0.69	0.01	−0.38	−0.07	−0.35
8	*Oskar* 20-5	17.71	8.58	8.88	4.98	0.89	0.84	0.84	0.73	−0.06	−0.06	−0.15	−0.20
9	*Oskar* 84-2	4.96	3.00	4.00	4.00	0.57	0.53	0.64	0.75	−0.74	−0.89	−0.01	−0.31
10	*Oskar* 93-5	12.89	10.00	5.74	3.00	0.82	0.89	0.65	0.48	0.04	−0.13	−0.32	−0.15
11	*Oskar* 60-5	6.00	5.00	5.83	3.00	0.58	0.68	0.73	0.59	0.15	0.23	0.14	0.20
12	*Oskar* 62-5	5.96	5.00	5.00	2.00	0.73	0.77	0.73	0.27	−0.07	0.23	0.02	−0.18
13	*Oskar* 2-5	13.87	8.67	11.76	4.00	0.86	0.83	0.88	0.68	0.07	−0.15	0.05	−0.15
14	*Oskar* 77-5	10.85	6.78	6.83	5.87	0.76	0.79	0.48	0.72	0.02	0.33	0.35	0.03
15	*Oskar* 21-5	6.92	10.87	13.41	6.00	0.67	0.90	0.81	0.70	0.10	−0.05	0.01	−0.44
16	*Oskar* 66b-	7.00	5.90	5.94	5.85	0.79	0.82	0.76	0.68	0.09	0.36	−0.09	0.10
17	*Oskar* 69-5	9.00	7.00	15.91	4.00	0.72	0.85	0.93	0.66	0.05	0.03	0.18	−0.21
18	*Oskar* 37-3	3.00	2.00	4.00	3.00	0.59	0.47	0.62	0.56	−0.38	0.21	−0.08	−0.32
19	*Oskar* 83-5	6.99	6.00	5.00	5.83	0.81	0.79	0.73	0.72	−0.08	−0.05	0.31	−0.04
20	*Oskar* 8-5	–	–	–	6.00	–	–	–	0.82	–	–	–	−0.13
21	*Oskar* 40-3	–	–	–	5.00	–	–	–	0.65	–	–	–	0.02
22	*Oskar* 31-5	–	–	–	1.88	–	–	–	0.03	–	–	–	0.00
23	*Oskar* 27-5	–	–	–	1.99	–	–	–	0.05	–	–	–	−0.01
24	*Oskar* 9-5	–	–	–	8.00	–	–	–	0.85	–	–	–	−0.14
25	*Oskar* 68-5	–	–	–	4.85	–	–	–	0.72	–	–	–	−0.26
	Mean	7.42	5.44	6.21	4.52	0.63	0.63	0.61	0.61	−0.09	−0.05	−0.02	−0.17

Note: *F_{IS} was calculated overall from the loci, and is not equivalent to the mean value. Allelic richness estimates were based on minimum samples of 32 individuals for Semangkok ($n = 36$); 78 for Pasoh ($n = 87$); 17 for Bukit Rengit ($n = 19$); and 35 for Maxwell Hill ($n = 41$). Population names are abbreviated as follows: Pasoh (Psh.); Bukit Rengit (Br.); Semangkok (Smk.) and Maxwell Hill (Tpg.). Number of voucher collections included in this analysis is as follows: Pasoh (8); Bukit Rengit (5); Semangkok (9) and Maxwell Hill (3).

represent hybrid zones for them. Cryptic or sibling species are often difficult to differentiate based on morphological characters alone. This is probably due to their rather recent origin that has not allowed them sufficient time to become morphologically divergent.

Molecular markers such as microsatellites, hence, are very useful in such situations because it can be used to quickly assess the species status of closely related populations either through the use of population genetics distances or genotype clustering patterns of their microsatellite data. Roy et al. (2006) utilised a combination of DNA markers which included microsatellites on *Cubitermes* sp. *affinis subaquartus* in Gabon, Africa, to detect the presence of distinct species clusters within this complex, some of which were found to occur in sympatry with one another. Palaeogeographic events were thought to be responsible for causing the isolation and the subsequent formation of the *Cubitermes* species inhabiting this region (Roy et al., 2006). This has similarly been suggested for a new species of *Reticulitermes* found in France and Italy (Uva et al., 2004).

In this study, it was evident that *O. srinakarinensis* and *O. denticulatus* were closely related because many of the microsatellite loci developed for *O. srinakarinensis* could be amplified in *O. denticulatus*; additionally, both species were even found to share some similar alleles. However, there were differences in terms of their distribution and the habitats they occupied. Lastly, it would be useful to date the split between these two species using a molecular clock on the phylogenetic tree or by calibrating the tree with a known palaeogeographic event as this would allow us to better understand some of the factors that may have been responsible for the origins of these species.

5. Conclusion

A total of 29 microsatellites were isolated from *O. srinakarinensis* sp. nov to investigate the presence of cryptic species within this clade of *Odontotermes*. Primers were then designed and screened on 41 termite individuals using three voucher samples that represented different colonies from Maxwell Hill (Perak, Malaysia). Except for one monomorphic locus, the rest were polymorphic with an average of 4.28 alleles per locus. One-hundred and fifty termite individuals from 23 other collecting sources representing members of *Odontotermes* from this cryptic species complex were then genotyped at these loci. Trees of relationships reconstructed from the proportion of shared-alleles between these samples, genetic distances between their colonies, and Bayesian clustering of their genotype data showed the presence of two clusters that corresponded to two different species, that is, *O. srinakarinensis* and *O. denticulatus*. Both species were genetically and morphologically different and showed almost no overlap in their distribution except in Bukit Kinta and Angsi F.R.s.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2012.12.084.

Acknowledgments

We thank Azmi Mahyudin, Mohd Shah Fadir Ishak, Saimas Ariffin, Shaiful Amri, and Wan Shaina Mazuin Wan Mamat for collecting termite samples and for their assistance in the laboratory. We thank Lee Soon-Leong for providing support during the marker development and genotyping phase of the study. Thanks are also due to Paul Eggleton (NHM) and Joanna Darlington (University of Cambridge) for permission to reference the termite collections at their respective institutes. The first author also wishes to specially acknowledge the financial assistance provided by Universiti Putra Malaysia and Mr. and Mrs. Cheng Poh-Heng which enabled him to travel to the NHM London and Cambridge Museum of Zoology for a one-month attachment in 2011 to complete this research for his doctoral degree. This study was funded by a grant from the Ministry of Natural Resources and Environment, Malaysia.

References

Ahmad, M., 1958. Key to the Indo-Malayan termites. Biologia 4, 33–198.

Brown, D.M., Brenneman, R.A., Koepfli, K.P., Pollinger, J.P., et al., 2007. Extensive population genetic structure in the giraffe. BMC Biol. 5, 57.
Cheng, S., Kirton, L.G., Panandam, J.M., Siraj, S.S., Ng, K.K.S., Tan, S.G., 2011. Evidence for a higher number of species of *Odontotermes* (Isoptera) than currently known from Peninsular Malaysia from mitochondrial DNA phylogenies. PLoS One 6, e20992.
Darlington, J.P.E.C., 1997. Comparison of nest structure and nest parameters of sympatric species of *Odontotermes* (Termitidae: Macrotermitinae) in Kenya. Insect. Soc. 44, 393–408.
Darlington, J.P.E.C., Benson, R.B., Cook, C.E., Walker, G., 2008. Resolving relationships in some African fungus-growing termites (Termitidae, Macrotermitinae) using molecular, morphology and field parameters. Insect. Soc. 55, 256–265.
Davison, D., Darlington, J.P.E.C., Cook, C.E., 2001. Species-level systematics of some Kenyan termites of the genus *Odontotermes* (Termitidae: Macrotermitinae) using mitochondrial DNA, morphology and behaviour. Insect. Soc. 48, 138–143.
Dyer, N.A., Ravel, S., Choi, K.S., Darby, A.C., Causse, S., et al., 2011. Cryptic diversity within the major trypanosomiasis vector Glossina fuscipes revealed by molecular markers. PLoS Negl Trop Dis 5, e1266.
Earl, D.A., vonHoldt, B.M., 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4, 359–361.
Elmer, K.R., Davila, J.A., Lougheed, S.C., 2007. Cryptic diversity and deep divergence in an upper Amazonian frog, *Eleutherodactylus ockendeni*. BMC Evol. Biol. 7, e247.
Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software **structure**: a simulation study. Mol. Ecol. 14, 2611–2620.
Feldheim, K.A., da Silva, J.M., Tolley, K.A., 2012. Isolation of novel microsatellite loci in dwarf chameleons from KwaZulu-Natal province, South Africa and their cross-amplification in other Bradypodion species. Conserv. Genet. Resour. 4, 205–211.
Förschler, M.I., Senar, J.C., Perret, P., Björklund, M., 2009. The species status of the Corsican finch *Carduelis corsicana* assessed by three genetic markers with different rates of evolution. Mol. Phylogenet. Evol. 52, 234–240.
Goldstein, D.B., Ruíz-Linares, A., Feldman, M., Cavalli-Sforza, L.L., 1995. An evaluation of genetic distances for use with microsatellite loci. Genetics 139, 463–471.
Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3), 2001, http://www.unil.ch/izea/softwares/fstat.html. Accessed 2011 January 5.
Holmgren, N., 1913. Termitenstudiesn. 4. Versuch einer systematischen Monographie der Termiten der orientalischen Region. Kungl Svenska Vetensk-Akad Handl 50, 1–276.
Kaib, M., et al., 2000. Microsatellite loci in *Macrotermes michaelseni* (Isoptera: Termitidae). Mol. Ecol. 9, 502–504.
Kijas, J.M., Fowler, J.C., Garbett, C.A., Thomas, M.R., 1994. Enrichment of microsatellites from the citrus genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. Biotechniques 16, 656–662.
Lee, S.L., Tani, N., Ng, K.K.S., Tsumura, Y., 2004. Isolation and characterisation of 20 microsatellite loci for an important tropical tree *Shorea leprosula* (Dipterocarpaceae) and their applicability to *S. parvifolia*. Mol. Ecol. Notes 4, 222–225.
Li, W., Godzik, A., 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinforma. Appl. Note 22, 1658–1659.
Liu, K., Muse, S.V., 2005. Powermarker: Integrated analysis environment for genetic marker data. Bioinformatics 21, 2128–2129.
Meglécz, E., 2007. MICROFAMILY: a computer program for detecting flanking region similarities among different microsatellite loci. Mol. Ecol. Notes 7, 18–20.
Nei, M., Tahima, F., Tateno, Y., 1983. Accuracy of estimated phylogenetic trees from molecular data. J. Mol. Evol. 19, 153–170.
Park, S.D.E., 2001. Trypanotolerance in West African cattle and the population genetic effects of selection. Ph.D. thesis. University of Dublin, Dublin, Ireland.
Pinheiro, F., Palma-Silva, C., Barros, F.D., Cozzolino, S., 2009. Cross-amplification and characterization of microsatellite loci for the Neotropical orchid genus *Epidendrum*. Genet. Mol. Biol. 32, 337–339.
Primmer, C.R., Painter, J.N., Koskinen, M.T., Palo, J.U., Merilä, J., 2005. Factors affecting avian cross-species microsatellite amplification. J. Avian Biol. 36, 348–360.
Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.
Roy, V., Demanche, C., Livet, A., Harry, M., 2006. Genetic differentiation in the soil-feeding termite *Cubitermes* sp. *affinis subarquatus*: occurrence of cryptic species revealed by nuclear and mitochondrial markers. BMC Evol. Biol. 6, 102.
Schmidt, A.M., Trindl, A., Korb, J., 2007. Isolation and characterization of 10 microsatellite loci in the magnetic termite, *Amitermes meridionalis* (Isoptera: Termitidae). Mol. Ecol. Notes 7, 1045–1047.
Takematsu, Y. *A taxonomic revision of the Macrotermitinae (Isoptera: Termitidae) in Thailand*. Manuscript submitted for publication.
Thiel, T., Michalek, W., Varshney, R.K., Graner, A., 2003. Exploiting EST databases for the development and characterisation of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). Theor. Appl. Genet. 106, 411–422.
Tho, Y.P., 1991. Termites of Peninsular Malaysia. Kirton L.G. (Ed.), Malayan Forest Records No. 36. Forest Research Institute Malaysia, Kuala Lumpur. pp 224.
Uva, P., Clement, J.L., Austin, J.W., Aubert, J., Zaffagnini, V., Quintana, A., Bagnères, A.G., 2004. Origin of a new *Reticulitermes* termite (Isoptera, Rhinotermitidae) inferred from mitochondrial and nuclear DNA data. Molecular Phylogenetics and Evolution 30, 344–353.
Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M., Shipley, P., 2004. Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes 4, 535–538.