

Table 1 Description of microsatellite loci for *Tetrao urogallus*; sequences and repeat type, PCR fragment length (based on the clone sequence), number of resolved alleles (number of analysed birds are in parentheses), optimal annealing temperature, heterozygosity, and GenBank accession nos of the clone sequences. Significant differences between the observed and expected heterozygosity are marked with an asterisk

Locus	Primer sequences (5'–3')	Repeat type	Size (bp)	No. of alleles	Annealing temp.	H_O	H_E	Accession no.
TUD1	F: ATTTGCCAGGAACTTGCTC R: AACTACCTGCTTGTGCTTGG	(CA) ₁₄	209	8 (19)	59	0.70	0.82	AF254644
TUD2	F: GTGACAACCTCAGCCCCTGTC R: AATAAGGGTGCGCATACACC	(CA) ₁₃	200	10 (19)	59	0.83	0.89	AF254645
TUD3	F: TCCAAGGGGAAAATATGTGTG R: TTCTTCCAGCCCTAGCTTTG	(TG) ₁₂	192	11 (19)	60	0.66	0.82	AF254646
TUD4	F: TTAGCAACCGCAGTGATGTG R: GGGAGGACTGTGTAGGAGAGC	(CA) ₂₁	167	11 (19)	60	0.61*	0.88	AF254647
TUD5	F: CCTTGCTGCACATTTTCTCC R: GGTGCTGAGCATGTACTAGGG	(GT) ₂₃	193	12 (19)	57	0.72	0.88	AF254648
TUD6	F: GGTGAGCAAGCCACAATAAC R: GAGGACTGCAGAACCCTG	(CA) ₂₁	210	13 (16)	58	0.69*	0.88	AF254649
TUD7	F: TGACACTGGGTCATTAGGC R: AACATGGGCAGGAGGAGAC	(CA) ₁₁	200	5 (19)	59	0.58	0.63	AF254650
TUD8	F: TGCAGCCTCCTCTAATTTTCG R: CTGACATCAGCAATCATGC	(GT) ₁₅	187	11 (17)	59	0.70*	0.85	AF254651
TUT1	F: GGTCTACATTTGGCTCTGACC R: ATATGGCATCCAGCTATGG	(CTAT) ₁₂	217	8 (20)	60	0.60	0.83	AF254653
TUT2	F: CCGTGTCAAGTTCTCCAAAC R: TTCAAAGCTGTGTTTCAITAGTTG	(GATA) ₁₂	160	9 (20)	60	0.70	0.76	AF254654
TUT3	F: CAGGAGGCTCAACTAATCACC R: CGATGCTGGACAGAAGTGAC	(TATC) ₁₁	154	8 (20)	60	0.50	0.80	AF254655
TUT4	F: GAGCATCTCCAGAGTCAGC R: TGTGAACCAGCAATCTGAGC	(TATC) ₈	179	7 (20)	60	0.80*	0.77	AF254656

people who provided feather samples, D. Begerow for sequencing, and S. Piertney for technical advice.

References

- Estoup A, Turgeon J (1996) *Microsatellite marker isolation with non-radioactive probes and Amplification, Version 12/1996, available via Website* <http://inapg.inra.fr/dsa/microsat/microsat.htm>.
- Piertney SB, Goostrey A, Dallas JF, Carss DN (1998a) Highly polymorphic microsatellite markers in the great cormorant *Phalacrocorax carbo*. *Molecular Ecology*, **7**, 138–140.
- Piertney SB, MacColl AD, Bacon PJ, Dallas JF (1998b) Local genetic structure in red grouse (*Lagopus lagopus scoticus*): evidence from microsatellite DNA markers. *Molecular Ecology*, **7**, 1645–1654.
- Primmer CR, Raudsepp T, Chowdhary BP, Müller AP, Ellegren H (1997) Low frequency of microsatellites in the avian genome. *Genome Research*, **7**, 471–482.
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*. 2nd edn. Cold Spring Laboratory Press, New York.
- Storch I (1997) The role of the metapopulation concept in conservation of European woodland grouse. Abstract. *Wildlife Biology*, **3**, 272.
- Storch I (2000) *Status Survey and Conservation Action Plan 2000–2004 Grouse*. 93-IUCN, Gland, Switzerland.

Identification of polymorphic microsatellite loci in the Formosan subterranean termite *Coptotermes formosanus* Shiraki

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Keywords: *Coptotermes formosanus*, genetic variability, microsatellites, population structure

Received 3 April 2000; revision received 4 June 2000; accepted 29 June 2000

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The Formosan subterranean termite *Coptotermes formosanus* Shiraki (Rhinotermitidae) is thought to have originated in China, but has spread to many areas around the world where it is a highly destructive pest of wood structures (Su & Tamashiro 1987). In the USA, this species is abundant in Hawaii, and has become established in many places on the mainland, particularly in the south-eastern and south central regions (Wang & Grace 1999).

Table 1 Primer sequences and characteristics of 15 microsatellite loci of *Coptotermes formosanus*

Locus	Core repeat*	Size (bp)	Concentration of primer (nM)	Primer sequence (5' → 3')	GenBank accession no.
Cf 4:1A2-4	(AAG) ₁₄	175	100	F: CTGTTTCTCGTAATCGGGGA†	AF247458
			200	R: TTCTTCAACGTCTTCGCCTT	
Cf 4:1A2-5	(CAA) ₁₀	141	200	F: TCGGACTCCAGGTACTACCAA	AF247459
			100	R: GATTGCCGTTCCCTCCTTCT†	
Cf 1-1	(TTA) ₇ (GTA) ₁₁	255	100	F: TTGCTCAGGATAGGGACAGG†	AF247460
			200	R: TGGTTAATCGTGCCACATA	
Cf 2-5	(CAT) ₇	175	100	F: CCTCAAATCCCTCACACAC†	AF247462
			200	R: GGACATTTTGTCTCCTCCAA	
Cf 4-4	(ACT) ₂₀	214	100	F: GCATAAACGAACCTGGAAA†	AF247463
			200	R: TGCCAAACATGTGTGCTTT	
Cf 4-9A	(TCA) ₁₁	283	200	F: GTGTGGGATTTGAGGTGGAC	AF247464
			200	R: GAAAAACAGCGACTGCCTTCC†	
Cf 4-10	(CAT) ₁₁	229	200	F: GCAAGTTTTCGCCCTGTGAGT†	AF247465
			200	R: GAAAAACAGCGACTGCCTTCC	
Cf 8-4	(CTA) ₉ (CTC) ₁₅	221	200	F: TCTGTGGAACGTGGTGTGAT†	AF247468
			200	R: CCTCTCTGTGCCTGCTTAGG	
Cf 10-4	(AGT) ₂₂	154	200	F: GCGCATGTGGACTGTAAAAA	AF247469
			200	R: TCCAAGTATGCTGATCGGGT†	
Cf 10-5	(GAT) ₈	295	200	F: CAGCTATATTTGGGCACAGCA†	AF247470
			200	R: CACGACGGACTGAAGTGGTT	
Cf 11-1	(GTA) ₁₀	204	200	F: CGTTCCTTCGAAACTTCTGC	AF247471
			200	R: TACCATCACCACCACCCT†	
Cf 12-4	(TAG) ₂₁	172	200	F: AGCGTCTAGCCTTGCACCTCT†	AF247472
			200	R: CTCCTCACAAAATCCGGTA	

*Sequenced allele. †Primer to which M13 forward tail is attached. F, forward; R, reverse.

Polymorphic genetic markers for *C. formosanus* would be useful in elucidating details of colony organization, population structure, and relationships among introduced and native populations. Previous studies employing allozymes have found limited genetic variation in this species (Korman & Pashley 1991; Strong & Grace 1993). To provide variable genetic markers for examining colony and population structure, we developed microsatellite markers for *C. formosanus*.

Microsatellite loci were isolated following the protocol of Glenn (1996) with modifications as described by Vargo (2000). The partial genomic library was constructed from pooled samples of heads of workers from eight colonies originating from Gretna, Lake Charles and New Orleans, Louisiana, USA. After grinding the tissue in liquid nitrogen, the DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). Fragments (300–700 bp) of *Sau3AI*-digested genomic DNA were ligated into pZErO-2 plasmids (Invitrogen), cut with *Bam*HI, and transformed into *Escherichia coli* TOP10 cells (Invitrogen). Approximately 20 000 clones were plated and lifted onto nylon membranes. The membranes were probed with the oligonucleotides (AAT)₁₀, (AAC)₈, (ATC)₈, (AAG)₈ and (ACT)₈, which were end-labelled with [³²P]-dATP. Southern blots confirmed 42 positive clones. Sequencing of these clones yielded 25 sequences containing five or more tandem repeats. We designed 22 primer pairs.

Microsatellite analysis was performed by fluorescent labelling according to the methods of Oetting *et al.* (1995).

Labelling was achieved by attaching the first 19 bp of the M13 forward sequencing primer (CACGACGTTGTAAAACGAC) to the 5' end of one of the specific primers in each pair as indicated in Table 1. A fluorescent-labelled M13 primer (M13F-29/IRD 800, Li-Cor) was included in the polymerase chain reaction (PCR), yielding labelled product which was detected in a Li-Cor 4000 automated sequencer.

Whole bodies of workers, either frozen alive and stored at –80 °C or killed and preserved in 95% ethanol, were pulverized in 1.5 mL Eppendorf tubes with a plastic pestle. DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). The PCR amplifications were carried out in a total volume of 10 µL, containing 4 µL (about 20 ng) template DNA, 10 mM Tris–HCl, 50 mM KCl and 0.1% Triton®X-100, 2 mM MgCl₂, 0.2 µg/µL BSA, 200 µM each dNTP, 32 nm labelled M13F primer, 0.4 U *Taq* DNA polymerase (Gibco BRL) and variable amounts of the specific primers (Table 1). All loci were amplified on a PTC-100 thermal cycler (MJ Research Inc., Littleton, Massachusetts, USA) using the following touch-down programme: initial denaturation step at 94 °C (30 s), followed by six cycles at 94 °C (30 s), 60 °C (30 s) and 72 °C (30 s), ramping down the annealing temperature 1 °C per cycle, and then 30 cycles at 94 °C (30 s), 54 °C (30 s) and 72 °C (30 s), with a final extension step at 72 °C (5 min). PCR products were separated by electrophoresis on polyacrylamide sequencing gels (8% Long Ranger, 7 M urea).

Table 2 Allele frequencies and heterozygosity levels for *Coptotermes formosanus* microsatellites for introduced and native populations

Locus	Origin	<i>n</i>	Number of alleles	Size* (bp) of most common allele	Frequency of most common allele	H_O	H_E	<i>P</i>
Cf 4:1A2-4	US and China	17	5	188	0.50	0.41	0.66	0.047
	US only	15	4	188	0.47	0.40	0.70	0.071
Cf 4:1A2-5	US and China	17	4	160	0.65	0.35	0.49	0.325
	US only	15	3	160	0.63	0.33	0.49	0.232
Cf 1-1	US and China	17	5	274	0.35	0.35	0.75	0.0001
	US only	15	4	274	0.33	0.40	0.73	0.0001
Cf 2-5	US and China	17	2	188	0.97	0.06	0.06	1.0
	US only	15	1	188				
Cf 4-4	US and China	17	6	245	0.40	0.59	0.78	0.190
	US only	15	6	245	0.40	0.53	0.77	0.059
Cf 4-9A	US and China	17	8	278	0.38	0.71	0.77	0.023
	US only	15	8	278	0.43	0.67	0.74	0.014
Cf 4-10	US and China	17	4	233, 242	0.41	0.53	0.64	0.070
	US only	15	3	233, 242	0.47	0.47	0.56	0.789
Cf 8-4	US and China	17	6	240	0.30	0.65	0.78	0.032
	US only	15	5	240	0.30	0.67	0.76	0.077
Cf 10-4	US and China	17	6	170	0.50	0.50	0.66	0.093
	US only	15	4	170	0.60	0.43	0.62	0.093
Cf 10-5	US and China	17	10	293	0.26	0.53	0.84	0.001
	US only	15	9	293	0.30	0.47	0.80	0.001
Cf 11-1	US and China	17	3	220	0.82	0.12	0.30	0.003
	US only	15	2	220	0.87	0.00	0.23	0.004
Cf 12-4	US and China	17	4	146	0.56	0.41	0.57	0.048
	US only	15	4	146	0.53	0.40	0.59	0.045

*Size includes the 19-mer M13F sequence. *n*, number of individuals examined (only one individual per colony was genotyped); H_O , observed heterozygosity; H_E , expected heterozygosity; *P*, probability by HW exact test in GENEPOP version 3.1b (Raymond & Rousset 1995).

Individuals from 17 different colonies were screened for variability. Colonies originated from the following locations: 11 from Louisiana, three from Florida, one from North Carolina and two from mainland China. Of the 22 primer pairs tested, 15 gave scorable products. Twelve of the 15 loci showed variation among the entire study population, with 2–10 alleles per locus (Table 2). One locus, Cf 2-5, was variable only in the samples from China. Among all 12 polymorphic loci, observed heterozygosity was less than the expected in all but one locus (Cf 2-5; Table 2), with significant deviations ($P < 0.05$, HW exact test in GenPop version 3.1b; Raymond & Rousset 1995) at Cf 4:1A2-4, Cf 1-1, Cf 4-9A, Cf 8-4, Cf 10-5, Cf 11-1 and Cf 12-4. Reduced heterozygosity is expected in subterranean termites which commonly form inbred colonies (Thorne *et al.* 1999).

The present set of microsatellite markers, with numerous polymorphic loci, provides a sensitive tool for investigating the colony and population genetic structure of both native and introduced populations of *C. formosanus*.

Acknowledgements

N.-Y. Su (University of Florida) kindly provided samples from Florida, and J. Powell (USDA, Mississippi State University, Mississippi, USA) generously donated samples from China. We thank the Forest Biotech Group at North Carolina State Univer-

sity (NCSU) for their generous assistance and support, especially D. O'Malley, D.M. Johnson, B.C. Lee, A. Myburg, and R. Whetten. K. Patel gave technical assistance. K. Nix coordinated shipment of termites from Louisiana to North Carolina. E.L.V. was supported by a Faculty Research and Professional Development Grant from NCSU and the W.M. Keck Program for Behavioural Biology. Funds from the Louisiana Department of Agriculture and Forestry supported G.H.

References

- Glenn T (1996) *Microsatellite Manual*, MsatManV6, ftp://onyx.si.edu/protocols/
- Korman AK, Pashley DP (1991) Genetic comparisons among U.S. populations of Formosan subterranean termites. *Sociobiology*, **19**, 41–50.
- Oetting WS, Lee HK, Flanders DJ, Wiesner GL, Sellers TA, King RA (1995) Linkage analysis with multiplexed short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. *Genomics*, **30**, 450–458.
- Raymond M, Rousset F (1995) GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.
- Strong KL, Grace JK (1993) Low allozyme variation in Formosan subterranean termite (Isoptera: Rhinotermitidae) colonies in Hawaii. *Pan-Pacific Entomologist*, **69**, 51–56.

- Su N-Y, Tamashiro M (1987) An overview of the Formosan subterranean termite (Isoptera: Rhinotermitidae) in the world. In: *Biology and Control of the Formosan Subterranean Termite* (eds Tamashiro M, Su, N-Y), pp. 3–15. College of Agriculture and Human Resources, University of Hawaii, Honolulu.
- Thorne BL, Traniello JFA, Adams ES, Bulmer M (1999) Reproductive dynamics and colony structure of subterranean termites of the genus *Reticulitermes* (Isoptera: Rhinotermitidae): a review of the evidence from behavioral, ecological, and genetic studies. *Ethology, Ecology and Evolution*, **11**, 149–169.
- Vargo EL (2000) Polymorphism at trinucleotide microsatellite loci in the subterranean termite *Reticulitermes flavipes*. *Molecular Ecology*, **9**, 817–829.
- Wang J, Grace JK (1999) Current status of *Coptotermes* Wasmann (Isoptera: Rhinotermitidae) in China, Japan, Australia and the American Pacific. *Sociobiology*, **33**, 295–305.

Isolation and characterization of polymorphic microsatellite loci in the common frog, *Rana temporaria*

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Keywords: DIG, frog, microsatellite, polymorphism, *Rana*

Received 3 May 2000; revision accepted 29 June 2000

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The common frog, *Rana temporaria*, is the most widespread amphibian species in Europe, and in many areas of northern and central Europe it is also the most common anuran species (Gasc *et al.* 1997). Consequently, it is a frequently studied organism used in ecological and evolutionary investigations (Beebee 1995; Miaud *et al.* 1999), as well as in conservation genetics studies (Reh & Seitz 1990; Hitchings & Beebee 1997;

Seppä & Laurila 1999). However, the lack of polymorphic microsatellite markers developed for the common frog has restricted its usage as a model organism in evolutionary studies, which generally require highly variable loci for estimation of relatedness among individuals (e.g. Lynch & Ritland 1999). With this in mind, we describe here the first microsatellite markers for the common frog.

Genomic DNA was digested with *Bam*HI and fragments between 400 and 1200 bp were purified from agarose gels using a QiaEx II gel extraction kit (QiaGen). Subsequently, DNA fragments were ligated to pUC18 plasmid vector and electroporated into *Escherichia coli* DH5 α cells. Transformed cells were plated and white colonies were transferred to new plates. Lifts were screened with ³²P-labelled (CA)₁₅ (12 000 colonies) and (CT)₁₅ (8000 colonies) probes, plus a cocktail including (AAAT)₇, (GATA)₇, (GAAT)₇ and (AAAG)₇, and one including (GGAA)₇, (GGAT)₇, (GACA)₇ and (AAAC)₇ oligonucleotide probes (4000 colonies), according to Sambrook *et al.* (1989). Another genomic library was also constructed using *Hind*III-digested DNA, ligated to pUC19 vector. Approximately 4000 colonies were screened with a DIG-labelled (CA)₁₅ probe according to instructions in the manufacturer's manual (Roche Molecular Biochemicals). Positive clones were sequenced with Big Dye terminator chemistry using M13 forward and reverse primers, and sequences were recorded on an ABI Prism 377 sequencer (Perkin-Elmer). Primers were designed using Oligo (Primer Analysis Software, MBI).

DNA was prepared from muscle tissue by proteinase K digestion and two phenol/chloroform and one chloroform extraction, followed by ethanol precipitation and resuspension in 20 μ L of water. The basic thermal cycling on a PTC-100 (MJ Research Inc.) comprised 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at the selected annealing temperature, 45 s at 72 °C, and finally 7 min at 72 °C. Two types of 'touch-down' programs were also applied. One set of primers (RtSB3) started with an annealing temperature of 60 °C which decreased by 0.5 °C per cycle for 10 cycles followed by 20 more cycles at 55 °C. The second set (RtSB14) started at 65 °C and then decreased by 1 °C per cycle for 10 cycles followed by 25 cycles at 55 °C.

Table 1 Characteristics of *Rana temporaria* microsatellites. Polymorphism was estimated from the genotypes of 19–44 individuals from one population

Locus	Repeat motif	Primers (5' → 3')	Number of alleles	n†	Allele size (bp)	H _E	H _O	T _a (°C)	GenBank accession no.
RtU4	(GT) ₂₃ (T) ₁₃	F: GGCTTCAAAGTAGAATAAAG R: AATCTTTTCCCTTACTGTAGC	10	31	75–108	0.71	0.39	50	AF257481
RtU7	(GATA) ₃₇	F: GCATTATTACAGCATTCTGGAT R: TTAATGGCTGGATAGATTATCC	22	46	152–295	0.89	0.91	50	AF257482
RtSB3	(GT) ₁₄ (CT)(GT) ₂	F: GAGATCCATGIGTATTTATCG R: CCCTGCGATTCTGACCTGTC	7	44	172–205	0.75	0.91	60–55*	AF257479
RtSB14	(GT) ₁₂ (GTT) ₁₆	F: TGTGTCCAGCAATGAATGTTA R: GCAGAGTTACAGCCAAGGAA	4	19	174–191	0.71	0.63	65–55*	AF257478
RtSB80	(TA) ₁₃	F: ACAGCTATATCCGACCAC R: GGAGGACATAAGTTCAATAA	9	45	199–250	0.75	0.78	58°	AF257480

†Number of individuals genotyped; T_a, annealing temperature; *Initial annealing temperature for RtSB3 and RtSB14).