Applications in Plant Sciences

PRIMER NOTE

Microsatellite markers for the prized matsutake mushroom (*Tricholoma matsutake*, Tricholomataceae)

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PREMISE OF THE STUDY: Novel and cost-effective microsatellite markers were developed to explore the population genetics, biogeographic structure, and evolutionary history of the prized Euro-Asian wild edible ectomycorrhizal fungus *Tricholoma matsutake* (Tricholomataceae).

METHODS AND RESULTS: Eighteen new polymorphic simple sequence repeat loci, detected from a microsatellite-enriched genomic library, were used to characterize 131 individuals from eight *T. matsutake* populations. The number of alleles ranged from two to 10, with averages of 1.42 to 3.22. Levels of observed and expected heterozygosity ranged from 0.00–1.00 and from 0.00–0.83, with mean values of 0.21 and 0.26, respectively. In total, 50% of the loci showed interspecific transferability and polymorphism in the related species *T. equestre*.

CONCLUSIONS: These newly developed markers will aid research into the genetic diversity and population structure of *T. matsutake*. They can also be used in other species of *Tricholoma*.

KEY WORDS capillary electrophoresis; population genetics; simple sequence repeat (SSR); *Tricholoma matsutake*; Tricholomataceae.

The ectomycorrhizal basidiomycete *Tricholoma matsutake* (S. Ito & S. Imai) Sing (Tricholomataceae) is one of the most expensive edible mushrooms (Wang et al., 1997). No fruiting body of *T. matsutake* has ever been artificially produced to date. Price fluctuations and the question of how to ensure the sustainable use of the resource in the context of climate change and varying management approaches are of major concern. For many mountainous communities in the Himalayas, harvesting the matsutake mushroom is a major source of household income. This has led to intensive harvesting of this fungus in its natural habitat, which places its sustainability at risk. Therefore, to ensure better management of *T. matsutake*, a thorough understanding of its biology, ecology, and population genetics is essential.

Tricholoma matsutake has a Eurasian distribution (Matsushita et al., 2005) and is mainly found in eastern Asia, the Himalayan region, and northern Europe. Previous molecular studies have focused on developing markers to track the geographic origin of the matsutake mushroom (Xu and Hong, 2007) and to differentiate between populations at regional levels (Lian et al., 2003). The rapid development of whole genomic sequencing has afforded researchers greater opportunities to explore more novel and cost-effective simple sequence repeat (SSR) markers. Moreover, capillary electrophoresis in combination with fluorescence-labeled SSR markers has made it possible to identify complex aneupolyploid hybrids and carry out genetic evaluations (Pan et al., 2003). In this study, we attempt to detect reliable SSRs by analyzing the whole genomic sequencing data of *T. matsutake* and the related species *T. equestre* (L.) P. Kumm. The results of the study will enable us to understand the biogeographic patterns and evolutionary processes of *T. matsutake*, as well as facilitate its sustainable utilization.

METHODS AND RESULTS

Ten polymorphic SSR markers (Appendix S1) of *T. matsutake* have been previously reported (Lian et al., 2003). However, only 60%

of these loci displayed polymorphism (Appendix S1) when tested against our 131 samples from Europe and Asia. We developed additional primers for *T. matsutake* using whole-genome sequencing data (175.76 Mbp) of *T. matsutake* published by the Genome Portal of the Department of Energy's Joint Genome Institute (National Center for Biotechnology Information [NCBI] accession no. PRJNA200596) to construct an enriched microsatellite library. The MIcroSAtellite Identification Tool (MISA; Thiel et al., 2003) was employed to detect SSRs with the criteria of eight, five, five, five, and five repeat units for di-, tri-, tetra-, penta-, and hexanucleotide motifs, respectively. In total, 7983 SSRs were identified from whole genome sequence data, the most common being trinucleotide repeats (87.9%, 7025), followed by hexanucleotides (11.1%, 885), dinucleotides (0.5%, 42), tetranucleotides (0.3%, 22), and pentanucleotides (0.1%, 9). A total of 48 primer pairs were designed by Primer Premier 5 software (PREMIER Biosoft International, Palo Alto, California, USA), using the following parameters: primer length 18–21 bp with the amplified product size set to range from 100 to 700 bp. Of these primer pairs, 21 (18 polymorphic and three monomorphic; Table 1) amplified successfully (GenBank accession no. KY986283–KY986303), and the remaining 27 failed to amplify (Appendix S2). These 18 polymorphic primer pairs were tested for polymorphism using 131 individuals collected from eight distinct geographic locations in Bhutan, China, the Republic of Korea, Japan, and neighboring populations in Finland and Sweden (Appendix 1). Fresh matsutake mushrooms were collected in the field where possible. However, to maximize geographic representation, we also purchased mushrooms from different collectors at surrounding township-level local markets.

TABLE 1. Characteristics of 21 microsatellite primers developed for *Tricholoma matsutake*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
LJW075	F: GGGAATGGAGATGCTGAG R: TTGTTGTGATGAGGGTAGGA	$(CAGGGA)_6$	387–405	55	KY986294
LJW068	F: GTGTCACCGCCGCCAGTAT R: TCGGAGACGCTCGGATGCT	$(TCCCAA)_5$	387–411	57	KY986293
LJW045	F: GGGAATGGAGATGCTGAG R: TTGTTGTGATGAGGGTAGGA	$(TCCAGC)_5$	387–405	63	KY986289
LJW089	F: AGAGCGTCATTGCTTGGG R: CTGTCGGATGCCTCGTAG	$(GGGACT)_6$	186–210	55	KY986298
LJW002	F: AGCCAAACACCAAAGCCCAACA R: CGCCCACAGCCGCATAAA	$(CCTCTC)_5$	215–233	56	KY986283
LJW014	F: CCGTATTCTTCCTTTCGTTG R: CTGCCTTCTTACCGCCAC	$(CAC)_{10}$	162–192	54	KY986285
LJW100	F: CAAGTCCACCTCGTTTCTC R: AATATCCATAACTACGCCTGA	$(CACAAC)_6$	284–338	59	KY986299
LJW018	F: GCAGATTCGCACCAGGAT R: CGCCCACAGCCGCATAAA	$(CCTCTC)_5$	303–327	50	KY986286
LJW036	F: CTTGACGGAAGAAAGAGTATGT R: CGTGAGCCGAGTGGTGAT	$(AGCAGG)_6$	315–339	54	KY986287
LJW052	F: GTGTCACCGCCGCCAGTAT R: TCGGAGACGCTCGGATGCT	$(TCCCAA)_5$	387–411	56	KY986290
LJW053	F: GTGCGGAACCATCTCAGTC R: CGTAGGAGCGTCCATAGTGT	$(CAA)_9$	365–380	54	KY986291
LJW077	F: ACAACACCAATGCCAACC R: CAAGAAATGAGAAACAAAA	$(CACCAA)_5$	328–358	48	KY986295
LJW079	F: GTGTCACCGCCGCCAGTAT R: TCGGAGACGCTCGGATGCT	$(TCCCAA)_5$	387–411	57	KY986296
LJW104	F: CCACCTAACACCCACTCTT R: GACAGCACGGAACCATCT	$(TCTCAC)_5$	291–309	50	KY986300
LJW005	F: TTGGTGAAGGCGGGAAGA R: CATGCCACTCATAGGCAGTA	$(GAGAGG)_6$	142–154	59	KY986284
LJW083	F: TCATCGTTCAACTGTGGCTTCT R: CGTTTGTGGCGGCTATTT	$(ACC)_5(CCT)_5$	201–213	50	KY986297
LJW145	F: CCCCTCCCAACTCAACAT R: CGGCGTAACTGCACTAACAT	$(GGTGTT)_7$	210–252	56	KY986302
LJW154	F: GCTTTGTCTCAGCCTTCAAAG R: AAGACAACCACAAATCCTCCC	$(GCTGGT)_6$	115–175	55	KY986303
LJW39*	F: TCACTTTGGGAGTCTGTC R: GTTTGCTTATTTGTTGGGTA	$(AAAGGG)_5$	394	54	MF318475
LJW76*	F: GAGGATTGCCTGAGTGAT R: AACCTGCTTATGTGGATTTT	$(GAAAGA)_{10}$	413	57	MF318484
LJW41*	F: TCACTTTGGGAGTCTGTC R: GTTTGCTTATTTGTTGGGTA	$(AAAGGG)_5$	227	54	MF318476

Note: T_a = annealing temperature.
[a]Values are based on 131 samples.
*Monomorphic loci.

TABLE 2. Results of initial primer screening for the 18 newly developed polymorphic loci, as well as 10 loci developed by Lian et al. (2003), in eight populations of *Tricholoma matsutake* and cross-species amplification in *T. equestre*.[a]

	T. matsutake																									
	BTN-TP (*n* = 15)			CN-TB (*n* = 14)			CN-SC (*n* = 15)			CN-YN (*n* = 15)			CN-NC (*n* = 15)			KOR-SE (*n* = 14)			JPN-NK (*n* = 13)			FIN-RM (*n* = 15)			*T. equestre* (*n* = 15)		
Locus	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e
LJW75	1	0.00	0.00	1	0.00	0.00	2	0.07	0.06	1	0.00	0.00	3	0.60	0.58**	1	0.00	0.00**	4	0.38	0.65**	0	0.00	0.00**	0	0.00	0.00**
LJW68	2	0.08	0.07	1	0.00	0.00	2	0.20	0.28	2	0.27	0.32	4	0.20	0.70***	3	0.25	0.32*	4	0.67	0.69	2	0.20	0.18	0	0.00	0.00
LJW45	2	0.07	0.06	2	0.10	0.10	1	0.00	0.00	1	0.00	0.00	3	0.33	0.62**	3	0.42	0.34	1	0.00	0.00	1	0.00	0.00	0	0.00	0.00
LJW89	5	0.20	0.30	4	0.38	0.56***	4	0.53	0.50***	5	0.67	0.56	3	0.50	0.54	4	0.50	0.62	3	0.50	0.41	1	0.00	0.00	0	0.00	0.00
LJW2	2	0.00	0.49***	2	0.00	0.17***	2	0.00	0.12***	2	0.00	0.12***	3	0.58	0.47	1	0.00	0.00	3	0.33	0.61	2	0.57	0.49	0	0.00	0.00
LJW14	3	0.09	0.31***	2	0.08	0.20**	2	0.07	0.06	4	0.13	0.29***	3	0.71	0.53	5	0.90	0.69	3	0.00	0.65***	4	0.18	0.38***	0	0.00	0.00***
LJW100	3	0.21	0.31	4	0.30	0.47***	4	0.15	0.21***	4	0.20	0.30**	5	0.29	0.41	4	0.14	0.26**	3	0.67	0.61	2	0.14	0.13	0	0.00	0.00
LJW18	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	4	0.53	0.54	3	0.50	0.46	3	0.46	0.41	2	0.13	0.44***	7	0.54	0.45
LJW36	1	0.00	0.00	2	0.09	0.09	3	0.43	0.54	3	0.27	0.53	3	0.53	0.64	3	0.79	0.63	5	0.38	0.71**	1	0.00	0.00**	5	0.87	0.68
LJW52	1	0.00	0.00	3	0.29	0.25	3	0.40	0.57	4	0.47	0.56	4	0.27	0.66***	3	0.57	0.52	5	0.69	0.76	1	0.00	0.00	6	0.29	0.78
LJW53	2	0.00	0.48***	2	0.07	0.07	2	0.20	0.18	2	0.07	0.28***	4	0.33	0.29	3	0.36	0.30	3	0.40	0.46	1	0.00	0.00	2	0.18	0.46**
LJW77	4	0.31	0.28	1	0.00	0.00	3	0.36	0.39	4	0.33	0.39**	4	0.27	0.34**	3	0.36	0.39	3	0.17	0.52**	1	0.00	0.00**	0	0.00	0.00**
LJW79	3	0.29	0.41	3	0.29	0.64**	4	0.40	0.68***	6	0.53	0.66	6	0.27	0.72**	4	0.43	0.64*	6	0.62	0.75	4	0.20	0.51***	6	0.11	0.72***
LJW104	2	0.60	0.46	1	0.00	0.00	1	0.00	0.00	2	0.13	0.23	3	0.67	0.54	3	0.86	0.64	3	0.31	0.32	1	0.00	0.00	3	0.38	0.32
LJW5	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.08	0.07	1	0.00	0.00	2	0.08	0.41***
LJW83	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.13	0.12	2	0.00	0.12***	1	0.00	0.00***	2	0.00	0.46***	1	0.00	0.00***	10	0.13	0.83***
LJW145	2	0.14	0.13	3	0.29	0.45**	4	0.27	0.34**	3	0.47	0.38	3	0.53	0.42	3	0.43	0.36	3	0.38	0.32	1	0.00	0.00	3	0.00	0.59***
LJW154	3	0.13	0.13	3	0.07	0.14***	5	0.33	0.35***	4	0.13	0.24**	4	0.14	0.20***	3	0.14	0.40**	2	0.00	0.43***	1	0.00	0.00***	0	0.00	0.00***
Trma01	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.27	0.23	2	0.08	0.07	—	—	—	2	0.00	0.12**	2	0.00	0.12**
Trma02	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.87	0.49***	2	1.00	0.50***	2	0.38	0.31	—	—	—	2	0.00	0.24***	0	0.00	0.00
Trma06	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.07	0.36***	2	0.15	0.43**	0	0.00	0.00	—	—	—	0	0.00	0.00	0	0.00	0.00
Trma07	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.07	0.36***	2	0.08	0.45***	0	0.00	0.00	—	—	—	0	0.00	0.00	0	0.00	0.00
Trma08	2	0.00	0.14**	1	0.00	0.00	1	0.00	0.00	3	0.00	0.34***	1	0.00	0.00	0	0.00	0.00	—	—	—	0	0.00	0.00	0	0.00	0.00
Trma10	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.07	0.07	—	—	—	1	0.00	0.00	0	0.00	0.00
Trma12	2	0.79	0.50	2	0.57	0.46	2	0.36	0.38	2	0.33	0.28	2	0.73	0.49	2	0.17	0.15	—	—	—	2	0.13	0.12	1	0.00	0.00
Trma13	1	0.00	0.00	0	0.00	0.00	1	0.00	0.00	2	0.33	0.28	2	0.20	0.28	2	0.25	0.22	—	—	—	2	0.38	0.31	0	0.00	0.00
Trma14	2	0.00	0.12**	1	0.00	0.00	2	0.00	0.13**	3	0.73	0.66	2	0.60	0.46	3	0.36	0.45	—	—	—	2	0.67	0.44	0	0.00	0.00
Trma16	2	0.93	0.50***	2	0.64	0.48	2	0.93	0.50***	2	1.00	0.50***	2	0.93	0.50***	2	1.00	0.50***	—	—	—	2	1.00	0.50***	2	1.00	0.50***

Note: — = monomorphic locus; *A* = number of alleles sampled; H_e = expected heterozygosity within populations; H_o = observed heterozygosity.

[a]Locality and voucher information are provided in Appendix 1.

[b]Significant deviation from Hardy–Weinberg equilibrium (*$P < 0.1$, **$P < 0.05$, ***$P < 0.01$).

Genomic DNA was extracted from silica-dried cap tissue following the cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1987). In addition, individuals of *T. equestre* (*n* = 15, collected from specimens deposited at the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences [KUN]) were chosen to test the cross-species amplification of polymorphic and monomorphic markers in *T. matsutake*. Voucher specimens were deposited at KUN (Appendix 1).

PCR amplification was performed in a 25-μL reaction mixture that consisted of 12.5 μL of 2× *Taq* Master Mix (total 1 mL of solution containing 100 units *Taq* polymerase, 0.5 mM dNTP, 20 mM Tris-HCl [pH 8.3], 3 mM $MgCl_2$; Vazyme Biotech Co., Nanjing, China), 1 μL of each primer, and 1.5 μL of genomic DNA (~50 ng/μL). The 5′ end primers of SSRs (Table 1) were labeled with two different fluorescent dyes (6-FAM and HEX) under the following conditions: 95°C for 3 min; 35 cycles of 95°C for 15 s, the appropriate annealing temperatures (Table 1) for 30 s, and 72°C for 16 s; and a final extension of 72°C for 5 min.

The product was analyzed by capillary electrophoresis on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, California, USA) at Beijing Microread Genetech Co. (Beijing, China) using GeneScan 500 ROX Size Standard (Applied Biosystems). SSR fragment lengths were analyzed by GeneMapper version 3.2 (Applied Biosystems). Aberrant peaks were not scored (Pan et al., 2003). We calculated the number of alleles, as well as observed (H_o) and expected heterozygosities (H_e) for each population. Hardy–Weinberg equilibrium was determined using GenAlEx version 6.4 (Peakall and Smouse, 2012).

Of the 48 primer pairs, 18 (37.5%; Table 1) were polymorphic when screened using 131 individuals from eight populations. The number of alleles ranged from two to 10 and averages ranged from 1.42 to 3.22. Levels of H_o and H_e varied from 0.00 to 1.00 and 0.00 to 0.83, respectively (Table 2), with mean values of 0.21 to 0.26. Eight out of the 18 polymorphic SSR loci showed significant deviations from Hardy–Weinberg equilibrium in different populations ($P < 0.05$) (Table 2). Within *T. equestre*, 50% of the SSR primer pairs were successfully cross-amplified, with levels of H_o and H_e ranging from 0.06–0.93 and 0.32–0.83 (Table 2).

CONCLUSIONS

The 18 newly reported polymorphic SSR markers for *T. matsutake* are reliable and will be used in the further study of the species. The interspecific transferability and polymorphism shown in the related species *T. equestre* suggest that these markers may also be applicable to the study of genetic diversity in other *Tricholoma* species.

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DATA ACCESSIBILITY

Sequence information for the developed primers has been deposited to the National Center for Biotechnology Information (NCBI); GenBank accession numbers are provided in Table 1.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

APPENDIX S1. SSR markers for *Tricholoma matsutake* reported by Lian et al. (2003).

APPENDIX S2. The 27 loci developed for *Tricholoma matsutake* that did not anneal in the samples after PCR product testing.

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APPENDIX 1. Collection information for all *Tricholoma* samples used in this study.

Species	Population code	Collection location[a]	Geographic coordinates	Voucher specimen accession no.	*N*
Tricholoma matsutake (S. Ito & S. Imai) Sing	BTN-TP	Paro dzongkhag, Kingdom of Bhutan	27°33′47″N, 90°53′46″E	X. F. Yang et al. 100087	15
	CN-TB	Nyingchi, Tibet Autonomous Region, People's Republic of China	29°56′38″N, 94°47′56″E	X. F. Yang et al. 100118	14
	CN-SC	Yajiang, Sichuan Province, People's Republic of China	30°01′52″N, 101°00′50″E	X. F. Yang et al. 100192	15
	CN-YN	Deqing, Yunnan Province, People's Republic of China	28°29′10″N, 98°54′40″E	X. F. Yang et al. 100219	15
	CN-NC	Antu, Jilin Province, People's Republic of China	43°06′47″N, 128°53′53″E	X. F. Yang et al. 100453	15
	KOR-SE	Cheongju Province, Republic of Korea	36°49′59″N, 127°45′18″E	X. F. Yang et al. 100479	14
	JPN-NK	Nagano, Japan	43°28′44″N, 129°19′48″E	—	13
	FIN-RM	Kalix Municipality, Norrbotten County, Sweden	65°51′00″N, 23°10′12″E	—	5
		Rovaniemi Municipality, Finland	66°30′00″N, 25°43′59″E	—	5
		Nuuksio National Park, Espoo, Finland	60°18′0.13″N, 24°27′59″E	—	5
Tricholoma equestre (L.) P. Kumm.	CN-YNE	Kunming, Yunnan Province, People's Republic of China	25°9′47″N, 102°43′48″E	Y. Q. Xiao et al. 49796	15

Note: N = number of individuals.
[a]The DNA samples from Japan and Sweden were contributed by Dr. Chunlan Lian and Niclas Bergius, respectively.