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Genetic diversity and population structure of *Armillaria luteo-virens (Physalacriaceae)* in Qinghai-Tibet Plateau revealed by SSR markers

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ABSTRACT

Armillaria luteo-virens is a type of ectomycorrhiza whose fruit body is widely used as an edible mushroom in the Qinghai-Tibetan Plateau. In total, 404 individuals of *A. luteo-virens* from 23 geographically separate populations were analyzed using eight pairs of SSR markers. The average number of alleles among nine populations was 28.65 with a range from 20 to 38. The mean observed (H_O) and expected (H_E) heterozygosity ranged from 0.446 to 0.501 and from 0.499 to 0.554, respectively. An analysis of molecular variance (AMOVA) showed that most genetic variation occurred within populations (83.32%). The mean value of F_{ST} (0.176) suggested low genetic differentiation among populations. The 23 studied populations were divided into two main clusters based on the NJ dendrogram, which was also confirmed by STRUCTURE analysis. This study may aid in not only the protection of this species but also the management of genetic resources for future artificial cultivation.

1. Introduction

The Qinghai-Tibet Plateau is the youngest and largest plateau in the world, and it covers most of the Tibet and Qinghai provinces with an average elevation exceeding 4500 m (Zheng, 1996). It is a global biodiversity hotspot because of the variety of its geographical and topographic features (Zhao et al., 2006). The organisms inhabiting this unique environment face a great challenge because of the harsh geographical and topographic environmental conditions. Whether or not a species can cope with these environmental challenges conditions largely depends on the maintenance of sufficient genetic variation to facilitate adaptation. Previous research studies of the genetic diversity of endangered alpine plants in the Qinghai-Tibet Plateau provided fundamental information for the conservation of these species (Chen et al., 2009; Zhu et al., 2009).

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However, the genetic diversity and population structure of ectomycorrhizal fungi in the Qinghai-Tibet Plateau have not been studied.

The genus *Armillaria*, or honey fungus, is a genus of parasitic fungi that mainly lives on trees and woody shrubs and includes more than 30 species distributed in Europe, North America and East Asia, most of which are economically deleterious pathogens with a broad host range (Guillaumin et al., 1993; Hasegawa et al., 2010; Kendra et al., 2011). *Armillaria luteo-virens* is unique within the *Armillaria* genus given its non-pathogenic nature and distribution in the meadows of the Qinghai-Tibet Plateau (Kuhle, 2001). The fruiting body of *A. luteo-virens* is edible and is well known in traditional Chinese cuisine. Because of its short fruiting season, high content of nutrients, therapeutic activity, lack of artificial cultivation, and the impact of alpine meadow degradation the natural abundance of this species has greatly decreased in recent years (Chen and Diao, 2011). Recently, many studies have evaluated the population patterns and phylogenetic structure of the genus using different molecular markers. *Armillaria mellea* in the eastern and western regions of the United States were investigated based on 10 SSR markers to reveal the patterns of population structure and genetic diversity. An absence of genetic differentiation within eastern subpopulations and western subpopulations suggests that spore dispersal within each region is sufficient to prevent geographic differentiation. In contrast to the western United States, more than one genetic cluster of isolates was found within the eastern United States ($K = 3$) through Bayesian assignment of multilocus genotypes in STRUCTURE (Baumgartner et al., 2010). The AFLPs among 153 *A. mellea* isolates from XX indicate low genetic diversity within the population. An analysis of molecular variation indicated high genetic differentiation, with 70% of the molecular variation explained at the site level within *Armillaria gallica* subpopulations (Brazee et al., 2012). According to Sun et al. (2012), 79 *A. gallica* isolates from China and Europe had experienced obvious genetic divergence, and several isolates had lower intraspecific similarity coefficients than isolates from other *Armillaria* biological species. As *A. luteo-virens* is an edible mushroom, most of investigators are interested in the chemical compounds of the fruiting body and cultivation conditions (Feng et al., 2006; Xu et al., 2010; Fu et al., 2011). However, no information is available about the genetic diversity and population structure of *A. luteo-virens*.

In the current study, eight EST-based SSR markers developed through 454 pyrosequencing (Table 1) were used to determine the genetic diversity and population structure of 23 wild populations of *A. luteo-virens*. The main objective was to (1) estimate the genetic diversity and population structure within and among the populations of *A. luteo-virens*, (2) to measure whether EST-SSRs could be a valuable tool to estimate genetic diversity and population structure of ectomycorrhizal fungi, and (3) obtain information regarding genetic structural data and background for future artificial cultivation.

2. Materials and methods

2.1. Sample collection

In total, individuals from 23 wild populations were collected. Populations were separated from each other by at least 50 km from one another, and the sampling was performed in Qinghai and Sichuan provinces, China during 2011–2013 (Table 2). All individuals from the same population were spaced at least 50 m apart.

2.2. DNA extraction and amplification

Total genomic DNA was extracted from the fruiting body of all of the individuals using the CTAB method (Doyle, 1987). The quality and concentration of the DNA were checked through electrophoresis on 1.0% agarose gels and the samples were compared to standardized lambda DNA size markers. Eight polymorphic EST-SSR primers with high PCR success were used

Table 1
Characterization of eight microsatellite loci used in this study.

Locus	Primer sequence	Fragment size	Repeat motif	T_a (°C)	GeneBank accession no.
AIV25	F:AGGTAGTGATGGGGGTT R:ACAACCTCCACAACCCTCAT	179–191	$(AGG)_6$	57	KC357679.1
AIV58	F:GGAGGTCTGGGATTCTG R:AGATGTGCAAGCTGCACATG	165–186	$(CT)_8$	54	KC357680.1
AIV45	F:CGTGTTAGGGTTTAATT R:TTGGATTCCACCTCTCTGCAT	117–129	$(GAT)_8$	54	KC594361.1
AIV48	F:AACGGATTCTACAAGTG R:TTTTCTTCCCTATCCCCTGTT	129–144	$(GT)_7$	56	KC357682.1
AIV61	F:CAATCCAGCTCAGATCC R:GGGAACCTCTGTTGATGATGA	106–124	$(ACC)_6$	54	KC357686.1
AIV64	F:TGAGGGGTTTGAAAGAA R:CACCACACCCTTTTCCACA	190–202	$(GGT)_6$	54	KC357687.1
AIV92	F:TGAGAACTTGGGCTCTA R:TGTGGTAGCCGCCATTCTT	267–276	$(AGG)_4$	56	KC594360.1
AIV96	F:GGTTTTGGTGATGATGA R:ACAACCTCCACAACCCT	348–372	$(GGT)_8$ $(GGT)_5$ $(AGG)_8$	57	KC357679.1

Note: F = forward; R = reverse; T_a = annealing temperature.

Table 2
Collection information and genetic parameters for *Armillaria luteo–virens* wild populations.

Population	*N*	Longitude/latitude	Altitude(m)	N_A	H_O	H_E	F_{IS}
AR	17	100°27′/38°04′	1881	31	0.475	0.525	−0.038
EB	15	100°52′/37°58′	1743	35	0.488	0.512	−0.047
YN	14	99°35′/38°25′	1755	28	0.479	0.521	0.008
YL	25	98°33′/38°29′	3777	27	0.464	0.536	−0.160
ML	16	100°79′/37°65′	3658	25	0.479	0.521	0.062
RS	20	100°28′/37°28′	3539	38	0.485	0.515	−0.062
YK	15	100°06′/37°33′	3260	38	0.459	0.541	−0.136
ME	24	99°56′/37°33′	3245	24	0.469	0.531	−0.135
HM	19	99°72′/36°74′	3201	26	0.486	0.514	−0.064
DT	14	100°86′/36°32′	3181	28	0.459	0.541	−0.159
XH	23	99°96′/35°55′	3348	33	0.474	0.526	−0.074
SL	20	100°64′/35°62′	3412	24	0.471	0.529	−0.068
TD	28	100°87′/35°40′	3513	33	0.477	0.523	−0.099
DR	4	99°48′/33°38′	4021	20	0.490	0.510	−0.048
HS	13	98°61′/35°05′	4028	30	0.501	0.499	0.073
MD	21	98°27′/34°61′	4356	31	0.495	0.505	0.020
QS	14	97°′33°77′	4121	26	0.499	0.501	0.074
CD	20	97°26′/32°41′	4318	29	0.499	0.501	0.107
NQ	9	96°46′/32°19′	4394	27	0.487	0.513	0.006
SQ	28	96°47′/33°04′	4169	26	0.478	0.522	−0.041
TJ	11	98°01′/38°80′	4012	33	0.497	0.503	0.112
QM	22	95°86′/34°15′	4312	23	0.464	0.536	−0.120
DL	12	98°06′/36°27	3892	24	0.446	0.554	−0.080
Mean			3528	28.65	0.479	0.521	−0.038

Sample size; N_A, number of different alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, fixation index.

for this study. Polymerase chain reactions were performed in a 15-μL volume containing 30 ng of genomic DNA, 200 mM 10×PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, each primer at 200 nM, and 1 U of Taq DNA polymerase (Takara, Dalian, China). The PCR program was set as an initial denaturation for 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at the annealing temperature, and 30 s at 72 °C, with a final extension at 72 °C for 10 min. Amplification products were sized on a QIAxcel Advanced system by the OM700 method.

2.3. Data analysis

Number of alleles per locus (N_A), H_O (observed heterozygosity), H_E (expected heterozygosity), F_{ST} (fixation index), and inbreeding coefficient (F_{IS}) to characterize the genetic diversity and distribution of the variation were calculated using the program POPGENE 4.0 (Yeh and Yang, 1999). All loci were tested for their fit to the Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium in GENEPOP Web Version 4.0.10 (Raymond and Rousset, 1995; Rousset, 2008). Microchecker software was used to identify various genotyping errors and to detect typographic errors (Van Oosterhout et al., 2004). Gene flow (N_m) was estimated using the equation $N_m = 0.25(1 - F_{ST})/F_{ST}$. To estimate variance components and to partition the variance within and between populations, an analysis of molecular variance (AMOVA) was implemented in Arlequin Version 3.5 (Excoffier et al., 2005). For hierarchical AMOVA analysis, populations were grouped into two lineages based on neighbor-joining tree analysis.

Genetic structure was determined by STRCTURE 2.3.4 (Pritchard et al., 2000) using both the admixture mode and non-admixture mode. In total, nine independent runs ($K = 1 - 25$) were performed with 100,000 Markov Chain Monte Carlo (MCMC) repetitions after a burn-in period of 100,000 interactions. The optimal value of K was estimated by calculating the $\triangle K$ value to identify the top level in the hierarchical structure. A correlation between Nei's genetic distance and geographical distance (Nei, 1987) was tested between populations, and a Mantel test and reduced major axis (RMA) evaluation were performed using IBDWS Version 3.23 (Jensen et al., 2005). To determine whether *A. luteo virens* populations have experienced a recent shrinkage of effective population size, bottleneck software was used under IAM, SMM, and TPM mode with a 10,000 simulation iteration (Piry and Cornuet, 1999).

In addition, relationships among the populations were constructed using Nei's D_A distance between all pairs of populations by the neighbor-joining (NJ) method (Saitou and Nei, 1987) in POPULATIONS Version 1.2.28 (http://bioinformatics.org/tryphon/populations/). Finally, the geographical distribution of the 23 populations was drawn on a map by MeteoInfo Version 1.1 (Wang, 2012).

3. Results

3.1. Microsatellite variation and genetic diversity

In total, 659 alleles across the 404 individuals within 23 wild populations were generated based on eight microsatellite loci. The total number of alleles per population ranged from 20 (DR) to 38 (RS, YK) with an average of 28.65 per population.

Table 3
Summary statistics for genetic variation of eight SSR markers in *Armillaria luteo–virens*.

Locus	Repeat motif	Tm (°C)	N_a	N_m	H_O	H_E	F_{IS}	F_{ST}
AIV25	$(AGG)_6$	57	10	1.045	0.451	0.549	−0.224	0.193
AIV45	$(GAT)_8$	54	8	1.511	0.466	0.534	−0.052	0.142
AIV48	$(GCT)_7$	53	10	1.833	0.471	0.529	−0.113	0.120
AIV58	$(CT)_8$	54	5	3.426	0.478	0.522	−0.260	0.068
AIV61	$(ACC)_6$	54	4	0.360	0.481	0.519	−0.056	0.410
AIV64	$(GGT)_6$	54	15	1.265	0.485	0.515	−0.013	0.165
AIV92	$(AGC)_5$	56	6	1.511	0.485	0.515	0.188	0.142
AIV96	$(GGT)_8$ $(GGT)_5$ $(AGG)_8$	54	12	1.238	0.457	0.513	−0.099	0.168
Mean			8.75	1.524	0.472	0.525	−0.079	0.176

Tm, annealing temperature (°C); H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, fixation index; F_{ST}, genetic differentiation.
$N_m = 0.25(1 - F_{st})/F_{st}$.

Observed heterozygosity (H_O) ranged from 0.446 to 0.501 with an average of 0.479, whereas expected heterozygosity (H_E) ranged from 0.499 to 0.554 with an average of 0.521, indicating polymorphism in all of the SSR loci. The coefficient of inbreeding (F_{IS}) ranged from −0.160 to 0.112 (Table 2). In addition, a significant bottleneck was only detected in AR, QM, and RS populations ($p < 0.05$) under the SMM assumptions of the Wilcoxon test (data not shown). The characteristics of eight SSR loci are shown in Table 3; N_m at each locus ranged from 0.360 (AIV61) to 3.426 (AIV58), with an average of 1.524 per locus, whereas H_O ranged from 0.451 (AIV25) to 0.485 (AIV64, AIV92) and H_E ranged from 0.513 (ALV96) to 0.549 (AIV25). We did not observe significant departures from HWE at all loci and in all populations.

3.2. Genetic differentiation among populations

Genetic distances between populations varied from 0.011 (YK, DT) to 0.342 (AR, SQ). The mean value of F_{ST} (0.176) suggested low genetic differentiation among populations. Similarly, AMOVA analysis grouped by lineages showed that 77.23% of total variation was within populations, 14.99% was between groups and only 7.79% was between populations. While for total populations 16.68% of the total molecular variance was attributable to between-population diversity ($p < 0.001$), whereas the rest (83.32%) was associated with differences within populations (Table S1).

Analysis of microsatellite data by the STRUCTURE clustering algorithm suggested that the most probable division was $K = 2$ (Fig. S1), which received the strongest support in terms of log-likelihood values under the data condition on K, Ln Pr ($X|K$) (Pritchard and Wen, 2004). The Mantel test showed a lack of significant relationship between genetic distance (F_{ST}) and geographical distance ($r = 0.4012$ $p = 1.000$) (Fig. S2). The NJ tree (Fig. 1) generated from the SSR data suggested that the 23 populations divided into two main sister clusters, and each cluster was separated into two sub clusters. The first cluster consisted of the populations from DR, AR, YN, EB, YL, RS, TD, YK, DT, ML, XH, MJ, and HM. The second cluster consisted of populations from QS, HS, SL, CD, NQ, SQ TJ, QM, and DL.

4. Discussion

Expressed sequence tag (EST)-based SSR markers have the advantage of being directly associated with the expressed regions and have been widely used to estimate genetic diversity and population structure in recent studies (Yeh, 1997). However, many studies have shown that EST-based SSRs could have lower polymorphism rates than SSRs in noncoding regions (Cho et al., 2000; Eujayl et al., 2002; Gutierrez et al., 2005). In this study, we used eight pairs of SSR markers to examine the population genetics and geographical variations of 23 wild populations in *A. luteo-virens*. Our data revealed that the genetic diversity of *A. luteo-virens* is higher (Table 2, $H_E = 0.521$ at population level) than that of the other ectomycorrhizal fungi species studied based on different molecular data (Pierluigi et al., 1998; Bernard and Ross, 2003; Xu et al., 2008). This result suggests that EST-SSRs can be successfully used for estimation of genetic diversity and population structure.

Gene flow is the transfer of genes or alleles from one population of a species to another. When genes are carried to a population where those genes previously did not exist, gene flow can be a very important source of genetic variation (Futuyma and Douglas, 1998). The most important factor affecting the rate of gene flow between different populations is mobility (Mayr, 1976). Two processes, the growth of dicaryophytic mycelia and the spread of basidiospores, are involved in the construction of ectomycorrhizal fungi populations. Regarding the ectomycorrhizal population, sexual spores may play a more important role than vegetative growth (Liang et al., 2004). It is widely accepted that for most basidiomycetes, a small number of basidiospores is transported by wind or animals over long distances, and most of them fall in the immediate vicinity of the basidiome (Stenlid, 1994). Spore dispersal studies of the forest pathogen *Fomes annosus* also suggested that a large number of spores fall within a few meters of the mushroom (Kallio, 1970). It is likely that the sporpcarps appear in a very limited region around the mushroom. However, many studies support that long-distance dispersal of the basidiomycete is possible (Hirst and Hurst, 1967; Mitakakis and Guest, 2001). *Pisolithus tinctorius* was found to be associated with *Kunzea ericoides* in New

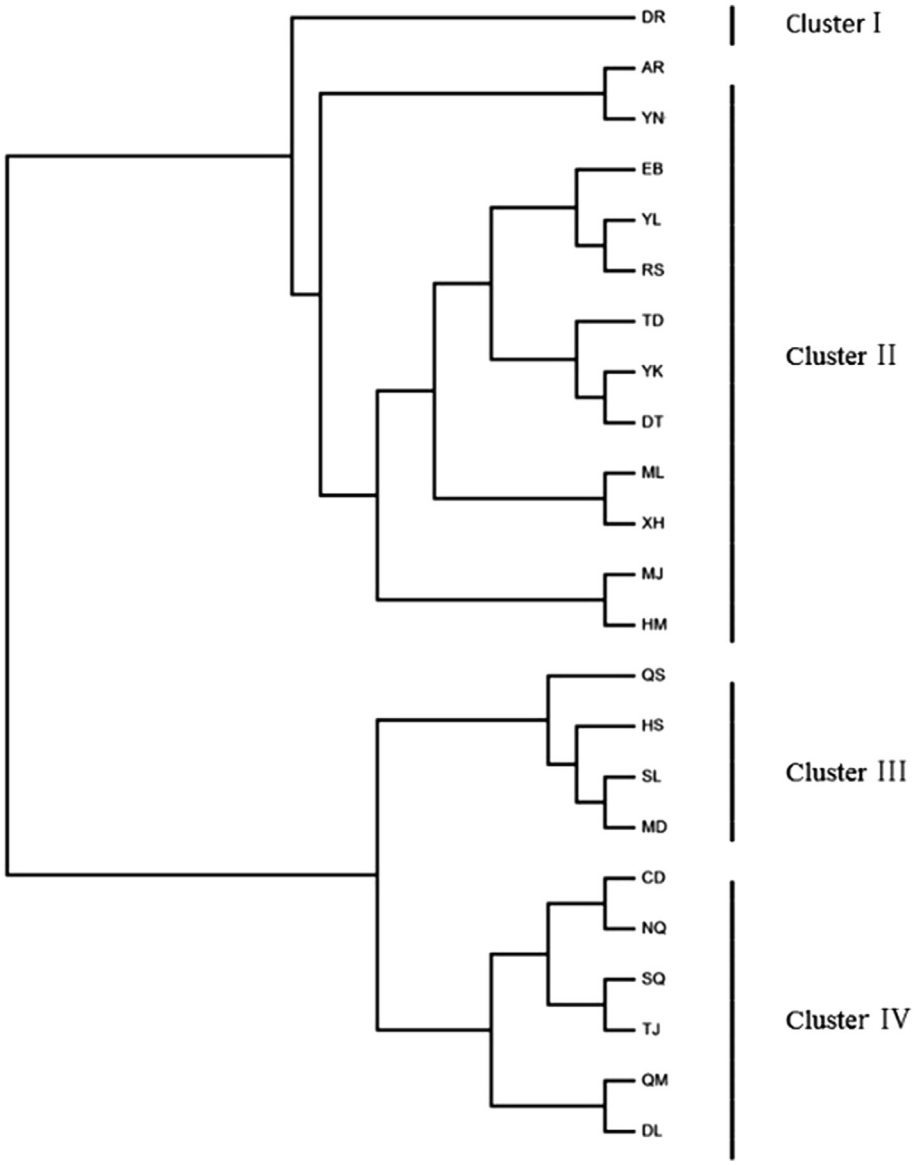

Fig. 1. Neighbor-joining (NJ) tree of genetic relationships among none populations in *Armillaria luteo–virens*.

Zealand as a result of basidiospore movement from Australia via air currents across the Tasman Sea in recent times (Fuhrer, 1985). In the current study, the value of F_{ST} (0.176) for this species suggests a low genetic variance. The AMOVA analysis also showed that only 16.68% of the total molecular variance was between populations, possibly because of a relatively higher gene flow ($N_m = 1.542$) between A. luteo-virens populations (geographic distance between populations of 51.1 km–751.5 km), relative to other ectomycorrhizal fungi (Robert, 1983; James et al., 1999; Urbanelli et al., 2003). This result indicates that basidiospores of *A. luteo-virens* could have been transported for a long distance in the Qinghai-Tibet Plateau by wind to decrease genetic differences among populations.

Genetic diversity plays an important role in evolution by allowing a species to adapt to a new environment. Individuals with more variation will possess variations of alleles that are suited for the changing environment. The population will be propagated because of the success of these individuals (Avise and Hamrick, 1996; Groom et al., 2006; Sahney et al., 2010). Maintaining genetic variation is a major objective for conservation of endangered species. We obtained valuable data on population genetics and structures from this study, and found that the major threat to the *A. luteo-virens* in recent years has been not only the decline of genetic diversity but also the widespread use of this species. To overcome this threat, two main strategies are necessary: first, to educate the inhabitants within the area on how to collect the fruiting body without damaging or exhausting mycelia, and second, to artificially cultivate the species. Ex situ artificial cultivation would be a

method to increase production according to consumer demand. If this species is not protected, it will become extinct as a result of high demand and severe climatic conditions, or it will diverge to an endangered species.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2014.04.006.

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