Genetic Variability of Flamulina velutipes Collections from Armenia

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Armenian collections of *Flammulina velutipes* were examined for DNA sequence variability within the ribosomal ITS1-5.8S-ITS2 region. Of 22 *F. velutipes* collections examined, several were heterozygous for two indels and were cloned to recover complete ribosomal ITS sequences. Genetic diversity was remarkably high in collections from Armenia when compared to collections from other parts of Eurasia. Haplotypes were assayed by phylogenetic analysis using maximum parsimony. At least 16 haplotypes were recovered (collections were defined as the same haplotype if they differed by no more than two base pairs). Most natural collections were heterozygous with respect to haplotype, suggesting an interbreeding, genetically divergent population of fungi. This high level sequence diversity may be a consequence of survival of ancient genetic variation in the Caucasus and in Armenia during periods of glaciation while in Europe, genetic variation was extirpated. A subset of Armenian genetic diversity is found in Europe.

1. Introduction

The genus *Flammulina* comprises several species commonly cultivated for food and for medicinal properties. Until 1970, *Flammulina* was thought to consist of a single species, *Flammulina velutipes,* which had a temperate Northern Hemisphere distribution (Buchanan 1993). *Flammulina* is collected throughout Europe as an edible, often in the winter, thus earning the epithet "the winter mushroom." and is widely cultivated in Asia as enoki-taki.

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The first species to be segregated from *F. velutipes* was *Flammulina ononidis* based on material from Germany growing on *Ononis spinosa* (Arnolds 1977). Bas (1983) proposed *F. fennae* and later (1995) summarized the known taxa of *Flammulina* in Europe as *F. ononidis*, *F. fennae* and *F. velutipes.* Based on morphology, Redhead and Petersen (1999) described *F. populicola*, *F. rossica* and a new combination, *F. elastica*. A New Zealand species, *Flammulina stratosa* was described (Redhead et al. 1998). Finally, *Flammulina mexicana* was assigned to a unique endemic growing at 9000' in the volcanic highlands of Mexico (Redhead et al. 2000). Petersen et al. (1999) examined crosses between and within these North Temperate taxa and identified mating groups as *F. velutipes*, *F. fennae*, *F. ononidis*, *F. populicola*, *F. mexicana*, and *F. rossica / F. elastica*. The latter two taxa are partially inter-compatible and inter-fertile but are separate morphological species. Morphospecies designations were confirmed by comparing DNA sequences for the ribosomal ITS1-5.8S-ITS2 (ITS) region using geographically diverse collections of each morphospecies where possible (Hughes et al. 1999). Following this, authentic *F. velutipes*, based on mating studies and DNA sequence information, has a pan-northern hemisphere distribution but is also found in Argentina, New Zealand and Australia where it is most likely an invasive species (Methven 2000).

The object of our study, *F. velutipes,* is a well-known medicinal mushroom. It contains different groups of active compounds (polysaccharides, protein-glucan complexes, sterols, lectins, phenolic compounds, etc) and substrate-specific enzymes. A large spectrum of pharmacological activity (immune-modulating, antitumor, antioxidant, etc.) and a number of medicinal properties have been attributed *F. velutipes*. Ethanol extracts of *F. velutipes* were reported to suppress hypersensitive immune responses such as inflammation in delayed allergy responses (Sano et al. 2002). Recently, a fruiting body protein from *F. velutipes*, Fve, was shown to possess immune-regulatory activity (Paavolainen et al. 2001; Wang et al. 2004). Polysaccharides (β-glucans) derived from putative *F. velutipes* have been shown in a number of studies to have significant anti-tumour activity (Yoshioka et al. 1973; Ikekawa 1995). Proflamin, a new antitumor glycoprotein was isolated from the culture mycelium of *F. velutipes* and was reported to be effective against the B-16 melanoma and adenocarcinoma (Ikekawa et al. 1982; Ikekawa 1995). Wine produced by fermentation using *F. velutipes* showed thrombosis-preventing activity, giving a prolonged thrombin clotting time

2.2-fold that of the control (Okamura 2001). Finally, antimicrobial compounds such as cuparene-type sesquiterpenes have been isolated from *F. velutipes* (Ishikawa et al. 2000; Ishikawa et al. 2001).

In our study of antibacterial activity of the cultural liquid of *F. velutipes* SBIII-2 and SB99 strains against *Staphyllococcus aureus* (209p) and *Salmonella typhimurium* (ATCC 1474), antiviral activity of fruiting body extract and obtained fractions against encephalomyocardial virus of mice have been observed (unpublished data). Antifungal/antagonistic activity of several mycelial strains against test-micromycetes pathogenic for men/animals (*Acremonium alternatum, Alternaria alternata, Aspergillus candidus, A. wentii, Chrysosporium keratinophilum, Fusarium tricinctum, Geotrichum candidum, Paecilomyces lilacinus, Penicillium aurantiogriseum, P. griseofulvum, Stachybotris chartarum, Verticillium lecani*), plants (*Bipolaris sorokiniana, Fusarium culmorum, Gaeumannomyces graminis* var. *tritici, Rhizoctonia cerealis*), as well as their antagonists (*Gliocladium roseum, Trichoderma viride, T. harzianum* and *T. pseudokoningii)* has been reported as well (Badalyan 2004). Antioxidant activity (AOA) of both mycelial and fruiting body samples of *F. velutipes* was detected (Badalyan 2003). Weak antiprotozoal activity (APA) and miotogenic effect (MGE) of cultural filtrate was also revealed (Badalyan and Sisakyan 2005). No APA was mentioned in mycelial and fruiting body extracts, as well as polysaccharide-protein fraction separated from fruiting body extract of *F. velutipes.* However, they were able to stimulate mitosis of *Paramecia* up to 2.2, 1.8 and 1.4 times, respectively.

The presence of APA, MGE and AOA completes the list of medicinal properties of in *F. velutipes* and makes it suitable for further development of new mushroom-based nutritional supplements with antiprotozoal, antioxidant and wound-healing properties. However, many reports concerning medicinal properties of fungi have not confirmed the species morphological identifications of their experimental materials, particularly mycelial cultures and have not retained voucher specimens. In many cases, incorrect species epithets have been used (unpublished data).

Screening of morphological and growth characteristics, fruiting body formation specificity in 21 different strains of *F. velutipes,* including Armenian collections, have been analyzed (Badalyan and Sakeyan, 2005). Two (A, B) species-specific morphological types of colony correlated with substrate nature and geographical origination of strains were described in *F. velutipes'*

collections. However, significant correlation between their genetic variability and colony morphology have not been revealed, yet (unpublished data).

We previously reported high genetic variability within the ribosomal ITS1-5.8S-ITS2 gene region in collections of *F. velutipes* from Armenia (Badalyan and Hughes 2004) when compared to collections from Europe and Asia. Here, we further explore this genetic variability using DNA sequences of the ribosomal ITS1-5.8S-ITS2 region.

2. Methods and Materials

Collections: Collections of *F. velutipes* used in this study are given in Table 1. Armenian samples were isolated from fruiting bodies collected in different part of country using a tissue culture method. To obtain polyspore cultures, a piece of the pileus from a fruiting body was suspended from the lid of a Petri dish and spores were deposited on malt extract agar (MEA: 15g/L Difco Malt Extract, 20g/L Difco Agar). Alternately, spores were deposited on paper and diluted in sterile water before plating on MEA. Cultures were stored on MEA slants at 10°C.

To obtain material for DNA extraction, dikaryotic cultures were grown in 30 mL PD broth (24 g/L Difco Potato Dextrose Broth) until the mycelial culture was ca. 2 cm in diameter. The culture was filtered through a fine mesh cloth and blotted to remove excess medium.

Approximately 0.3g tissue was ground in 750 uL Carlson lysis buffer (Carlson et al. 1991) and incubated at 75oC for 30 mins. Tissue debris was removed by centrifugation and the supernatant extracted with 750 uL chloroform: iaoamyl alcohol (24:1). The top layer was removed and measured, and an equal volume of isopropanol alcohol added to precipitate DNA. DNA was washed with 200 uL 70% cold ethanol, air dried and suspended in TE buffer. PCR amplification of the ribosomal ITS1-5.8S-ITS2 region was carried out with primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Cycle parameters were as described by White et al. (1990). PCR products were visualized by gel electrophoresis in 1.5% TBE agarose gels. PCR products were sequenced with an automated ABI 3100 DNA sequencer (ABI Prism Dye Terminator cycle sequencing; Applied Biosystems, Foster City, California, USA) with primers ITS5, ITS4, ITS3 and ITS2 de-

pending on the position of insertions and deletions (White et al. 1990). Sequences of each gene were manually corrected and aligned using the SEQLAB program in the Genetics Computer Group package (GCG 2000). Some collections were heterozygous for several indels and were cloned to obtain an accurate sequence. Cloning was accomplished using the pGEM-T vector system) in JM109 high efficiency competent *E. coli* cells (Promega, Madison, Wisconsin, USA following manufacturer's directions. Colonies containing an insert were subcloned, plasmid DNA was extracted and PCR amplification was performed using primers ITS1F and ITS4 as described above. Products were analysed by gel electrophoresis as described above. Plasmids which contained the ITS insert were sequenced as described above.

Data Analysis: Maximum parsimony analysis was performed using Paup* version 10 (Swofford 2001). Gaps were few and were informative. For this reason, gaps were treated as a 5th base. One-hundred bootstrap replicates were performed. Trees were visualized in Tree View (Page, 1998). Because cloning may occasionally introduce an aberrant base, collections differing by 1-2 base pairs were considered to be the same haplotype.

3. Results

In previous studies, we attempted to determine the number of identical haplotypes which existed in Eurasia based on specific variable sites in the ribosomal ITS1 and ITS2 regions. As sequence data accumulated, it became clear that Armenian populations represented too much variability to be conveniently described by a discrete number of haplotypes. For this reason, parsimony analysis was used to group the haplotype sequences derived from individual collections. An unrooted phylogram representing relationships among haplotypes is given in Figure 1. Cloned ITS1-5.8S-ITS2 sequences of *F. velutipes* collections may appear in different clades. Cloned ITS sequences of collection SBI-2, collected near Abovian City are found in Figure 1, clades 4, 5, 6, 7 and 8. Base pair differences between sequences of SBI-2 are given in Figure 2. The SBIII-2 also collected near Abovian City appears in clade 6.

Cloned sequences of SBI'-4, collected near Kharberd appear in clades 9, 10 and 11 (Fig. 1). These clades are monophyletic, appearing on a common

stem. Cloned sequences of SBII-3 appear in divergent clades 3 (clone 12) and 7 (clone 11). Not all of the cloned haplotypes were recovered. Cloned sequences of SB99 appear in the same clade and the second haplotype, present in the dikaryon culture, was not recovered.

Collections from the Netherlands, the Ural Mountains (Russia) and England (the type location for *F. velutipes*) are together in a large clade together with Armenian haplotypes represented by SBII'-1, SBII-2 clone 10 and others but there is considerable genetic variability in the Armenian collections that is not present in the European collections. Clades 5 through 15 differ from European collections. A collection from France appears to be unique. Collections from China are most like SB99 from Armenia however, the Chinese collections represent a long divergent branch (data not shown).

4. Discussion

Ribosomal ITS sequences are maintained in a tandem repeat and are subject to recombination (Schlotterer and Tautz 1994; Hughes and Petersen 2001). Further, the ribosomal repeat is subject to an unknown homogenization process in which all copies of the ribosomal repeat become identical (Hillis, Moritz et al. 1991; Linares, Bowen et al. 1994; Schlotterer and Tautz 1994; Sanget al. 1995; Wendel et al. 1995; Cronn et al. 1996; Franzke and Mummenhoff 1999). The time required for homogenization is unknown but may be relatively quick, within a few generations (Hughes and Petersen 2001). In hybrids representing two different haplotypes, without recombination or homogenization, only two haplotypes should be recovered. Clones of collections from Armenia appear in more than two clades suggesting that; 1) the ribosomal repeat is undergoing recombination, and 2) following recombination, the ribosomal repeat has not completely homogenized. The cloning process will occasionally result in an aberrant base substitution. These can tentatively be identified by comparison to the parent sequence or absence of the "mutation" from all other sequences in a data set.

Cloning artifacts were identified in the data set but the observed natural genetic variability in cloned ITS sequences is in specific ITS1 or ITS2 variable regions and appears in several clones from different collections. In some instances, we were able to get corrected sequences without cloning. We con-

clude that cloning artifacts account for only a very small portion of the recovered sequence variability.

Only a subset of the observed genetic variability in Armenia is present in collections from Western Europe (represented by collections from Netherlands, Eastern Russia and England). One explanation for this genetic diversity is that Armenia was a refugium during periods of glaciation in the Pleistocene and that current genetic diversity in Armenia represents an old and divergent gene pool. Michaux et al. (2004) examined populations of the yellow-necked fieldmouse (*Apodemus flavicollis*) and demonstrated unique haplotypes in Turkey, the Near East and Middle East area. The authors suggested that *Apodemus flavicollis* survived glaciation in this region and was blocked from moving north after glaciation by the Caucasus and the Black Sea. Haynes et al. (2003) studied the common vole (*Microtus arvalis*) and demonstrated a unique genetic lineage in Armenia not present in Western European populations. They also suggested this region as a possible glacial refugium. The possibility that some of the observed genetic divergence in Armenia also may result from post-glacial migration into this region from other regions cannot be excluded. Another potential contributing factor could be rare hybridization between *F. velutipes* and other species of *Flammulina* leading to some level of introgressive hybridization. Studies by Petersen and Hughes have demonstrated that, while rare, interspecific hybridization is possible (Hughes and Petersen 2001).

The genetic variability of ITS sequences for Armenian collections of *F. velutipes* suggests that this is a region in which there is considerable variability, enhanced by interbreeding and recombination. Much of the genetic variability is not seen in western European populations. Regardless of the source of genetic variability, it would appear that Armenian collections offer a pool of unusual genetic variability that may be mined for medicinal purposes or used for strain improvement.

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Table 1
Collections of *Flammulina velutipes*

Culture Designation	*Country*	*Location*
SB F-1	France	Paris
SB R-9	Russia	Near Moscow
CBS 771.81	Netherlands	–
7200	England	London
9887	Russia	Ural Mountains
SB I-2 – Clone 1 – Clone 2 – Clone 3 – Clone 4 – Clone 8 – Clone 9	Armenia	Near Abovian City
SB I'-4 – Clone 31 – Clone 32 – Clone 33 – Clone 37 – Clone 46	Armenia	Near Kharberd
SB II'-1		
SB II-2 – Clone 9 – Clone 10		
SB II-3 – Clone 11 – Clone 12	Armenia	Yerevan
SB II-4 – Clone 29 – Clone 30		
SB III-2	Armenia	Near Abovian City
SB III'-3 – Clone 39 – Clone 40	Armenia	Near Kharberd

Culture Designation	*Country*	*Location*
SB IV'-1 – Clone 1 – Clone 8		
SB V-3 – Clone 41 – Clone 42	Armenia	Yerevan
SB VI-1 – Clone 13 – Clone 17	Armenia	Dilijan National Park
SB IX-1 – Clone 22 – Clone 27		
SB 10	Armenia	Yerevan
SB 20	Armenia	Dilijan National Park
SB 99 – Clone 37 – Clone 42	Armenia	Yerevan