Interspecific Hybrids of *Flammulina*

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Fig. 1. *Flammulina* hybrid fruiting in culture

Introduction

Flammulina (Basidiomycetes; Agaricales, Tricholomataceae) is a popular edible mushroom that has been cultivated for centuries in Japan and marketed worldwide under the name *enoki-take*. Until the 1960s *Flammulina* was thought to consist of a single species with a pan-Northern Hemisphere distribution. As a result the name *Flammulina velutipes* (Curt.: Fr.) Singer (FIG 1) was more or less uniformly applied to all collections in the genus whether commercially or naturally produced (Buchanan 1993). Beginning in the 1960s a series of papers that described new *Flammulina* taxa or reported incompatability between strains cast doubt on the uniform application of the epithet *velutipes*. Based on the hypothesis that additional taxa might be sheltered within the epithet *velutipes*, a project was conceived to examine *Flammulina* from three directions: Morphology, compatability studies, and molecular data. In the ensuing studies approximately 200 collections of *Flammulina* were identified to species based on morphology (Redhead and Petersen 1999), mating studies (Petersen et. al. 1999), and RFLP patterns (Table I; Methven et. al. 2000). These species designations were subsequently confirmed by ITS sequences using geographically diverse collections of each morphospecies (Hughes et. al. 1999).

Petersen et. al. (1999) revealed partial compatability between interspecific crosses of *F. onondidis* and *F. elastica*, *F. onondidis* and *F. populicola*, *F. onondidis* and *F. velutipes*, *F. populicola* and *F. velutipes*, and *F. rossica* and *F. velutipes* (Table II) and maintained several laboratory generated interspecific hybrids in culture. A number of these hybrids have been fruited in culture (Fig. 1) and produced more or less normal basidiomata with viable basidiospores (Petersen and Methven, unpublished data). Hughes and Petersen (2001) recently documented an apparent hybridization event in nature between *F. velutipes* and *F. rossica* that resulted in a homogenized ribosomal repeat that contains elements of both parents. To further document the potential patterns of ITS RFLPs in *Flammulina* hybrids, we surveyed several laboratory generated interspecific hybrids (Petersen et. al. 1999) in this genus. Comparison of the RFLP signatures of hybrids to their parents demonstrated a complicated pattern of ITS evolution; additivity and concerted evolution were observed in the ITS hybrids.

Materials and Methods

Procedures for isolation and maintenance of cultures are given by Hughes et. al. (1999). Interspecific hybrids used in this study (Table II) were generated in the laboratory by Petersen et. al. (1999). DNA extractions and PCR amplifications followed techniques outlined by Hughes et. al. (1999). RFLP digestions adhered to the protocol utilized by Methven et. al. (2000). For restriction digestions ca 200 ng of amplified ITS1-5.8S-ITS2 DNA were digested with 1-10 units of restriction enzyme following manufacturer's directions. Digestion products were electrophoresed on a 1.5% agarose gel in 1X TBE buffer (Sambrook et. al. 1989), stained for 20 minutes in ethidium bromide, and visualized on a UV transilluminator. Gels were documented using the Kodak EDAS system (Eastman Kodak).

Results

The *F. onondidis x F. elastica* f. *longispora* hybrid is
olates exhibited concerted evolution in both the *Hae* III and *Bst* F51 restriction sites (Fig. 3, lane 6) and produced a *Hae* III and *Bst* F51 pattern characteristic of *F. onondidis* (three *Hae* III restriction sites and four bands; no *Bst* F51 site) rather than *F. elastica* f. *longispora* (three *Hae* III restriction site and four bands; one *Bst* F51 restriction site and two bands).

The *F. onondidis x F. populicola* hybrid isolates exhibited concerted evolution in the *Hae* III restriction sites (Fig. 2, lane 12) and produced a *Hae* III pattern characteristic of *F. populicola* (two *Hae* III restriction sites and three bands) rather than *F. onondidis* (three *Hae* III restriction sites and four bands). The *F. onondidis x F. velutipes* var. *velutipes* hybrid isolates demonstrated additivity in the *Hae* III restriction sites (Fig. 2, lanes 6-7) with four bands characteristic of the three *Hae* III restriction sites of *F. onondidis* and two bands characteristic of the single *Hae* III restriction site in *F. velutipes* var. *velutipes*.

Table I. RFLP Signatures - Northern Hemisphere *Flammulina* Taxa

	RFLP Signature	*Hae* III Restriction Sites	Calculated *Hae* III Fragment Sizes	*Bst* F51 Restriction Sites	Calculated *Bst* F51 Fragment Sizes
F. elastica* f. *longispora	**3,1**	**3**	**437, 218, 102, 53 bp**	**1**	**499, 334 bp**
F. fennae	**1,1**	**1**	**579, 234 bp**	**1**	**514, 299 bp**
F. mexicana	**1,0**	**1**	**598, 227 bp**	**0**	
F. onondidis	**3,0**	**3**	**437, 219, 103, 55 bp**	**0**	
F. populicola	**2,0**	**2**	**437-438, 227, 159-161 bp**	**0**	
F. rossica	**2,1**	**2**	**436-437, 246, 155-156 bp**	**1**	**506, 288-290 bp**
F. velutipes var. *lactea*	**1,0**	**1**	**607, 225 bp**	**0**	
F. velutipes var. *lupinicola*	**1,0**	**1**	**607, 225 bp**	**0**	
F. velutipes var. *velutipes*	**1,0**	**1**	**601-605, 222-226 bp**	**0**	

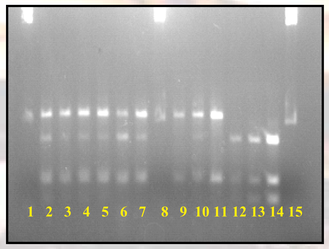

Fig. 2. *Hae* III digestions of *Flammulina* hybrids.
Lanes 1, 8, 15 = λ DNA/*Hind* III marker
Lanes 2-5 = *F. populicola x F. velutipes* var. *velutipes*
Lanes 6-7 = *F. onondidis x F. velutipes* var. *velutipes*
Lane 9 = *F. populicola x F. velutipes* var. *velutipes*
Lane 10 = *F. populicola x F. velutipes* var. *lupinicola*
Lane 11 = *F. rossica x F. velutipes* var. *velutipes*
Lane 12 = *F. onondidis x F. populicola*
Lanes 13-14 = *F. populicola x F. velutipes* var. *velutipes*

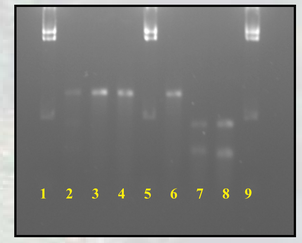

Fig. 3. *Bst* F51 digestions of *Flammulina* hybrids.
Lanes 1, 5, 9 = λ DNA/*Hind* III marker
Lane 2 = *F. rossica x F. velutipes* var. *velutipes*
Lane 3 = *F. onondidis x F. velutipes* var. *velutipes*
Lane 4 = *F. populicola x F. velutipes* var. *velutipes*
Lane 6 = *F. onondidis x F. elastica* f. *longispora*
Lane 7 = *F. rossica x F. velutipes* var. *velutipes*
Lane 8 = *F. fennae*

Table II. RFLP Signatures of Interspecific Hybrids in *Flammulina*

Interspecific hybrid	Number of Isolates	RFLP Signature	Estimated *Hae* III Fragment Sizes	Estimated *Bst* F51 Fragment Sizes	Additivity or Concerted Evolution
F. onondidis x F. elastica* f. *longispora	**2**	**3, 0**	**440, 230, 100, 55 bp**	**814-832 bp**	**Concerted evolution in *Hae* III and Bst F51 for *F. onondidis***
F. onondidis x F. populicola	**3**	**2, 0**	**440, 230, 150 bp**	**814-823 bp**	**Concerted evolution in *Hae* III for *F. populicola***
F. onondidis x F. velutipes* var. *velutipes	**4**	**3+1, 0**	**580, 440, 230, 100, 50 bp**	**814-830 bp**	**Additivity in *Hae* III**
F. populicola x F. velutipes* var. *lupincola	**1**	**1, 0**	**580, 230 bp**	**823-832 bp**	**Concerted evolution in *Hae* III for *F. velutipes***
F. populicola x F. velutipes* var. *lupincola	**5**	**2+1, 0**	**580, 440, 230, 150 bp**	**823-832 bp**	**Additivity in *Hae* III**
F. populicola x F. velutipes* var. *velutipes	**2**	**2, 0**	**440, 230, 150 bp**	**823-830 bp**	**Concerted evolution in *Hae* III for *F. populicola***
F. populicola x F. velutipes* var. *velutipes	**1**	**1, 0**	**580, 230 bp**	**823-830 bp**	**Concerted evolution in *Hae* III for *F. velutipes***
F. populicola x F. velutipes* var. *velutipes	**4**	**2+1, 0**	**580, 440, 230, 150 bp**	**823-830 bp**	**Additivity in *Hae* III**
F. rossica x F. velutipes* var. *velutipes	**1**	**1, 0**	**580, 230 bp**	**823-830 bp**	**Concerted evolution in *Hae* III and *Bst* F51 for *F. velutipes***
	1	**2+1, 1**	**580, 440, 230, 150 bp**	**520, 300 bp**	**Additivity in *Hae* III and *Bst* F51**
	1	**2+1, 0**	**580, 440, 230, 150 bp**	**823-830 bp**	**Additivity in *Hae* III but not *Bst* F51**

Low molecular weight bands are poorly resolved and not photographically enhanced

Results

In the *F. populicola x F. velutipes* var. *lupinicola* hybrid isolates, both additivity and concerted evolution in the *Hae* III restriction sites were documented. One *F. populicola x F. velutipes* var. *lupinicola* hybrid revealed concerted evolution in the *Hae* III restriction sites and produced a *Hae* III pattern characteristic of *F. velutipes* var. *lupinicola* (one*Hae* III restriction site and two bands) instead of *F. populicola* (two *Hae* III restriction sites and three bands). The remaining five *F. populicola x F. velutipes* var. *lupinicola* hybrids revealed additivity in the *Hae* III restriction sites with three bands characteristic of the two *Hae* III restriction sites in *F. populicola* and two bands characteristic of the single *Hae* III restriction site in *F. velutipes* var. *lupinicola* (Fig. 2, lane 10).

In the *F. populicola x F. velutipes* var. *velutipes* hybrid isolates both additivity and concerted evolution in the*Hae* III restriction sites were documented. Two of the *F. populicola x F. velutipes* var. *velutipes* hybrids revealed concerted evolution in the Hae III restriction sites (Fig. 2,lanes 13-14) and produced a *Hae* III pattern characteristic of *F. populicola* (two *Hae* III restriction sites and three bands) rather than *F. velutipes* var. *velutipes* (one *Hae* III restriction site and two bands). A single *F. populicola x F. velutipes* var.*velutipes* hybrid also revealed concerted evolution in the *Hae* III restriction sites (Fig. 2, lane 9) but produced a *Hae* III pattern characteristic of *F. velutipes* var. *velutipes* (one *Hae* III restriction site and two bands) instead of *F. populicola* (two *Hae* III restriction sites and three bands). The remaining four *F. populicola x F. velutipes* var. *velutipes* hybrids revealed additivity in the *Hae* III restriction sites (Fig. 2, lanes 2-5) with three bands characteristic of the two *Hae* III restriction sites in *F. populicola* and two bands characteristic of the single *Hae* III restriction site in *F. velutipes* var. *velutipes*.

In the *F. rossica x F. velutipes* var. *velutipes* hybrid isolates both additivity and concerted evolution were observed. One *F. rossica x F. velutipes* var. *velutipes* hybrid displayed concerted evolution in both the *Hae* III and *Bst* F51 restriction sites (Fig. 2, lane 11; Fig. 3, lane 2) and yielded a *Hae* III and *Bst* F51 pattern characteristic of *F. velutipes* var. *velutipes* (one *Hae* III restriction site and two bands; no *Bst* F51 restriction site) rather than *F. rossica* (two *Hae* III restriction sites and three bands; one *Bst* F51 restriction site and two bands). The second *F. rossica x F. velutipes* var. *velutipes* hybrid showed additivity in the *Hae* III restriction sites with three bands characteristic of *F. rossica* and two bands characteristic of *F. velutipes* var. *velutipes* as well as additivity in the *Bst* F51 restriction sites (Fig. 3, lane 7) with two bands characteristic of *F. rossica*. The third *F. rossica x F. velutipes* var. *velutipes* hybrid exhibited additivity in the *Hae* III restriction sites with three bands characteristic of *F. rossica* and two bands characteristic of *F. velutipes* var. *velutipes*. However, this hybrid lacked additivity in the *Bst* F51 restriction site and did not produce the two bands characteristic of *F. rossica*. We hypothesize that a point mutation has eliminated the *Bst* F51 site in this hybrid although sequencing is required to demonstrate whether or nor a point mutation has occurred.

Discussion

Ribosomal repeats are tandemly repeated and the paralogs are usually identical due to a poorly understood process of concerted evolution (Sanderson and Doyle 1992). Proposed mechanisms of concerted evolution include unequal crossing over (Schlotterer and Tautz 1994) and biased gene conversion (Hillis et. al. 1991). Within-individual diversity for the ribosomal ITS region has been observed and, in some cases, been attributed to hybridization (Sang et. al. 1995). In higher plants, three different outcomes have been reported for the ribosomal repeat following hybridization: 1) Both parental ITS sequences can be retained (Kim and Jansen 1994); 2) The ribosomal repeat may be homogenized to one parental type (Wendel et. al. 1995a); and/or 3) The ribosomal repeat could be homogenized but contained scattered elements of both parents (Wendel et. al. 1995b). This study reports evidence for recombination or gene conversion within the ribosomal repeat followed by homogenization of the repeat in interspecific hybrids of *Flammulina*. Although rare, interspecific hybrids have been reported for Dutch elm disease (Brasier 2001). While hybrids have been suspected in basidiomycetes based on morphology, few have been confirmed by molecular means. This study suggests that rare hybridization events are possible in mushrooms and that when hybridization occurs, recombination and gene conversion in the ribosomal repeat can result

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