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Phylogeography and evolution in matsutake and close allies inferred by analyses of ITS sequences and AFLPs

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Abstract: Matsutake are commercially important ectomycorrhizal basidiomycetes in the genus *Tricholoma.* Despite their importance, the systematics of this species complex have remained elusive and little is known about their origin and biogeography. Using DNA analyses on a worldwide sample of matsutake, we present here the first comprehensive definition of natural groupings in this species complex. We infer patterns of migration and propose Eocene origins for the group in western North America by a transfer from an angiosperm-associated ancestor to an increasingly specialized conifer symbiont. From these origins, matsutake appear to have followed migratory routes parallel to those of coniferous hosts. Patterns of vicariance between eastern North America and eastern Asia are resolved and their origins are suggested to stem from migration through Beringia. Using an analysis of genetic dissimilarity and geographical distance, we reject both the possibility that migration into Europe and Asia occurred through Atlantic bridges and the connection between matsutake populations in the Mahgrebi Mountains and those from Europe. Instead, African and European matsutake appear to be the most recent ends of a westward expansion of the domain of these fungi from North America.

Key words: basidiomycetes, Bering Strait, disjunct distributions, ectomycorrhiza, biogeography, *Pinus, Tricholoma,* vicariance

INTRODUCTION

Systematic principles have provided useful insight into the biogeography of basidiomycetes. The geographical distribution of morpho- physio- or DNA-types has been used to determine diversification areas (Kerrigan 1995, Kerrigan et al 1993), historical movement of germplasm (Methven et al 2000), or large-scale dispersal (Hughes et al 1999). Up to now, methodological restrictions have favored studies focusing mostly on saprotrophic and pathogenic taxa. Biogeographical systematic studies of symbiotic basidiomycetes are scarce, despite the pre-eminent role of the symbiotic way of life in fungi (Wu et al 2000).

Systematic conundrum and economic imperative.—The term ''matsutake'' refers to a loosely defined circumboreal ''species complex'' in the genus *Tricholoma* whose mycelia form an extensive ''white domain'', or ''Shiro'', as they establish a unique ectomycorrhizal relationship with conifer and broadleaf trees (Gill et al 2000, Gill et al 1999, Yamada et al 1999). Ectomycorrhizal members of the genus *Tricholoma* have been distinguished by Moncalvo et al (2002, 2000) from the saprotrophic fungi assigned to the same genus under earlier taxonomic arrangements, placing matsutake in coincidence with Kühner's and Singer's nonclamped forms of the genus (Singer 1986). Matsutake and their morphological allies grade in an apparent continuum (Bon 1984, Pilz and Molina 1997, Redhead 1997) contained in the Singerian system under the subg. *Tricholoma,* especially in sects. *Tricholoma* and *Genuina* (Singer 1986). In recognition of the difficulty of establishing natural groups in this species complex, Pilz and Molina (1997) proposed the informal name ''matsutakes'', to refer to those fungi recognized as valuable by the Japanese market, with other denominations for specific geographical provenances or morphological types (Redhead 1997, Yun et al 1997). It currently is recognized that European specimens of *T. nauseosum* and the Asian *T. matsutake* are to be considered conspecific (Bergius and Dannell 2000). From an evolutionary perspective, these species both represent the so-called true matsutake in reference to the economic value attached to native matsutake in Japan. There is strong support for the conservation of the *T. matsutake* binomial to include both species (Ryman et al 2000, Gams 2002). In this paper we use the term ''matsutake-ally'' to include those species that, although traditionally considered close relatives of the commercially sold taxa, are not accepted by the Japanese market. While Bon (1984) provided a detailed discussion of morphological characters in an attempt to establish species boundaries, Singer (1986), in his defini-

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tive treatise of the order Agaricales, simply characterized the group as ''awaiting future research''. The problem has eluded solution even under the scope of recent DNA-based studies, which have shown that sequence data from the large ribosomal DNA subunit cannot resolve the conundrum (Hwang and Kim 2000).

Despite systematic uncertainties, matsutake have enormous economic importance, not only because of their market value as gourmet mushrooms but also because they epitomize nontimber forest products (NTFP) in coniferous forests (Amaranthus et al 1998, Pilz and Molina 1997, Redhead 1997, Yun et al 1997). It has been argued that the value of nontimber forest products in mixed forests in the Pacific Northwest of the United States outstrips the economic value of the timber in the same forest and matsutake represent an important component of such analyses, providing a forceful incentive for forest conservation.

Matsutake are known from Europe and Northern Africa, from the sub-Himalayan region and the easternmost countries of Asia, and also from the Pacific Rim in North America (Kytövuori 1989; Redhead 1997). Further matsutake locations include the Rocky Mountains, the Great Lakes and the east coast of the United States. Matsutake have not been recorded from Africa outside the Atlas Range, from Australasia or from South America, despite long and intensive search efforts (Dunstan et al 2000). In general, the range of matsutake roughly matches the distribution of coniferous genera such as *Pinus, Pseudotsuga, Tsuga, Picea, Cedrus* and *Abies* (Singer 1986, Yun et al 1997). Nonetheless, several forms of matsutake frequently are found in mixed forests where associations with broadleaf trees cannot be ruled out. *T. magnivelare* in the Pacific Northwest coast of North America often is found in pure stands of *Lithocarpus densiflora* (tanoak) (Arora 1986). *T. bakamatsutake,* a matsutake-ally species, is sympatric with the Japanese *T. matsutake* but is believed to be associated with *Castanopsis, Fagus, Pasania* and *Quercus* spp., despite its occurrence in forests where conifers also are present (Imazeki et al 1988, Lee et al 1999, Pegler 2000, Terashima 1993). A similar case in North America applies to the matsutake-ally *T. caligatum* from the U.S.A. and Mexico, where this fungus is believed to associate with angiosperm hosts, even though it is found in mixed forests, often in sympatry with the commercialized matsutake (Chapela, unpubl data).

Much of the basic biology of matsutake remains obscure although some auto-ecological studies have been undertaken in the Pacific Northwest of the United States (Hosford 1996) and in Japan (Redhead 1997, Terashima et al 1993). In this study, we attempt to define the presence and geographic distribution of phylogenetic groupings within matsutake and species traditionally regarded as their close relatives. Second, we attempt to define phylogeographic patterns for matsutake, including biogeographical information such as the definition of centers of origin and potential routes of species dispersal.

MATERIALS AND METHODS

Specimens.—Materials were obtained in a diversity of forms: fresh from the field, as herbarium specimens, as cultures on artificial media or as DNA sequences (TABLE I). For most field collections, DNA was extracted from specimens in the field. If DNA extraction had to be delayed, 2 mm thick slices were cut with a clean razorblade and placed in a 50% ethanol : water (v:v) mixture for at least 8 h, after which a dehydration series of 75%, 80%, 90% and 100% ethanol was carried out, keeping tissues in each of the increasing ethanol concentrations for at least 8 h. To store longer than 3 mo, tissues in 100% ethanol were drained and lyophylized and maintained at −80 C.

DNA analyses.—DNA from approximately 50–75 mg of mycelium was extracted using a simple CTAB miniprep protocol (Gardes and Bruns 1996). Extracted DNA was resuspended in 100 μL Tris-EDTA buffer (1 M Tris; 0.25M EDTA, pH 8.0). This stock DNA template was diluted in distilled water 1:100 for use in a polymerase chain reaction (PCR). The internal transcribed spacers (ITS) 1 and 2 of the nuclear rDNA were chosen as sequences whose mutations are presumed to be neutrally selected and variable enough for the depth of resolution required to resolve species groupings. PCR reactions were run on a PTC-100 thermocycler and included 1 denaturation cycle at 95 C for 85 s, 35 cycles of denaturation at 94 C for 35 s, annealing for 55 s, and extension at 72 C for 50 s + 5 s/cycle and a final extension cycle at 72 C for 10 min before parking at 9 C. Primers ITS1-f (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) amplified all isolates at 53 C annealing temperature. The PCR reaction cocktail contained these reagents: 1× PCR buffer (1 M Tris; 0.5 M $MgCl_2$; gelatin; KCl), 0.2 mM/dNTP, 0.5 μM of each primer, and 0.025 units/μL of Taq DNA Polymerase (Promega). Both DNA strands of amplicons were sequenced using dideoxy chain termination chemistry on an ABI automatic sequencer at the DNA Sequencing Facility of UC Berkeley. Additional sequences were obtained from the GenBank database (TABLE I). Multiple sequences were aligned using Sequencher 3.0, with additional hand-adjustments as needed. ITS sequences were easily aligned across all taxa studied and 5.8S sequences were not included in the analysis. Short regions of uncertain alignment due to insertions/deletions were excluded from the analysis. Alignments were exported and processed in PAUP 4.0d64 (Swofford 1998). Maximum parsimony (MP), maximum likelihood (ML) and neighbor joining (NJ) trees were constructed using sequences separately and together (Hillis et al 1996). Heuristic searches were performed with these settings: MAXTREES set to auto-increase in the broader da-

TABLE I. Specimens

No.[1]	Name[1]	Origin	Specimen Location[2]		Material Type[3]	Coll./Leg.
1	*Clitocybe lateritia*	n/a	GenBank U66431	U66431	S	n/a
2	*T. argyraceum*	n/a	GenBank AF062612	AF062612	S	n/a
3	*T. bakamatsutake*	Japan	GenBank D87339	D87339	S	n/a
4	*T. bakamatsutake*	Japan	GenBank D86583	D86583	S	n/a
5	*T. bakamatsutake*	Japan	ICh	AF309542	D	T. Nakai
6	*T. caligatum*	Costa Rica	NY Halling 7321	AF309520	H	R. Halling/G. Mueller
7	*T. caligatum*	Oaxaca, Mexico	ICh Capulalpam 15	AF309518	P	J. Toro
8	*T. caligatum*	Oaxaca, Mexico	ICh Xiacuí 25	AF309519	P	J. Toro
9	*T. caligatum*	North Carolina	DUKE HN2633	AF309522	H	K.M. Shanks
10	*T. caligatum*	Tuolomne Co., CA	SFSU MGW 48	AF309533	H	n/a
11	*T. caligatum*	Yuba Co., CA	SFSU HDT 48319	AF309523	H	n/a
12	*T. focale*	Yuba Co., CA	SFSU HDT 48327	AF309534	H	n/a
13	*T. focale*	Calabria, Italy	TO 941118-33DZT	AF309535	H	Levorato
14	*T. fulvocastaneum*	n/a	GenBank D87340	D87340	S	n/a
15	*T. fulvocastaneum*	n/a	GenBank D86585	D86585	S	n/a
16	*T. imbricatum*	n/a	GenBank AF062608	AF062608	S	n/a
17	*T. nictitans*	n/a	GenBank AF062611	AF062611	S	n/a
18	*T. orirubens*	n/a	GenBank AF062620	AF062620	S	n/a
19	*T. portentosum*	n/a	GenBank AF241517	AF241517	S	n/a
20	*T. pseudonictitans*	n/a	GenBank AF062622	AF062622	S	n/a
21	*T. robustum*	n/a	GenBank AF62634	AF062634	S	n/a
22	*T. scalpturatum*	n/a	GenBank AF062623	AF062623	S	n/a
23	*T. terreum*	n/a	GenBank AF062618	AF062618	S	n/a
24	*T. sejunctum*	n/a	GenBank AF062630	AF062630	S	n/a
25	*T. magnivelare*	North Carolina	TENN 22474	AF309516	H	n/a
26	*T. caligatum*	H.-Savoie, France	TO ZT 73-330	AF309521	H	n/a
27	*T. caligatum*	Morocco	GenBank D86572	D86572	S	n/a
30	*T. caligatum*	Morocco	ICh	AF309532	D	T. Nakai
31	*T. magnivelare*	Guerrero, Mexico	ICh	AF309525	F	J. Nikaido
32	*T. magnivelare*	Marin Co., CA	ICh	AF309529	F	T. Horton
33	*T. magnivelare*	Mendocino Co., CA	ICh	AF309528	F	D. Geiser
34	*T. magnivelare*	Mendocino Co., CA	ICh	n.s.*	F	I. Chapela
35	*T. magnivelare*	Estado de México	ICh	AF309527	F	J. Nikaido
36	*T. magnivelare*	Estado de México	ICh		F	J. Nikaido
37	*T. magnivelare*	New England	ICh	AF309539	F	n/a
38	*T. magnivelare*	Tennessee	TENN 22716	AF309524	D	n/a
39	*T. magnivelare*	Oaxaca, Mexico	ICh San Andrés	AF309530	P	J. Toro
40	*T. magnivelare*	Oaxaca, Mexico	ICh Ixtepeji	AF309531	P	J. Toro
41	*T. magnivelare*	Oregon	ICh	AF309540	D	C. Lefebvre
42	*T. magnivelare*	Sierra Nevada, CA	SFSU no number		H	n/a
43	*T. magnivelare*	Washington State	SFSU DE5372	AF309541	H	n/a
44	*T. magnivelare*	Colorado	DUKE HN206	AF309526	H	J. Parkinson
45	*T. matsutake*	Japan	ATCC 64715	AF309536	C	n/a
46	*T. matsutake*	Kitsurin, China	ICh	AF309537	D	T. Nakai
47	*T. matsutake*	S. Korea	ICh		D	T. Nakai
48	*T. matsutake*	Unnan, China	ICh	AF309538	D	E. Yei
49	*T. matsutake*	Unnan, China	ICh		D	T. Nakai
50	*T. nauseosum*	Finland	IB 70-112	AF309517	H	Moser

1: Names as identified prior to this study; code names and sequential numbers from this Table are used in all figures. 2. Represents location of physical material or sequence data: ATCC, American Type Culture Collection; GB, GenBank database; IB, Herbarium Universität Innsbruck, Austria; TENN, Herbarium, University of Tennessee, Knoxville; SFSU, San Francisco State University Herbarium; DUKE, Herbarium, Dept. of Botany, Duke University; NY, Herbarium, New York Botanical Gardens; TO, Erbario, Dipartimento di Biologia Vegetale, Università degli Studi di Torino; ICh, personal collection, Ignacio Chapela, GenBank submission number. 3. Type of material used: C, culture; D, dried basidiocarp; F, fresh basidiocarp; H, herbarium specimen; P, pickled basidiocarp (absolute ethanol); S, sequence data. n/a, not available (in the case of New England, collector requested anonymity).

* n.s.: not submitted to GenBank, but sequence identical to accession AF309528.

taset and set to 30.500 for the 26-taxa dataset, TBR, random taxa addition sequence, MULTREES on, zero length branch collapsed, gaps treated as missing, and steepest descent option not in effect. Maximum parsimony was carried out using a heuristic search of 1000 random addition sequence replicates, saving 100 trees each cycle. One thousand bootstrap replications were performed for all trees. Outgroup analysis was used for the combined final tree, but MP was used on the ingroup only to test for topology changes. The Kishino-Hasegawa model was used for ML analysis (Kishino 1989, Hillis 1996). Third-base-pair changes were not weighted because the sequences used are not translated. Including gaps as characters in the analysis did not change the statements presented here. *Clitocybe* is a genus that appears consistently near the mycorrhizal *Tricholoma* in the phylogenetic arrangement of the Agaricales produced by Moncalvo et al (2000, 2002). We therefore selected the species *Clitocybe lateritia* as an acceptable basal outgroup to polarize the phylogenies.

Amplified fragment length polymorphism (AFLP) analysis was performed following the protocols of Vos et al (1995), modified by Mueller and Milgroom (1996), using primers as described in Gibco BRL catalog No. 10717-015, 10719-011. Genomic DNA was digested with EcoR1 and Mse1 restriction enzymes. Final amplifications to obtain AFLPs were performed using these five primer sets: Mse1 + CTC/EcoR1 + TC (EcoR1; p l0 = GACTGCGTACCAATTC, Mse1 + 0 = CGATGAGTCCTGAGTAAC); Mse1 + CTC/EcoR1 + AC; Mse1 + CTC/EcoR1 + TA; Mse1 + CTC/EcoR1 + AG; and Mse1 + CTC/EcoR1 + GTG. Jaccard's similarity index JI was applied to AFLP data as a proxy for genetic similarity between specimens (Hillis et al 1996). The presence of a common AFLP band for two specimens was scored as 1, while no coincidence in bands was scored as 0, accounting for the noncodominant nature of AFLP markers. From these matrices, Jaccard's similarity index (JI_{AFLP}) was calculated.

Analysis of phylogeographic and evolutionary hypotheses.—Geographical distance between provenances (D_{GEO}) was calculated using the geod program, available at http://www.indo.com/distance/ (June 2000). Distances were calculated following presumed migratory routes (e.g., overland) for matsutake but ignoring changes due to tectonic plate movement. The Mentel's test (Sokal and Rohlf 1995) was used to test the significance of association between genetic dissimilarity of the AFLP data and corresponding geographic distances between single specimens.

The Kishino-Hasegawa constraint test (Kishino and Hasegawa 1989) was used to compare the fit of the best ML tree with trees constructed with specific stated constraints, as detailed in the results section. Trees containing the respective constraints were constructed leaving dichotomies internal to the constrained branching unresolved. Constrained trees were compared to the best ML tree using maximum likelihood parameters.

Molecular clock analysis (Avise 1994, Hillis et al 1996) was used in our 26-taxa analysis to provide approximate dating of major events in the evolutionary history of matsutake. Data were tested for clock-like behavior using the infrastructure of PAUP 4.0d64 (Swofford 1998), with 24 degrees of freedom. Molecular clock behavior could not be rejected ($P = 0.389$) using this method. Graphic and statistical analysis of geographical distance and genetic similarity was performed using JMP IN 3.2.1 (SAS Institute).

RESULTS

A total of 50 sequences were used in this study, of which 34 were produced by the authors and 16 were obtained from GenBank. Two separate phylogenetic analyses were performed using 40 and 26 sequences, respectively (FIGS.1, 2). In addition, AFLP profiles were obtained for 18 samples of matsutake with worldwide distribution

Molecular systematic groupings.—Our DNA-based phylogenetic analysis defines several natural groups within matsutake and their close relatives. Twelve equally parsimonious trees were produced from our MP analysis of sequence data from a 546 bp region of the ITS sequences for 40 samples, including matsutake and their allies. All significant aspects discussed here were retained in a consensus tree obtained from these equally parsimonious trees (FIG. 1), as well as from trees produced using NJ and ML methods. A summary of statistics for the analyses is presented in TABLE II.

The matsutake.—Matsutake recognized by the Japanese market are shown consistently as a monophyletic entity (MP bootstrap value of 82%). Fungi with close morphological and chemical resemblance to matsutake, such as *T. caligatum* from Mesoamerica, California or the eastern United States, clearly were distinct in our analysis from the commercial matsutake. Although these "false matsutake" often are suggested as valuable and even consumed locally (Gong et al 1999), they are rejected by the market (Jorge Nikaido, pers comm 1998). Such is the case of *T. bakamatsutake* in Japan (Japanese "fool's matsutake") and the darker *T. caligatum* from the Americas, which apparently represents at least two distinct groupings (FIG. 1). "False matsutake", such as *T. bakamatsutake* and the North American *T. caligatum,* share the ecological character of being associated with broadleaf trees, rather than with the conifers, as normally observed for the matsutake (Yun et al 1997).

Clearly distinguishable lineages in the matsutake suggest these groupings: Western North America (WNA), Mesoamerica (MX), and Circumboreal (CB) (Asia-Europe, eastern North America). While ITS and AFLP analyses are congruent for all isolates, placement of one eastern North American (ENA)

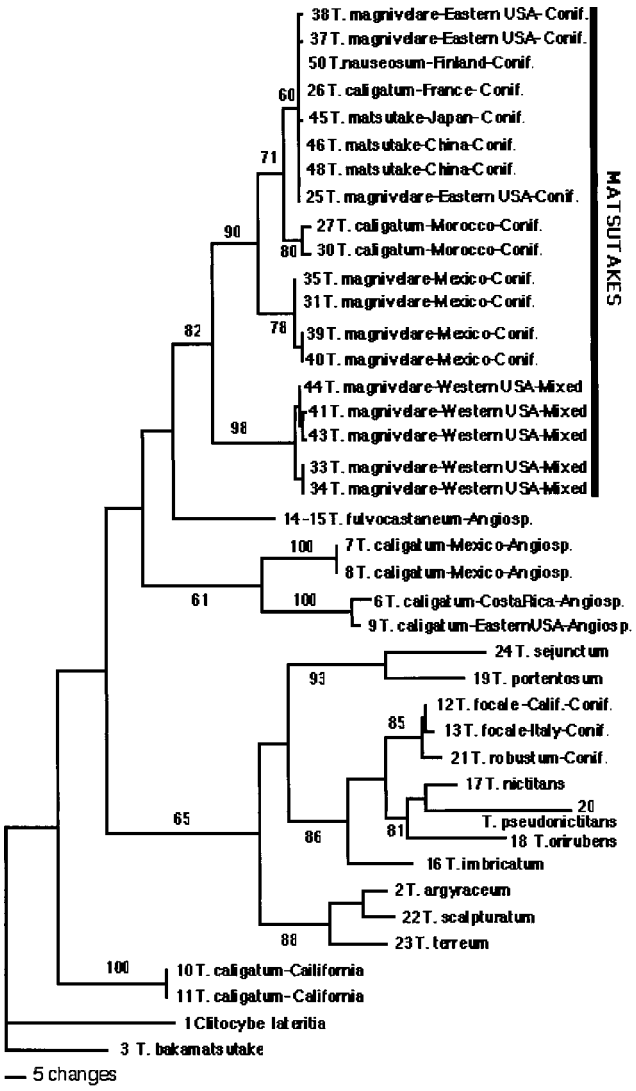

FIG. 1. Phylogram representing one of 12 most parsimonious trees obtained from maximum parsimony analysis of ITS sequences from matsutake specimens, related *Tricholoma* species and *Clitocybe lateritia* as outgroup. Specimens are identified according to sequential number and code-name from TABLE I, followed by the current nomenclature, provenance and host association when known. Bootstrap values above 50% are provided near appropriate branches. For further resolution within the commercial matsutake see FIG. 2.

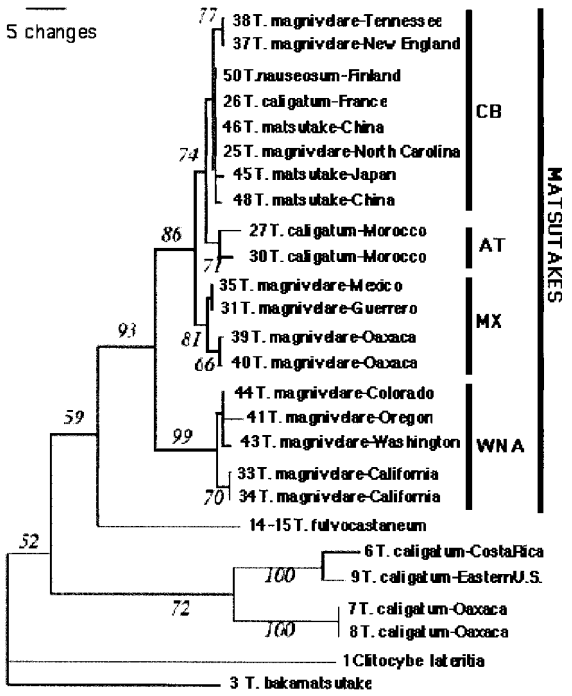

FIG. 2. Phylogram representing one of 30 500 most parsimonious trees obtained from maximum parsimony analysis of ITS sequences from matsutake specimens and *Clitocybe lateritia* as outgroup. Specimens are identified according to sequential number and code-name from TABLE I. Branch lengths are proportional to the number of changes connecting each node. Bootstrap values above 50% are provided in italics.

isolate was incongruent between the two, as discussed below.

Finer resolution was obtained by considering a larger sequence dataset of 638 bp in the ITS region for a subsample of 26 isolates (FIG. 2). This coverage was not possible for the 40 samples included in FIG. 1 because of incomplete alignments for the larger dataset. In this finer analysis, a substructuring of the CB group reveals a separation between the European/Asian/eastern North American specimens and those obtained from the Atlas Mountains (AT). In the MX group, a separation becomes evident between more northerly specimens from central Mexico and those from the southern end of the known

TABLE II. Summary of phylogenetic analysis statistics

DNA analysis	Total/ Informative characters	Number of taxa	Most parsimonious trees	CI/RI/RC
ITS sequence	546/164	40	6	0.740/0.813/0.601
ITS sequence	638/111	26	30500	0.854/0.866/0.740
AFLP (6 primer combinations)	239/n/a	18	n/a	n/a

n/a: not applicable.

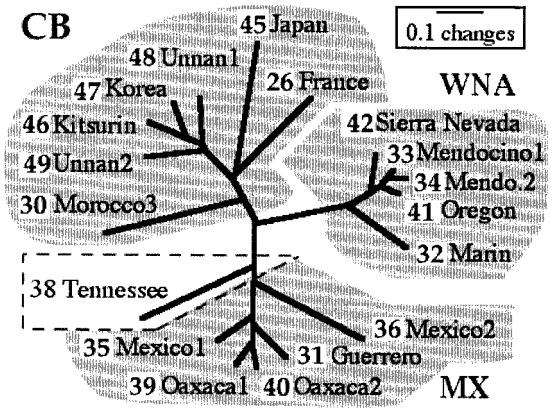

FIG. 3. Unrooted network illustrates the groupings and relationships of 18 matsutake of various provenances, as revealed by analysis of amplified fragment length polymorphism (AFLP) data. Data from five different primer combinations are included for a total of 239 characters. Branch length in the network is proportional to phenetic distance between terminals. Groupings, relationships and distances were derived from a neighbor-joining analysis. Shaded areas represent various groups discussed in text (labeled in boldface).

matsutake range in Oaxaca. In western North America (WNA), California specimens clearly become distinguishable from those obtained from Oregon, Washington or Colorado. *T. caligatum* from Mesoamerica, a *Quercus* symbiont, continues to appear outside the matsutake group, and an association becomes evident between Costa Rican and eastern United States specimens of this taxon, which appear distinct from those in southern Mexico. These results were supported strongly by both MP and NJ analyses.

Amplified fragment length polymorphisms (AFLP) results on the matsutake (FIG. 3) support all groupings proposed on the basis of rDNA sequence data, except for the placement of one specimen from the eastern USA (specimen 38, Tennessee; TABLE I). The multilocus AFLP analysis places the latter specimen in consistent association with the MX group (FIG. 3), while ITS sequence data assigns this same specimen to the CB provenance (FIG. 2). A Kishino-Hasegawa test applied to ITS sequence data could not reject the placement of the Tennessee specimen within the MX clade, further supporting their affiliation, although any placement of the Tennessee specimen that was not strictly basal within this clade was rejected ($P > 0.05$) by this test.

Sister groups.—Potential sister groups of the matsutake are (FIG. 1): (i) *T. caligatum* of the western United States, often found associated in mixed forests containing *Lithocarpus densiflora*/*Quercus* spp.; (ii) the *Quercus*-associated *T. caligatum* of Mesoamerica and the eastern United States, each a strongly supported subgroup; (iii) the Fagaceae-associates *T. fulvocastaneum* and *T. bakamatsutake,* for which little resolution can be obtained; or (iv) a very diverse monophyletic group containing specimens such as *T. portentosum, T. sejunctum, T. argyraceum, T. scalpturatum, T. terreum, T. focale, T. robustum, T. nictitans, T. pseudonictitans, T. orirubens* and *T. imbricatum.* It might be possible to suggest these subgroups within the latter group (iv): *T. sejunctum/T. portentosum*; *T. terreum/T. argyraceum/T. scalpturatum,* with *T. argyraceum/ T. scalpturatum* forming a subgroup; *T. robustum/T. focale* (here our limited sampling still allows a suggestion of support for the *focale* epithet, inasmuch as Italian and Californian specimens strongly group together); *T. orirubens/T. nictitans/T. pseudonictitans,* with a strong suggestion that *T. nictitans* and *T. pseudonictitans* might represent a unique taxonomic unit or two extremely closely related ones. It should be noted that precise taxonomic positioning within this large sister clade cannot be inferred by our analysis, which is based mostly on GenBank sequences, without the possibility of direct confirmation of the identity of each isolate. The establishment of a definite sister group of matsutake and further detail on the structure of this complex monophyletic group awaits more extensive taxon sampling and morphological confirmation.

Phylogenetic arrangement.—Rooting of the phylograms with *C. lateritia* produced important implications. Coniferous associates appear to have derived at least twice from angiosperm associates (FIGS. 1, 2). *T. fulvocastaneum,* or a closely related taxon not included in this analysis, emerge as the closest relative to a common matsutake ancestor, and the WNA group as ancestral to the rest of the true matsutake

Geographical distance and genetic similarity.—Genetic similarity between specimen pairs (JI_{AFLP}) and their corresponding geographical distance (D_{GEO}) showed the expected trend of increased dissimilarity corresponding to increasing geographic distance (FIG. 4a, b). This relationship was highly significant ($P < 0.001$) when using the Mentel test. However, when plotted for all data together it was evident that comparisons between some pairs of specimens within relatively short geographic distances were inordinately dissimilar. Closer inspection revealed that the association was not monotonic across all specimen pairs but showed a clearly biphasic pattern. When data points were divided with the aid of our phylogenetic analysis into within-group and across-group pairs, according to the three major groups defined above (CB, MX, WNA), it became clear that across-group pairs overwhelmingly fall in a D_{GEO}-insensitive dataset, while within-group comparisons showed a strong

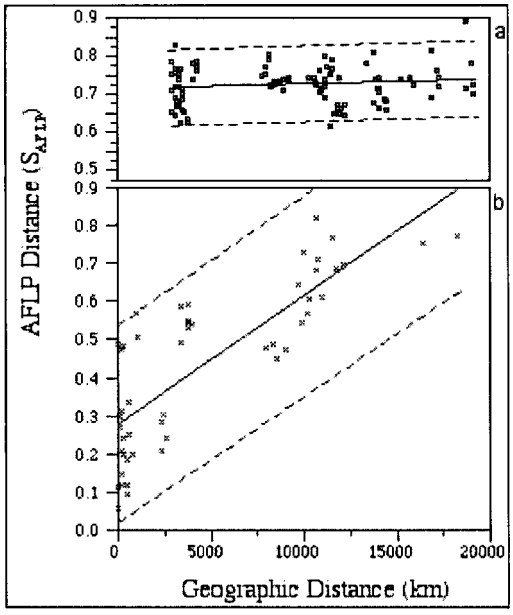

FIG. 4. Comparison between geographic distance (D_{GEO}) and multilocus genetic dissimilarity (JI_{AFLP}) among specimens of matsutake included in FIG.3. See text for description of methods to calculate distances. The Beringian and Near East routes were the ones used for this dataset (see text), and the eastern North American sample was regarded as belonging to the Mesoamerican group (see text). Upper panel (a) contains ''across-group'' comparisons, lower panel (b) within-group comparisons, with groups defined as in FIG. 3. Solid lines represent regressions fitted with data from each panel (see text). Dotted lines represent 95% confidence limits for regressions.

dependence of JI_{AFLP} on D_{GEO} (FIG. 4a). In other words, increasing genetic difference following increasing geographical distances could be shown only for within-group pairs, while across-group pairs appear to be ''saturated'' in their genetic divergence at $JI_{AFLP} \approx 0.7$. The distribution of within-group pairs fits a linear equation:

Equation 1: $JI_{AFLP} = 4 \times 10^{-5} D_{GEO} + 0.27545$ ($r^2 = 0.625$, $P < 0.001$, n = 48),

denoting a strong correlation between geographical and genetic distances, while the equivalent equation for across-group pairings is:

Equation 2: $JI_{AFLP} = 3.1 \times 10^{-6} D_{GEO} + 0.69348$ ($r^2 = 0.063$, $P < 0.009$, n = 105),

where genetic distance is relatively constant, irrespective of the geographical distance between the specimens considered in each comparison (low r^2 value).

DISCUSSION

Phylogenetic inferences.—The coarsest resolution in our analysis discriminates among matsutake and their allied taxa in almost exactly the same way that the Japanse market has done for at least 100 yr. This congruence is striking in light of the reported morphological and organoleptic similarity of taxa such as *T. bakamatsutake,* which often is confused with the sympatric *T. matsutake* of Japan and China (Gong et al 1999, Imazeki et al 1988) and *T. caligatum* from Mesoamerica and the eastern United States, which shares morphological traits and boasts the highly prized scent of *T. matsutake* (I.H. Chapela and Jorge Nikaido unpubl). Beyond this supraspecific agreement with established practice, our results show that much confusion has arisen throughout history on the species systematics of this group.

Our results show that previous assumptions of a taxonomic organization based on continental provenance (e.g., *T. magnivelare* including all specimens from North America, and a split between the northern European *T. nauseosum,* the southern European *T. caligatum* and the Asian *T. matsutake*) is incorrect. The western North American group is distinguished from the eastern North American and the Mesoamerican groups on the basis of our analysis, which also is supported by recent studies using RAPD markers (Murata et al 1999). Finally, the Mesoamerican and the eastern North American populations also appear to be distinguishable based on ITS sequence analysis, although AFLP analyses might suggest a continuum between populations of these two regions. Intensive sampling is necessary to fully resolve the taxonomic relationship between populations from these two regions. By comparison, the CB group, including Asian, European, African and some eastern North American individuals, show relatively little genetic variation, as measured by either sequence or AFLP analyses. The great similarity between European and eastern Asian populations also is suggested by DNA fingerprinting of Swedish specimens (Bergius and Danell 2000). Our data suggest that, with the potential exclusion of the Moroccan (AT) specimens, true matsutake may include the entire CB group from Europe, Asia and the eastern U.S.A.

A similarly novel picture emerges when other matsutake-related fungi are considered in our analysis. Of particular interest is the designation *T. caligatum.* The binomial *T. caligatum* (Viv.) Ricken originally was coined for the European specimens that are indistinguishable from the *T. nauseosum* included here (FIG. 1) and often have been classified as conspecific with *T. matsutake* (Kytövuori 1989, Bon 1984). We found that specimens of these fungi ranging from Japan to Europe and including our two Moroccan specimens are hardly differentiated on the basis of either rDNA or AFLP markers. The continuity in the matsutake across Eurasia also is supported by recent

studies showing the molecular congruence of Korean and Japanese matsutake based on RAPD analysis (Lee et al 1999) and the similarity between Swedish and Japanese matsutake shown by rDNA sequence analysis (Bergius and Danell 2000). One or both of these hypotheses may explain the data: (i) *T. matsutake* recently spread rapidly across this vast if also relatively continuous range; and (ii) there are continued and significant recombination events across metapopulations in this range. Although a postglacial radiation has to be invoked, a recent and anthropogenically mediated colonization of this vast region by these fungi seems unlikely because we have detected significant rDNA sequence and AFLP variation within the CB group. More careful studies of populations (Pannell and Charlesworth 2000) are necessary to differentiate between a recent evolutionary colonization and an anthropogenic introduction.

T. caligatum specimens from North America conversely were not placed in the same clade as matsutake but in at least two separate clades, suggesting that the name *caligatum* is being used to describe a polyphyletic group.

Our evidence points consistently to the existence of three main groups of matsutake: western North America (WNA), Meoamerica (MX) and Asia-Europe-North Africa-eastern North America (CB), with further substructuring within each group. These groups do not coincide with currently used nomenclature.

Species-like status and geographical relationships among matsutake groups.—We are aware of a growing debate over the validity of various species concepts, as well as the concept of species altogether (Benton 2000, Brasier 1997, Hull 1997, Withgott 2000). Because we focused here on a species-complex our data and analysis can help clarify some confusion surrounding this debate.

Although our sample size is too limited to perform population analyses, the crucial question for us is to determine whether there is demonstrable genetic continuity or exchange between and within each of the groups defined in our phylogenetic analysis. We used our observations on the correlation between geographical distance (D_{GEO}) and multilocus dissimilarity measurements (JI_{AFLP}) to address this question.

The existence of a monotonic correlation (FIG. 4; Equation 1) between geographic and multilocus similarity within the groups outlined in our phylogenetic analysis strongly suggests that these groups indeed do form relatively isolated taxa with intrinsic genetic continuity among their populations. These groups thus can be recognized as species-like. It cannot be established on the basis of our data whether the apparent genetic continuity over large geographical ranges is a reflection of current genetic exchange within a metapopulation (Pannell and Charlesworth 2000) or simply a result of common ancestry and/or previous sympatric evolution of populations within each group. Resolving this quandary would be essential for a biologically sound conservation and management strategy for matsutake (Etienne and Heesterbeek 2000, Hibbett and Donoghue 1996, Hibbett et al 1998). The generalized relationships within and across species-like groups defined above also allow for a statistical treatment of these evolutionary hypotheses:

Hypothesis 1: Beringian migration from North America. Data points corresponding to comparisons between eastern U.S. specimens and those from Morocco and France fell outside the 95% confidence limits set on the S_{AFLP} versus D_{GEO} regressions (FIG. 4b) when D_{GEO} was calculated through the shortest route over the Atlantic. This lack of conformity to the rest of the data was corrected when D_{GEO} was calculated following a route along the Bering Strait. This observation supports the idea that matsutake migrated from their North American diversification areas through Beringian, rather than Atlantic land bridges. A Beringian migration is compatible with a more recent colonization of the CB region from North America, which in turn is consistent with the lack of large diversification in the CB group shown by our sequence data and those of others (Bergius and Danell 2000, Lee, et al 1999, Terashima and Nakai 1996).

Hypothesis 2: Isolation across the Strait of Gibraltar. Our data suggest that the connection between European and Atlas Mountains specimens traces back to a common ancestor in the Anatolian region rather than to a direct exchange of genetic material across the Mediterranean (e.g., through the Strait of Gibraltar). This finding is counterintuitive, given the proximity of Moroccan and European populations. Further supporting this hypothesis, we note that the matsutake of the Atlas region (Morocco) are associated with *Cedrus atlantica*, which also has evolutionary connections back to the Anatolian and Himalayan foothills (Abourouh and Najim 1995, Farjon 1990, Nezzar-Hocine et al 1998). A Turkish specimen (kindly provided by Dr M. Intini, Italy) recently was sequenced and placed by ITS analysis between the North African and the European specimens (data not shown), further supporting this hypothesis.

Hypothesis 3: Eastern United States as an evolutionary bridge between Mesoamerican and Circumboreal groups. Data points in our D_{GEO} vs JI_{AFLP} regression corresponding to comparisons between one eastern North American (ENA) and Mesoamerican (MX)

specimens fall outside the 95% confidence limits for Equation 2 if they are considered across-group, as would be suggested by sequence data. By contrast, these same data points fall well within the 95% confidence limit for within-group (Equation 1, FIG. 4b) comparisons, suggesting that it is most parsimonious to include at least some eastern North American individuals as part of a possible group that is evolutionarily continuous with the MX matsutake. Consistent with this interpretation, data points corresponding to comparisons between the same eastern United States specimens and those from the WNA group fall well within the 95% confidence limit for the across-group trend (FIG. 4a, Equation 2). Further supporting the affiliation of some ENA specimens with MX, the Kishino-Hasegawa test using ITS sequence data cannot reject their basal placement within the MX group ($P > 0.5$). Our interpretation of these observations is that ENA populations represent an intermediate between the Circumboreal and the Mesoamerican groups, either because of their ancestral relation to the MX group or because they might be located at an hybridization zone between the two groups.

Origins and migrations of matsutake.—Results indicate that the matsutake clade derived from angiosperm-associated ancestors similar to the extant American *T. caligatum* and *T. fulvocastaneum.* Consistent with this hypothesis, *T. magnivelare,* which forms conifer and angiosperm symbioses in western North America, always maps as basal to matsutake elsewhere in the world, which have an apparent obligate relationship with coniferous hosts. Careful comparative host-specificity, host-selectivity and host-preference experiments should be necessary to confirm this suggestion, given that, with the exception of tanoak-associated California specimens, it is practically impossible to find matsutake that are in true geographical isolation from either angiospermous or coniferous hosts in nature. Although our analysis of the sister group was based mostly on GenBank assessions (FIG. 1), again the basal taxa include angiosperm associates such as *T. nictitans, T. pseudonictitans, T. orirubens* and *T. imbricatum* while the coniferous-associates *T. focale* and *T. robustum* (Singer 1986, Bon 1984, Breitenbach and Kränzlin 1984, Viviani 1834) consistently are placed as derived.

From these observations we suggest that angiosperm associations predate conifer mycorrhization in the evolutionary history of matsutake. The host shift could have taken place in the WNA group, given its basal status with respect to the CB and MX groups (FIGS. 1, 2), its relatively high genetic diversity (FIGS. 2, 3), its dual angiosperm/conifer associations and its clear geographic isolation from other matsutake. This evolutionary scenario is compatible with the receding front of coniferous forests into Eocene refugia (Axelrod 1986, Millar 1993, Millar and Woolfenden 1999, Richardson 1998), resulting in repeated and abundant opportunities for the angiosperm-based ancestor to adapt to the new coniferous habitat. Mixed forests containing matsutake would have receded to the coniferous refugia before drier, colder and more seasonal climatic patterns settled in, inducing the new radiation of conifers throughout mid latitudes in the Tertiary (Millar et al 1988). One of the recognized conifer refugia in the Eocene was in western North America, where the WNA group is now found (Axelrod 1986, Millar 1993). From this initial period of adaptation to the coniferous symbiotic state, matsutake could initiate a migratory trajectory following conifers such as *Pinus, Tsuga* and *Cedrus.* It is significant that in a detailed study of *Suillus,* Kretzer et al (1996) found that association with *Pinus* was a strong organizing principle in this ectomycorrhizal genus, potentially providing another example of comigration similar to that of matsutake.

Following this hypothetical scenario, we can suggest a date for the shift from an angiosperm-associated mycorrhizal ancestor of matsutake to the earliest conifer associates within the true matsutake. In our more resolved phylogenetic tree with 26 individuals (FIG. 2), this would date the node separating *T. fulvocastaneum* and the true matsutake at about 55 MaBP (at the beginning of the Eocene). Taking this date as a basis for a molecular clock analysis, the following putative evolutionary history for the matsutake emerges. First, the separation of *T. magnivelare* from other matsutake is dated at 28 ± 2 MaBP, coincident with the rain-shadow effect of the newly formed Rocky Mountains. Within the WNA region, a sequence can be observed placing the Rocky Mountain specimens (Colorado) as basal to the Oregon and Washington state specimens and the group containing all these three provenances being ancestral to the specimens from California (Mendocino County). The separation of the derived California group from the more northerly and easterly *T. magnivelare* is dated at 5–6 ± 0.4 MaBP, coincident with the rise of the Sierra Nevada and the consequent desertification of the Great Basin as a major geographical barrier.

The derivation of the MX group is further dated at 10–12 ±1.5 MaBP. This time widely is believed to have generated conditions for the isolation and differentiation of pine and other coniferous forests in present-day Mexico, deriving from both a southern refuge as well as from migration from northern forests over the newly formed mountain ridges (Farjon 1996, Perry 1991, Richardson 1998).

Whether the MX group migrated at all through

the eastern seaboard of North America (a Caribbean migration hypothesis) (Lamb 1997) rather than through connections between the Rocky Mountains and the Sierra Madre Occidental (a Sierran migration hypothesis) remains to be assessed. It seems most likely that the latter, more recent route might have been taken, as has been discussed for pine forests (Farjon 1996, Martinez 1963, Perry 1991). The hypothesis of a Sierran migration route for the Mexican matsutake is further supported by the consistent placement of central Mexico specimens as basal to those from southern Mexico (FIG. 2), suggesting a geographical directionality in the migration from north to south, which should be reversed under the Caribbean hypothesis. Fine-scale differentiation of the southernmost populations (Oaxaca) from the central Mexico specimens is dated according to our molecular clock estimates at 6.5–8 MaBP, a period of high orogenic and volcanic activity in this region. A similarly recent separation between the CB specimens and those in the Atlas Mountains of Morocco is dated using the same molecular clock calculation at between 5.3 and 6.4 ±1.9 MaBP. This date matches the significant climatic changes leading to the desertification of lowlands on the Mediterranean coast of north Africa.

It is possible that this long history of retreat, adaptation and radiation will continue as the biological complex formed by matsutake and coniferous forests continues its southward expansion. To date, no human-mediated reproduction of matsutake has been reported and it seems likely that the profitable market for matsutake, the flagship for nontimber forest products, will continue to depend on the judicious management of the naturally occurring communities that have maintained them since their presumed start in the Eocene. Understanding the ecological, evolutionary and phylogeographical details of the matsutake will be essential for the long-term maintenance of these valuable resources.

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