

BIOSYSTEMATIC STUDIES ON *PHLEBIA ACERINA*, *P. RUF*A, AND *P. RADIATA* IN NORTH AMERICA

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ABSTRACT

Three closely related species, *Phlebia rufa*, *P. acerina*, and *P. radiata*, were analyzed with molecular, morphological, genetic, and physiological methods. Emphasis was placed on the phenotypically similar taxa *P. rufa* and *P. acerina*. *Phlebia acerina* is common and widely distributed in North America, but *P. rufa* occurs only in Washington, Oregon, and British Columbia. Although often considered conspecific, *P. acerina* and *P. rufa* are intersterile. In addition, differences in basidioma and cultural traits support their separation. At the molecular level, these species differ slightly in sequence of an internal transcribed spacer of the nuclear ribosomal DNA repeat and a part of the large mitochondrial rRNA gene. Restriction maps of the small mitochondrial rRNA gene and flanking regions provide further evidence that the species are distinct but closely related. This report includes basidioma and cultural descriptions of both species. *Phlebia radiata* is genetically distinct but shares many morphological and molecular traits with *P. acerina* and *P. rufa*. Cladistic analyses of molecular data showed that the three taxa are closely related relative to the outgroup taxa *P. ludoviciana* and *P. subochracea*.

Key Words: Corticiaceae, internal transcribed spacer, mitochondrial rRNA gene, mating tests

Phlebia Fries, a genus of wood decay fungi, includes approximately 70 species that are distributed worldwide. Typically small and inconspicuous, these fungi are quite common in the temperate zone, although not well known. Keys and descriptions by Jülich and Stalpers (1980) and Eriksson et al. (1981) have facilitated the identification of *Phlebia* species; however, many taxonomic problems remain. Like many genera in the Corticiaceae, *Phlebia* is poorly defined and delimited (Donk, 1957; Eriksson et al., 1981). The simple form and structure of *Phlebia* and other corticioid fungi frequently result in species complexes involving genetically distinct but phenotypically similar taxa (Boidin, 1977; Hallenberg, 1987). Although compatibility tests (Boidin, 1986) and careful morphological studies of

basidiomata and cultures can resolve many species complexes, these methodologies are often inadequate. Cladistic analysis of morphological features is often used to address taxonomic problems in fungi, plant, and animal systems. However, corticioid fungi have very few distinguishing morphological characters, and these characters frequently exhibit convergence. In species complexes where morphology and compatibility tests are insufficient or unreliable, molecular techniques can be extremely helpful. These techniques have been used successfully to study the phylogenetic relationships and taxonomy of many different organisms, including basidiomycetes (see Bruns et al., 1991, for review).

In this report, we examine three closely related taxa: *P. radiata* Fr., *P. rufa* (Pers.:Fr.) M. P. Chris., and *P. acerina* Peck. Because of the extensive variation in the basidioma and hymenial configuration, these species are sometimes difficult to distinguish. *Phlebia radiata* and *P. rufa* are well-known taxa that differ in basidiospore shape and size (Ginns, 1976; Eriksson et al., 1981).

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They are distributed worldwide and are reported throughout North America (Cooke, 1956; Ginns, 1976). The name *P. acerina* represents a taxon that is phenotypically similar to *P. rufa* and in the past, they were considered conspecific. Ginns (1976) first showed that the concept of *P. rufa* was too broad when he paired monosporous cultures from France and the United States. The cultures from France paired readily with those from Washington but not with those from New York. Hallenberg (1984) reported a similar phenomenon in which cultures from Europe were compatible with those from British Columbia but not from Ontario.

We demonstrate that the three taxa are distinct based on morphological characters of the basidiomata and cultures, compatibility tests, temperature growth studies, and DNA sequence and restriction site maps. Restriction maps of the small mitochondrial rRNA gene and flanking regions and sequences from one of the internal transcribed spacer regions of the ribosomal DNA repeat and large mitochondrial rRNA gene are analyzed using cladistic methods to infer phylogenetic relationships. Finally, we evaluate the utility of molecular techniques in the study of closely related taxa.

MATERIALS AND METHODS

Fungal strains.—TABLE I lists dikaryotic fungal strains of polysporous origins used in the temperature growth and molecular studies. Collection data for strains of *P. acerina* and *P. rufa* are recorded in the section Description of Taxa. All cultures are on deposit at the Center for Forest Mycology Research (CFMR) at the USDA Forest Service, Forest Products Laboratory.

Morphological studies.—The methods for studying basidiomata and cultures are described in Nakasone (1990b), except that basidiospores were measured in 2% (w/v) KOH and 1% (w/v) phloxine. Species codes are based on the work of Nobles (1965) as modified by Nakasone (1990a). Color notations are from Kernerer and Wanscher (1978).

Temperature growth studies.—Seven strains each of *P. acerina* and *P. rufa* and six strains of *P. radiata* were grown on 1.5% malt extract (Difco) agar (MEA), pH adjusted to 4.5 with 1 M hydrochloric acid, for 1 wk at 25 C. These starter cultures were used to inoculate 90-mm-diam MEA plates at the side. Three replicates of each strain were grown at 16, 20, 24, 26, 28, 30, 32, and 36 C. Colony radius was measured after 8 da.

Genetic studies.—Sexuality was determined by pairing presumptive monosporous cultures of a basidioma in all combinations on MEA. After 4 wk, the cultures were examined for clamp connections. To test for in-

TABLE I
POLYSPOROUS STRAINS USED IN TEMPERATURE
GROWTH AND MOLECULAR STUDIES

Species and collection no.	Location	Study ^a
<i>Phlebia acerina</i>		
GB 553	Quebec	L S M T
GB 568	Quebec	T
JLL 10595	Oregon	S T
HHB 10607	Virginia	T
HHB 11096	Arizona	T
HHB 11146	Wisconsin	M T
JLL 15744	New York	T
<i>Phlebia rufa</i>		
Kropp 4	Oregon	L S T
GB 1199	Norway	S M T
GB 1790	Spain	M T
JLL 8213	Washington	M T
FP 133432	Oregon	M T
FP 133684	Oregon	T
Aho 74-86-B	Oregon	T
<i>Phlebia radiata</i>		
GB 1792	Spain	S M T
GB 1940	Denmark	M T
JLL 13127	Costa Rica	T
JLL 15608	New York	L S M
FP 70836	Maryland	M T
FP 101840	Wisconsin	M T
FP 133493	Oregon	M T
<i>Phlebia ludoviciana</i>		
HHB 9640	Florida	M
FP 101738	Wisconsin	L S M
<i>Phlebia subochracea</i>		
HHB 8494	Arizona	S

^a L and S denote strains in which a segment of the large mitochondrial rRNA gene and ITS-II region, respectively, were sequenced. M indicates strains in which the small mitochondrial rRNA gene region was mapped. T denotes strains used in the temperature growth study.

traspecific and interspecific compatibility, monokaryons of a strain, representing each mating type, were paired in all combinations with monokaryons of another strain. The pairings were examined after 3 wk of incubation at 25 C for the presence of clamp connections which indicates compatibility.

Fungal growth and DNA extraction.—Starter cultures were grown on 2% MEA for 1 wk in a 25 C incubator. From these cultures, nine agar plugs were used to inoculate flasks containing 50 ml of 2.5% (w,v) malt extract broth. The flasks were capped with small beakers, sealed with Parafilm, and incubated for 3-4 da at 25 C without shaking. Fungal cultures were placed in a stainless steel semimicrocontainer and macerated at high speed for 1 min with a Waring Blender. This slurry was used to inoculate 500 ml of enriched broth [10 g glucose, 3 g malt extract, 1 g yeast extract, 1 g

L-asparagine, and 2 drops antifoam-A (Sigma A-5633)² per liter; Tim Flynn, pers. comm.]. Filtered air was bubbled through the broth to keep macerated mycelium suspended. After 4- to 7-da incubation at room temperature, mycelia were collected with a büchner funnel and lyophilized. Mycelial samples were kept in tightly capped vials at -20°C. Total DNA was extracted from 1-2g of mycelia using the procedure of Specht et al. (1982) with some modifications. Toluene and sodium perchlorate were not added to the extraction buffer, and α -amylase and RNase enzyme treatments were eliminated. Instead, the samples were deproteinized with an equal volume of phenol (pH 8) and chloroform : isoamyl alcohol (CIA) (24: 1, v/v), then extracted once more with CIA alone. The DNA samples were banded in a cesium chloride gradient to separate a polysaccharide component.

Restriction mapping. — Following single and double digestions with restriction endonucleases, DNA fragments were separated in 0.8%, 1%, or 1.2% horizontal agarose gels in TBE (45 mM Tris, 45 mM boric acid, 5.5 mM EDTA; pH 8). The fragments were denatured, neutralized, then blotted onto nylon membrane filters. Filters were probed with heterologous mitochondrial ribosomal RNA (rRNA) gene clones. The clones were from *Suillus* spp. and contained a 0.6-kb *Hind* III fragment that included the 3'-end of the 16.5-like small rRNA gene and a 0.9-kb *Sst* I/*Hind* III fragment from the 5'-end of the small rRNA which may include some Ranking region (clones 11 and 10, respectively; Bruns and Palmer, 1989). Clones were labeled by nick translation and hybridized to filters for at least 20 h at 65°C. The filters were washed twice for 15 min at 65°C [$2 \times$ saline sodium citrate (SSC), 0.2% sodium lauryl sulfate (SDS), $1 \times$ Denhardt's], washed twice for 30 sec at 25°C and twice again for 30 min at 38°C ($2 \times$ SSC, 0.1% SDS), and wrapped in clear plastic wrap. Filters were placed on Kodak X-OMAT film for 3-7 da at -70°C for visualization of probe-specific DNA fragments.

Restriction sites were mapped for 2-6 strains of each species. After aligning the maps, each restriction site was scored as present (1), absent (0), or unknown (?). The occurrence of unknown character states is due to the use of small gene clones. Only one restriction site can be detected beyond each end of the clone specific region. Restriction sites past these sites will only be visualized if the closer restriction site is lost (Williams et al., 1988). The data were analyzed using Wagner parsimony and character-state weighting in PAUP 3.0s (Swofford, 1991). In Wagner parsimony, site gains and site losses are given equal weight (Farris, 1970). BRANCH AND BOUND and EXHAUSTIVE searches were done along with MULPARS, COLLAPSE, ACCTRAN, and DELTRAN options. Character-state weighting approach was used as described by Albert et al. (1992). Site gains were weighted against site losses by factors of 1.1, 1.2, 1.5, and 2.0 to 1 with the USER-

TYPE STEPMATRIX option. This weighting scheme favors the occurrence of convergent site losses and gain/loss while discriminating against convergent gains and loss/gains. *Phlebia ludoviciana* (Burt) Nakas. & Burds. was used as the outgroup taxon to root the trees (Watrout and Wheeler, 1981). Bootstrap resampling technique was used to estimate support for monophyletic clades (Felsenstein, 1985). While recognizing the limitations of the bootstrap method (Felsenstein, 1985; Sanderson, 1989; Swofford and Olsen, 1990), we believe that bootstrap values are valuable in indicating probable monophyletic groups. Decay analysis (Bremer, 1988) was performed to gauge the strength of monophyletic clades.

Amplification and sequence of DNA segments. —Primer pairs ML7-ML8 and ITS3-ITS4 were used to generate single-stranded DNA of a portion of the large mitochondrial rRNA gene and the internal transcribed spacer region (ITS-11) in the nuclear rDNA repeat between the 5.8S rRNA and large rRNA subunit genes, respectively (White et al., 1990). In the first amplification step, instructions provided by the manufacturer (Perkin-Elmer Cetus, Norwalk, Connecticut) were followed except that 25- μ l reactions and 5 ng (1 ng/ μ l solution) of total DNA were used. Samples were subjected to 30 cycles (denature for 30 sec at 93°C, anneal for 2 min at 53°C, extend for 2 min at 72°C) in a Perkin-Elmer Cetus DNA Thermocycler. In the last cycle, the extension time was increased to 10 min. After amplification, 5-10 μ l of the DNA product was run through a 1.5% low melting agarose (Sigma A 4018) minigel in TBE for 1 h at 100 V. After staining in dilute ethidium bromide, a band about 350 base pairs (bp) was excised, placed in 500, 700, or 900 μ l of sterile, distilled water (depending on the intensity of the band), and dissolved at 65°C for 10 min. In the subsequent asymmetric amplification, 10 μ l of this solution and primer ratios of 1:50 and 50:1 pmoles (ML7:ML8 or ITS3:ITS4) were used in 50- μ l reaction samples. Samples were subjected to the described cycling parameters to produce the complementary strands. To determine the quality and quantity of the single-stranded DNA product, 5 μ l were run on a 1.5% agarose minigel in TBE. After removal of mineral oil with 50 μ l of CIA, excess primers and nucleotides were removed with Centricon-30 microconcentrators (Amicon, Danvers, Massachusetts) or by precipitation with 2 M ammonium acetate. The DNA was dissolved in a final volume of 7 or 15 μ l, depending on the yield, then sequenced using [α -³⁵S] dATP and a commercial kit (Sequenase, United States Biochemicals, Cleveland, Ohio); manufacturer's instructions were followed except that the labeling reactions were incubated on ice. The DNA was separated in acrylamide gels (6% acrylamide: 8 M urea) in TBE and sodium acetate (Sheen and Seed, 1988). Gels were run from 3-6h at 1700 V, fixed in 12% acetic acid and 10% ethanol, transferred to chromatography paper, dried under vacuum, and then exposed to Kodak X-OMAT film at room temperature.

Sequences were aligned manually with Genetics Computer Group Sequence Analysis Software Program, version 7 (Devereaux et al., 1983). Gaps were introduced to increase the similarity among the taxa. The aligned sequences were entered into PAUP gaps

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were treated as missing characters. Wagner and character-weighted (2: I and 3: I transversions to transition ratios) parsimony analyses were conducted under ACCTRAN and DELTRAN optimizations. Support for monophyletic groups was estimated by the bootstrap method and decay analysis.

DESCRIPTION OF TAXA

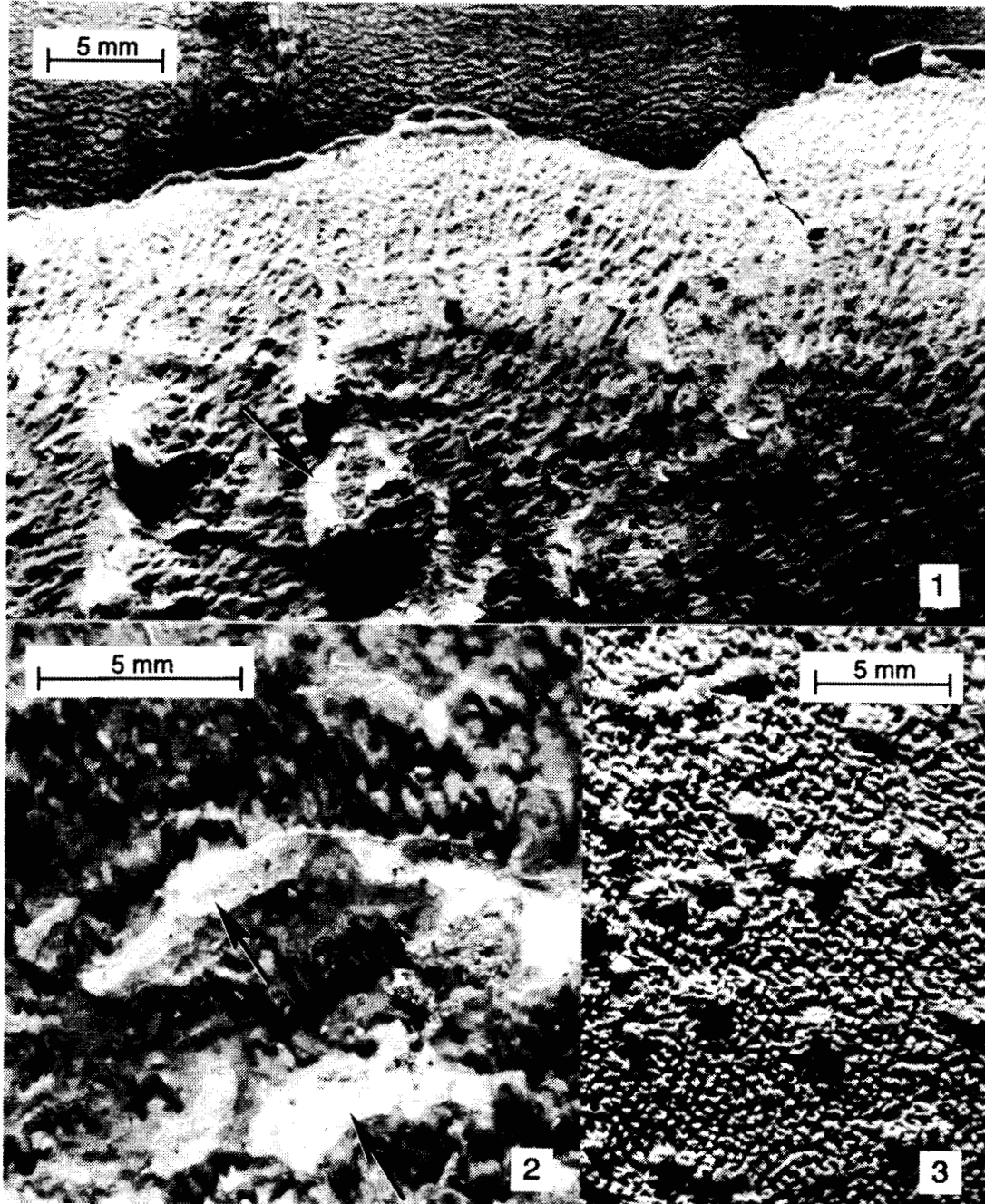
Phlebia acerina Peck. New York State Bot. Rep. **42**: 27, 1889. FIGS. 1-4

- = *Merulius pruni* Peck, New York State Bot. Rep. **105**: 25, 1906.
- = *Phlebia merulioides* G. C. Lloyd, Mycological Notes **39**: 537. Mycol. Writ. **4**: 1915.
- = *Merulius pilosus* Burt in Zeller, Mycologia **14**: 184, 1922.
- = *Merulius gelatinosus* Petch, Ceylon J. Sci., Sect. A. Bot. **9**: 315, 1925.
- = *Merulius interruptus* Bres., Mycologia **17**: 72, 1925.
- = *Merulius nothofagi* G. H. Cunn., New Zealand Dept. Sci. Industr. Res., Pl. Dis. Bull. **83**: 7, 1950.
- = *Phlebia vassilkovii* Parm., Bot. Mater. Otd. Sporo. Rast. Bot. Inst. Komarova Akad. Nauk S.S.S.R. **15**: 130, 1962.
- ≡ *Phlebia radiata* Fr. var. *vassilkovii* (Parm.) Parm., Izv. Akad. Nauk **Ēstonsk. S.S.R.**, Ser. Biol. **16**: 393, 1967.

Basidiomata annual, effuse, often starting as obicular areas that become confluent, up to 100 × 70 mm and 1.5 mm thick, waxy, unchanged or staining light brown (7D4-7D7) in 2% KOH; hymenia continuous but dried specimens frequently interrupted by areas of white, felty to woolly subicular mycelia (Figs. 1, 2), typically central area well-developed and composed of raised folds that develop into dissepiments or teeth, teeth may remain single or become fused at the base. folds often anastomosing to form irregular reticulations or pits, one to three pits per mm, folds and teeth often translucent, light orange (6A5) to greyish orange (6B5), brownish orange or reddish orange (6C7-6C8), light brown (6D5), brown (7E7-7E8), or dark brown (8F4-8F5), but fertile areas between folds often paler, light orange (5A5), greyish orange (5B5-5B6), or orange (5A6) to brownish orange (6C6-6C7), then toward margins folds becoming more appressed, slightly broader, and forming shallow pits, irregular reticulations, and warts; in other specimens, hymenia mostly smooth with warts and scattered dissepiments, greyish orange to brownish orange (6B6-6C6) throughout: *margins* smooth or with warts and shallow pits or reticulations, concolorous but often paler than central area. light yellow (4D4), light orange (5A4, 6A4), greyish or-

ange (5B6, 6B4-6B6), brownish orange (6C6), or white. loosely attached or detached from substrate, sometimes curling away from substrate. entire, occasionally with white and fibrillose edges; *context* bicolored. consisting of white to pale cream-colored, thicker lower layer and thinner upper layer that is concolorous with the hymenium.

Hyphal system monomitic. *Subiculum* 300-1600 μm thick, differentiated into three parts. Lower subiculum next to substrate. which may not be present, a loose *textura intricata*, 70-100 μm thick, hyphae 4.5-5.5(-7.5) μm diam. hyaline, even, nodose septate. moderately branched, sometimes branching from clamp connections, walls thin to slightly thick. Middle subiculum a dense *textura porrecta* at base and changing gradually to a dense *textura intricata*. 180-1000 μm thick, hyphae 4.5-7(-9) μm diam, hyaline, even or irregular because of numerous knobs, nodose septate with scattered adventitious septa, occasionally branched, often forming H-connections, walls slightly thick to thick, up to 2.5 μm thick, frequently coated with resinous materials; hyphae agglutinated and arranged more or less parallel to substrate, with small crystal clusters scattered throughout. Upper subiculum a loose *textura intricata*, 150-350 μm thick, composed of brownish yellow, resinous materials, subicular cystidia (sometimes absent), and subicular hyphae, these hyphae 2.5-3.5 μm diam. hyaline, nodose septate, moderately branched, also forming H-connections, walls thin to slightly thick, coated with resinous materials. In some specimens, subicular-subhymenial interface distinguished by a dense layer of pale yellow, resinous materials. *Subhymenium* thickening, 25-80 μm thick, consisting of a few cystidia and densely packed, vertical hyphae, these 2-3.5 μm diam, hyaline. nodose septate (often forming medallion clamps), walls thin. sometimes coated with resinous materials. Hymenium 25-30 μm thick, tightly packed with basidia and a few hymenial cystidia. easily separated from subiculum but difficult to isolate individual elements. *Cystidia* of two types: 1) subicular cystidia large, clavate, (40-)50-120(-250) × 9-16 μm, 5-6 μm diam at base, with a basal clampconnection, occasionally with adventitious septa. with thin walls, arising from subiculum, abundant in upper subiculum especially near margins, occasionally penetrating the subhymenium and lower hymenium, apparently lacking or disintegrated in old or well-de-



FIGS. 1-3 Hymenial configuration and margins of *Phlebia* species. 1. *P. acerina*, FP 8 1 178. 2. *P. acerina*, FP 101055. 3. *P. rufa*, GB 1790. Arrows indicate white, felty patches characteristic of *P. acerina*.

veloped parts of specimen; 2) hymenial cystidia smaller, clavate to spathulate, $20-45(-60) \times 7-10 \mu\text{m}$, $2-3.5 \mu\text{m}$ diam at base, with a basal clamp, occasionally septate to form an empty,

apical cell, arising from hymenium, abundant or rare in hymenium, walls thin, smooth but occasionally covered with yellow, resinous materials. *Basidia* narrowly clavate. (19-)25-30(-38)

× (3.5–)4.5–7 μm, hyaline, with a basal clamp connection, walls thin, four-sterigmate. *Basidiospores* cylindrical to ellipsoid. (4.3–)4.7–5.2(–5.8) × 2.0–2.2(–2.5) μm, hyaline, at base of adaxial side slightly concave, smooth, negative in Melzer's reagent.

HABITAT. on bark and wood of angiosperms, especially *Acer*, rarely on gymnosperms.

DISTRIBUTION. Austria. Yugoslavia, Iran, Tanzania. Sri Lanka, Pakistan, Taiwan, China, USSR, Australia. New Zealand, Argentina, Canada, USA (FIG. 5).

REPRESENTATIVE SPECIMENS EXAMINED. ARGENTINA. Tierra del Fuego, Lago Escondido, 60 km NE of Ushuaia, on *Nothofagus* sp., 21 Feb. 1982. *L. Ryvardeen 19374* (O). AUSTRALIA. VICTORIA: Sedwich near Bendigo, on bark, 19 Jun. 19—E. J. Semmens 32787 (BPI). AUSTRIA. Steiermark, Jogerberg, on hardwood, 2 Oct. 1983, *R. L. Gilbertson 14392* (ARIZ). CANADA. ALBERTA: Edmonton, on *Populus balsamifera* L., 9 Aug. 1931, *E. H. Moss 2446* (DAOM). BRITISH COLUMBIA: Vancouver Island, Lake Cowichan, on *Acer* sp., 7 Sept. 1967, *J. H. Ginns 966* (BPI, DAOM). MANITOBA: Riding Mtn. Nat. Park, 14 mi. E Clear Lake, along Hwy. 19, Norgate Lookout, on *Betula* branch, 19 Aug. 1979, *J. H. Ginns 4254* (BPI, DAOM). ONTARIO: Algonquin Provincial Park, near west gate, Hwy 60, on hardwood log. 20 Sept. 1982, *N. Hallenberg and J. H. Ginns, GB 755* (GB). QUEBEC: Gatineau Nat. Park, Kingsmere, on *Acer* sp., 23–24 Jul. 1982, *L. and N. Hallenberg, GB 553* (GB) and NW of Lake Meach, on bark of *Acer*, 25 Jul. 1982, *L. and N. Hallenberg, GB 568* (GB). SASKATCHEWAN: Candle Lake, on *P. balsamifera* L., 16 Jun. 1949, *C. G. Riley 125268* (DAOM). CHINA. Chansi, Yao chan, 2178 m, 2 Sept. 1935, *E. Licent 4168* (PRM); Jilin Prov., Chang Bai Shan Forest Reserve, Huang Song Pu, 1200 m, on *Acer* sp., 11–17 Sept. 1983, *L. Ryvardeen 21524* (O). IRAN. Gorgan, Jangale Shast Kalatech (20 km SW Gorgan), on fallen branch, 3 Jul. 1976, *L. and N. Hallenberg and D. Ershad, NH 1435, GB 23683* (GB). NEW ZEALAND. Otago, Routeburn Valley, 450 m, on *Nothofagus solandri* (Hook.f.) Oerst., Feb. 1948, *J. M. Dingley 6849* (ISOTYPE of *Merulius nothofagi*, BPI). PAKISTAN. Charehan, Murree Hills, on decayed wood, 16 Jul. 1983, *S. Ahmad 28272* (O). SRI LANKA. Peradeniya, on dead mango branch, Jun. 1922, *no. 6468* (HOLOTYPE of *Merulius gelatinosus*, K). TAIWAN. Miaoli, Taian Hsiang, Kuanwu, alt. 1900 m, on hardwood branch, 24 Aug. 1988, *S. H. Wu 880824-40* (TAI). TANZANIA. Kilimanjaro Prov., Mt. Kilimanjaro, W. slope of Lemosho Glades, 2400 m, 3°1–2'S, 37°9'E, 12 Jan. 1970, *L. Ryvardeen 5082* (O). USA. ARIZONA: Cochise Co., Chiricahua Nat. Forest, Turkey Creek Recreation Area, on *Quercus hypoleucoides* A. Camus, 7 Sept. 1972, *R. L. Gilbertson 10760* (ARIZ, CFMR); Santa Cruz Co., Coronado Nat. Forest, Madera Canyon, on *Q. arizonica* Sarg., 11 Jul. 1980, *H. H. Burdsall, Jr. 11096* (CFMR) and on *Q. hypoleucoides*, 7 Aug. 1980, *H. H. Burdsall, Jr. 11049* (CFMR). CALIFORNIA. Santa Catalina Island, Big Wash Canyon, on *Quercus* log, 14 Jun. 1921, *L. W. Nuttall 1128* (BPI); Smith River, on hardwood. 30 Oct. 1958, *J. L. Lowe and R. L. Gilbertson 10571* (CFMR). MARYLAND: Sug-

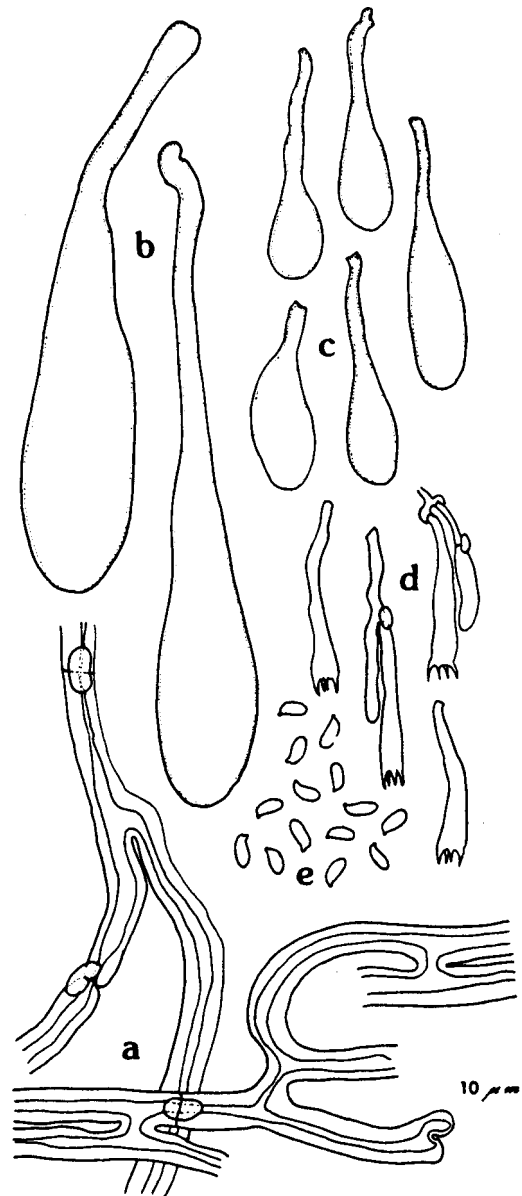


FIG. 4. Line drawings of basidioma characters of *Phlebia acerina* (FP 101090): a) thick-walled subicular hyphae, b) subicular cystidia (from type specimen), c) hymenial cystidia, d) basidia, e) basidiospores.

ar Loaf Mtn., on *Castanea dentata* (Marsh.) Borkh., 24 Jul. 1932, *R. W. Davidson, FP 57020* (CFMR). MICHIGAN: Marquette Co., Big Bay, Huron Mtn. Club, SE of Breakfast Roll, on *Acer saccharum* Marsh., 8 Aug. 1978, *H. H. Burdsall, Jr. 10437* (CFMR) and N slope of Iver Hill, on *A. spicatum* Lam., 7 Aug. 1974, *H. H. Burdsall, Jr. 8203* (CFMR). NEW HAMPSHIRE: Waterville, Osceola Branch Trail, on bark of *Prunus*

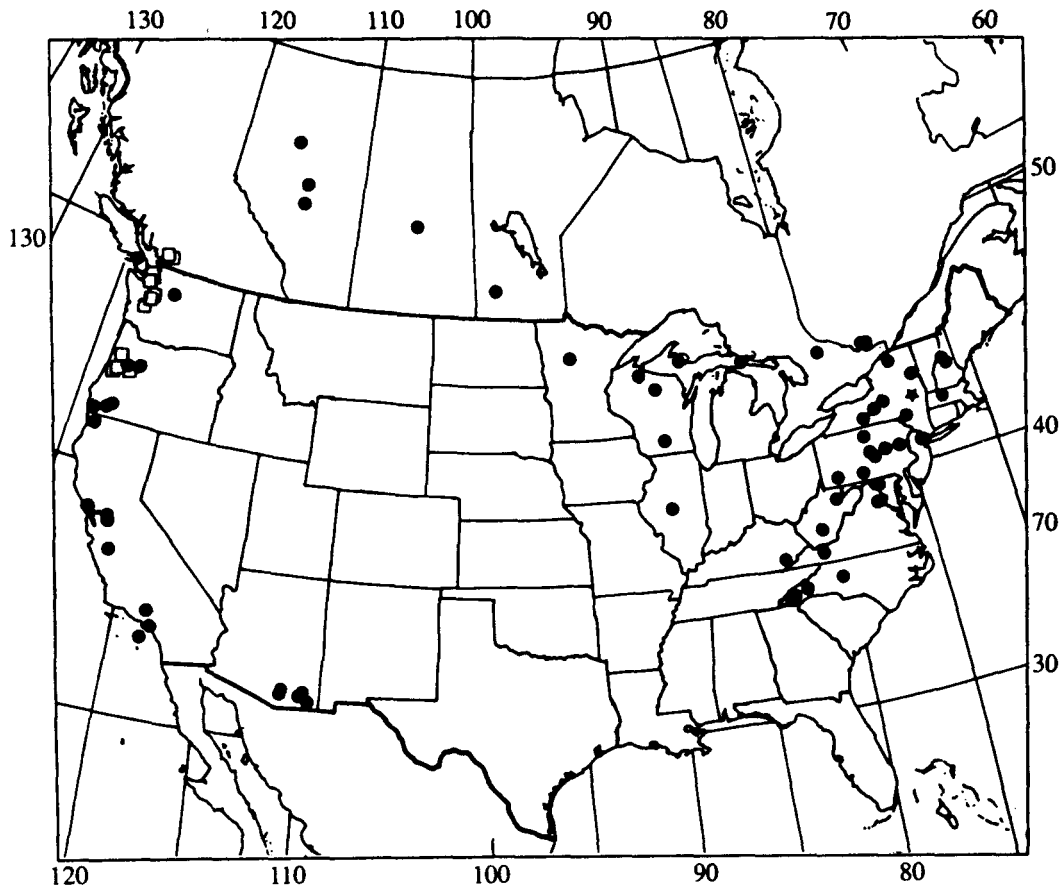


FIG 5. Distrigution of *Phlebia acerina*; and *P. Rufa* in North America. Circles, *Phlebia acerina*; star, location of type specimen; squares, *Phlebia rufa*.

pennsylvanica L. fil, 19 Aug. 1937, H. C. Eno, FP 81178 (TRTC). NEW YORK: Fayetteville, on *Acer* sp., 20 Jun. 1978, J. L. Lowe 15744 (CFMR); Mechanicsville, on maple, 20 Jul. 1988, C. H. Peck (HOLOTYPE of *Phlebia acerina*, NYS); Warren Co., Horicon, on bark of *P. pennsylvanica* (probably *Betula* sp.), 25 Jul. 1905, C. H. Peck (HOLOTYPE of *Merulius pruni*, NYS; ISOTYPE, BPI). NORTH CAROLINA: Haywood Co., Great Smoky Mtns. Nat. Park, Cataloochee, 300 m, on hardwood bark, 1 I Jul. 1988, M. Rajchenberg 4280 (CFMR). OREGON: S of Corvallis, on rotted, decorticated frondose wood, 28 Sept. 1919, S. M. Zeller 1772 (HOLOTYPE of *Merulius pilosus*, BPI; ISOTYPE, NY, FH); Brookings, on hardwood, Oct. 1958, J. L. Lowe and R. L. Gilbertson 10595 (CFMR). VIRGINIA: Arlington Cemetery, on *Liriodendron tulipifera* L., 16 Apr. 1922, C. L. Shear 4013 (HOLOTYPE of *Merulius interruptus*, BPI); Giles Co., Mountain Lake Biological Station, Moonshine Dell, on *Acer* sp. bark, 24 Jul. 1979, H. H. Burdsall, Jr. 10607 (CFMR). WEST VIRGINIA: Eglon, on hardwood, 5–10 Sept. 1915, C. G. Lloyd 32765 (HOLOTYPE of *Phlebia meruloides*, BPI).

WISCONSIN: Ashland Co., Copper Falls State Park. Brownstone Falls, on *A. saccharum*, 25 Jun. 1981, H. H. Burdsall, Jr. 11146 (CFMR); Oneida Co., Minocqua, Kemp Biological Station, on bark of *Betula papyrifera* Marsh., 10 Aug. 1973, F. F. Lombard and R. G. Payne, FP 101138 (CFMR). USSR. Siberia, Tara District, on *Populus tremula* L. trunk, Aug. 1929, Ziling, no. 156137 (PRM); Chabarovsk, Jadrino, on bark of *Quercus* sp., 9 Aug. 1961, E. Parmasto, TAA 18-019 (PRM); Kazakstan, Alma Ata, on *Picea schrenkiana* Fisch. & Mey., Sep 1935, Krawtzen, no. 25150 (PRM); Mari, Sidelnikovsk, on *Tilia cordata* Mill. 21 Jul., B. Vassilkov, LE 22536 (HOLOTYPE of *Phlebia vassilkovii*, LE). YUGOSLAVIA. Near Kostanjevica, Krakorsã gozel, on trunk of *Corylus avellana* L., 2 July 1978, M. and S. Tortiã (60-78 (H).

Cultural description. — Growth on MEA rapid, (35–)45–55mm radius at 1 wk, > 90 mm radius at 2 wk; mats white, some isolates around inoculum moderately thick, appressed and sub-

felty, then becoming thinner toward margins, in other isolates slightly raised and farinaceous around inoculum, then becoming appressed and subfelty toward margins at 1 wk. by 2 wk white. occasionally inoculum plugs and raised mycelia orange white (5A2) to light orange (5A5), moderately thick, slightly raised, downy to thick, raised, woolly, sometimes developing cottony mounds at mat periphery, growing vigorously up sides of dish at 2 wk, by 6 wk white to light orange (5A5) with occasional brownish yellow (5C6) patches, moderately thick, slightly raised, felty to tomentose or raised, thick, felty to woolly with small or large, thin, appressed, subfelty areas, occasionally with scattered tufts of mycelia; fertile areas greyish orange (5B5–5B6) to brownish orange (5C4), tuberculate, scattered over mat surface; margins even to slightly uneven, appressed, fimbriate; odor absent at 1 wk, absent to sour at 2 wk, absent, sour or sweet at 6 wk; agar unchanged or bleached under inoculum at 1, 2, and 6 wk: occasionally fruiting by 4 or 6 wk. On gallic acid agar (GAA) reaction positive (+ + to + + + +), no growth or 30–65 mm diam at 1 wk, no growth or 63–90 mm diam at 2 wk mats at 2 wk mostly submerged with scanty, thin, arachnoid to downy, with white aerial mycelia. On tannic acid agar (TAA) reactions positive (+ + + +), 30–50 mm diam at 1 wk, 52–84 mm diam at 2 wk; mats at 2 wk white, moderately thick, raised and woolly with scattered cottony patches, in other isolates thin, appressed to slightly raised, subfelty to downy, margins even and appressed.

Microscopic characters. Marginal hyphae 4–8(–10) μm diam, simple septate, sparsely to moderately branched, walls thin at first, then becoming thick, up to 1.5 μm thick. Submerged hyphae: a) similar to marginal hyphae; b) 2–6 μm diam, nodose septate with adventitious septa, moderately to frequently branched, walls thin to slightly thick. Aerial hyphae 1.5–8 μm diam, nodose septate with adventitious septa, sparsely to moderately branched, walls thin to slightly thick, occasionally becoming roughened, often coated with hyaline to pale yellow, resinous globules that become more copious with age, a few segments heavily encrusted with small, hyaline crystalline materials, occasionally becoming agglutinated. Cystidia cylindrical to clavate, 25–60(–90) \times 3.5–8 μm , 2–3.5 μm diam at base, with a basal clamp connection, terminal, walls thin to slightly thick, smooth or coated with resinous globules that dis-

appear in 2% KOH, scarce or numerous in aerial mats at 1, 2, and 6 wk.

Species code. 2,4,13,21,27,32,36,(38),(40),42,(48),(50),(53),54,(55),59.

Key pattern. A-P-F-1-10-16.

Cultures studied. GB 553, GB 568, GB 755, MR 4280, HHB 8203, HHB 10437, JLL 10571, JLL 10595, HHB 10607, RLG 10760, HHB 11049, HHB 11096, HHB 11146, JLL 15744, FP 101138.

Other cultural descriptions of *P. acerina* are found under the name *P. rufa* in Lombard and Chamuris (1990, p. 71), Nakasone (1990a, p. 260). Ginns (1976, p. 145) included cultures of both *P. acerina* and *P. rufa* in his description.

Remarks.—*Phlebia acerina* is the earliest name for this taxon. Because of its variable hymenial configuration and wide distribution, this taxon was described many times. In addition, because of their morphological similarity to *P. rufa*, most specimens of *P. acerina* were misnamed *P. rufa*. Cooke (1956), however, considered *P. acerina* synonymous with *P. radiata*. Other descriptions and illustrations of *P. acerina* are available in type studies of *Merulius* and *Phlebia* species published by Ginns (1968, 1969, 1970, 1971) and as *P. rufa* by Wu (1990). Because the basidiomata of *P. acerina* are variable, the photographs in the following reports may be helpful in identifying the species: Cunningham (1950, Fig. 3); Hallenberg (1984, Fig. 14A, C); Lloyd (1915, Fig. 738), Zeller (1922, Fig. 1).

Phlebia acerina is known from all the continents except Antarctica, whereas *P. rufa* appears to be limited to Europe and the Pacific Northwest region of the United States. The report of *Phlebia rufa* from Turkey by Hallenberg (1991) is probably *P. acerina* since it is not compatible with *P. rufa* or *P. radiata*.

Although very similar, *P. acerina* and *P. rufa* can be differentiated by hymenial color, reaction of the hymenium to potassium hydroxide, width of hymenial folds, attachment and edge morphology of margins, microstructure of basidiomata, and presence of hymenial cystidia. Typical specimens of *P. acerina* have hymenia that are yellowish brown and are unchanged in potassium hydroxide, whereas the hymenia of *P. rufa* are reddish brown and turn even darker in potassium hydroxide. The folds in *P. acerina* are broader and thus have a coarser appearance than the folds in *P. rufa*, which are narrow and often form dis-

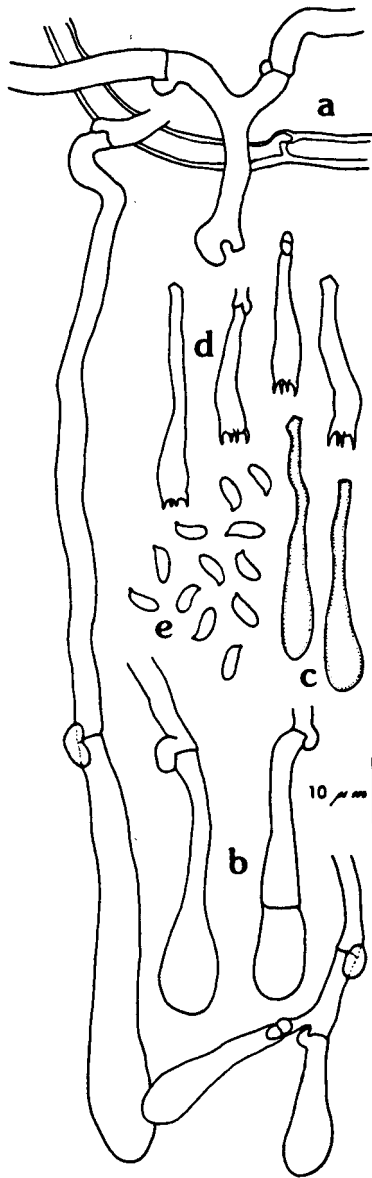


FIG. 6. Line drawings of basidioma characters of *Phlebia rufa* (FP 133432). See legend to FIG. 4 for description of characters a-e.

tinct, well-developed pits. The margins of *P. acerina* are usually entire and often detach from the substrate (FIGS. 1-3), whereas those of *P. rufa* are fibrillose and closely appressed. Mature, well-developed specimens of *P. acerina* often develop a differentiated subiculum with conglutinated parallel hyphae next to the substrate and a loose upper layer that contains abundant, yellow,

amorphous substances; this is never present in *P. rufa*. Hymenial cystidia are usually present in *P. acerina* and are difficult to separate from the agglutinated hymenial elements. In *P. rufa*, however, hymenial cystidia are rare or absent, and hymenial elements are not agglutinated and thus easier to separate. In addition, the hymenial surface is interrupted by white, felty to woolly mycelia in many specimens of *P. acerina* (FIGS. 1-3). These two species may also be distinguished by distribution; *P. acerina* occurs throughout North America whereas *P. rufa* is limited to western Washington and Oregon and southwestern British Columbia (FIG. 5). Cultures of *P. acerina* and *P. rufa* are nearly identical except that the latter sometimes develop arthroconidia.

Phlebia rufa Pers. (Fr.) M. P. Chris., Dansk Bot. Ark. 19(2): 194. 1960. FIGS. 3, 6

= *Merulius rufus* Pers. Synop. Meth. Fung. p. 498, 1801.

= *Merulius rufus* Pers.: Fr., Syst. Mycol. I: 327, 1821.

= *Xylomyzon isoporum* Pers., Mycol. Europe 2: 33, 1825.

= *Merulius pallens* Berk., Ann. Mag. Nat. Hist., ser. 1, 6: 357. 1841.

= *Merulius lividus* Bourd. & Galzin, Bull. Soc. Mycol. France 39: 104. 1923.

= *Phlebia sublivida* Pam., Izv. Akad. Nauk Estonsk. S.S.R., Ser. Biol. 16: 393. 1967.

= *Merulius phlebioides* Bourd. & Galzin, Bull. Soc. Mycol. France 39: 104. 1923.

= *Phebia erecta* Rea, Trans. Brit. Mycol. Soc. 5: 252. 1916.

Basidiomata annual, waxy, effuse, often starting as circular areas, becoming confluent, up to 160 × 60 mm and 0.4 mm thick, rarely up to 1 mm thick, ceraceous, often turning pinkish red to brownish red in 2% KOH; *hymenia* continuous, translucent, often pruinose, developing folds up to 1 mm in height, folds often forming irregular reticulations and deep pits. 1-3 per mm, folds also becoming interrupted, gyrose-plicate to dentate-raduloid, sometimes developing into tooth-like structures that aggregate into clusters or wart-like structures up to 3 mm in height, in well-developed central area reddish orange (7B7), brown (7D7, 7E8), or dark brown (8F4) to reddish brown (8D8, 8E6), occasionally greyish orange (5B6), brownish orange (6C6), light brown (6D5-6D6), or brown (6E6); other specimens with shallow pits and reticulations throughout, hymenium brown (6D7) to greyish orange (6B6);

margins smooth to slightly warted, concolorous or paler than central area. up to 2 mm wide. closely attached to substrate. with a white. fibrillose edge: context bicolored with upper part concolorous with hymenium and basal part next to substrate pale yellow, cream-colored. or white.

Hyphal system monomitic. *Subiculum* 250-400(-1000) μm thick, often with brownish yellow, resinous materials throughout, a dense *textura intricata*. but at margins a *textura porrecta*, subicular hyphae 3-5.5(-7.5) μm diam, nodose septate, moderately branched, often anastomosing, walls thin to slightly thick, rarely up to 1.5 μm thick. In folds subiculum a *textura porrecta*, hyphae 3.5-5.5 μm diam, nodose septate, walls thin to slightly thick. heavily encrusted with tiny, brownish yellow crystalline materials. *Subhymenium* thickening, up to 150 μm thick, a *restura intricata*. hyphae 2-3 μm diam, nodose septate, moderately branched. walls thin and smooth. *Hymenium* composed of basidia and cystidia. often coated with resinous materials. *Cystidia* of two types: 1) subicular cystidia large, clavate, 20-60 \times 6-12 μm . 3.5 μm diam at base, with a basal clamp connection, arising from subiculum, rare in subiculum but abundant in margin, walls thin. smooth; 2) hymenial cystidia narrowly clavate. 30-40 \times 5-7 μm , 1.5-2.5 μm diam at base, with a basal clamp connection, smooth, arising from subhymenium. rare or absent. walls thin. *Basidia* narrowly clavate, 19-37 \times 4-5.5 μm , with a basal clamp connection. walls thin. four-sterigmate. *Basidiospores* cylindrical, (4.5-)5-5.5(-5.8) \times 2-2.2 μm , hyaline, walls thin, smooth, negative in Melzer's reagent.

HABITAT. On bark and wood of angiosperms and occasionally on gymnosperms.

DISTRIBUTION. Great Britain, Denmark (Christiansen, 1960), Finland, Sweden, Spain, France, Switzerland (Breitenbach and Kränzlin, 1986), Poland, Czechoslovakia, Bulgaria, Iran, Canada (British Columbia), United States (Oregon, Washington) (Fig. 5).

REPRESENTATIVE SPECIMENS EXAMINED, BULGARIA. Eminska planina, in valle supra Vlas pr. Nesebar, *Carpini orientalis*. 8 Sept. 1984. K. Kotlaba 836120 (PRM). **CANADA. BRITISH COLUMBIA:** Vancouver Island, Lake Cowichan, on *Acer* sp., 7 Sept. 1967, J. H. Ginns 956 (DAOM); South Burnaby, on *Alnus* sp., 7 Aug. 1957, S. J. Hughes, no. 137806 (DAOM); Vancouver, Univ. British Columbia Endowment Land. on *Acer rubra* Bong., 24 Sept. 1967, R. Bandoni, B. and J. Eriksson 8464 (DAOM. GB); Vancouver Island, Victoria, Goldstream Park, on *Tsuga* sp., 15 Oct. 1971, J. H. Ginns 1939 (DAOM). **CZECHOSLOVAKIA.** Carpati Rossici, in silvis ad rivum Kosovská Rjeka, prope Jalinka, ad ligna *Fagi silvaticus* L.. Jul. 1930, A.

Pilat, Fungi Carpatici Lignicoli Exsiccati no. 131 (NY). **FINLAND.** Etelä-Häme, Lahti, Mukkula, Ritamäki, on *Pinus sylvestris* L., 28 Oct. 1984, V. Haikonen 5220 (H); Varsinais-Suomi, Kemiö, Wigksgård, on *Quercus robur* L., 12 Sept. 1970. T. Niemelä (H). **FRANCE.** Gallia, Herb no. 190.277-360 (HOLOTYPE of *Xylomyzon isoporum*, L): Aveyron, sur chêne, 21 Jun. 1920. Herb. H. Bourdot 30180 (HOLOTYPE of *Merulius lividus*, PC); Aveyron, sur saule, Sept. 1907, Herb. H. Bourdot 14458 (HOLOTYPE of *Merulius phlebioides*, PC). **GREAT BRITAIN. SCOTLAND:** Murthley. on hardwood. 9 Aug. 1923. C. L. Shear. US 0257188 (BPI). **ENGLAND:** Nottinghamshire and Grace Dieu Woods, ex herb. Berkeley, Herbarium Hookerianum, 1867 (LECTOTYPES of *Merulius pallens*. K); Warwickshire, Whitechurch, Crimscoote Downs, north of Paradise, 25 Oct. 1915, herb. Carleton Rea (LECTOTYPE of *Phlebia erecta*. K). **IRAN.** Mazanderan, Sangdeh, S. of Pol-e-Sefid, alt. 1300-1800 msm, on fallen branch of *Fagus. L. and N. Hallenberg and E. Danesh-Pashuuh* 2750 (GB). **POLAND.** Kraków province, Ojcow Nat. Park. on *F. sylvatica*, 18 Sept. 1978, T. Niemelä 1347 (H). **SPAIN.** Huesca, Ordesa Nat. Park, on *Fagus* branch. 8 Nov. 1986. N. Hallenberg 9783 (GB). **SWEDEN.** Vastergotland, Marböck par.. Sjögnannavsko, on deciduous wood, 25 Jul. 1977. K. Hjortstam 7834 (BPI). **USA. OREGON:** Finley Nat. Wildlife Refuge, on hardwood bark. Oct. 1982, B. R. Kropp 4 (CFMR); Lincoln Co., Cougar Mtn. on conifer. 3 Oct. 1973. M. J. Larsen FP 133684 (CFMR); Sinuslaw Nat. Forest. Marys Peak, on hardwood, 6 Nov. 1971. M. J. Larsen FP 133014 and FP 133432 (CFMR). **WASHINGTON:** Olympic Peninsula. Hump-tulips River. 1000 ft., on *Alnus* sp., 5 Sept. 1957. J. L. Lowe 8213 (CFMR); Mason Co., Twanoh State Park, on *Pseudotsuga menziesii* (Mirb.) Franco, 22 Oct. 1950, W. B. and V. G. Cooke 27244 (DAOM).

Cultural description. —Growth on MEA moderately rapid, 25-40(-52) mm radius at 1 wk. >90 mm radius at 2 wk: mats white, around inoculum thin to moderately thin, appressed, subfelty, rarely farinaceous. becoming thinner toward margins at 1 wk. by 2 wk unchanged except a few isolates with pale orange (5A3) mycelia on and around inoculum plugs and occasionally woolly around inocula. by 6 wk white, with scattered areas greyish orange (5B4-5C5) or light orange (5A4), mostly thin, appressed, subfelty to farinaceous. with peripheral areas becoming slightly raised and cottony, rarely thick, raised and woolly throughout; fertile areas brown (6E6-6E7), warted, in small patches over inocula and adjacent to dish sides: margins slightly uneven. appressed to submerged, fimbriate: odor absent at 1 wk, faintly sweet at 2 wk, and absent at 6 wk; agar unchanged at 1 wk, unchanged or slightly bleached at 2 and 6 wk: rarely fruiting by 6 wk. On GAA reaction positive (+ + + to + + + +), no growth or 20-38 mm diam at 1 wk.

no growth or 36–63 mm diam at 2 wk; mats at 2 wk mostly submerged, with a scanty, thin, white to brown, arachnoid aerial mycelia. On TAA reaction positive (+ + +), growth 15–30 mm diam at 1 wk, 43–58 mm diam at 2 wk mats at 2 wk white, typically appressed, thin and subfelty throughout, a few isolates with slightly raised, arachnoid to downy aerial mycelia around inoculum, then becoming appressed and subfelty toward margins.

Microscopic characters. Marginal hyphae (2–)2.5–6.5(–7.5) μm diam, simple septate, occasionally with clamp connections, sparsely to moderately branched, walls thin at first, then becoming slightly thick to thick, up to 3.5 μm thick. Submerged hyphae: a) similar to marginal hyphae; b) 1.5–4.5 μm diam, nodose septate and with adventitious septa, moderately to frequently branched, walls thin. Aerial hyphae 1.5–4(–5.5) μm diam, nodose septate and with adventitious septa, moderately branched, sometimes becoming agglutinated, walls thin to thick, up to 1.5 μm thick, often coated with refractive, resinous globules. Cystidia clavate to spatulate, rarely fusiform, 20–100 \times 4.5–9 μm , 1.8–3.5 μm diam at base, with a basal clamp connection, occasionally developing adventitious septa in stalk, terminal or lateral, with thin walls, staining in phloxine, often lightly coated with resinous materials, scattered to abundant in aerial mat at 1 and 2 wk, absent at 6 wk; infrequently cystidia developing another cystidium or nodose septate hyphae from apex. Arthroconidia cylindrical to ellipsoid, 4–7 \times 2–2.5 μm , hyaline, with thin walls, absent or numerous in aerial mat at 1, 2, and 6 wk.

Species code. 2.4.13.21.27.(35).36.38.(40).42.(48).(50).54.55.59.

Key patterns. A-P-F-1-10-16; A-P-F-1-4-10-16.

Cultures studied. Kropp 4, GB 1199, GB 1790, JLL 8213, FP 133014, FP 133432, FP 133684, Aho 74-58-B, Aho 74-86-B, and Aho 74-598-B. Aho strains isolated from twigs of *Abies grandis* (Dougl. ex D. Don) Lindl., Little Baldy and De-arorff Creeks, Malheur National Forest, Oregon, Jul. 1973.

Other cultural descriptions. Boidin (1958, p. 179, as *Merulius porinoides* Fr.); Kühner and Yen (1947).

Remarks. - *Phlebia rufa* is known only from Washington, Oregon, and British Columbia in

North America (FIG. 5) but is apparently widespread in Europe. Generally, basidiomata of *P. rufa* are thinner and less robust than those of *P. acerina*. See remarks under *P. acerina* for further discussion. Other descriptions and illustrations of *P. rufa* are available in Eriksson et al. (1981, p. 1157). Breitenbach and Kränzlin (1986, p. 166), and Hallenberg (1984, Figs. 14B, D). Descriptions of *P. rufa* by Burt (1917) and Ginns (1976), however, are composites of *P. acerina* and *P. rufa*, whereas Lindsey and Gilbertson (1978) describe *P. centrifuga* P. Karst. The description of *P. rufa* by Wu (1990) is actually *P. acerina*. Although *P. rufa* is reported by Pilát (1934, 1940) to occur in Siberia and China, the specimens cited are *P. acerina* or undeterminable species. Similarly, specimens of *P. rufa* from Argentina and China (Hjortstam and Ryvarden, 1985, 1988) and Iran (Hallenberg, 1978) are in fact *P. acerina*.

An attempt was made to examine the 15 type specimens listed as synonyms under *P. rufa* by Ginns (1976). We agree with Ginns (1976, 1984) that *X. isoporium*, *M. pallens*, *M. lividus*, *M. phlebioides*, and *P. erecta* are facultative synonyms of *P. rufa*; however, seven other types are synonyms of *P. acerina*. The identity of *Phlebia castanea* G. C. Lloyd is not known for it is neither *P. acerina* nor *P. rufa* but represents another species. Type specimens of *P. butyracea* Rick and *Merulius rubrotremellosus* Corner were not available for study.

Phlebia radiata Fr., Syst. Mycol. I: 427. 1821.

This species is well known in North America and Europe. It is adequately described and illustrated by Ginns (1976, p. 139), Eriksson et al. (1981, p. 1153), and Breitenbach and Kränzlin (1986, p. 166). Cultural descriptions are available in Boidin (1958, p. 172), Ginns (1976, p. 141), and Nakasone (1990a). The name *Phlebia merismoides* Fr.: Fr. may have priority, but it is not widely used.

RESULTS

Sexuality and intraspecific pairings. —Nineteen presumptive monokaryotic isolates from *P. acerina* (GB 568) were paired in all combinations. Two mating types were recovered: $A_1 = 1, 3, 4, 7, 10, 12, 13, 15, 16, 17, 18, 19, 20, 23, 24, 25$; $A_2 = 2, 6, 22$. Similar results were obtained from pairing 14 monokaryons of JLL 10595: $A_3 = 1, 2, 12, 16, 17$; $A_4 = 3, 5, 7, 8, 10, 13, 14, 15, 18$.

TABLE II
INTRASPECIFIC AND INTERSPECIFIC PAIRINGS BETWEEN MONOKARYONS OF *PHLEBIA ACERINA*, *P. RADIATA*,
AND *P. RUFA*^a

Pairing					
Species and strain no.	Monokaryons	Species and strain no.	Monokaryons	Total pairings	Positive pairings
<i>P. rufa</i>		<i>P. acerina</i>			
FP 133684	6	GB 568	12	72	0 ^b
	12	JLL 10595	6	72	0
		<i>P. radiata</i>			
	25	JLL 15608	6	150	1 ^c
<i>P. acerina</i>		<i>P. acerina</i>			
GB 568	12	JLL 10595	4	48	47
		<i>P. radiata</i>			
	12	JLL 15608	5	60	0

^a At least two strains of each mating type were used in each set of pairings.

^b In six different pairings, pseudoclamps were observed.

^c Pseudoclamps observed in one pairing.

Monokaryotic cultures from these two strains readily paired and indicate that multiple alleles occur at the mating type locus. Thus, we confirm similar results reported by Ginns (1976, p. 145) who found that strains JHG 575 and JHG 577, under the name *P. rufa*, were unifactorial and multiallelic.

Yen (1947) and Ginns (1976, isolate JLL 82 15) reported that *P. rufa* is unifactorial. We confirm their findings after pairing 29 monokaryotic cultures of FP 133684: A₁ = 1, 2, 4, 5, 6, 7, 9, 10, 16, 21, 22, 25, 26, 27, 28; A₂ = 3, 8, 11, 12, 13, 14, 15, 17, 18, 19, 20, 23, 24, 29. *Phlebia rufa* is also multiallelic at the mating type locus, as reported by Boidin (1958, p. 180) and Ginns (1976, p. 146).

Phlebia radiata is also unifactorial (Nobles et al., 1957, as *P. merismoides* Fr.; Terra, 1953, as *P. aurantiaca* (Sowerby) P. Karst.). Our results concur with published reports. Two mating types were identified after pairing 16 monokaryotic cultures of JLL 15608: A₁ = 1, 3, 4, 5, 8, 11, 14, 17, 18; A₂ = 2, 6, 9, 12, 13, 15, 16. Ginns (1976, p. 142) reported that *P. radiata* is multiallelic.

Interspecific pairings. —As shown in TABLE II, monokaryons of *P. acerina* are compatible with other monokaryons of this species but not with those of *P. rufa* or *P. radiata*. Furthermore, *P. rufa* is not compatible with *P. radiata*. These pairings show that the three taxa are genetically distinct.

Effect of temperature on linear growth. —All three taxa have similar growth responses to temperature, with a temperature optimum of 26 C (FIG. 7). *Phlebia acerina* grew significantly faster than *P. rufa* from 24 C to 32 C.

Restriction maps and analysis. —The restriction-site maps of *P. radiata* (6 strains), *P. acerina* (2 strains), *P. rufa* (4 strains), *P. ludoviciana* (2 strains) are shown in FIG. 8. The small mitochondrial rRNA gene is highly conserved within each species. Although the three ingroup species share a number of restriction sites in the mitochondrial rRNA gene, this is not the case in the flanking regions. Even fewer sites are shared with the outgroup species, *P. ludoviciana*, which was difficult to align properly with the other taxa. All four taxa share a *Hind* III site within the gene. Of 97 restriction sites mapped, 85 are phylogenetically informative while 12 are not informative (11 sites not variable and one site autapomorphic). A site is phylogenetically informative when each character state (0.1) is present in at least two strains. The data matrix is presented in TABLE III. The shortest possible tree is 85 steps; however, cladistic analysis generated two most parsimonious Wagner trees of 93 steps that required six convergent site losses (28, 58, 70–72, 92), one parallel gain (21), one autapomorphic site, and has a consistency index (Kluge and Farris, 1969) of 92.5% and a homoplasy rate of 7.5%. Excluding the autapomorph, the tree is 92

TABLE III
DATA MATRIX OF RESTRICTION SITES IN THE SMALL MITOCHONDRIAL rRNA GENE AND RANKING REGIONS OF *PHLEBIA* SPECIES^a

	1 2 3 4	5 6 7 8 9	1 1 1 1 1 0 1 2 3 4	1 1 1 1 5 6 7 8	1 2 2 2 2 2 9 0 1 2 3 4 5	2 2 2 2 3 3 3 6 7 8 9 0 1 2	3 3 3 3 3 3 4 5 6 7	3 3 4 4 4 4 4 4 4 8 9 0 1 2 3 4 5 6 7
	<i>Sal</i> I	<i>Eco</i> RI	<i>Cla</i> I	<i>Xba</i> I	<i>Eco</i> RV	<i>Sca</i> I	<i>Bgl</i> II	<i>Hind</i> III
	a a a a 1 2 3 4	b b b b b 1 2 3 4 5	c c c c c 1 2 3 4 5	d d d d 1 2 3 4	e e e e e e e 1 2 3 4 5 6 7	f f f f f f f 1 2 3 4 5 6 7	g g g g g 1 2 3 4 5	h h h h h h h h h 1 2 3 4 5 6 7 8 9 10
<i>P. radiata</i> 15608	? ^b 1 1 0	? 1 0 0 0	1 0 0 0 0	1 1 0 0	1 1 0 1 0 0 0	1 1 0 ? 0 0 0	0 0 0 0 0	1 1 1 1 ? ? 0 0 0 0
70836	? 1 1 0	? 1 0 0 0	1 0 0 0 0	1 1 0 0	1 1 0 1 0 0 0	1 1 0 ? 0 0 0	0 0 0 0 0	1 1 1 1 ? ? 0 0 0 0
101840	? 1 1 0	? 1 0 0 0	1 0 0 0 0	1 1 0 0	1 1 0 1 0 0 0	1 1 0 ? 0 0 0	0 0 0 0 0	1 1 1 1 ? ? 0 0 0 0
133493	? 1 1 0	? 1 0 0 0	1 0 0 0 0	1 1 0 0	1 1 0 1 0 0 0	1 1 0 ? 0 0 0	0 0 0 0 0	1 1 1 1 ? ? 0 0 0 0
1792	1 0 1 0	1 0 1 0 0	1 0 0 0 0	1 1 0 0	1 1 1 ? 0 0 0	1 1 0 ? 0 0 0	0 0 0 0 0	1 1 1 1 ? ? 0 0 0 0
1940	1 0 1 0	1 0 1 0 0	1 0 0 0 0	1 1 0 0	1 1 1 ? 0 0 0	1 1 0 ? 0 0 0	0 0 0 0 0	1 1 1 1 ? ? 0 0 0 0
<i>P. acerina</i> 553	0 0 1 0	0 0 0 0 0	0 1 1 0 0	? 0 1 0	0 0 0 0 0 0 0	1 0 1 1 0 0 0	1 ? 0 0 ?	0 1 1 1 1 0 0 0 0 0
11146	0 0 1 0	0 0 0 0 0	0 1 1 0 0	? 0 1 0	0 0 1 ? 0 0 0	1 0 1 1 0 0 0	1 0 0 0 ?	0 1 1 1 1 0 0 0 0 0
<i>P. rufa</i> 1199	0 0 1 0	0 0 ? 0 0	0 0 0 1 0	? 0 1 0	0 1 ? ? 1 0 0	1 ? 1 ? 1 0 0	0 1 0 0 0	0 1 1 1 1 0 0 0 0 0
1790	0 0 1 0	0 0 ? 0 0	0 0 0 1 0	? 0 1 0	0 1 ? ? 1 0 0	1 ? 1 ? 1 0 0	0 1 0 0 0	0 1 1 1 1 0 0 0 0 0
8213	0 0 1 0	0 0 ? 0 0	0 0 0 1 0	? 0 1 0	0 1 ? ? ? 1 0	1 ? 1 ? 1 0 0	0 1 0 0 0	0 1 1 1 1 0 0 0 0 0
133432	0 0 1 0	0 0 ? 0 0	0 0 0 1 0	? 0 1 0	0 1 ? ? ? 1 0	1 ? 1 ? 1 0 0	0 1 0 0 0	0 1 1 1 1 0 0 0 0 0
<i>P. ludoviciana</i> 9640	? ? 0 1	? ? ? 1 1	0 0 0 0 1	? 0 ? 1	? 0 0 0 0 0 1	0 0 0 0 0 1 1	0 ? ? 1 1	0 0 1 ? ? 1 1 1 1 1
101738	? ? 0 1	? ? ? 1 1	0 0 0 0 1	? 0 ? 1	? 0 0 0 0 0 1	0 0 0 0 0 1 1	0 ? 1 1 1	0 0 1 ? ? 1 1 1 1 1

^a Strains as listed in TABLE I. Each enzyme site is also shown in FIG. 8.
^b Sites are coded as present (1), absent (0), or not known (?).

TABLE III
CONTINUED

	44555 89012	5555 3456	555666666 789012345	666677777 7 7 678901234 5 6	777888888 8 8 8 8 789012345 6 7 8 9	999999999 01234567
	<i>Pst I</i>	<i>Sst I</i>	<i>Cfo I</i>	<i>Msp I</i>	<i>Pal I</i>	<i>Afl II</i>
	iiii 12345	jjjj 1234	kkkkkkkkkk 123456789	llllllllll 1 1 1234567891011	nnnnnnnnnn n n n n 12345678910111213	oooooooo 12345678
<i>P. radiata</i> 15608	11100	1100	11111?0?0	110100000 0 0	1111?0000 ? ? 0 0	1111??0?0?
70836	11100	1100	11111?0?0	110100000 0 0	1111?0000 ? ? 0 0	1111??0?0?
101840	11100	1100	11111?0?0	111?00000 0 0	1111?0000 ? ? 0 0	1111??0?0?
133493	11100	1100	11111?0?0	111?00000 0 0	1111?0000 ? ? 0 0	1111??0?0?
1792	11000	1100	11111?0?0	110100000 0 0	1111?0000 ? ? 0 0	1111??0?0?
1940	11000	1100	11111?0?0	110100000 0 0	1111?0000 ? ? 0 0	1111??0?0?
<i>P. acerina</i> 553	00?11	?010	11111?0?0	10??11100 0 ?	000011111 1 0 0 0	01111?0?0?
11146	00?11	?010	11111?0?0	10??11100 0 ?	000011111 1 0 0 0	01111?0?0?
<i>P. rufa</i> 1199	00000	0001	10?1011?0	?0??11110 0 ?	1111?0000 ? ? 0 0	0101010?0?
1790	00000	0001	10?1011?0	?0??11110 0 ?	1111?0000 ? ? 0 0	0101010?0?
8213	00000	0001	10?1011?0	?0??11110 0 ?	1111?0000 ? ? 0 0	0101010?0?
133432	00000	0001	10?1011?0	?0??11110 0 ?	1111?0000 ? ? 0 0	0101010?0?
<i>P. ludoviciana</i> 9640	00000	0000	0000?0011	?0??000?1 1 1	0000?0111 ? 1 1 1	?000???11
101738	00000	0000	0000?0011	?0??000?1 1 1	0000?0111 ? 1 1 1	?000???11

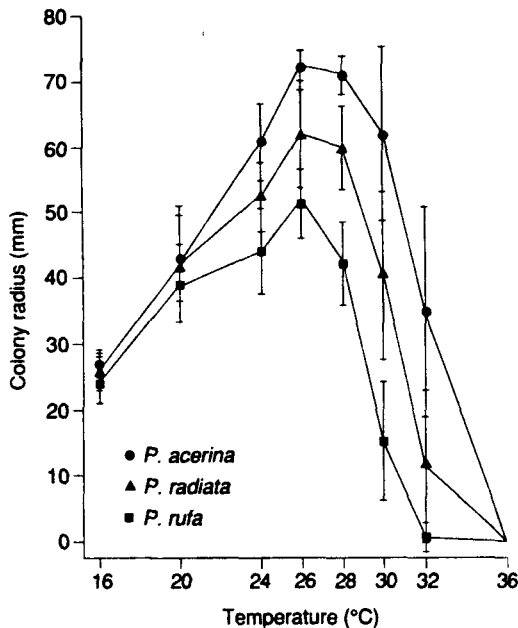


FIG. 7. Growth response of *Phlebia* species to different temperatures after 8 da. Vertical bars indicate one standard deviation.

steps with a CI of 92.4% and homoplasy of 7.6%. The strict consensus tree (FIG. 9) places the strains of each taxon into a monophyletic clade, and *P. radiata* and *P. rufa* clades are grouped together. *Phlebia radiata* consists of two lineages, one from North America and the other from Europe. This basic tree topology was maintained in the decay analysis when trees one to three steps longer were examined. The only change was that the resolution of the strains within each taxon was gradually lost. At 97 steps, or four steps longer than the most parsimonious trees, the three ingroup taxa were reduced to a trichotomy. The cladograms obtained from character-weighted parsimony were identical to the strict consensus tree (FIG. 9) with one exception. When convergent site gains were weighted against convergent site losses by 2:1, *P. rufa* and *P. acerina* were paired together. The majority-rule bootstrap tree of 1000 replicate trees was also similar to the strict consensus tree (FIG. 9). Bootstrap values of 100% were obtained for each of the ingroup taxon clades and 89% for the *P. radiata* and *P. rufa* clade. Only minor differences in bootstrap values and restriction site placement were observed between analyses using ACCTAN (reversals preferred over parallelisms) and DELTRAN (parallelisms

preferred over reversals) options. Otherwise, results of all analyses were identical with DELTRAN and ACCTAN options.

Sequence analysis of ITS-II region. —Although attempts were made to sequence both strands of DNA, sequence was often generated with the ITS4 primer only. Two strains of each ingroup taxon were sequenced as a check. Sequences could be read 20 to 30 nucleotides from the end of the primer. The ITS-II spacer is 180–187 bp long (FIG. 10). The flanking 5.8S and large rRNA genes are highly conserved as the ingroup taxa differ from the outgroup taxa, *P. ludoviciana* and *P. subochracea*, in only 4 (of 92) nucleotides while the ITS-II spacer is somewhat more variable. There are 49 variable sites including 35 informative and 14 autapomorphic sites. Sites with gaps were treated as missing data. Wagner and character-weighted parsimony analyses were performed on 35 sites only. Because at two of these sites (102, 116) three character states are present, the shortest possible tree is 37 steps long. Using the EXHAUSTIVE search option, two most parsimonious Wagner trees, each 39 steps long, were recovered (FIG. 11). However, only four of these sites (102, 116, 231, 251) can be used to infer relationships among the ingroup taxa. In one tree, *P. radiata* is paired with *P. acerina* and in the other tree *P. radiata* is paired with *P. rufa*. These trees have a consistency index of 97.4% and a homoplasy index of 2.6%. In the strict consensus tree, the ingroup species form a trichotomy. A tree of 40 steps was found that is also topologically identical to the strict consensus tree. Identical trees were obtained with character-weighted parsimony analysis. Bootstrap analysis of 1000 replicates also supports a trichotomy of the ingroup species because the values are similar for the branches that pair *P. radiata* with *P. acerina* (47%) and *P. radiata* with *P. rufa* (46%) with ACCTAN option and 46% and 48%, respectively, with DELTRAN (FIG. 11). Different sequence alignments were tried in which the gaps at the 3'-end of the spacer were rearranged. The resulting trees were identical in topology to those in FIG. 11. Similarly, when the gaps were included in the analysis as separate sets of characters (insertion/deletion data) with equal or one-half the weight of sequence data, the trees obtained were unchanged except in one case. Where *P. acerina* and *P. rufa* shared a common nucleotide (T) at position 258 (FIG. 10) in alternative

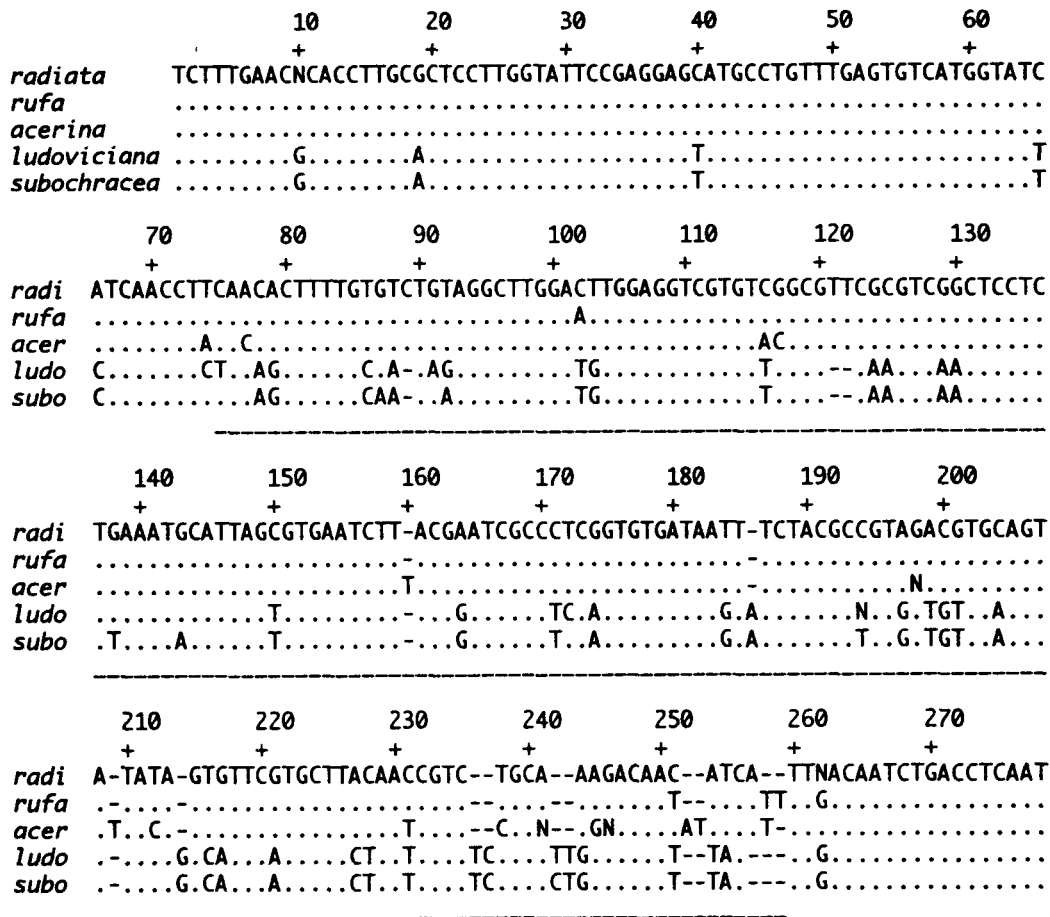


FIG. 10. Aligned sequences of internal transcribed spacer and flanking 5.8S and large rRNA genes of five *Phlebia* species. The ITS-II spacer is underlined. Dots denote identity to sequence in the first row. Dashes denote gaps, and N indicates ambiguous residues.

only 18 variable sites and four insertion/deletion sites. Parsimony analysis was not done on this data set as phylogenetically informative sites are lacking.

DISCUSSION

Morphological and molecular studies. —Wedem demonstrate that *P. acerina*, *P. rufa*, and *P. radiata* are separate species based on morphology, genetic incompatibility, growth response to temperature, restriction map of the small mitochondrial rRNA gene, and sequence of the ITS-II region and portion of the large mitochondrial rRNA gene. Morphologically, *P. radiata* is easily identified by its narrow, cylindrical basidiospores and plicate hymenial surface. Differenti-

ating between *P. acerina* and *P. rufa* is more problematical because basidia, cystidia, and basidiospores are very similar in shape and size. *Phlebia acerina* is characterized by yellowish brown, coarsely warted or folded hymenia, detached margins, and small, clavate hymenial cystidia. On the other hand, *P. rufa* has a reddish brown, pitted hymenium that turns darker in KOH, attached margins, and lacks hymenial cystidia. In culture, however, the three species are indistinguishable. Perhaps, the most reliable method to identify cultures is by intraspecific and interspecific pairings with appropriate haploid cultures. In addition, colony growth at 30 C may be used to distinguish cultures of *P. acerina* from *P. rufa*. Additionally, the three taxa can be identified by differences in the restriction sites of the

small mitochondrial DNA and sequence of the mitochondrial large rRNA gene and ITS-II region.

Molecular analyses demonstrate a close evolutionary relationship among the three taxa. Results from cladistic analyses based on restriction sites of the mitochondrial small rRNA gene region place *P. radiata* and *P. rufa* together in a clade with one exception. However, analysis of ITS-II sequence data place *P. radiata*, *P. rufa*, and *P. acerina* in a trichotomy. The restriction data included sites from both a conserved area in the small mitochondrial rRNA gene (~1.6 kb in size) and larger and more variable areas flanking the gene (~4-5 kb on either side). Eighty-five phylogenetically informative sites were available in this data set, and 52 sites could be used to determine relationships among the ingroup taxa. On the other hand, the ITS-II region, which is about 270 bp long, has 35 phylogenetically informative sites of which only four were useful for determining relationships among the ingroup taxa. Nevertheless, there is insufficient data to determine with any confidence the sister species relationship within the ingroup taxa. While the restriction site data provide the strongest molecular evidence for considering *P. radiata* and *P. rufa* as sister species, morphological similarities of basidioma characters suggest that *P. rufa* and *P. acerina* are sister species.

Topological differences of the trees obtained from the ITS and mtDNA data are not unexpected. It is important to recognize that each tree represents the phylogeny of a homologous segment of DNA and represents a gene tree. A species tree, on the other hand, is the actual phylogeny of a group of populations or species. Species trees contain many gene trees, and the topology of individual gene trees can differ from that of the species tree (Tateno et al., 1982; Pamiolo and Nei, 1988; Avise, 1989). Five factors can account for the different results: sample size, rate of evolution, sorting of polymorphic alleles, introgressive hybridization (Avise, 1989), and uniparental inheritance of genomes. Sample size, rate of evolution, and uniparental inheritance of mitochondria are probably the most influential factors in our study. Saito and Nei (1986) and Felsenstein (1987) show that when species are closely related, as in the human-chimpanzee-gorilla group, between 2.5 and 5 kb of sequence are required to obtain the correct species tree. Therefore, the probability of obtaining the correct tree

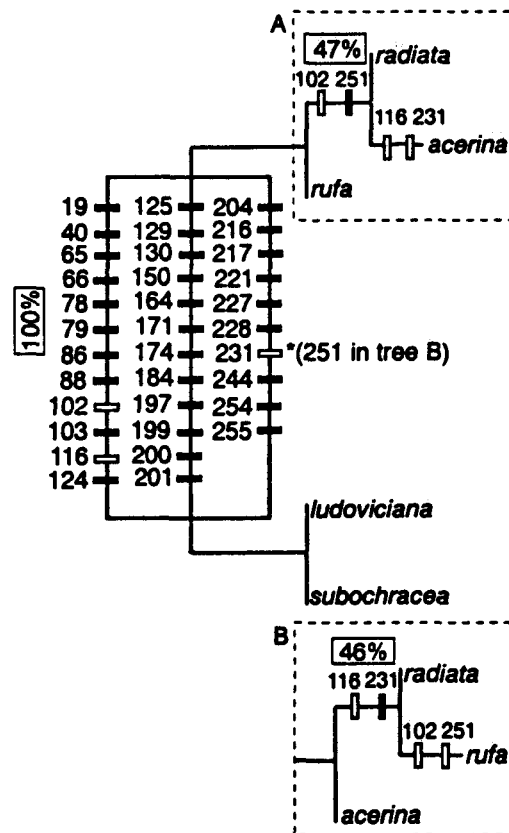


FIG. 11. Phylograms of most parsimonious Wagner trees based on ITS-II sequence data. Numbers along branches refer to nucleotide positions in FIG. 10. Solid bars represent unique nucleotide positions and open bars indicate more than one base change per site. Bootstrap values using ACCTRAN option are enclosed in boxes. Tree B is similar to tree A except for arrangement of the ingroup clade and the position marked with an asterisk.

from the ITS-II region is small. In addition, the taxa may have separated recently so that besides being too short, the region has not accumulated many mutations. Different modes of inheritance of the nuclear rDNA (biparental) compared to the mitochondria (uniparental) can also result in disparate phylogenies. While laboratory matings suggest that mitochondria in basidiomycetes are biparentally inherited (Hintz et al., 1988; May and Taylor, 1988; Smith et al., 1990), in nature only one parental-mitochondrial genotype is recovered (Smith et al., 1990).

Utility of molecular techniques in fungal systematics. —In addition to clarifying the *P. rufa* com-

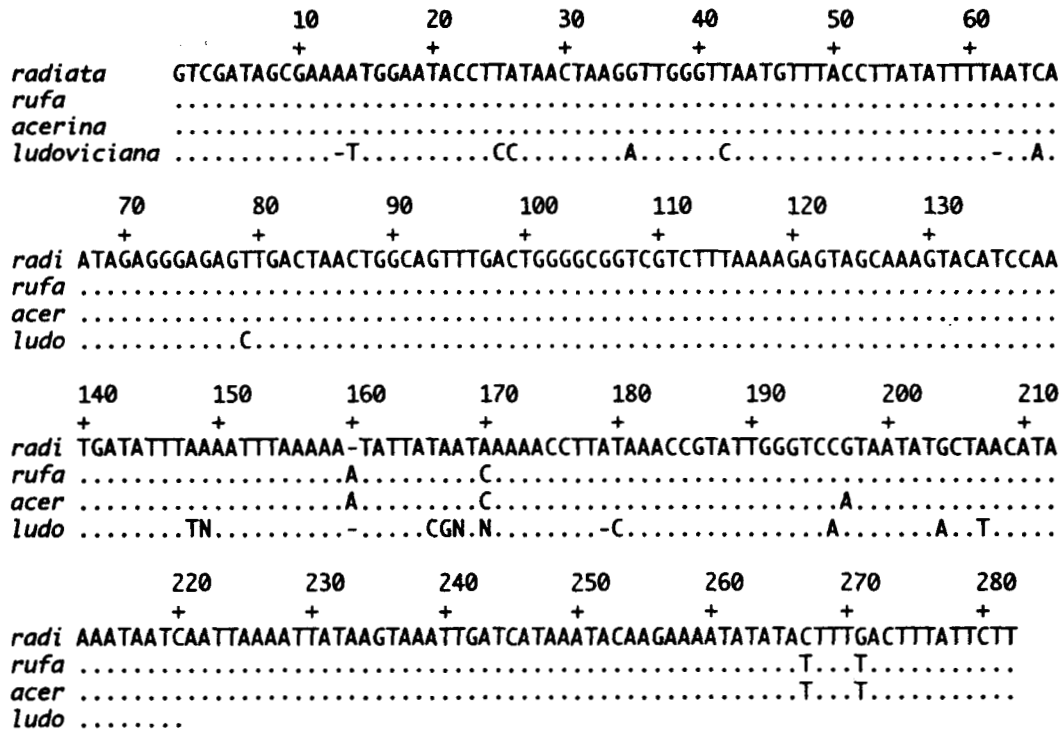


FIG. 12. Aligned sequences of segment of large mitochondrial rRNA gene amplified with ML7-ML8 primers. Dots denote identity to sequence in the first row, dashes indicate gaps, and N denotes ambiguous residues.

plex, we wanted to evaluate how standard morphological, physiological, and genetic methods compare with molecular techniques when examining closely related species. In this study, all the methods could be used to identify and delimit the three taxa. No single method, however, would have convinced a skeptical taxonomist that *P. acerina* is a distinct taxon. For simplicity and cost effectiveness, the standard morphological, genetic, and physiological methods are excellent for identifying these taxa. By comparison, molecular techniques are faster but also more expensive. In addition to confirming the distinctness of each taxon, however, molecular data can be used also to infer their phylogenetic relationships. Although sequence data were easily obtained, sequences were highly conserved among the taxa and provided only a few informative sites in the regions we sampled. More informative sites were obtained by mapping the small mitochondrial rRNA gene region. However, this method is tedious and only feasible when the species are very closely related since it becomes very difficult, if not impossible, to align restriction maps of distantly related species.

To obtain the best estimated phylogenetic relationships, future studies of closely related fungal species will require sequencing of several independent and rapidly evolving genes or noncoding loci (Tateno et al., 1982; Pamilo and Nei, 1988). The nontranscribed spacer region of the rDNA repeat evolves rapidly and may be a suitable region for further study (Jorgensen and Cluster, 1988; Schaal and Learn, 1988; Vilgalys and Gonzalez, 1990; Anderson and Stasovski, 1992). Fine restriction mapping or RFLP (restriction fragment length polymorphism) analysis of rapidly evolving genome segments, after DNA amplification, may be an alternative to sequencing (Vilgalys and Hester, 1990). Additionally, RFLP analysis with random, single-copy DNA probes can be used to study phylogenetic relationships in closely related fungi (Castle et al., 1987; Hulbert et al., 1988).

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