

## A phylogenetic reconstruction and emendation of *Agaricus* section *Duploannulatae*

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**Abstract:** *Agaricus* section *Duploannulatae* comprises the group of species allied with *A. bisporus* and *A. bitorquis*. Disagreement exists in the literature regarding the composition of this group. We used DNA sequence data from the ITS segments of the nuclear ribosomal DNA region, in a sample of European and North American isolates, to identify characters shared by this group, to further delimit species-level taxa within the section, and to develop a phylogenetic hypothesis. Shared polymorphisms that suggest a natural limit for section *Duploannulatae* were found. ITS1 data were assessed using parsimony, distance and maximum likelihood methods of phylogeny. The section *Duploannulatae* comprised six robust clades. Five clades corresponded to well characterized species from the temperate Northern Hemisphere (*A. bisporus*, *A. subfloccosus*, *A. bitorquis*, *A. vaporarius*, *A. cupressicola*). The sixth clade encompassed an *A. devoniensis* complex. Species concepts, nomenclature, and relationships are discussed and compared with prior reports.

**Key Words:** *Agaricus*, cultivated mushroom, champignon, phylogeny, systematics

### INTRODUCTION

A review of the twentieth-century literature on *Agaricus* L. : Fr. emend Karst. finds a diversity of opinion on the circumscription of natural infrageneric groups and on the relationships of species within and among the proposed groups (for a review see Cap-

PELLI 1984). The group of species most closely related to the economically important, cultivated species *A. bisporus* (Lange) Imbach is no exception (TABLE I). Many authors have accommodated the uncertainties surrounding infrageneric hierarchy in *Agaricus* by referring only to provisional 'groups,' rather than proposing or revising formal, ranked taxa.

There has been an increasing trend toward grouping species on the basis of biochemical features of the sporocarps: color changes, odor, spot-test reactions, and distribution of laccase and tyrosinase activities (Kerrigan 1986). While this appears to be the most sound of the traditional approaches to the classification of these species, it is not always consistently applied, and may have inherent limitations. A related problem is that these features are generally not observable in herbarium specimens. Morphological features are also of limited value as unifying characters in *Agaricus*; many are the result of single gene differences (Elliott 1979, Callac et al 1998a, b) or are subject to environmental influences (Kerrigan 1986), and could have arisen from convergent developmental tendencies.

The use of DNA sequence data permits the testing of pre-existing phylogenetic hypotheses and the formulation of new hypotheses that may have a more objective basis. We have begun a project with the ultimate goal of providing a phylogenetic framework for the entire genus *Agaricus*, in which well-documented species provide points of reference, and new taxa may be placed as opportunities arise. We are pursuing a 'tip-down' strategy, in which distal groups (species, sections) are defined and aggregated into a generic whole, rather than initially attempting a skeletal 'big tree' of the genus, tribe, or family. We are also investigating whether there is any phylogenetic signal at the population (e.g., amphioceanic) level.

We further appreciate, from prolonged exposure to the problem, how difficult field and even laboratory identification of fresh specimens of *Agaricus* can sometimes be. Misidentifications, even by experts, are hardly extraordinary in our experience. For this reason, we have sampled multiple collections for each species. Further, as we think that DNA sequences may ultimately provide the most reliable characters for *Agaricus* systematics, we have emphasized such characters in the discussions of the included species.

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TABLE I. Comparison of infrageneric classifications including *A. bisporus* and allied species

Reference & taxon:	Species <sup>a</sup> :	BISP	SFLC	SBPR	DEVO	BITQ	VAPO	BERN	Oth- er <sup>b</sup>
Moller 1950/ <i>Edulis</i> group		in <sup>c</sup>	in	out <sup>c</sup>	in? <sup>c</sup>	in	out	in	1
Heinemann 1978/subsection <i>Hortenses</i>		in	in	in	out	out	in	out	4
Cappelli 1984/section <i>Bitorques</i>		in	out	out	in	in	out	in	3
Wasser 1989/section <i>Duploannulatae</i>		in	in	in?	—	in	out	in	1
Challen <i>et al.</i> (this manuscript)/section <i>Duploannulatae</i>		in	in	in	in	in	in	out	1

<sup>a</sup> Species abbreviations: BISP = *A. bisporus*; SFLC = *A. subfloccosus s.l.*; SBPR = *A. subperonatus*; DEVO = *A. devoniensis*; BITQ = *A. bitorquis*; VAPO = *A. vaporarius*; BERN = *A. bernardii*.

<sup>b</sup> Number of other included species: in the present emendation, *A. cupressicola*. Authors including *A. bernardii* also included *A. maleolens*.

<sup>c</sup> in = species included in classification; out = excluded; in? = position ambiguous but apparently included.

The first segment of our project, reported here, examines the section *Duploannulatae* Wasser ex Wasser 1980. This group is more commonly referred to as section *Hortenses* (Heinem.) Bon. However, Wasser *et al.* (1976) were the first to publish a taxon (validated in Wasser 1980), at the sectional level, that (1) had a type [*A. bitorquis* (Quél.) Sacc.] properly included in the phylogenetically determined 'group of interest' while (2) excluding the type of the genus (*A. campestris* L.: Fr.). In representations of the section *Duploannulatae*, Wasser *et al.* (1976) and Wasser (1989) include the following descriptors: stipe with two rings, occasionally with one peronate; cheilocystidia narrow, cuspidate, clavate, spores globose-ovate; handled sporocarps are unchanged or stain pinkish/reddish; exposed flesh becomes pale-pink, pink, red, carmine-red; odor acid; cross reaction with Schaefer's reagent, as a rule, negative.

The most familiar species in section *Duploannulatae* is *A. bisporus* (often synonymized with *A. brunnescens* Peck), the commonly cultivated 'button mushroom' of western cuisine. Lange (1926) originally proposed two sister taxa (*P. hortensis* var. *subfloccosa* and var. *subperonata*) at the varietal level; these were later elevated to species rank (Lange 1939). However, beyond this trio, few additional close relationships have been consistently proposed (see TABLE I).

The utility of nuclear ribosomal sequences in resolving *Agaricus* taxa has been investigated by Mitchell and Bresinsky (1999) and Calvo-Bado *et al.* (2000). In this study we use ITS1, 5.8S, and ITS2 DNA sequences to propose a natural limit for section *Duploannulatae*. Below, we discuss sequence-level, morphological, biochemical, and other characters of the section. We also discuss each included species in the context of prior and current classification frameworks.

#### MATERIALS AND METHODS

*Sampling.*—Specimens of *Agaricus* were obtained as opportunities occurred, beginning in 1975. Cultures were preferentially prepared from explants of pileus tissue on agar-based media. In some cases, spore prints were made, or lamellae were air-dried, and cultures were made by germinating the spores on agar-based media. In most cases, after notes were taken and/or photographs were made, dried voucher specimens were deposited in herbaria. Samples included in this analysis are listed in TABLE II.

*ITS amplification & sequencing.*—Genomic DNAs for ITS amplification were prepared from mycelia and purified using QIAquick PCR spin columns (Qiagen Ltd, Crawley, UK) and the protocols described by Calvo-Bado *et al.* (2000). Alternatively, DNAs were purified using the CTAB mini-prep procedure of Zolan and Pukkila (1986), or with Chelex 100 (Biotechnology Grade; Bio-Rad, Hercules, California) via the following protocol: Five 3-mm-diameter plugs were cut from the periphery of an agar culture; the thin top layers harboring the mycelia were sectioned using a scalpel and transferred to a micro-centrifuge tube containing 1 mL of 3% w/v Chelex in 1 mM Tris-HCl (pH 8) and ca 200 mg of glass beads (0.1 mm dia.). Samples were subjected to three cycles of vortexing at room temperature for 1 min, freezing in liquid nitrogen for 1 min. and boiling in a water bath for 1 min. The final boiling was extended to 5 min before samples were transferred to 55 C for 30–40 min. After micro-centrifugation (11 000 × g, 5 min), a 100 μL aliquot of supernatant was recovered, without disturbing the Chelex resin. Supernatants were used directly for ITS amplifications or purified further using QIAquick PCR columns.

The internal transcribed spacer region (= ITS, comprising ITS1 + 5.8S + ITS2) of the nuclear rDNA was amplified using the ITS1extB and ITS4 extA primers and thermal cycling parameters previously defined (Calvo-Bado *et al.* 2000). Double stranded sequence was generated for ITS1, or in some cases the entire ITS region, using, as required, the primers ITS1, ITS2, ITS3, and ITS4 described by White *et al.* (1990). Cycle-sequencing reactions were performed using ABI PRISM<sup>®</sup> BigDye Terminator Cycle Sequencing

TABLE II. Origin of isolates in the ITS1 DNA sequence analysis

Species/Isolate	Origin area	Date	Collector <sup>d</sup>	Habitat	Isolation <sup>b</sup>	Collection
<i>A. bisporus</i>						
<i>var. bisporus</i>						
Bs364	France, Dinard	10/93	JC-PH	<i>Cupressus macrocarpa</i>	TC	INRA/CTC
Bs368	France, Dinard	11/93	JC-PH	<i>C. macrocarpa</i>	TC	INRA/CTC
Bs369	France, Dinard	11/93	JC-PH	<i>C. macrocarpa</i>	TC	INRA/CTC
Bs572	Greece, Crete, Zourva	04/98	IT-JG-PC	<i>Cupressus sempervirens</i>	VC	INRA
Bs584 <sup>c</sup>	Italia, Sicily, Manfria	12/97	M. Contu	<i>Acacia, Eucalyptus</i>	SSI	INRA
KP1 <sup>c</sup>	USA, MA, Boston area	09/99	K. Peterson	Unknown	SSI	ARP
C90	Commercial culture (Somycel A12)	—/—	M.P. Challen	Commercial grain spawn	VC	HRI
RWK1737 <sup>e</sup>	Canada, ONT, Willowdale	10/90	RWK	Parking lot	TC	RWK
RWK1885 <sup>e</sup>	Denmark, Copenhagen	10/92	RWK	Horse stable sweepings	TC	RWK
RWK1420	U.S.A., CA, Moss Beach	01/86	RWK	<i>C. macrocarpa</i>	TC	RWK
RWK1525	U.S.A., CA, Pt. Lobos	01/88	RWK	<i>P. * radiata/C. macrocarpa</i>	TC	RWK
RWK1547	Canada, ALTA, Banff	07/89	RWK	<i>Picea glauca</i>	TC	RWK
RWK1549	Canada, ALTA, Banff	07/89	RWK	<i>Picea glauca</i>	TC	RWK
RWK1629	Canada, ALTA, Banff	08/90	RWK	<i>Picea glauca</i>	TC	RWK
RWK1631	Canada, ALTA, Banff	08/90	RWK	<i>Picea glauca</i>	TC	RWK
I2	Israel	—	R. Kenneth	Unknown	SSI	ATCC 34840
I3	Israel	—/86	R. Kenneth	Unknown	SSI	RWK
I4	Israel, Palmachim	01/90	R. Kenneth	<i>Eucalyptus</i>	SSI	ARP
<i>var. burnethii</i>						
JB2	U.S.A., CA, Riverside	11/89	J. Burnett	<i>Prosopis</i>	MSI	ARP
JB3	U.S.A., CA, Riverside	12/90	J. Burnett	<i>Prosopis</i>	MSI	ARP
homothallic genet						
Bs423	France, Olonne-sur mer	11/94	JG-PC	<i>C. macrocarpa</i>	TC	INRA/CTC
Bs514	Greece, Larissa	01/97	IT	<i>C. sempervirens</i>	TC	INRA
<i>A. subfloccosus</i> complex						
lowland entity						
RWK1397	U.S.A., CA, Pt. Lobos	01/89	RWK	<i>C. macrocarpa</i> , trail	TC	RWK
Sf5	France, Dinard	10/92	PC	<i>C. macrocarpa</i>	TC	INRA
FS5 <sup>c</sup>	U.S.A., CA, San Francisco	03/89	F. Stevens	<i>C. macrocarpa</i>	TC	ARP
FS10	U.S.A., CA, San Mateo	02/90	F. Stevens	<i>C. macrocarpa</i>	SSI	ARP
FS13	U.S.A., CA	02/90	F. Stevens	<i>C. macrocarpa</i>	TC	ARP
WAT1	U.K., Scotland	—	R. Watling	Garden or park	SSI	ATCC 34843
highland entity						
SUB1	Switzerland ZH Bäretswit	10/90	R. Stadelmann	<i>Picea abies</i>	TC	INRA
RWK1542	Canada, AB, Banff	07/89	RWK	<i>Picea</i> , trail	MSI	RWK
RWK1552	Canada, AB, Banff	09/90	RWK	<i>Picea</i>	TC	RWK
RWK1553	Canada, AB, Banff	09/90	RWK	<i>Picea</i>	TC	RWK
RWK1441 <sup>c</sup>	U.S.A., CO, Denver	08/84	G. Pickett	Urban park, under lilacs	TC	RWK

TABLE II. Continued

Species/Isolate	Origin area	Date	Collector <sup>s†</sup>	Habitat	Isolation <sup>b</sup>	Collection
<i>A. devoniensis</i> complex						
Dv1 <sup>c</sup>	France, Ile d'Oléron	11/97	JG	Dune without trees	TC	INRA
Dv2 <sup>c</sup>	France, Soulac-sur-mer	11/97	JG	Dune without trees	TC	INRA
Dv6 <sup>c</sup>	France, Plouharnel	10/92	PB-PC-RWK	<i>C. macrocarpa</i>	TC	INRA
Dv7 <sup>c</sup>	Greece, Velestino	01/99	IT	<i>C. sempervirens</i>	TC	INRA
Dv8 <sup>c</sup>	France, Bordeaux	10/99	JG-PC-RWK	<i>Cupressocypris leylandii</i>	TC	INRA
RWK1800 <sup>c</sup>	U.S.A., CA, Pebble Beach	04/91	RWK	<i>C. macrocarpa</i> , roadside	MSI	RWK
RWK1899 <sup>c</sup>	U.S.A., CO, Mizpah Co.	07/95	RWK	<i>Picea</i>	TC	RWK
RWK1900 <sup>c</sup>	U.S.A., CO, Mizpah Co.	07/95	RWK	<i>Picea</i>	TC	RWK
PDD68573 <sup>c</sup>	NZ, Canterbury, Lincoln <sup>d</sup>	10/96	A. Mitchell	<i>C. macrocarpa</i>	VO	—
<i>A. bitortiquis</i>						
VR1	Nederland, Venray	10/96	RWK	Urban cement sidewalk	TC	RWK
K26	Commercial culture (Somycel K26)	1978	M.P. Challen	Commercial grain spawn	VC	HRI/ATCC 56055
RWK1379	U.S.A., CA, Goleta	12/85	RWK	Landscaped roadside	TC	RWK
RWK1389	U.S.A., CA, Goleta	01/86	RWK	Landscaped <i>Pinus</i>	TC	RWK
RWK1462 <sup>c</sup>	U.S.A., CA, Goleta	03/87	RWK	Landscaped area	TC	RWK
RWK1476	U.S.A., CA, Goleta	11/87	RWK	Landscaped area	TC	RWK
Ag4	U.S.A., IL [both parents]	—	GIAR	—	X	ATCC 24666
Ag9	Canada, ON	—	D.W. Malloch	Lawn	SSI	ATCC 56926
<i>A. vaporarius</i>						
Vp3 <sup>c</sup>	France, Villenave d'Ornon	06/00	M.M. Fernandez	<i>Pinus</i>	TC	INRA
RWK1701 <sup>c</sup>	Canada, ONT, Toronto Zoo	09/90	RWK	<i>Pinus</i>	TC	RWK
RWK1733 <sup>c</sup>	Canada, ONT, Willowdale	10/90	RWK	Sand, edge of parking lot	TC	RWK
<i>A. cupressicola</i>						
Cp1 <sup>c</sup>	France, Olonne-sur-Mer	11/92	JG	<i>C. macrocarpa</i>	TC	INRA
Cp2 <sup>c</sup>	Grèce, Larissa	01/97	IT	<i>C. sempervirens</i>	TC	INRA
<i>A. gennadii</i>						
Gn17 <sup>c</sup>	France, Quiberon	11/00	JG-PB	<i>C. macrocarpa</i>	TC	INRA
<i>A. bernardii</i>						
Bn1	France, Léognan	10/92	PC	Rabbit manure	TC	INRA
Bn2	France, Ile d'Oléron	10/99	G. Dupuis	<i>C. macrocarpa</i>	TC	INRA
ARP173 <sup>c</sup>	U.S.A., NY, Bronx	11/92	B. Wu	Open grassy area	TC	ARP
<i>A. campestris</i>						
WIH	England, York	—/84	—	Grassland	TC	HRI
<i>A. pattersonnae</i>						
RWK1415	U.S.A., CA, San Francisco	01/86	RWK	<i>C. macrocarpa</i>	TC	RWK

TABLE II. Continued

Species/Isolate	Origin area	Date	Collector <sup>a†</sup>	Habitat	Isolation <sup>b</sup>	Collection
<i>A. xanthodermus</i>						
W3I	England, East Preston	09/89	J.F. Smith	Unknown	TC	HRI
<i>A. arvensis</i>						
93.10	England, Bedham	10/93	R.H. Gaze	Composting grass clippings	TC	HRI

<sup>a</sup> IT = I. Theochari; JC = J. Callac; JG = J. Guinberteau; PB = P. Boisselet; PC = P. Callac; PH = P. Hérissou; RWK = R. W. Kerrigan; CIAR = Campbell Institute for Agricultural Research.

<sup>b</sup> TC = Tissue Culture; SSI = Single Spore Isolate; VC = Vegetative Culture; MSI = Multi Spore Isolation; VO = Voucher only, no culture; X = hybrid formed between local wild strains.

<sup>c</sup> Originally determined as *A. bisporatus* by Contu.

<sup>d</sup> Originally determined as *A. subperonatus* by Mitchell.

<sup>e</sup> Isolates for which full-length sequences were obtained and analyzed.

Ready Reaction kits (Applied Biosystems, Perkin-Elmer Corp., North Warrington, UK) as previously described (Calvo-Bado et al 2000). Sequencing gels were run through facilities at the University of Durham UK, at HRI-Wellesbourne, or at the Pennsylvania State University. All reads for each sequence were then manually inspected and assembled using the SeqManII module of the Lasergene software package (DNASTar Inc., Madison, Wisconsin). Primer sequences were excluded from the amplified ITS product sequences. For the ITS1 region 13 bases from the 3' end of the 18S rDNA gene and 29 bases from the 5' end of the 5.8S rDNA gene were retained. The full-length ITS sequences also included the entire 5.8S gene and 37 bases at the 5' end of the 25S rDNA gene. In a few cases, we obtained only single strand data for short regions within the highly conserved 18S or 25S genes; these agreed with double-strand data for conspecific isolates. When length heterogeneity was present in a single isolate we used the longer sequence. 'Ambiguous' bases that we report in a few species were due to reproducible single-base character heterogeneities; heteroallelism is one conventional explanation. In summary, 63 ITS1 and 28 full-length ITS sequences were produced (TABLE II).

Three species from other traditionally accepted sections of the genus were selected as outgroups: *Agaricus arvensis* J. C. Schaeffer: Fr., *A. campestris*, and *A. pattersonnae* Peck (sections *Arvenses*, *Agaricus*, and *Sanguinolenti*, respectively). In diverse sequence analyses those three species were most often most distant from *Duploannulatae*. *Agaricus xanthodermus* Genevier was included but not given outgroup status when its proximity to *Duploannulatae* was revealed through our analyses. *Agaricus gennadii* (Chatin & Boudier) Orton and *A. bernardii* (Quélet *apud* Cooke & Quélet) Saccardo were also included as possible members of section *Duploannulatae*.

*ITS data analysis.*—The MegAlign package (Lasergene v4.05–6) was used to prepare multiple sequence alignment files (MSF) via the Clustal V algorithm (Higgins and Sharp 1988) and to calculate pairwise distance or similarity values (as percentages) for 63 ITS1 products. Clustal alignments were manually adjusted in 3 short (2–4 bp) regions where overlapping nucleotide gaps (= 'indels') were evident. The adjustments were performed so as to produce the shortest alignments, the minimum number of character states summed over positions and, where alternatives were equal by those measures, the fewest unique gaps. Ultimately one ambiguously aligning region of 3 bp was excluded. The 19 indels were appended to the MSF as coded binary characters (Hibbett et al 1995) using either of two models. In the first, unique gaps were considered to be contiguous and non-overlapping. In the second, unique gaps overlapped. These models of gap evolution are considered to be conservative and yield the minimum number of evolutionary events necessary to account for the observed gaps. The resulting ITS1 data sets were primarily evaluated using the maximum parsimony criterion in PAUP\* (v 4.0b8; Swofford 2000) with the following heuristic search settings: random addition sequence, one tree held at each step during stepwise addition using the tree-bisection-reconnection algo-

rithm, branch collapsing if maximum branch length was zero, gaps treated as 'missing', step-wise descent option not in effect, topological constraints not enforced, bootstrapping ( $\eta = 10\ 000$ ) and maxtrees = 100. Alternative phylogenies were developed using the Phylogeny Interface Environment at the UK Human Genome Mapping Project Bioinformatics Resource Centre (HGMP-RC; Rysavy et al 1992) and algorithms from the PHYLIP (Felsenstein 1993) or TreePuzzle (v5.0; Schmidt et al 2000) packages. MSF alignments were bootstrapped ( $\eta = 100$ ) and jumbled ( $\eta = 10$ ) using Seqboot; parsimony analysis was conducted using DNAPars and Consense. Alternatively with TreePuzzle, the ITS1 MSF files were analysed using the quartet maximum-likelihood method (Strimmer and von Haeseler 1996).

The smaller set of 28 full-length ITS sequences (TABLE II) was also aligned as described above. The resulting MSF was used to identify and evaluate diagnostic polymorphisms useful in circumscribing varietal, species, and sectional taxa.

*Nucleotide sequence and MSF accession numbers.*—The ITS1 sequences used in this study are available within the EMBL/GenBank databases under accession numbers AF432877–432904, AJ418715–418776, and AJ419899. The ITS1 alignment file is available in the EMBL-Align database as accession ALIGN.000242. The full length ITS files have accession numbers AF432880–AF432904 and their alignments are available in TreeBase under SN1000. MSF, indel coding, data and various tree files from the various ITS analyses are available for reference at <http://members.lycos.co.uk/mchallen> and/or <ftp://ftp.hri.ac.uk> (username/password: mushroom).

## RESULTS

All the phylogenetic analyses used in this study provided strong support values for a clade corresponding to section *Duploannulatae* (FIGS. 1 and 2). The trees were less well resolved with respect to the relatively more basal groups. The analyses also revealed six robust clades within the *Duploannulatae*.

Five well-defined species were resolved as clades with strong bootstrap support in PAUP\* parsimony analysis (FIG. 1). These were *A. bisporus* (78%), *A. subfloccosus* (J. Lange) Pilát *s.l.* (95%; where the two incipient species (Kerrigan et al 1999) were themselves clearly resolved with 64% support), *A. bitorquis* (99%), *A. vaporarius* (Persoon) Cappelli (90%), and *A. cupressicola* Bon & Grilli (100%). The remaining clade, with 93% support, comprised a less well resolved complex including *A. devoniensis* (Wakefield & Pearson) Orton and the New Zealand collection PDD68573 that was originally reported as *A. subperonatus* (J. Lange) Singer. This latter clade/complex of apparent morpho-species is more clearly resolved when ITS2 data are used (Kerrigan unpubl).

We obtained strict consensus trees that were congruent at and above the species level regardless of

how gap data were coded, or whether they were included at all. A phylogenetic representation based on the PAUP\* parsimony analysis using non-overlapping gap-character coding is presented in FIG. 1. Bootstrap support for taxa branch points was high and ranged between 81–100% in all cases (excluding potential, unpublished taxa within the *A. devoniensis* complex).

Separation of the 55 *Duploannulatae* samples into six clades was further supported by the alternative PHYLIP and TreePuzzle analyses. Using DNAPars (data not shown), tree topology was remarkably consistent with the PAUP\* analysis. Bootstrap support ranged from 84–100% for all six *Duploannulatae* clades. Using DNAPars, polytomy and paraphyly were again evident among the *A. devoniensis* and '*A. subperonatus*' collections. The maximum likelihood analysis (FIG. 2) provided further support for the six *Duploannulatae* clades with support values ranging from 51–99%. As with other analyses, TreePuzzle indicated that the *A. devoniensis* might comprise a complex of taxa.

Within the ITS regions, the section *Duploannulatae* appears to be delimited by a number of distinctive sequence polymorphisms, described below. This group is difficult to circumscribe using any other single character. *Agaricus bitorquis* seldom exhibits rufescent context. *Agaricus cupressicola* (as do some specimens of other included taxa) has a pendant rather than subperonate or peronate veil. Rufescence and peronate veils are found outside of the section; for example, *A. bernardii* exhibits both. While there is a tendency in this section toward short-statured, broad-capped sporocarps with inrolled pileus margins, this habit is neither universal among nor consistent within included taxa, nor is it absent among excluded taxa. Below we provide new characters to delimit the included taxa, and commentary on various problems and features of the species.

Distance analysis indicated that outgroup species were appropriately distinct from the *Duploannulatae*. Outgroup sequence identities with the section *Duploannulatae* were as follows: *Agaricus arvensis* 84.9–87.2%, *A. campestris* 86.3–88.3%, *A. pattersonae* 86.9–89.2%. Among pairs of isolates within *Duploannulatae*, sequence identities ranged from 92.2% for pairings between *A. cupressicola* and RWK1379, and up to 100% within taxa. It was interesting to note that *A. xanthodermus* shared relatively high identity with section *Duploannulatae*, e.g., with JB3 (89.8%) and Vp3 (90.7%). Other ITS data (unpubl) from section *Xanthodermatei* Singer also indicate that this section is proximate to but distinct from *Duploannulatae*; this unexpected result merits further investigation. *Agaricus bernardii* (87.8–90.1%) and *A. gennadii* (86.6–88.9%) were relatively more dissimilar to section *Du-*

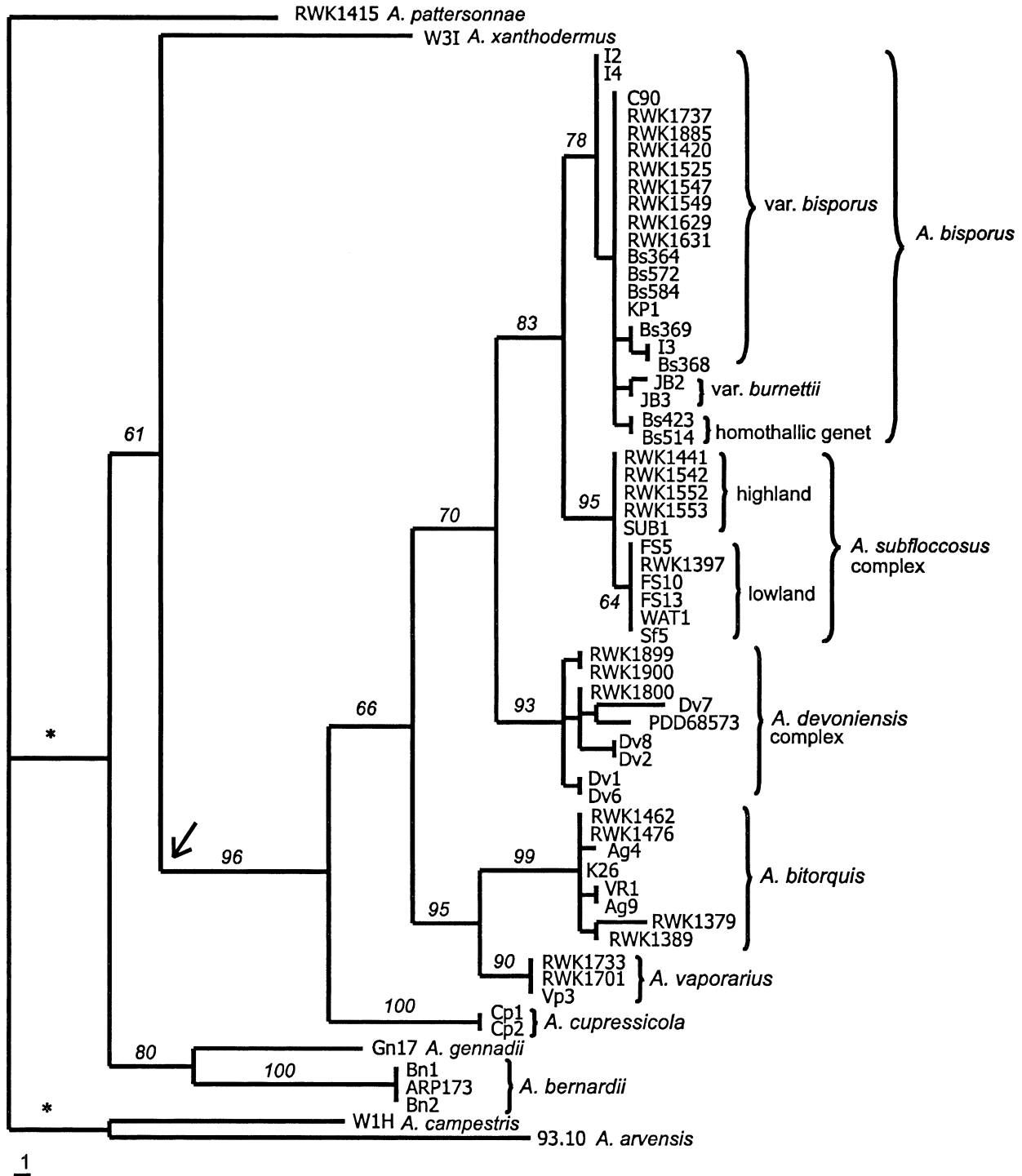


FIG. 1. Phylogeny of *Agaricus* section *Duploannulatae* inferred from ITS1 sequences. A single most-parsimonious tree was found using PAUP\* maximum parsimony criterion (zero length branches were collapsed) with three nominated outgroup species: *A. campestris*, *A. pattersonnae*, and *A. arvensis*. Non-overlapping unique gap character data were included. Bootstrap values (% of 10 000 replications) are shown in italics; branches on the MP tree that had less than 50% support are indicated by asterisks. Arrow indicates the branch defining section *Duploannulatae*. Unresolved terminal branches result from identical ITS sequences. Scale bar indicates tree-building steps. The following index measures were determined for this tree: Length = 192 steps; CI = 0.724; RI = 0.907; RC = 0.657; HI = 0.276. Bs584 is *A. bisporatus* and is conspecific with *A. bisporus*. PDD68573 is the New Zealand collection tentatively identified as *A. subperonatus*.

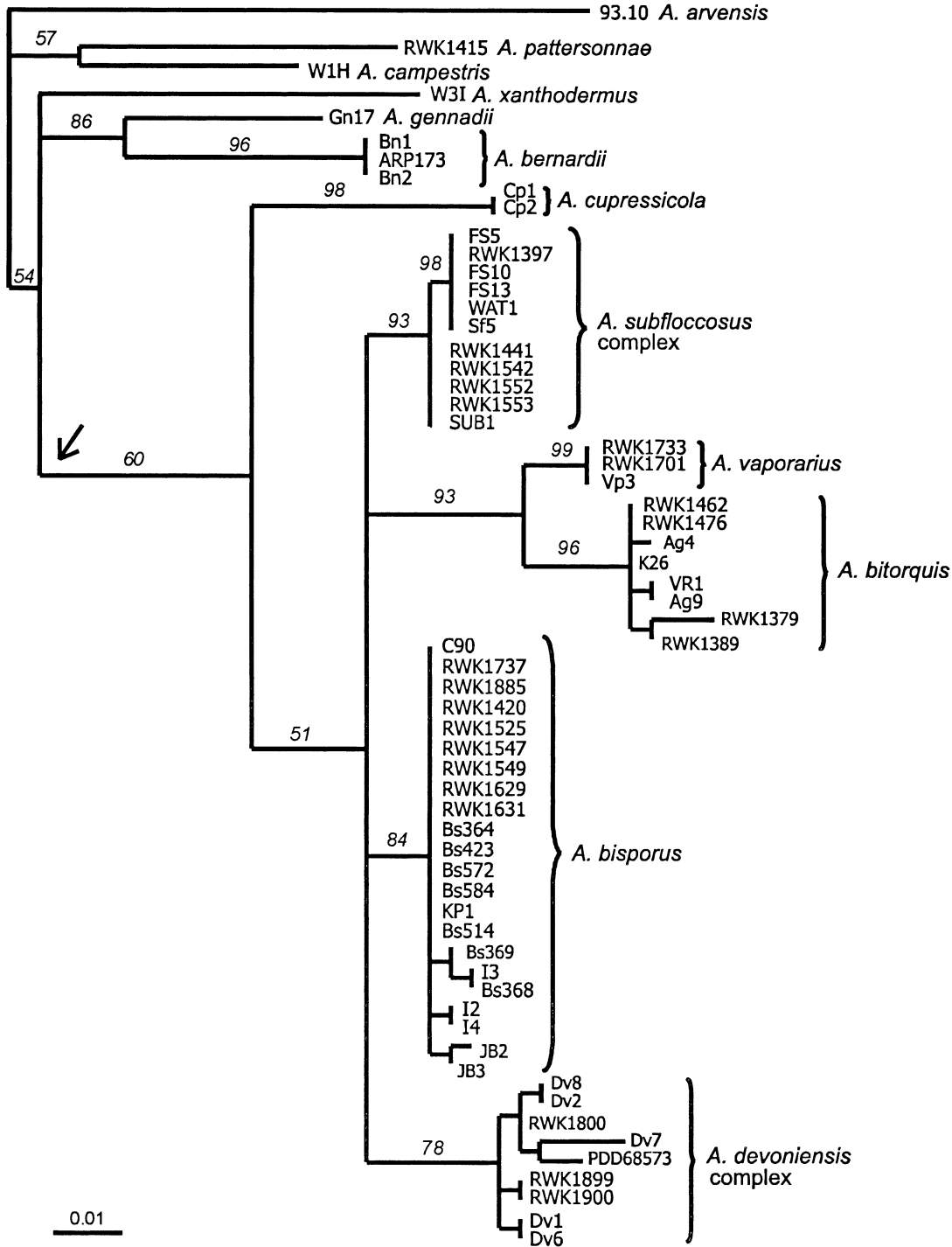


FIG. 2. Quartet puzzling tree with maximum likelihood branch lengths for ITS1 sequences within *Agaricus* section *Duploannulatae*. Branch lengths were computed using the HKY model (Hasegawa et al 1985) for substitution and rate heterogeneity. Support values (%) for 25 000 puzzling steps are shown in italics. Arrow indicates the branch defining section *Duploannulatae*. Scale bar indicates number of substitutions per site. Nominated outgroup species was *A. arvensis*.

*ploannulatae*, and formed a branch basal to the *Xanthodermatei-Duploannulatae* clade. Whether there is a monophyletic group of species comprising *A. bernardii*, *A. gennadii*, and perhaps other species, and

what molecular, morphological, or other characters might unify such a group, are questions to be addressed in further studies.

We note that we evaluated sequences provided by

A. Mitchell, or generated by us from DNA provided by Mitchell, or deposited in GenBank and reported on by Mitchell and Bresinsky (1999). While we have observed considerable congruence between corresponding regions of their ITS2+28S[pp] rDNA tree and our own ITS-based trees (unpubl), a few placements merit comment. Their '*A. impudicus*' and '*A. spissicaulis*' grouped respectively within our collections of sequences from *A. bitorquis* and *A. bisporus*. Both the specimens and the sequences (which only partially overlap ours) merit further study.

*Taxa, circumscription, phylogeny, and commentary.*—Position data given below are based on a full length ITS alignment space of length 726 bp, which was determined from a Clustal alignment of all *Agaricus* sequences produced in this study. Characters that were unique (within our sample) to all representatives of an individual taxon are indicated by uppercase italics and are given with flanking sequence on both sides, to facilitate comparisons of sequences in different alignments. Characteristic deletions are shown with an integer representing their length (i.e., -n-); +x+ indicates an insertion.

#### TAXONOMY

*Delimitation of section.*—*Duploannulatae*. *Characteristic ITS polymorphisms*: cttt(k/-)tCaggta @ 120–121, at(r)tAag(r)a @167, ttat(Y)atac @311, attaTattc @473, c(y)c(y)(R)atac @488, (y)cgtCtgcg @580, and mag[-8]gaca @663–670.

*Biochemical features.* In most species, the context is rufescent when injured or exposed, and the presence of tyrosinase can be demonstrated, for example, by a purple reaction with *o*-toluidine (however, these features are absent or atypical in *A. bitorquis*, the type of the section, in contrast to Wasser's circumscription of *Duploannulatae*). KOH and Schäffer's reaction are negative. Odors are 'mushroomy' (i.e., like the cultivated *A. bisporus*), never clearly (or primarily) like brine, phenol, or almond/anise extracts.

*Morphological features.* Veils of several species tend to show peronate or subperonate development. Pileus margins are usually strongly inrolled. Pileus diameters often approach the length of the stem. Lamellar margins are usually sterile (cheilocystidia are present). Spores lack an apical pore. No toxic species are known from this section. Many species from section *Duploannulatae* can grow and reproduce on compost prepared for commercial *A. bisporus*. Their life cycles are quite variable: homothallism for *A. subfloccosus*, heterothallism for *A. bitorquis* and *A. devoniensis*, amphithallism and pseudohomothallism for *A. bisporus* var. *bisporus*. The features detailed above are

quite distinct from the *Xanthodermatei*, the apparent sister group to *Duploannulatae*, where KOH and Schäffer's reactions are positive and the *o*-toluidine reaction is universally blue rather than purple, the species are flavescent rather than rufescent and exhibit characteristic phenolic odors. Many species from *Xanthodermatei* are toxic and their sporocarps are mostly erect, with pendant annuli.

*Taxa included in section Duploannulatae*

*Agaricus bisporus* (J. E. Lange) Imbach 1946. Mitt. naturf. Ges. Luzern 15:15.

= *Psalliota hortensis* Cooke emend. Lange var. *bispora* Lange 1926. Dansk Bot. Arkiv 4(12):8.

*Key synonyms*: *A. campester majusculus* Peck 1912, *A. hortensis* (Cooke) Pilát 1951a, *A. bisporatus* Contu 1993, [*A. brunnescens* Peck 1900?]

*Characteristic ITS polymorphisms*: tctg[-2-]atgt @637–638.

*Discussion.* Jacob Lange (1926) was among the first to call attention to the bisporic basidia present in members of European (and most other) populations of this species (see also Atkinson 1906). Previously, this widely cultivated mushroom had most often been called *A. campestris* L.: Fr., an error that occasionally persists to this day in textbooks, medical studies, and other non-specialist literature. *Agaricus campestris*, a tetrasporic species, is difficult if not impossible to cultivate, and has biochemical and micromorphological features (the absence of cheilocystidia, the presence of an apical pore on the spores, etc.) that clearly distinguish it from *A. bisporus* and section *Duploannulatae*.

An early American synonym of *A. bisporus* is *A. campester majusculus* Peck (Peck 1912). Examination of the holotype (NYS), which includes good photographs, revealed no points of distinction. Spores of the type averaged  $7.1 \times 5.6 \mu\text{m}$ , within the range of *A. bisporus*.

The best-known proposed synonym is *A. brunnescens* (Peck 1900, Isaacs 1967, Malloch 1976). While Peck's type appears to belong to section *Duploannulatae*, unusually small spores and aberrant basidial morphology, discussed by Kerrigan (1987), lead to some reservations about accepting the proposed synonymy with *A. bisporus* unequivocally. A call for conservation of the epithet *bisporus* has been made, but not yet acted upon (Edwards 1990). A neotype should be selected for *A. bisporus*.

We have found no evidence to support the existence of bisporic species distinct from *A. bisporus*. *Agaricus hortensis* (Cooke) Pilát appears to be applied to pallid individuals of *A. bisporus* (Pilát 1951a, Essette 1964, Moser 1983). Alleles for white or pale brown pilei have been found in five of five studied

populations, at various frequencies; the genetics of this trait is understood, at least with respect to the primary locus and the most common phenotypic effects (Callac et al 1998b, Kerrigan 2000). Material of *A. bisporatus* Contu (Contu 1993), authenticated by Contu, has been cultivated and studied by us. ITS sequence data confirm our impression that Contu's species is conspecific with *A. bisporus* (FIG. 1). This was also supported by other morphological studies made on this material in cultivation and by genetic studies with alloenzymatic markers (Callac et al 2000).

We were not able to distinguish between American and European isolates of *A. bisporus* var. *bisporus* based on ITS1 data. However, we were able to distinguish the two following tetrasporic infraspecific entities:

(1) *A. bisporus* var. *burnettii* Kerrigan & Callac (Callac et al 1993), which forms what is apparently a geographically isolated population in the extreme southwest of the USA (the extent of its range is unknown), and which is distinguished by a combination of tetrasporic basidia and predominantly heterothallic reproduction. Within the species *A. bisporus*, the var. *burnettii* appeared to be delimited by a single G>A substitution; cttAaatg @261.

(2) the rare homothallic tetrasporic genet known from France and Greece (Callac et al 1998a, 2000). In the two strains sequenced, a single T deletion, cttt[-3]tcag @118, delimited the homothallic genet from the consensus *A. bisporus* cttt[-2]Ttcag.

In some analyses, there were indications that the southeastern Mediterranean population tended to have the most divergent sequences (see I2, I4, FIG. 1).

***A. subfloccosus*** (J. E. Lange) Pilát 1951a. Acta Musei Nat. Pragae VIIIB(1):49.

= *P. hortenses* Cooke emend Lange var. *subfloccosa* Lange 1926. Dansk Bot. Arkiv 4(12):8.

*Key synonyms*: sometimes confounded with *A. subperonatus*. See also *A. cappellianus* Bohus.

*Characteristic ITS polymorphisms*: tggaCtctt @217, ccct[-1]gctt @539, and acaaAttct @676.

*Discussion*. This species was first proposed as a variety of *Psalliota hortensis*, then elevated by Lange (1939) to species rank. Our data indicate that these two species are indeed closely related, and can be considered to be 'sister species'. Our ITS data further support the contention of Kerrigan et al (1999) that *A. subfloccosus* itself comprises two 'sister' entities (putative species) that are phylogenetically distinct (FIG. 1) see also atgt[C/T]attg @267 (where C = highland and T = lowland). Problems of nomencla-

ture and typification were discussed in Kerrigan et al (1999).

Cappelli (1984) placed this species among the 'fusco-fibrillosus group' of section *Sanguinolenti*. His concept was somewhat elastic (compare his plates 26 and 26 bis.) and might possibly have been based on heterogeneous material. Our experience of the 'highland' and 'lowland' entities is of a pair of fairly homogeneous organisms. ITS trees support this conclusion and the inclusion of *A. subfloccosus* in *Duploannulatae*.

P. Boisselet (pers comm) has suggested that the montane entity studied by Kerrigan et al may be *A. cappellianus* (Bohus 1993, 1995). However, Bohus placed this species in section *Sanguinolenti* 'gruppo Fusco-fibrillosi'. We suspect that the species described by Bohus and studied by Cappelli is not closely related to *A. subfloccosus*; ITS sequence data, when available, should clarify the relationship.

***A. devoniensis*** (Wakefield & Pearson) Orton 1960. Trans. Br. Mycol. Soc. 43(2):173

= *P. arenicola* Wakefield & Pearson 1946. Trans. Br. Mycol. Soc. 29:205;

= *A. arenophilus* Huijsman 1960. Persoonia 1(3):324

*Characteristic ITS polymorphisms*: cctgCctgg @72, tgagTgaag @131, ctctGatc @488, and gtctGagga @660.

*Discussion*. *A. devoniensis* sporocarps are usually smaller than those of *A. bisporus*. They are found in sand dunes without trees, or under *Cupressus macrocarpa* or *C. sempervirens*, along the coast of Western Europe (France, England, Denmark, Italy, Greece) and California (Monterey Co.). Cappelli (1984) provides a review of synonymy and suggests that *A. littoralis* (Wakefield & Pearson) Pilát (1951b) may be another synonym.

Similar species deserve critical study. *Agaricus devoniensis* has spores 5–7 × 4.5–5.5 µm, in agreement with our own observations on Californian (avg. 6.0 × 5.1 µm) and French (avg. 6.9 × 5.5 µm) material (both of which we have cultivated). *Agaricus gennadii*, which is excluded from section *Duploannulatae* (FIG. 1), is known from coastal Southern and Western Europe (Cypress, France, England); it has spores (7–)8–10(–11) × (5–)6–7 µm. The *A. gennadii* material studied by us had such spores (avg. 9.2 × 6.1 µm). *Agaricus cellaris* (Bres.) Konrad & Maublanc has spores 8–13 × 6–8 µm. Orton (1960) placed the latter two species in synonymy and Cappelli (1984) agreed. However, this would be a very broad range of spore sizes for an *Agaricus* species. *Agaricus gennadii* as described and figured by Courtecuisse and Duhem (1994) would be difficult to distinguish from *A. devoniensis* based on macroscopic features; the fi-

nal form of the veils is quite likely to be variable due to environmental influences. The French collections from dunes intermingled, with respect to ITS sequence affinities, with those collected under *Cupressus*. It is noteworthy that the two *A. devoniensis* collections from high elevations in Colorado could be uniquely distinguished by *gtgaTaaca* @185 and were separable from other *A. devoniensis* collections by *actcActtg* @540. The Colorado collections appear to belong to a lineage sufficiently distinct to merit taxonomic recognition at some level, pending further study (Kerrigan unpubl).

The data provided by Mitchell and Bresinsky (1999) suggest that closely related taxa exist in Australasia (see also *A. subperonatus*, below). Also in this *devoniensis* clade, on a branch immediately basal to those of the other sequences, was an Australasian *A. devoniensis* [Bresinsky 1995 Tasmania] (based on an ITS2 sequence in GenBank). Which species can be successfully delimited within the '*A. devoniensis* complex' is a good question for further study.

***A. subperonatus*** (J. E. Lange) Singer 1951. *Lilloa* 22: 432.

= *P. hortensis* Cooke emend. Lange var. *subperonata* Lange 1926. *Dansk Bot. Arkiv* 4(12):8.

Although this species is widely reported in Europe, we have been unable to locate authentic material for ITS sequence analysis or cultural studies. Cultures and sequences we have studied have been consistent with those of *A. bisporus*, *A. subfloccosus* (lowland type), and the *A. devoniensis* complex. Lange (1926) placed *A. subperonatus* as a sister taxon to taxa that later became *A. bisporus* and *A. subfloccosus*. However, other authors have placed it closer to *A. vaporarius* (Möller 1950, Essette 1964, Cappelli 1984), or in heterogeneous groupings (Moser 1983). Descriptions of the species are constant regarding the radially fibrillose character of the brown pileipellis. The sources above, and Pilát (1951a), Bon (1988) and Courtecuisse and Duhem (1994) provide descriptions and figures. It is curious to have not encountered this widely reported species in Europe or North America.

The New Zealand collection (PDD68573) of Mitchell generally agrees with the particulars of the species; it has a radially fibrillose dark brown pileus cuticle, a peronate veil, and spores averaging  $6.4 \times 5.1 \mu\text{m}$ . However the ITS sequence groups among the *A. devoniensis* sequences, and the spore size is also consistent with the latter species. The cheilocystidia of PDD68573 are narrow and semi-cylindrical, about  $17\text{--}18.5 \times 6\text{--}7.5 \mu\text{m}$ , consistent with *A. devoniensis* (and *A. litoralis*) but not with *A. subperonatus* (at  $32\text{--}60 \times 9\text{--}13 \mu\text{m}$ , per Möller 1950). We need to study

more material of these entities before proposing circumscriptions or placements for them.

***A. bitorquis*** (Quélet) Saccardo 1887. *Syll. Fung.* V: 998.

= *P. bitorquis* Quélet 1883. *Assn. Fran. Avanc. Sci.* p. 500

*Key synonyms:* *A. edulis* (Vittadini) Möller & Schäfer 1938; *A. rodmani* Peck 1894.

*Characteristic ITS polymorphisms:* *gcacTtttt* @102, *cagtTtacc* @144, and *taccCttga* @205.

*Discussion.* This species provides the best-known example of a peronate veil, adorning the lower stipe and sometimes forming a volva, which arises from a combination of basal/near-basal origin, apical dehiscence, and/or greater elongation of the upper stipe. The species is cosmopolitan and prefers sites impacted by human activity, such as roadsides and hard-packed soils. It is cultivated in some tropical areas (e.g., India). Some tropical populations exhibit partial reproductive isolation from temperate Northern Hemisphere populations (Martinez Carrera et al 1995). Based on the ITS data, its closest relative may be *A. vaporarius*. *Agaricus bitorquis* is unusual in the section for exhibiting no rufescence of the cuticle or context; neither does it become purple with the *o*-tolidine reagent (Kerrigan 1986). Of interest, therefore, is *Agaricus bitorquis* (Quélet) Saccardo var. *validus* (Möller) Kerrigan comb. nov. [= *P. edulis* (Vitt.) Buchw. var. *valida* Möller 1950. *Friesia* 4:14], which becomes "vivid flesh color ('Buff Pink')" when broken (Möller 1950). We have not obtained material of this variety for study. On the other hand, the rufescent *A. bernardii*, which has peronate veils and otherwise resembles *A. bitorquis*, is not, based on ITS data, a close relative, nor even a member of section *Duploannulatae*.

***A. vaporarius*** (Persoon) Cappelli 1984. *Agaricus*. Saronno, Italy: Libreria editrice Biella Giovanna.

*Key synonyms:* An old taxon, sometimes identified with others (e.g., *A. villaticus* Brond.).

*Characteristic ITS polymorphisms:* *tgaacTatg* @19, and *aagtAgtca* @137.

*Discussion.* This is a robust species of Europe and northeastern North America (Michigan, Southern Ontario). The figure of '*A. pattersonae*' in Smith (1971) is probably this species; *A. pattersonae* is a distinctive species placed in section *Sanguinolenti*, known from *C. macrocarpa* groves in coastal California (Kerrigan 1979). In Toronto and in Copenhagen *A. vaporarius* grows under pines in landscaped areas. We have observed a copious growth of a zygomycetous fungus on over-mature lamellae of sporocarps from both locations, but have never noted it on other species of *Agaricus*. Based on ITS data, *A. vaporarius* may be most closely related to *A. bitorquis*; notably,

Smith (1971) compared aspects of the two species. It should also be noted that the name *Agaricus vaporarius* (Persoon) Cappelli 1984 may require conservation against *A. vaporarius* Schrank 1789 (a species of *Coprinus*; cf. Wuilbaut 2000).

The collection from France and the two collections from Toronto had the same ITS sequence.

**A. cupressicola** Bon & Grilli in Bon 1987, Documents Mycologiques XVII(67): II.

*Key synonyms*: none.

*Characteristic ITS polymorphisms*: aagcGgtgc @189, cttttTctgt @226, tgtaGagga @503, and aggaTtacc @710.

*Discussion*. This species morphologically differs from all other species of the section *Duploannulatae* by the following combination of characters: a pileus that is uniformly livid brown-grey and globose to subcampanulate when young, a pileus diameter smaller than the length of the stem, conferring a slender silhouette, and a relatively strongly rufescent context and a pendant veil, which led previous authors to place it in the section *Sanguinolenti* (Grilli 1988). *Agaricus cupressicola*, recently described for specimens collected in Italy under *C. sempervirens*, appears to be relatively frequent in France under *C. macrocarpa* along the Atlantic coast (Guinberteau et al 1998); it is also found under *Juniperus phoenicea* in Sardinia and under *Taxus baccata* in Belgium (Contu 1992). We note that the clade corresponding to this species seems absent, or has not yet been found, in North America, while the five other robust clades of the section *Duploannulatae* are represented on both continents.

*Taxa of undetermined affinities, similar to members of section Duploannulatae*.—Other taxa with peronate veils, such as *A. cellaris* (Bresadola) Konrad & Maublanc, *A. pequinii* (Boudier) Konrad & Maublanc, *A. maleolens* Möller, and *A. vinaceovirens* Kerrigan are expected to have greater affinities to *A. bernardii* and *A. gennadii* than to section *Duploannulatae*. Boisselet (1988) tentatively placed *A. boisseletii* Heinemann in section *Hortenses*, but our (unpubl) sequence data on this species exclude it from *Duploannulatae*. We have similarly excluded *A. spissicaulis* Möller. It will be interesting to place *A. bresadolianus* Bohus and *A. romagnesii* Wasser within the phylogenetic framework of *Agaricus*.

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