Ascobotryozyma cognata sp. nov., a new ascomycetous yeast associated with nematodes from wood-boring beetle galleries

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A new species of Ascobotryozyma, A. cognata sp. nov. (anamorph Botryozyma cognata), was isolated from beetle galleries in Idaho, USA. A. cognata was found on the surface of free-living nematodes, Panagrellus dubius, collected from galleries created by the long-horned beetle Saperda calcarata in Populus (aspen), and the weevil Cryptorhynchus lapathi in Salix (willow). A. cognata isolates were collected from similar habitats and in relatively close proximity to those of A. americana, the only species described from North America. The recognition of A. cognata as a distinct species was supported by morphological and molecular data. Thallus cells of A. cognata were significantly shorter than those of A. americana. Low DNA reassociation values, notably different randomly amplified polymorphic DNA (RAPD), inter-sequence simple repeat (ISSR), and amplified fragment-length polymorphic (AFLP) fingerprints, and sequence divergence in both the D1/D2 domain of the nuc-LSU rDNA and an additional unidentified region were all consistent with the recognition of a new species.

INTRODUCTION

Botryozyma is an anamorphic ascomycetous yeast genus characterized by its unique thallus morphology, which features determinate growth and a branched basal cell that attaches to nematodes (Smith et al. 1992). The genus contains two species, B. nematodophila and B. americana, both found attached to free-living nematodes of the genus Panagrellus. The type species, B. nematodophila, was discovered in Verona, Italy, from the disease complex sour-rot of grapes, vectored by Panagrellus redivivooides nematodes associated with Drosophila spp. (Shann 1987). No teleomorph has been reported for B. nematodophila. Ascobotryozyma americana, the teleomorph of B. americana, was discovered on P. dubius nematodes from beetle galleries in Populus tremuloides Michx. (trembling aspen) in eastern Washington (Kerrigan et al. 2001). The sexual state is characterized by globose asci bearing four lunate ascospores; ascii develop directly from thallus cells after two or more thalli have fused (Kerrigan et al. 2001). A. americana differs from B. nematodophila in its distribution, habit, nematode host, cell sizes, DNA reassociation values, and inability to grow at 37 °C (Kerrigan et al. 2001).

The fungus has a commensal relationship with the nematodes, apparently taking nutrition from the nematodes’ environment rather than the nematode itself. Thalli are attached to the nematode cuticle by branched cells that do not penetrate the cuticle (Kerrigan et al. 2001). Thallus cells of the anamorph alone are more commonly encountered and can be grown in culture. Asci, when present, are only produced shortly after isolation and cannot be maintained in pure culture; ascospore germination has not been observed (Kerrigan et al. 2001).

Additionally Ascobotryozyma isolates were obtained from beetle activity in Populus and Salix species in western Idaho, relatively close to where A. americana was found. The objectives of this research were to investigate the genetic relationships among A. americana isolates from Washington and Ascobotryozyma isolates from Idaho. The new species Ascobotryozyma cognata is described based on morphological, molecular, and physiological data.

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MATERIALS AND METHODS

Fungal isolation and culture conditions

Ascobotryozyma isolates were collected in eastern Washington and western Idaho (Fig. 1) from Populus and Salix species showing signs of beetle infestation. Infestations were identified by the presence of expelled frass and/or oozing slime near beetle entrance sites. Wood plugs were extracted from within and adjacent to entrance sites with the aid of an increment hammer; associated frass also was collected. To sample from galleries, trees with heavy infestations were felled and split, and wood and frass were collected. Samples were plated onto 2% corn meal agar (CMA; Difco, Sparks, MD) acidified with lactic acid and maintained at ambient room temperature (22–25°C). Plates were examined daily under a compound microscope and serial transfers of nematodes bearing Ascobotryozyma thalli were made until cultures were free of contaminants except for organisms necessary for the nematodes’ nutrition, mainly other yeasts. Pure cultures were obtained by transferring thalli to plates of potato dextrose agar (Difco) with 0.05% (w/v) yeast extract (PDYA). Single thallus isolates were generated by streaking out thalli and transferring a single thallus to PDYA. Specimen numbers, isolation information, and accession numbers are listed in Table 1.

Morphological examinations

Thallus dimensions from ten isolates from North America, including at least one representative isolate from each collection site (CBS 8461, CBS 8560, CBS 8706, CBS 8707, CBS 8708, CBS 8709, CBS 8750, CBS 8752, CBS 8753, and CBS 8754), and both isolates from Italy (CBS 7426, and CBS 7442) were measured. Some of the isolates produced the teleomorph after being plated, but only the anamorph was present when cells were measured. Isolates were grown on PDYA, and from each isolate five individual subcultures were generated by collecting cells from five different locations in the culture. For examinations, cells were collected with a loop from two different locations in each subculture, and each mounted separately in a drop of water on a glass microscope slide. Five mature thalli were randomly selected from each drop with yeast; a thallus was arbitrarily determined to be mature if at least five cells were present. From each thallus the length of the branched cell and the next two apical cells, called the first and second cells, were measured. To analyze statistical differences among isolates, Fisher’s analysis of variance and a two-sample t-test were performed using a 95% confidence level.

For scanning electron microscopy (SEM), nematodes bearing thalli were placed in a fixative of 2.5% glutaraldehyde and 0.1 M KPO₄ buffer (pH 7.2) and, after approximately 30 min, the nematodes were cut with a razor blade to reduce shrinkage. The samples and fixative were poured into a Prep-Eze (Pelco, Redding, CA) mesh tissue basket, rinsed three times in a similar phosphate buffer, post-fixed in 1% osmium tetroxide and 0.05 M KPO₄ buffer (pH 7.2) for 2 h at room temperature, rinsed with distilled water, and dehydrated in a graded ethanol series. Specimens were critical-point-dried, carefully affixed to mounts with conductive adhesive tabs, coated with 150–200Å of gold, and observed with a Hitachi S-570 scanning electron microscope operated at 20 kV accelerating potential.

Molecular analyses

DNA extractions

Single thallus cultures were grown for 10–14 d on PDYA at room temperature. Specimen numbers for cultures included in the analyses are listed in Table 1 and in the appropriate figures. To determine DNA base compositions and DNA–DNA reassociation values, DNA was isolated and purified according the protocol described by Smith et al. (1995). For RAPD- and ISSR-PCR analysis, DNA was isolated according the method of Möller et al. (1992) and the concentration...
was evaluated by gel electrophoresis. For AFLP and sequence analyses, DNA was extracted following the protocol of Lee & Taylor (1990) with the elimination of 2-mercaptoethanol in the lysis buffer. DNA was quantified with a Fluorolite 1000 (Dynatech Laboratories, Chantilly, VA) fluorometer using a Fluorescent DNA Quantitation Kit (Bio-Rad, Hercules, CA).

DNA base composition and rehybridization

The mol% G+C of isolates was measured following the procedure of Smith et al. (1995). The levels of DNA–DNA homology were determined spectrophotometrically using the method by Seidler & Mandel (1971) as modified by Kurtzman et al. (1980). The optimal reassociation temperature (Tm) of 25 °C, 50 °C, was determined according to the method of Kurtzman et al. (1980).

RAPD and ISSR

The core 10-mer primer OPA-13 (Operon Technologies, Alameda, CA) was used for RAPD-PCR and three microsatellite primers (ATG)5 (Garbelotto et al. 1993), (GTG)5 (Welsh & McClelland 1990), and M13 (Vassart et al. 1987) were used for ISSR-PCR analysis. Amplification reactions were performed in a final volume of 50 nl containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, 0.2 mM of each dNTP (Boehringer Mannheim, Mannheim), 10 pm of each primer, 1 U of Super-therm DNA polymerase (ITK Diagnostics, Withorn) and 20–30 ng of genomic DNA. The thermocycler (BioMed Thermocycler 60; BioMed, Thews) was programmed for 35 cycles for primers M13 and (GTG)5: denaturation of 20 s at 94 °C, annealing of 1 min at 55 °C, and extension of 2 min at 72 °C. Forty amplification cycles were performed with primers (ATG)5 and OPA-13: 1 min at 94 °C, 1 min at 48 °C for primer (ATG)5, or 1 min at 34 °C for primer OPA-13, followed by 2 min at 72 °C. The PCR products were analyzed by electrophoresis in a 1% agarose gels chilled at 14 °C. To avoid ambiguous results, the amplification reactions of all strains were processed simultaneously from stock solution of premixed reagents in a single PCR assay, as suggested by Messner et al. (1994).

RAPD- and ISSR-PCR bands were scored using a binary code (1, 0) corresponding to positive and null alleles, respectively, and patterns of each isolate were combined in a composite fingerprint with the computer program GelCompar 4.0 (Applied Maths). Genetic similarities were calculated using the Pearson product-moment correlation and cluster analysis was performed using the unweighted pair-group method with arithmetic average (UPGMA) clustering algorithm.

AFLP

The AFLP procedure outlined by Vos et al. (1995) was followed, with the exception the restriction enzyme...
Grand Island, NY), 2.5 M MgCl₂ reaction contained 1× PCR buffer (Life Technologies, Grand Island, NY). 2.5 mM MgCl₂, 2.5 mM each dNTP (Life Technologies), 3.75 μM of each primer, 0.5 U of Taq polymerase (Life Technologies), and 10 μl of template DNA. Thermocycling conditions consisted of an initial hold at 72 °C for 1 and 4 min, respectively, followed by 44 cycles with an annealing temperature of 65 °C for 30 s, the temperature reduced by 1 °C for the first 9 cycles, and an extension temperature of 72 °C for 1 min. All amplifications were performed in a PE-9700 thermocycler (Perkin Elmer, Norwalk, CT). Following amplification, 5 μl of formamide loading dye was added to each reaction and denatured for 4 min at 94 °C and cooled on ice. Three to four μl of each sample were loaded and separated on 4% acrylamide gels buffered in TBE (1.35 mM Tris pH 8.0, 0.45 mM boric acid, 25 mM EDTA) for approximately 2 h at 1800–1900 volts using a Bio Rad Sequi-Gen GT Sequencing Cell (Bio-Rad Laboratories). The gels were silver-stained by the method of Bassam, Caetano-Anolles & Gresshoff (1991) and dried overnight before scoring bands visually.

AFLP bands were scored using a binary code (1, 0) corresponding to positive and null alleles, respectively. The data were analyzed with NTSYS-pc 2.02 (Exeter Software, Setavket, NY). Genetic distance was calculated with the similarity for qualitative data function (SIMQUAL) using the Dice (1945) coefficient. Cluster analyses were performed by means of the UPGMA clustering algorithm.

**DNA sequencing**

A portion of the divergent D1/D2 domain (Guadet et al. 1989) at the 5′ end of the nuc-LSU rDNA gene was amplified and sequenced by means of the nuclear large primers NL-1 and NL-4 (O’Donnell 1993). Amplification of the 5′ end of the mt-LSU rDNA gene was attempted with the mitochondrial large universal primers ML1 and ML6 (White et al. 1990). After preliminary analysis showed informative sequence divergence among isolates, a reverse primer was designed to generate a sequence of approximately 500–600 bp for analysis in a single run. The reverse primer ML1R (5′-AGCGCAATAAGTCCGATTCC) was designed with the Primer3 website (Rozen & Skaletsky 1998). PCR amplification was performed in 40 μl reactions containing 4 μl of a 25-fold dilution of genomic DNA. PCR and thermocycling conditions are as described above with the exception that a constant annealing temperature of 52 °C for 30 cycles was used. PCR products were electrophoresed in 1% agarose gel containing ethidium bromide, illuminated using UV light, and clean reactions were purified using a QIAquick PCR Purification Kit (Quiagen, Valencia, CA). Sequencing reactions with both forward and reverse primers for both gene regions were performed following the manufacturers instructions using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). PCR products were purified with Centriflex Gel Filtration Cartridges (Edge Biosystems, Gaitherburg, MD). DNA sequencing reactions were analyzed on an Applied Biosystems model 377 DNA Sequencer (PE Biosystems) by the Laboratory for Biotechnology and Bioanalysis, School of Molecular Biosciences, Washington State University.

Sequence data were aligned by means of the multiple alignment program ClustalX 1.8 (Thompson et al. 1997). Unaligned regions were checked for accuracy manually by comparing the computerized sequence read data with the chromatographs. Phylogenetic relationships were calculated with PHYLIP version 3.5c (Felsenstein 1993). All data inputs were randomized, and bootstrap tests were performed in 1000 replicates using SEQBOOT. Analyses were performed with maximum parsimony (DNAPARS) (1000 data sets analyzed), maximum likelihood (DNAML) (100 data sets analyzed), and distance matrix (DNADIST) with neighbor-joining (NEIGHBOR) (1000 data sets analyzed, each) as the criteria. A majority-rule consensus tree was generated for each analysis using CONSENSE. *Pichia stipitis* (Kurtzman & Robnett 1997) and *Trigonopsis variabilis* (Kurtzman & Robnett 1995) were used as outgroups for the nuc-LSU. No organism aligned with the region amplified with the ML1 and ML1R primer pair with enough similarity for inclusion in the analyses. Phenograms were drawn with TreeView 1.6.5 (Page 1996).

**Physiological characterization**

The physiological characteristics of isolates were determined following the methods of Yarrow (1998). Growth tests were performed at least three times for each isolate.

**RESULTS**

**Isolates**

*Ascohotryzyzma* isolates were collected from beetle entrance sites and galleries in *Populus* and *Salix* species from seven locations in western Idaho (Fig. 1). Isolates from *Populus* trees were found in two locations in northern Idaho and were collected from galleries that appeared characteristic of those created by the poplar borer, *Saperda calcarata* (*Coleoptera: Cerambycidae*), a long-horned, wood-boring beetle whose larvae carve out galleries for two or more years (Metcalf & Flint 1939). Isolates from *Salix* were found in five locations, spanning a distance of about 125 km. Willows were infested by the poplar and willow borer, *Cryptorrhynchus lapathi* (*Coleoptera: Curculionidae*), an exotic,
ubiquitous beetle commonly found in shrubby willows (Smith & Stott 1964). All isolates were found associated with the free-living nematode *Panagrellus dubius*.

**Morphology**

The sizes of thallus cells from the Idaho isolates (from *Salix* and *Populus*) (Figs 2–3) were significantly shorter \( (P < 0.001) \) than those from the Washington isolates (from *Populus*) (Table 2). The first and second cells apical to the basal cell measured, respectively, 10.6–13.0 μm and 12.0–14.8 μm for the Idaho isolates and 12.0–14.5 μm and 13.9–16.6 μm for the Washington isolates. Basal cell sizes were not significantly different. The two isolates from *Populus* spp. in Idaho were intermediate in size between those from *Populus* in Washington and *Salix* in Idaho. These incremental cell lengths were generated through calculation of means and standard deviations; sizes could not be measured at a precision greater than 0.5 μm.

Isolates from Idaho (CBS 8460, CBS 8705, CBS 8707, CBS 8709, and CBS 8753) formed mature asci that appeared similar to those of *Ascobotryozyma americana* (from Washington), with globose asci and lunate ascospores (Figs 4–7). In isolates CBS 8460 and CBS 8753, however, the teleomorph differed morphologically, bearing multiple asci from one cell (Figs 8–10) rather than only one ascus per cell as in *A. americana* (Kerrigan et al. 2001). Ascus-bearing cells were sometimes inflated and irregular in shape (Fig. 10). No regular order of ascus production was noted, as young and mature asci occurred in different orientations. Ascospore germination has not been observed, and it is not known whether this yeast is heterothallic or homothallic. After the teleomorph stopped forming, thalli often exhibited irregular morphologies as previously observed in *A. americana* (Kerrigan et al. 2001).

**Molecular Data**

**DNA rehybridization**

DNA reassociation values of 99–100% were observed among representative isolates from Idaho, which were collected from both *Cryptorhynchus lapathi* damage in *Salix* and *Saperda calcarata* damage in *Populus*. Isolates from Idaho had low reassociations values with those from Washington (*Ascobotryozyma americana*) (47–63%) and Italy (*Botryozyma nematodophila*) (48–62%) (Fig. 11). Values ranging from 39–54% were reported between *A. americana* and *B. nematodophila* (Kerrigan et al. 2001).

**RAPD, ISSR, and AFLP**

Three distinct clades were found based on clustering analysis using RAPD and ISSR (Fig. 12) and AFLP (Fig. 13) markers. All isolates from Idaho clustered together, as did the *Ascobotryozyma americana* isolates from Washington and the two *Botryozyma nematodophila* isolates from Italy.

**DNA sequence data**

Sequencing the D1/D2 domain of the nuc-LSU rDNA yielded a 555 bp region for alignment. Sequences of *Botryozyma nematodophila* (from Italy) from this study were identical to those deposited in GenBank (accession no. U40105) from the same isolate (Kurtzman & Robnett 1995). Sequences from this domain were highly conserved and sequences were identical among isolates within each region. *B. nematodophila* differed from *Ascobotryozyma americana* (from

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Figs 2–3. *Botryozyma cognata*. **Fig. 2.** A nematode covered with thalli. Bar = 25 μm. **Fig. 3.** One thallus. Bar = 5 μm.
(1.80%) and from the Idaho isolates by six nucleotides (1.08%). Washington isolates had four nucleotide substitutions (0.72%) when compared with the Idaho isolates. Bootstrap values from all phylogenetic analyses, including maximum parsimony, maximum likelihood, and neighbor joining, strongly supported the division of isolates into three clades representing distinct species (Fig. 14).

The region amplified with the ML1 and ML1R primer pair yielded a 569 bp region. A BLAST similarity search of the sequences did not reveal significant similarity to other fungal sequences; therefore, the identity of these sequences is not known. This region was found to be informative for elucidating phylogenetic isolate relationships among the *Ascobotryozyma* isolates and was congruent with the nuc-LSU rDNA region.

### Table 2. Summary of *Ascobotryozyma* and *Botryozyma* species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Nematode</th>
<th>Plant host</th>
<th>Vector</th>
<th>Thallus cell sizes (µm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>americana</em></td>
<td>Washington, USA</td>
<td><em>P. dubius</em></td>
<td><em>Populus</em></td>
<td><em>Saperda calcarata</em></td>
<td>6.8–9.5 12.0–14.5 13.9–16.6</td>
</tr>
<tr>
<td><em>cognata</em></td>
<td>Idaho, USA</td>
<td><em>P. dubius</em></td>
<td><em>Populus, Salix</em></td>
<td><em>Saperda calcarata, Cryptorhynchus lapathi</em></td>
<td>6.5–9.4 10.6–13.0 12.0–14.8</td>
</tr>
<tr>
<td><em>nematodophila</em></td>
<td>Verona, Italy</td>
<td><em>P. redivivoides</em></td>
<td><em>Vitis vinifera</em></td>
<td><em>Drosophila</em></td>
<td>9.0–10.3 12.8–15.9 15.4–18.6</td>
</tr>
</tbody>
</table>

* Incremental cell lengths generated through statistical analysis.

Figs 4–10. *Ascobotryozyma cognata*. Bar = 5 µm. Fig. 4. Fused thalli with developing asci (A) on the cuticle of a nematode (N). Fig. 5. An ascus formed from two fused thalli, regions of fusion indicated with arrows. Fig. 6. Two fused (arrows) thalli bearing two asci (A) with portions of ascospores visible inside. Fig. 7. Three ascospores. Fig. 8. A thallus with four asci, two immature and two deliquesced, formed from one cell. Fig. 9. SEM of thalli with numerous asci (A), intact and deliquesced. Fig. 10. An inflated thallus cell with four asci of different ages.

Washington) by 10 nucleotides (1.80%) and from the Idaho isolates by six nucleotides (1.08%). Washington isolates had four nucleotide substitutions (0.72%) when compared with the Idaho isolates. Bootstrap values from all phylogenetic analyses, including maximum parsimony, maximum likelihood, and neighbor joining, strongly supported the division of isolates into three clades representing distinct species (Fig. 14).
Sequences of the isolates from Italy were identical to each other. Sequence data from the Washington isolates were identical except for one isolate (CBS 8462) that differed by two nucleotides. This isolate was collected in 1992 and has been maintained in pure culture; it no longer forms branched cells. The Idaho isolates were identical except for one isolate (CBS 8706) that differed by three base pairs; it is the only isolate from a different *Populus* species and the most northern collection. This region was more divergent than the D1/D2 domain, exhibiting a greater number of nucleotide substitutions between groups. Isolates again broke down into three well-supported clades (Fig. 15) using all phylogenetic analyses. Washington and Idaho isolates exhibited the greatest difference, with 13.34% sequence divergence. Isolates from Washington and Italy differed in 11.42% of their basepairs, and Idaho and Italy differed in 9.67%.

**Physiology**

Physiological capacities, listed in Table 3, are limited, as with the Washington (*Ascobotryozyma americana*) and Italian (*Botryozyma nematodophila*) isolates (Kerrigan et al. 2001). The production of carbon dioxide from glucose was not observed, and only nine out of 46 tested carbon compounds and two of nine tested nitrogen compounds were utilized (Table 3). Utilization profiles of the isolates from Idaho were the same as those for *A. americana* and *B. nematodophila* except none of the Idaho isolates were able to utilize trehalose and, like *A. americana*, the ability to grow at 37°C was variable.

**Taxonomy**

*Ascobotryozyma cognata* J. Kerrigan, M. T. Sm. & J. D. Rogers, sp. nov. (Figs 4–10).

*Etym.*: ’cognata’, referring to the relatedness of the species.

Thalli ad paginam nematodorum ab cellulis basaliibus affixi. Cellulae basales obovoideae, corporibus 5–12 × 2–4 μm, basibus furcatis. Cellulae etarum subcyllindriceae vel sub-ovoideae, 6.5–19 × 2–4.5 μm, ad apicem pulullantes. Asci globosi vel late ellipsoidei, tetraspori, 7–9 × 6–8 μm, uni usque ad quattuor e lateribus et apicibus cellularum pullulantes. Ascosporea hyalinae, lunatae, leves, 7–8.5 × 1.5–2 μm. A *Ascobotryozyma americana* differt in amplitudinibus minoribus cellularum et in structura DNA.

Fermentatio et cultura in carbonibus et in nitrogenis et additiciis characteribus in Tabula 3 monstrantur.

**DISCUSSION**

Our results clearly support the designation of a new species, *Ascobotryozyma cognata*, and also resolve and support the taxonomic separation of *A. americana* from *Botryozyma nematodophila* (Kerrigan et al. 2001). Molecular and morphological data revealed, surprisingly, that isolates separated into three distinct groups based on location: Italy (*B. nematodophila*), Washington (*A. americana*), and Idaho (*A. cognata*). The separation of isolates from Idaho is noteworthy because these isolates were collected from different tree genera, *Salix* and *Populus*, and were associated with different beetles, *Cryptorhynchus lapathi* and *Saperda calcarata*, respectively. All isolates were collected from two nematode species in the same genus, which inhabit similar environments. It was hypothesized if genetic separations had evolved, they would have been along tree-beetle taxonomic lines. This apparently has not occurred or at least has not been detected. Isolates from Idaho spanned a relatively long distance, about 250 km on a north-south axis, whereas all isolates from Washington were from one site about 50 km west of the nearest Idaho site (Fig. 1). There are no geographical barriers between the two states, and some Idaho sites were more distant from each other than from the Washington site. It is likely the distribution of the two species overlaps, but further sampling is needed to determine the ranges of the two species.

*A. americana* differs from *B. nematodophila* in its distribution (different continent), habit (different plant host and disease/damage), nematode host (different species), cells sizes (shorter), DNA reassociation values
& A. americana based on the unidentified region, sequences between RAPD, ISSR, and AFLP analyses also show the A. americana and A. cognata Ramos & Leoncini 1999). In this region, 1995, 1997, 1998, Kurtzman 1992, 1995, Valente, species within genera (Kurtzman & Robnett 1991, to be reliable for discerning ascomycetous yeast sequenced for over 500 species and has been shown The D1/D2 domain of the nuc-LSU rDNA has been the division of isolates into three taxonomic groups. Additional criteria were examined.

Of special note is molecular evidence suggesting A. cognata and A. americana are independently co-evolving with the nematode from which they were isolated. Nematodes from each Ascobotryozyma isolation site were sent to S. Patricia Stock (University of California at Davis Nematode Collection) for identification and were also included in a study on the morphological and molecular characterization of the genus Panagrellus. All isolates were identified as P. dubius. Based on sequence data from approximately 1000 bp of the nuc-LSU (28S) rDNA, the nematode isolates clustered by state, Washington (where A. americana was isolated) and Idaho (where A. cognata was isolated), and the two branches formed a single clade with 100% bootstrap support (S. P. Stock & S. A. Nadler, unpubl.). Sequence divergence among the nematode isolates was the same as among the Ascobotryozyma isolates, with the separation coinciding with yeast speciation. This provides additional evidence of a genetic separation of the yeast and its nematode associate based on location, and bears upon the coevolution of these two organisms. Thalli of A. americana were able to adhere to nematodes from which A. cognata was

Fig. 15. Phylogenetic tree calculated from neighbor-joining analysis of an unknown genomic region amplified with primers ML1 and ML1R. Branch lengths are proportional to nucleotide differences, as indicated in the scale bar (%).

(39–54%), and inability to grow at 37 ° (Kerrigan et al. 2001). A. cognata differs from A. americana and B. nematodophila in two of these criteria, cell sizes and reassocation values. Although cell sizes are not a standard character used to differentiate yeast species, the size differences are visually notable and these data serve as one criterion in support of the designation of a new species. Reassociation values were 47–63% between Idaho and Washington isolates and 48–63% between Idaho and Italian isolates. Values of similar magnitude have been used to support the differentiation of species. However, in a study on nuclear DNA reassociation studies of various heterothallic ascomycetous yeasts, Kurtzman (1987) reported isolates with reassociation values of 40–70% are often considered varieties of the same species. Since the North American isolates were collected in relatively close proximity to each other and all were from wood-boring beetle galleries and the same nematode species, additional criteria were examined.

Molecular investigations show strong support for the division of isolates into three taxonomic groups. The D1/D2 domain of the nuc-LSU rDNA has been sequenced for over 500 species and has been shown to be reliable for discerning ascomycetous yeast species within genera (Kurtzman & Robnett 1991, 1995, 1997, 1998, Kurtzman 1992, 1995, Valente, Ramos & Leoncini 1999). In this region, A. cognata and A. americana isolates were most similar; however, based on the unidentified region, sequences between A. cognata and A. americana were the most different. RAPD, ISSR, and AFLP analyses also show the division of isolates into three distinct clusters that correlate with A. americana, B. nematodophila, and the newly recognized A. cognata.

### Table 3. Summary of phenotypic characteristics of Ascobotryozyma cognata strains.

<table>
<thead>
<tr>
<th>Fermentation:</th>
<th>Gas formation not detectable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth on:</td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>v D-Mannitol w/+</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>– Galactitol –</td>
</tr>
<tr>
<td>L-Sorbose</td>
<td>v myo-Inositol –</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>– D-Glucono-6-lactone –</td>
</tr>
<tr>
<td>D-Ribose</td>
<td>– 2-Keto-D-gluconate +</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>– D-Gluconate –</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>– D-Glucuronate –</td>
</tr>
<tr>
<td>D-Arabinoose</td>
<td>– D-Galacturonic acid –</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>– DL-Lactate w/+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>– Succinate +</td>
</tr>
<tr>
<td>Maltose</td>
<td>– Citrate –</td>
</tr>
<tr>
<td>α,α-Trehalose</td>
<td>– Methanol –</td>
</tr>
<tr>
<td>Me-α-D-glucoside</td>
<td>– Ethanol v</td>
</tr>
<tr>
<td>Cellulobiose</td>
<td>– Propylene 1,2 diol –</td>
</tr>
<tr>
<td>Salicin</td>
<td>– Butane 2,3 diol –</td>
</tr>
<tr>
<td>Arbutin</td>
<td>– Quinic acid –</td>
</tr>
<tr>
<td>Melibiose</td>
<td>– Saccharate –</td>
</tr>
<tr>
<td>Lactose</td>
<td>– Galactonic acid –</td>
</tr>
<tr>
<td>Raffinose</td>
<td>– Nitrate –</td>
</tr>
<tr>
<td>Mlezitose</td>
<td>– Nitrite –</td>
</tr>
<tr>
<td>Inuline</td>
<td>– Ethylamine +</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>– L-Lysine +</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+ Cadaverine –</td>
</tr>
<tr>
<td>meso-Erythritol</td>
<td>– Creatine –</td>
</tr>
<tr>
<td>Ribitol</td>
<td>– Creatinine –</td>
</tr>
<tr>
<td>Xylitol</td>
<td>– Glucosamine –</td>
</tr>
<tr>
<td>L-Arabinitol</td>
<td>– Imidazol –</td>
</tr>
<tr>
<td>D-Glucitol</td>
<td>w/ + 0.1% Cycloheximide +</td>
</tr>
</tbody>
</table>

**Growth at:**

| 37°          | v | 40° | – |

w, weak; v, variable.
isolated and vice versa; therefore, the fungus is not nematode-specific, at least at the species level. The beetles are believed to passively vector nematodes, but nothing is known about the population genetics of these beetles. Perhaps speciation is driven by distance, which is governed by the distribution of the beetle vectors.

It appears Ascobotryozyma is widely dispersed, but not densely, in habitats where conditions are suitable for both the yeast and nematode. An additional Botryozyma isolate was obtained from southern Oregon. It was collected from slime flux from hybrid poplars, P. deltoides × trichocarpa, infested by the dusky sap beetle, Carpophilus lugubris (Coleoptera: Nitidulidae) (Melody Putnam, pers. comm.). Based on AFLP banding patterns and nuc-LSU sequence data this isolate differs from the other three species, but more isolates are needed to examine its relationship in greater detail. Although isolates have been found from different substrates in different regions, all have been associated with Panagrellus nematodes and a rich microbial biota. Investigations of similar environments will most likely yield additional isolates and contribute to a better understanding of the distribution and speciation of this yeast.

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REFERENCES


Ascobotryozyma cognata sp. nov.


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