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Unravelling the *Phellinus pini* s.l. complex in North America: a multilocus phylogeny and differentiation analysis of *Porodaedalea*

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Summary

Phellinus sensu lato (s.l.) is a complex of segregate genera that act as aggressive pathogens of woody plants. Nearly all of the genera in this complex have unresolved taxonomies, including *Porodaedalea*, which is one of the most important trunk rot pathogens of coniferous trees throughout the northern hemisphere. In an attempt to elucidate the species within *Porodaedalea*, a multilocus phylogenetic analysis was performed with partial sequences from four loci (internal transcribed spacer, nuclear large subunit, *tef1* and *rpb2*) using 41 isolates that originated from North America and Europe. For reference, we analysed the neotype isolates of *Porodaedalea pini* and *P. chrysoloma*. Our results confirmed that *Porodaedalea pini* s.s. and *P. chrysoloma* s.s. are unique phylogenetic species that do not occur in North America. We detected two discrete clades of *Porodaedalea* originating from the southwestern and southeastern United States. Isolates from these regions grouped with significant statistical support and represent undescribed taxa. With the exception of *P. cancriformans*, our analyses revealed monophyly among 28 isolates originating from the northern United States, Canada and Fennoscandia, a group we have labelled the 'Holarctic group'. Holarctic group isolates were collected from *Larix*, *Picea*, *Pinus*, *Pseudotsuga* and *Tsuga* and were presumed to represent at least four morphological species (*P. gilbertsonii*, *P. laricis*, *P. pini* s.l. and *P. piceina*). Tests of gene flow and genetic differentiation detected significant differences among Holarctic group isolates by region of origin, and three subgroups were designated: (i) Atlantic-Boreal; (ii) Interior; and (iii) Pacific. Neutrality tests using the Holarctic group demonstrated significant departures from the standard neutral model of evolution and could indicate that a diversifying selection has maintained rare phenotypes in the population, which has fostered taxonomic confusion in *Porodaedalea*.

1 Introduction

Phellinus sensu lato (s.l.) is universally considered to be one of the most important groups of wood-decaying fungi in temperate forest ecosystems (Sinclair and Lyon 2005). Members of this group are pathogenic to a wide array of hardwoods and conifers and cause significant volume losses in older forests (Hepting 1971). Because of the ecological and economic significance of *Phellinus* s.l. on such a large number of hosts, numerous competing names, varieties and types were assigned within the genus (Fiasson and Niemelä 1984). At one point, *Phellinus* s.l. comprised over 150 unique species, with over 50 varieties and types further subdividing the genus (Larsen and Cobb-Poulle 1990). The advent of molecular techniques provided a means to reassess *Phellinus* s.l., and the genus was deconstructed to reveal numerous segregate genera, leaving *Phellinus* sensu stricto (s.s.) as a trunk rot pathogen of hardwoods only (Wagner and Fischer 2001, 2002; Fischer and Binder 2004; Larsson et al. 2006). Trunk rot pathogens of conifers previously described as *Phellinus pini* s.l. now belong to *Porodaedalea* (Fiasson and Niemelä 1984; Wagner and Fischer 2002; Larsson et al. 2006).

Trunk rot fungi such as *Porodaedalea* species have a fundamental role in forest ecosystems, where they function as important disturbance agents (Hansen and Goheen 2000). These actions are directly responsible for accelerating stand development through gap formation, altering forest composition and successional pathways, creating coarse woody debris and wildlife habitat and recycling organic matter (Hennon 1995; Hansen and Goheen 2000; Worrall et al. 2005). *Porodaedalea pini* s.l. is widespread in North America in nearly all conifer-dominated forest types (Sinclair and Lyon 2005). The primary hosts (*Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga* and *Tsuga*) are colonized predominantly through branch stubs and wounds (Sinclair and Lyon 2005). Damage associated with trunk rot from *P pini* s.l., typically referred to as red ring rot, white pocket rot and red heart, was described by Boyce as 'far exceed[ing] those from any other [trunk rot] decay' (p. 389; 1961). Proper identification of *P. pini* s.l. is essential to determine its geographical range, host specificity and pathogenicity so that researchers can better understand its ecological significance.

The tremendous phenotypic variation in the basidiocarps has, for decades, led forest pathologists to suspect that species complexes exist within *Porodaedalea* (Owens 1936; Boyce 1961). Currently, four species of *Porodaedalea* are formally recognized in North America: *P. cancriformans*, *P. gilbertsonii*, *P. piceina* and *P. pini* s.l. (Larsen and Cobb-Poulle 1990; Fischer 1996; Larsen 2000). *Porodaedalea piceina* inhabits more northerly regions of North America and is known to occur only on *Picea* species (Niemelä 1985). In addition, *P. piceina* produces annual to perennial, resupinate to effused–reflexed basidiocarps (Larsen and Cobb-Poulle 1990). It is also believed that *P. piceina* is very closely related to European *P. chrysoloma*, which is also known only from *Picea* (Niemelä 1985; Fischer 1994, 1996). Yet, this hypothesis has been based on morphological characters (Niemelä 1985; Larsen and Cobb-Poulle 1990) or intersterility and molecular testing that included only a single isolate of *P. piceina* from Canada (Fischer 1994, 1996).

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Porodaedalea cancriformans is morphologically related to *P. piceina* in that it produces effused–reflexed to shelf-like, annual to perennial fruiting bodies, but is restricted to *Abies* species in northwestern North America (Larsen et al. 1979; Sinclair and Lyon 2005). Another western North American species, *P. gilibertsonii*, is known only from coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*) in California (Larsen 2000), and at present, it is unclear whether this species occurs on additional hosts in North America. An additional species known from Europe and Asia, *P. laricis*, was recently determined to attack *Larix*, *Picea* and *Pinus* (Tomšovský et al. 2010). Phylogenetic analysis detected significant intraspecific variation within *P. laricis*, and it is currently unknown whether this species occurs in North America (Tomšovský et al. 2010).

Previous studies suggest that additional, undescribed *Porodaedalea* species exist in North America. Fischer (1994, 1996), using both intersterility tests and PCR-RFLP, reported five to seven unique taxa of *Porodaedalea* in North America, with the majority showing host specificity. Fischer described these North American taxa as N-II to N-VII, and all were found to be unique from isolates of *P. pini* s.s. and *P. chrysoloma* s.s. originating from Europe (Fischer 1994, 1996). Considering all available literature, there could be as many as eight unique species within the North American population of *Porodaedalea*: *P. cancriformans*, *P. gilbertsonii*, *P. piceina* and *Porodaedalea* N-II and N-IV to N-VII, with N-III suspected to be conspecific with *P. gilbertsonii* (Larsen 2000). The evidence would also suggest that *P. pini* s.s. and *P. chrysoloma* s.s. do not occur in North America (Fischer 1994, 1996; Larsen and Melo 1996; Larsen and Stenlid 1999; Larsen 2000).

Therefore, the primary goal of this study was to elucidate species complexes within *Porodaedalea* on coniferous hosts in North America. We hypothesized that: (i) *Porodaedalea* isolates would group into strongly supported clades primarily by host; (ii) several undescribed species would be differentiated from the currently known species (*P. cancriformans*, *P. piceina* and *P. gilbertsonii*) using the phylogenetic species concept; and (iii) *P. pini* s.s. and *P. chrysoloma* s.s. do not occur in North America, as previously suspected.

2 Materials and methods

2.1 Isolates used

Isolates used in this study originated from throughout North America and northern Europe and were chosen based on host diversity, geographic disparity and availability of living cultures (Table 1). An attempt was made to analyse isolates from representative hosts in all regions of North America, although some regions (e.g. southwestern and southeastern United States) are poorly represented due to a lack of available cultures. For reference to North American isolates, we included the neotype specimens of *Porodaedalea pini* s.s. and *P. chrysoloma* s.s., which originated from Portugal and Sweden, respectively (Larsen and Melo 1996; Larsen and Stenlid 1999). Isolates used in this study are currently housed at the USDA Forest Service, Northern Research Station, Center for Forest Mycology Research (CFMR) in Madison, Wisconsin (Table 1).

2.2 DNA extraction, PCR and sequencing

DNA was isolated from lyophilized mycelium and live cultures on 2% MEA using a modified version of the protocol found in Lindner and Banik (2009). Mycelia were transferred to 200 µl of cell lysis solution in 8-well PCR strip tubes and frozen at −80°C followed by placement in a 65°C water bath for two hours. The samples were then centrifuged at 10,000 rcf for 5 min, after which 100 µl of supernatant was removed and transferred to a new strip tube. Next, 150 µl of ice-cold 2-propanol (isopropanol) was added to each supernatant, tubes were inverted to mix, cooled at −80°C for 15 min and then centrifuged at 10 000 rcf for 20 min at 0°C. Supernatants were discarded, 175 µl of 75% ethanol (v/v) was added, and tubes were centrifuged at 16 000 rcf for 5 min at room temperature. Next, supernatants were removed, and pellets were air-dried at room temperature for 10 min and then resuspended in 45 μl of molecular-grade water. DNA in aqueous solution was then cleaned using the GeneClean III kit (MP Biomedicals, Solon, OH, USA) following the manufacturer's protocol with the following modifications. First, 45 µl of aqueous DNA solution was combined with 135 µl of NaI solution and 5 μl of glassmilk. Tubes were then agitated continuously for 5 min followed by centrifugation at 16 000 rcf for 8 seconds. The supernatant was discarded, and the pellet was washed once using 175 µl of 'New Wash' solution. After removal of 'New Wash', pellets were air-dried for 15 min and template DNA was eluted in 50 µl of molecular-grade water.

PCR conditions used in this study have been described previously (Lindner and Banik 2008). To generate PCR amplicons, the following primer pairs were used: ITS1-F and ITS4 (Gardes and Bruns 1993) for the internal transcribed spacer (ITS); LR0R (Rehner and Samuels 1994) and LR5 (Hopple and Vilgalys 1999) for the nuclear large subunit (nLSU) domains one to three; and bRPB2-6F and bRPB2-7.1R (Matheny 2005) for the RNA polymerase II (*rpb2*) domains six to seven. To generate partial sequences of the translation elongation factor 1 alpha (*tef1*) gene, primers 983F and 2218R (Rehner and Buckley 2005) were used for a subset of isolates originating from across North America and Europe. After the initial sequence analysis, it was determined that a homopolymer repeat ($T_{7\text{-}9}$) at the 5′ end of the *tef1* amplicon was terminating the BigDye sequencing reaction. As a result, a new forward primer was developed using PRIMER3 (Untergasser et al. 2007) that annealed downstream of the homopolymer repeat, generating a 731-bp amplicon for *Porodaedalea* isolates (1487F: 5′ GCTGGACAAAGGAGACGAAG 3′).

Prior to sequencing, PCR products were visualized on 1% agarose gels stained with ethidium bromide to confirm the presence of a single amplicon and then diluted 1:10 with molecular-grade water. Isolates were sequenced using the BigDye

Table 1. Isolate information and GenBank accession numbers for Porodaedalea.

Species & isolate code	Host	State/Province	Country	Collector	GenBank accession nos. ITS	nLSU	*tef1*	*rpb2*
Porodaedalea chrysoloma s.s.								
FP-102121-T	*Picea abies*	Sumava	Czech Republic	A. Cerny	JX110031	JX110075	JX109987	JX109945
FP-135951[1]	*Picea abies*	Uppsala	Sweden	J. Stenlid	JX110032	JX110076	JX109988	JX109946
FP-135952	*Picea abies*	Uppsala	Sweden	J. Stenlid	JX110033	JX110077	JX109989	JX109947
NJB2011-Fin1	*Picea abies*	Uusimaa	Finland	N.J. Brazee & T. Niemelä	JX110034	JX110078	JX109990	JX109948
Porodaedalea pini s.s.								
74-64/2	*Pinus sylvestris*	Svealand	Sweden	F. Roll-Hansen	JX110035	JX110079	JX109991	JX109949
FP-102122-T	*Pinus pallasiana*	Crimea	Ukraine	A. Cerny	JX110036	JX110080	JX109992	JX109950
No-6170-T[1]	*Pinus pinaster*	Lisbon	Portugal	I. Melo & J. Cardoso	JX110037	JX110081	JX109993	JX109951
Porodaedalea sp. 1								
FP-103366-T[2]	*Pinus virginiana*	Georgia	USA	A.S. Rhoads	JX110038	JX110082	JX109994	JX109952
FP-71757[2]	*Pinus virginiana*	Virginia	USA	N.E. Hawes	JX110039	JX110083	JX109995	JX109953
Porodaedalea sp. 2								
AZ-10-T[2]	*Pinus strobiformis*	Arizona	USA	D. Rizzo	JX110040	JX110084	JX109996	JX109954
AZ-14-T[2]	*Pinus strobiformis*	Arizona	USA	D. Rizzo & Berhandt	JX110041	JX110085	JX109997	JX109955
Porodaedalea cancriformans								
1-Sp	*Abies concolor*	California	USA	L.R. Carpenter	JX110042	JX110086	JX109998	JX109956
FP-133112-R	*Abies magnifica*	Oregon	USA	M.J. Larsen	JX110043	JX110087	JX109999	JX109957
Porodaedalea Holarctic Group								
1470/5[3]	*Pinus sylvestris*	Akershus	Norway	Unknown	JX110044	JX110088	JX110000	JX109958
Aho-61-49[2]	*Pinus contorta*	Oregon	USA	P.E. Aho	JX110045	JX110089	JX110001	JX109959
Colo-247-R[2]	*Pinus contorta*	Colorado	USA	R.W. Davidson	JX110046	JX110090	JX110002	JX109960
DLL2009-120[4]	*Picea* sp.	Minnesota	USA	D.L. Lindner & S. Fraver	JX110047	JX110091	JX110003	JX109961
FP-104211-R[2]	*Pinus lambertiana*	California	USA	R.W. Davidson	JX110048	JX110092	JX110004	JX109962
FP-105533-R[2]	*Larix occidentalis*	Oregon	USA	R.W. Davidson	JX110049	JX110093	JX110005	JX109963
FP-133091-Sp[4]	*Picea sitchensis*	Oregon	USA	M.J. Larsen	JX110050	JX110094	JX110006	JX109964
FP-133157-Sp[2]	*Pinus strobus*	Wisconsin	USA	M.J. Larsen	JX110051	JX110095	JX110007	JX109965
FP-133623-Sp[4]	*Picea sitchensis*	Oregon	USA	M.J. Larsen	JX110052	JX110096	JX110008	JX109966
FP-134681-Sp[2]	Unknown Conifer	Idaho	USA	M.J. Larsen	JX110053	JX110097	JX110009	JX109967
FP-135418-T[2]	*Pseudotsuga menziesii*	Idaho	USA	M.J. Larsen	JX110054	JX110098	JX110010	JX109968
FP-135670[2]	*Pseudotsuga menziesii*	Idaho	USA	M.J. Larsen	JX110055	JX110099	JX110011	JX109969
FP-135936-T[5]	*Pseudotsuga menziesii*	California	USA	M.J. Larsen	JX110056	JX110100	JX110012	JX109970
FP-135945-T[5]	*Pseudotsuga menziesii*	California	USA	M.J. Larsen	JX110057	JX110101	JX110013	JX109971
FP-59059-T[2]	*Pinus strobus*	Virginia	USA	R.W. Davidson	JX110058	JX110102	JX110014	JX109972
FP-71112[4]	*Picea mariana*	Wisconsin	USA	R.C. Lorenz	JX110059	JX110103	JX110015	JX109973
FP-71681-T[2]	*Larix larcina*	Minnesota	USA	C.C. Christensen	JX110060	JX110104	JX110016	JX109974
FP-96506-T[4]	*Picea rubens*	Nova Scotia	Canada	P.S. Spaulding	JX110061	JX110105	JX110017	JX109975
FP-97385-T[4]	*Picea engelmannii*	Colorado	USA	R.W. Davidson	JX110062	JX110106	JX110018	JX109976
HHB-12766-T[4]	*Picea mariana*	Alaska	USA	H.H. Burdsall, Jr.	JX110063	JX110107	JX110019	JX109977
HHB-17351-T[2]	*Tsuga mertensiana*	Alaska	USA	H.H. Burdsall	JX110064	JX110108	JX110020	JX109978
HHB-3585-Sp[4]	*Picea* sp.	Michigan	USA	H.H. Burdsall	JX110065	JX110109	JX110021	JX109979
KTS-26[4]	*Picea rubens*	New Hampshire	USA	K.T. Smith	JX110066	JX110110	JX110022	JX109980
L-15572-Sp[4]	*Picea mariana*	New York	USA	J.L. Lowe	JX110067	JX110111	JX110023	JX109981
L-15665-Sp[4]	*Picea rubens*	New York	USA	J.L. Lowe	JX110068	JX110112	JX110024	JX109982

Table 1. Continued

Species & isolate code	Host	State/Province	Country	Collector	GenBank accession nos.			
					ITS	nLSU	*tef1*	*rpb2*
NJB2011-Fin2[3]	*Picea abies*	Hame	Finland	N.J. Brazee & T. Niemelä	JX110069	JX110113	JX110025	JX109983
NJB2011-RS1[4]	*Picea rubens*	Massachusetts	USA	N.J. Brazee	JX110070	JX110114	JX110026	JX109984
NJB2011-RS2[4]	*Picea rubens*	Massachusetts	USA	N.J. Brazee	JX110071	JX110115	JX110027	JX109985
Onnia tomentosa								
Bud-551-C-1	*Tsuga canadensis*	Ontario	Canada	G.E. Englerth	JX110072	JX110116	JX110028	JX109986
'Phellinus' coronadensis								
RLG-9387-T	*Pinus strobiformis*	Arizona	USA	R.L. Gilbertson	JX110073	JX110117	JX110029	—
RLG-9396-T[1]	*Pinus strobiformis*	Arizona	USA	R.L. Gilbertson	JX110074	JX110118	JX110030	—

[1]Neotype isolate.
[2]Initially presumed to represent *P. pini* s.l.
[3]Initially presumed to represent *P. laricis.*
[4]Initially presumed to represent *P. piceina.*
[5]Initially presumed to represent *P. gilbertsonii*.

Sequencing Kit v. 3.1 on an ABI 3130xl capillary sequencer (Applied Biosystems, Foster City, CA, USA) at the University of Wisconsin Biotechnology Center, Madison, Wisconsin. GenBank accession numbers are listed in Table 1.

2.3 Sequence alignment and phylogenetic analysis

Sequences were edited using BIOEDIT v. 7 (Hall 1999) and aligned with MAFFT v. 6 using the FFT-NS-i option (Katoh et al. 2005). Alignment gaps were treated as missing data in all analyses. Phylogenetic analysis of complete ITS and partial nLSU, *tef1* and *rpb2* sequences was performed using MEGA v. 5 (Tamura et al. 2011) with the following steps taken. For maximum likelihood (ML), the best-fit nucleotide substitution model was chosen using Akaike information criterion (AIC) values generated in JMODELTEST (Guindon and Gascuel 2003; Posada 2008), along with log-likelihood ($-ln\ L$) scores generated within MEGA. For all data sets, the model that produced the lowest AIC score in JMODELTEST and highest $-ln\ L$ score in MEGA was the general time reversible (GTR+I+G) substitution model (Tavaré 1986). The gamma shape parameter value was estimated directly from the data within MEGA. For the maximum parsimony (MP) analysis, the phylogeny was reconstructed using the close-neighbour-interchange (CNI) heuristic search method with the widest search level (level 3) to find the most parsimonious trees. The initial trees were obtained with the random addition of sequences (10 replicates). Confidence for internal branches was obtained through bootstrap analysis (1000 replicates) (Felsenstein 1985). Bootstrap support (BS) values >70% were considered significant in this study. To determine whether significant differences in substitution rates exist between the individual data sets, the disparity index (I_D) test of pattern heterogeneity (Kumar and Gadagkar 2001) with 1000 Monte Carlo replications was performed in MEGA.

Onnia tomentosa was chosen as an outgroup based on previous studies (Wagner and Fischer 2002; Tomšovský et al. 2010) and ITS sequence alignments using representative isolates of *Fomitiporia*, *Fuscoporia*, *Phellinidium*, *Phellinus* s.s. and *Onnia* sequenced from the CFMR culture collection. We also included '*Phellinus*' *coronadensis* as an outgroup species in this study. While this species is known only from coniferous hosts in the southwestern United States, a previous phylogenetic analysis using partial nuclear small subunit sequences showed that '*P.*' *coronadensis* grouped distal to *P. pini* s.l. isolates from western North America (Rizzo et al. 2003). Because of the ecological similarities, we were interested to determine how closely related '*P.*' *coronadensis* is to *Porodaedalea* using a multilocus data set.

2.4 Nucleotide diversity and neutrality tests

Differentiation analyses, performed in DNASP v. 5.10 (Librado and Rozas 2009) unless otherwise noted, were used to examine differences among isolates in the Holarctic group (described in Results section 3.2). Sites with gaps were excluded from all analyses. Nucleotide diversity was determined by calculating the total number of polymorphic sites (S), haplotype diversity (H_d, Nei 1987), nucleotide diversity per site between two sequences (π, Nei 1987) and the average number of pairwise nucleotide differences between two sequences (k, Tajima 1983). Haplotypes were reconstructed using PHASE v. 2.1 (Stephens and Donelly 2003), as implemented in DNASP.

Departures from the standard neutral model of evolution were tested using Tajima's D test (Tajima 1989), Fu and Li's D and F test (Fu and Li 1993) and Fay and Wu's H test (Fay and Wu 2000). Significance for each test was determined using 1000 coalescent simulations assuming an intermediate recombination rate and a mutation rate equal to θ. Recombination rates were generated using Hudson's R test (Hudson 1987). Under the infinite-sites model of neutrality, all test values should equal zero (Kimura 1968). When a population is experiencing a balancing or diversifying selection, a large number of high-frequency variants are observed, which is indicated in Tajima's D and Fu and Li's D and F test with positive values, while for Fay and Wu's H test, this is indicated by a negative value. When population growth introduces rare alleles, or conversely natural selection operates through a purifying or directional selection, low-frequency variants are expected in larger number, and Tajima's D and Fu and Li's D and F test values are negative, while Fay and Wu's H value is positive (Fu and Li 1993; Fay and Wu 2000; Hartl and Clark 2007). For the Holarctic group (described in Results section 3.2), we used *P. pini* s.s. as the outgroup species in Fu and Li's D and F test and Fay and Wu's H test, while for *P. pini* s.s., we used *Porodaedalea* sp. 1 from the southeastern United States.

Population size changes, illustrated by the distribution of pairwise nucleotide site differences (also known as the mismatch distribution), were also calculated. Models for expected values were based on: (i) a constant population size with no recombination and (ii) a population growth–decline model using an initial and final mutation rate ($\theta = 4N\mu$, where N is the effective population size and μ is the mutation rate) and the growth or decline of the population in mutational time ($\tau = 2\mu t$, where μ is the mutation rate per generation and t is the time in generations).

2.5 Gene flow and genetic differentiation

To determine rates of gene flow among *Porodaedalea* species (described in Results section 3.2), F_{ST} estimates were generated based on comparisons of frequencies at polymorphic sites (Hudson et al. 1992b). The distance matrix of F_{ST} values was then imported into TREEFIT (Kalinowsi 2009), which compares observed distances between populations with fitted distances using the neighbour-joining (NJ) (Saitou and Nei 1987) and UPGMA (Sokal and Michener 1958) methods, and expresses that relationship using the r^2 statistic. Based on this analysis, the NJ method ($r^2 = 0.982$) was found to best describe the distance data compared to UPGMA ($r^2 = 0.935$). The distance matrix was then imported into MEGA where the NJ method was used to produce a dendrogram representing genetic distances among *Porodaedalea*.

To assess genetic differentiation, Hudson's test of geographical subdivision was performed (Hudson et al. 1992a), which is based on the average number of nucleotide differences between sequences. Specifically, K_{ST} is equal to $1 - (K_S/K_T)$, where K_S is a weighted mean of nucleotide differences between sequences in two subpopulations, while K_T is the mean number of differences in the total population. When K_S is low and K_{ST} approaches a value of 1, then the null hypothesis of no genetic differentiation can be rejected at $p = 0.05$ (Hudson et al. 1992a). However, like Wright's F_{ST}, values less than 0.5 can signify very significant geographical subdivision (Hudson et al. 1992a). The test was performed using three subgroups within the Holarctic group based on geographic origin and polymorphisms within the sequence alignment (see Results section 3.2). To assess significance, we used the permutation test with 1000 replicates and a pseudorandom number seed (Hudson 2000).

3 Results

3.1 PCR and disparity index test

Amplicon sizes for *Porodaedalea* isolates ranged from: 748–765 bp for ITS; 975–979 bp for nLSU; 731 bp for *tef1*; and 894 bp for *rpb2*. The new forward primer (1487F) developed to generate partial *tef1* sequences for *Porodaedalea* also successfully amplified *Onnia tomentosa*, *Fuscoporia* spp. and '*Phellinus*' *coronadensis*. However, alignment of partial *tef1* sequences using numerous *Fomitiporia* species (Decock et al. 2007) highlights a single nucleotide polymorphism (SNP) that would inhibit amplification for certain species. Therefore, this primer will require modification before it has utility for all *Phellinus* s.l. species.

Results of the I_D test showed significant ($p < 0.05$) differences in substitution patterns among the loci utilized in this study. Two loci (ITS and *tef1*) evolved with significantly different substitution patterns compared to every other locus. Meanwhile, the remaining two loci (nLSU and *rpb2*) showed no significant differences in substitution patterns in all pairwise comparisons. Phylogenetic reconstructions using ML and MP were carried out separately for each locus, and there were no significant differences in tree topology (BS values $\geq$ 70% at the nodes) between any individual data sets (results not shown). Therefore, we are presenting the results from the concatenated (ITS+nLSU+*tef1*+*rpb2*) data set.

3.2 Phylogenetic analysis

The number of total characters and parsimony informative characters in the aligned data sets are as follows: ITS (765, 30); nLSU (979, 12); *tef1* (688, 33); *rpb2* (851, 22); and ITS+nLSU+*tef1*+*rpb2* (3248, 97). Results of the phylogenetic analysis revealed that isolates of *P. chrysoloma* s.s. and *P. pini* s.s. from Europe grouped separately from North American *Porodaedalea* isolates (Fig. 1). While isolates of *P. chrysoloma* s.s grouped with very strong BS, values for *P. pini* s.s. were slightly below the threshold for significance (Fig. 1). We detected two unique clades, both supported with significant BS, among isolates that originated from the southwestern (Arizona) and southeastern (Virginia and Georgia) United States, respectively (Fig. 1). A group of 30 isolates originating from the northern United States, Canada and Fennoscandia grouped together with no significant BS (Fig. 1). Within this group, *P. cancriformans* was positioned distal to all other isolates. The remaining 28 isolates, labelled here as the 'Holarctic group', were collected from *Larix*, *Picea*, *Pinus*, *Pseudotsuga* and *Tsuga* and originated from locations with tremendous geographic disparity (Table 1). There was no significant BS in the grouping of Holarctic group isolates by host or region of origin (Table 1, Fig. 1).

Within the Holarctic group, three subgroups were designated based on sequence alignments and phylogenetic grouping (Fig. 1). Despite a lack of significant BS, the subgroups are roughly divided by region of origin and are described as follows: (i) Atlantic-Boreal (eastern North America, Fennoscandia and interior Alaska); (ii) Interior (Colorado, Idaho and interior Oregon); and (iii) Pacific (California, coastal Oregon and coastal Alaska) (Table 1, Fig. 1).

For isolates of '*Phellinus*' *coronadensis*, partial sequences were successfully generated for the ITS, nLSU and *tef1* regions. However, *rpb2* amplicons could not be generated using primers bRPB2-6F and bRPB-7.1R, which we suspect is due to a SNP in either the forward or reverse primer. We did not attempt subsequent amplifications with additional *rpb2* primers, and therefore, this species was not included in the complete data set. When analyses were performed using the combined ITS+nLSU+*tef1* data set, '*P.*' *coronadensis* grouped distal to all *Porodaedalea* species and *Onnia tomentosa* (results not shown). Subsequent phylogenetic analysis of ITS and nLSU sequences of *Fomitiporia* spp., *Fuscoporia* spp., *Onnia tomentosa*, *Phellinus* (s.s.) spp. and *Porodaedalea* spp. established that '*P.*' *coronadensis* is most closely related with *Porodaedalea* and *O. tomentosa* (results not shown).

3.3 Nucleotide diversity and neutrality tests

Tests showed that the average nucleotide diversity and the average number of pairwise differences per sequence for all Holarctic group isolates were higher in partial *tef1* sequences compared to ITS, nLSU and *rpb2* sequences (Table 2). The average total nucleotide diversity (π) for the four gene regions studied was $\pi = 0.0047$ (Table 2). Overall, haplotype diversity was very high within the Holarctic group, ranging from 0.747 to 0.975 (Table 2), with considerable variation in values by locus and subgroup (Table 2). Across all loci, the average nucleotide diversity and average number of pairwise differences among the three Holarctic subgroups were as follows: (i) Atlantic-Boreal ($\pi = 0.0041$, $k = 13.16$); (ii) Interior ($\pi = 0.0019$, $k = 6.00$); and (iii) Pacific ($\pi = 0.0032$, $k = 10.31$).

Results of the neutrality tests demonstrated significant departures from the standard neutral model, primarily within the ITS, *tef1* and *rpb2* data sets (Table 2). For all Holarctic group isolates, positive selection was indicated within the ITS and

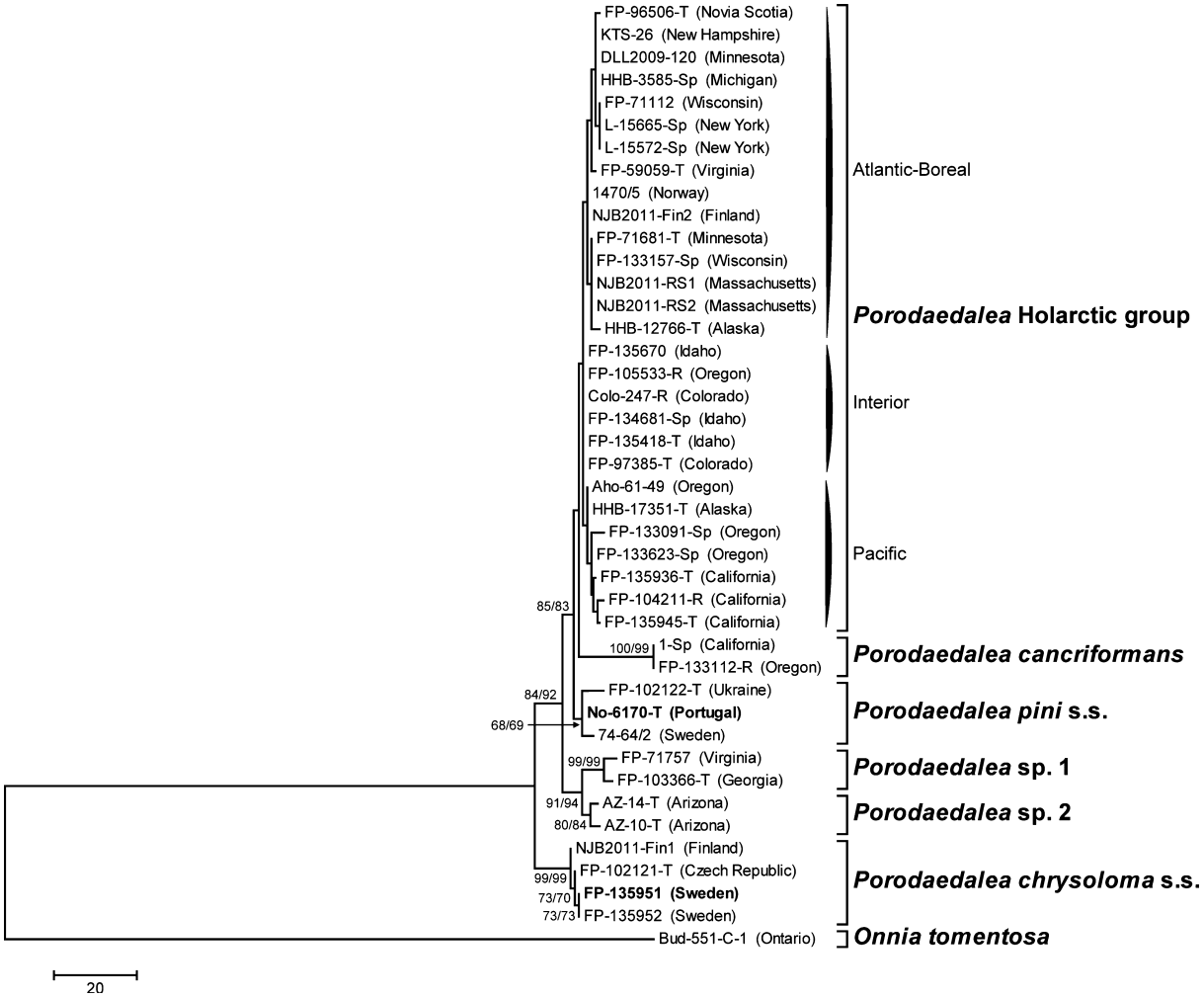

Fig. 1. Consensus tree of the concatenated data set of partial sequences (ITS+nLSU+*tef1*+*rpb2*) for *Porodaedalea* using MP, with gaps and missing data excluded from the analysis. Consensus BS values (1000 replicates) for ML and MP with values greater than 60% are listed next to the nodes. Isolate codes in bold indicate the specimen is the neotype. GenBank accession numbers are listed in Table 1.

tef1 data sets with significantly positive values using Tajima's *D* and Fu and Li's *D* and *F* tests and significantly negative values (for *tef1* only) using Fay and Wu's *H* test (Table 2). Conversely, for the *rpb2* data set, departures using Tajima's *D* and Fu and Li's *F* were significantly negative (Table 2). These trends were largely driven by the Atlantic-Boreal subgroup, yet were also observed in the Pacific subgroup (Table 2). *Porodaedalea pini* s.s. also demonstrated a similar pattern of significant departures from the neutral model within the ITS and *tef1* data sets (Table 2).

Using the combined Holarctic group data set, the mismatch distribution displayed a pattern consistent with a population undergoing size expansion based on the population growth–decline model (initial $\theta = 4.49$, final $\theta = 1000$ and $\tau = 10.59$) (Fig. 2). When performed separately with each individual data set, this pattern was consistently displayed (results not shown).

3.4 Gene flow and genetic differentiation

Comparison of the three Holarctic subgroups demonstrated limited gene flow ($0.312 < F_{ST} < 0.445$) and moderate rates geographical subdivision ($0.162 < K_{ST} < 0.254$, $p < 0.001$) (Table 3). The NJ analysis, based on F_{ST} estimates, illustrated this clustering by subgroup compared to all other *Porodaedalea* species (Fig. 3). Overall, the three Holarctic subgroups were most closely related to *P. pini* s.s., which is in agreement with the results of our phylogenetic analysis (Table 3, Fig. 3). Furthermore, estimates of F_{ST} between the three Holarctic subgroups and *P. cancriformans* approached near complete separation, ranging from 0.812 to 0.904, providing support for the species status of *P. cancriformans* (Table 3, Fig. 3).

4 Discussion

The primary goal of this study was to elucidate the complex of *Porodaedalea* species occurring on coniferous hosts in North America using a multilocus phylogeny and differentiation analyses. We have confirmed that *P. chrysoloma* s.s. and *P. pini*

Table 2. Nucleotide diversity and neutrality tests for Porodaedalea Holarctic group and P. pini s.s. haplotypes.

			Nucleotide polymorphisms			Neutrality tests[1]			
Group & Data set[2]	h[3]	H_d[4]	S[5]	π[6]	k[7]	D_{Tajima}	$D_{Fu\ \&\ Li}$	F	H
Holarctic Group: All (n = 56)									
ITS	23	0.951 (0.013)	18	0.0043	3.28	−0.51	1.76**	1.13	0.88
nLSU	14	0.747 (0.057)	8	0.0020	1.91	0.26	1.33	1.16	0.26
tef1	16	0.866 (0.031)	17	0.0084	5.75	1.70**	1.73**	2.07**	−2.03
rpb2	33	0.975 (0.008)	28	0.0049	4.14	−1.04*	−1.28	−1.43*	0.52
ITS+nLSU+*tef1*+*rpb2*	52	0.997 (0.004)	71	0.0047	15.08	−0.08	0.84	0.56	−0.42
Holarctic Group 1: Atlantic-Boreal (n = 30)									
ITS	12	0.901 (0.030)	6	0.0031	2.34	1.55*	1.26	1.60*	0.27
nLSU	7	0.670 (0.077)	4	0.0009	0.89	−0.29	0.01	−0.09	−0.08
tef1	12	0.862 (0.048)	12	0.0083	5.72	2.90**	1.58*	2.41**	−0.62
rpb2	20	0.968 (0.017)	17	0.0049	4.21	−0.07	0.52	0.39	0.73
ITS+nLSU+*tef1*+*rpb2*	28	0.995 (0.010)	39	0.0041	13.16	1.24**	1.22*	1.49**	0.17
Holarctic Group 2: Interior (n = 12)									
ITS	6	0.864 (0.072)	5	0.0021	1.62	−0.08	1.32*	1.13	1.00
nLSU	2	0.303 (0.147)	1	0.0003	0.30	−0.19	0.70	0.57	0.54
tef1	1	0	0	0	0	0	0	0	0
rpb2	10	0.970 (0.044)	12	0.0048	4.08	0.11	−0.01	0.03	0.88
ITS+nLSU+*tef1*+*rpb2*	12	1.000 (0.034)	18	0.0019	6.00	0.03	0.59	0.52	1.06*
Holarctic Group 3: Pacific (n = 14)									
ITS	8	0.912 (0.049)	9	0.0042	3.18	0.47	1.53*	1.47*	1.16*
nLSU	7	0.846 (0.074)	6	0.0021	2.07	0.34	1.37*	1.29	−3.01**
tef1	4	0.780 (0.061)	3	0.0017	1.20	0.83	1.08	1.19	0.82
rpb2	9	0.934 (0.045)	14	0.0046	3.87	−0.49	−1.00	−1.03	0.24
ITS+nLSU+*tef1*+*rpb2*	12	0.978 (0.035)	32	0.0032	10.31	0.10	0.59	0.54	−0.11
P. pini s.s. (n = 6)									
ITS	2	0.600 (0.129)	1	0.0008	0.60	1.45**	0.88	1.13	0
nLSU	3	0.600 (0.215)	3	0.0014	1.40	0.34	1.34	1.32	−1.70
tef1	4	0.867 (0.129)	16	0.0113	7.80	0.70	1.95**	1.99**	−1.93*
rpb2	4	0.800 (0.172)	4	0.0024	2.00	0.77	0.63	0.77	0.70
ITS+nLSU+*tef1*+*rpb2*	6	1.000 (0.096)	24	0.0036	11.80	0.77	1.84**	1.91**	−1.52*

[1]Neutrality Tests: Tajima's D, Fu and Li's D and F and Fay and Wu's H (normalized by SD). Significant departures from standard neutral model are noted as: *$p \leq 0.05$ and **$p \leq 0.01$.
[2]Number of haplotypes in parenthesis.
[3]h: total number of haplotypes.
[4]H_d: haplotype diversity with SD in parenthesis.
[5]S: total number of polymorphic sites.
[6]π: nucleotide diversity per site between two sequences.
[7]k: average number of pairwise differences per sequence.

s.s. do not occur in North America, as originally proposed by Fischer (1996). We also detected two undescribed taxa of *Porodaedalea* in the southern United States. However, the story remains unresolved for isolates originating from the northern United States, Canada and Fennoscandia, an assemblage we have labelled the Holarctic group. This group displayed monophyly in our phylogenetic analysis but exhibited significant genetic differentiation based on tests of population subdivision. The possibility that the Holarctic group represents multiple cryptic species warrants further evaluation with additional loci.

The basal group in our phylogenetic analysis was *P. chrysoloma* s.s., which is in agreement with the results of Tomšovský et al. (2010). The next most distal group consisted of two clades that represent unique phylogenetic species (*Porodaedalea* sp. 1 from the southeastern United States and *Porodaedalea* sp. 2 from the southwestern United States). While fruiting bodies of the isolates used in our analyses are available, we believe that formal description is premature until more isolates are examined from across the southern United States. Isolates of *P. pini* s.s., which originated from across Europe, grouped adjacent to North American *Porodaedalea* isolates, confirming the close relationship between these two groups. The *P. pini* s.s. clade was supported by BS values slightly less than 70%, but was monophyletic using isolates with considerable geographic disparity. *Porodaedalea cancriformans* grouped distal to the Holarctic group, yet BS separating this species was weak. However, *P. cancriformans* can be distinguished from the Holarctic group based on host specificity, as this species is known only from *Abies* spp. in northwestern North America (Larsen et al. 1979). In addition, *P. cancriformans* colonizes the sapwood of infected trees creating sunken cankers, while other *Porodaedalea* species primarily colonize the heartwood (Sinclair and Lyon 2005).

It was surprising that the 28 isolates comprising the Holarctic group, which derived from across northern North America and Fennoscandia and were collected from five genera in the Pinaceae, could not be accurately subdivided with a four-

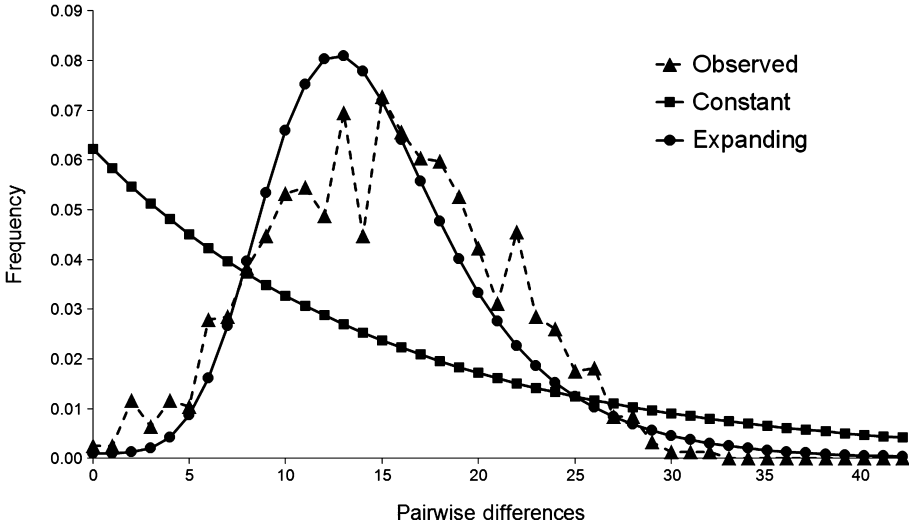

Fig. 2. Mismatch distribution for *Porodaedalea* Holarctic group haplotypes (n = 56). Observed frequencies of pairwise nucleotide site differences (triangles with dashed line) compared with frequencies expected under a constant population size model (squares with solid line) and a growth–decline model (circles with solid line).

Table 3. Gene flow (F_{ST}) and genetic differentiation (Hudson's test of geographical subdivision) between Porodaedalea haplotypes.

			Hudson's test		
Group A[1]	Group B	F_{ST}	K_S	K_T	K_{ST}[2]
Atlantic-Boreal	Interior	0.348	11.11	13.25	0.162***
Atlantic-Boreal	Pacific	0.445	12.25	16.43	0.254***
Atlantic-Boreal	*P. pini* s.s.	0.451	12.93	15.85	0.184***
Atlantic-Boreal	*Porodaedalea* sp. 1	0.679	12.86	18.24	0.295***
Atlantic-Boreal	*Porodaedalea* sp. 2	0.620	12.75	16.73	0.238***
Atlantic-Boreal	*P. cancriformans*	0.812	11.61	17.92	0.352***
Atlantic-Boreal	*P. chrysoloma* s.s.	0.801	10.99	22.02	0.501***
Interior	Pacific	0.312	8.32	10.23	0.187***
Interior	*P. pini* s.s.	0.559	7.93	13.25	0.401***
Interior	*Porodaedalea* sp. 1	0.769	7.17	18.25	0.607**
Interior	*Porodaedalea* sp. 2	0.678	6.92	13.52	0.488***
Interior	*P. cancriformans*	0.904	4.50	15.83	0.716**
Interior	*P. chrysoloma* s.s.	0.885	4.74	21.98	0.784***
Pacific	*P. pini s.s.*	0.551	10.76	16.76	0.358***
Pacific	*Porodaedalea* sp. 1	0.750	9.29	20.03	0.536**
Pacific	*Porodaedalea* sp. 2	0.665	9.07	15.83	0.427***
Pacific	*P. cancriformans*	0.856	8.02	19.20	0.583***
Pacific	*P. chrysoloma* s.s.	0.845	7.60	24.94	0.695***
P. pini s.s.	*Porodaedalea* sp. 1	0.697	11.35	25.13	0.549**
P. pini s.s.	*Porodaedalea* sp. 2	0.634	10.95	20.87	0.475***
P. pini s.s.	*P. cancriformans*	0.848	7.08	24.64	0.713**
P. pini s.s.	*P. chrysoloma* s.s.	0.822	6.69	24.54	0.727***
Porodaedalea sp. 1	*Porodaedalea* sp. 2	0.593	10.17	18.64	0.455*
Porodaedalea sp. 1	*P. cancriformans*	0.887	5.33	29.29	0.818*
Porodaedalea sp. 1	*P. chrysoloma* s.s.	0.861	5.46	25.76	0.788**
Porodaedalea sp. 2	*P. cancriformans*	0.890	4.83	27.21	0.822*
Porodaedalea sp. 2	*P. chrysoloma* s.s.	0.867	5.13	24.88	0.794**
P. cancriformans	*P. chrysoloma* s.s.	0.973	1.90	26.67	0.929**

[1]Atlantic-Boreal represents isolates from eastern North America, Fennoscandia and interior Alaska; Interior represents isolates from Colorado, Idaho and interior Oregon; and Pacific represents isolates from California, coastal Oregon and coastal Alaska.
[2]Significant K_{ST} values are noted as: *p $\leq$ 0.05; **p $\leq$ 0.01; and ***p $\leq$ 0.001.

locus data set. This group was presumed to represent at least three morphological species in North America (*P. gilbertsonii*, *P. pini* s.l. and *P. piceina*). Moreover, North American isolates believed to represent *P. piceina* (those occurring on *Picea*) did not cluster with European isolates of *P. chrysoloma* s.s., which challenges the notion that the two are sister taxa (Niemelä

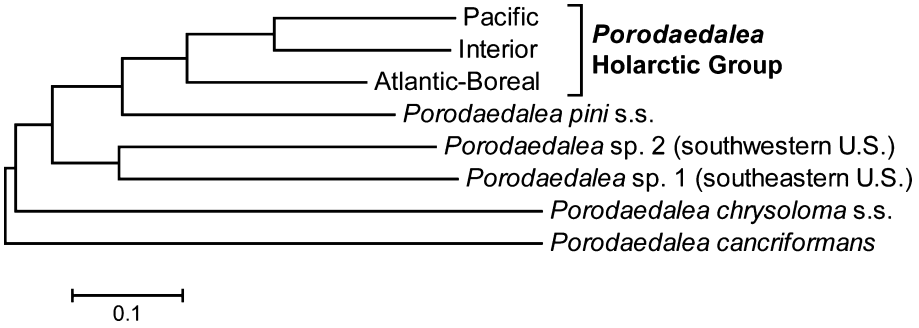

Fig. 3. Neighbour-joining dendrogram of genetic distances based on F_{ST} estimates for haplotypes of *Porodaedalea*.

1985; Fischer 1996). These results disprove our original hypotheses that North American *Porodaedalea* isolates would group primarily by host, and that we would be able to distinguish among isolates presumed to represent *P. gilbertsonii*, *P. piceina* and Fischer's N-II to N-VII (Fischer 1996).

Two isolates in our Holarctic group were collected from Finland (NJB2011-Fin2) and Norway (1470/5), respectively. The basidiocarp from Finland was analysed microscopically following its collection and was identified as *P. laricis* based on morphological characters (T. Niemelä, personal communication). For decades, *P. laricis* was confused with *P. chrysoloma* s.s. and was thought to be restricted to *Larix* species (Niemelä et al. 2005), but is now known to attack *Larix*, *Picea* and *Pinus* in Europe and Asia (Tomšovský et al. 2010). Because of the potential relationship between the Holarctic group and *P. laricis*, ITS sequences of *P. laricis* generated by Tomšovský et al. (2010) were combined with our ITS data set and the phylogenetic analysis was performed again. Isolates of *P. laricis* originated from the Czech Republic, Finland, France, Kazakhstan, Norway, Russia, Slovakia and Sweden. The results showed that all presumed *P. laricis* isolates grouped together with the Holarctic group with no significant statistical support. If the Holarctic group is truly conspecific with *P. laricis*, it would indicate that *P. laricis* has the largest geographic range of any *Porodaedalea* species and can attack most, if not all, members of the Pinaceae.

Nucleotide diversity levels detected at each locus (ITS, nLSU, *tef1* and *rpb2*) for the Holarctic group were similar to levels detected within North American populations of the mycorrhizal fungus *Amanita muscaria* (Geml et al. 2008) and the root and butt rot pathogen *Heterobasidion irregulare* (Dalman et al. 2010). Neutrality tests performed using the Holarctic group and *P. pini* s.s. showed there were significant departures from the standard neutral model of evolution, although the results were not consistent by subgroup or locus. For all isolates in the Holarctic group, neutrality tests at two loci (ITS and *tef1*) suggest that a balancing or diversifying selection may be acting to maintain heterozygous genotypes in the population (Fu and Li 1993). This trend was primarily influenced by the *tef1* data set in the Atlantic-Boreal subgroup, but was also observed in the Interior and Pacific subgroups, in addition to *P. pini* s.s. Diversifying selection is believed to favour extreme phenotypes (Hartl and Clark 2007), which could explain why the Holarctic group is thought to represent numerous morphological species in North America. If the positive departures represent a balancing selection, it could suggest the Holarctic group represents a newly divergent species (Hartl and Clark 2007).

When considering all Holarctic group isolates, one data set (*rpb2*) produced an excess of low-frequency variants, illustrated in the neutrality tests with significant, negative departures. These results could suggest that population growth is introducing rare alleles into the population. This hypothesis is supported by the mismatch distribution, which displayed a pattern of population expansion across all loci, and not the pattern typically displayed by a population with a constant size. However, population expansion is strongly dependent upon varying rates of recombination and would need to be assessed across a larger number of loci (Fay and Wu 2000; Hartl and Clark 2007). Furthermore, negative departures from the standard neutral model can indicate that a purifying or directional selection is taking place, which would shift the population towards a single, advantageous phenotype (Hartl and Clark 2007).

Significant levels of genetic differentiation were detected within the Holarctic group, and three subgroups were established, in part, by region of origin (Atlantic-Boreal, Interior and Pacific). Altogether, *Porodaedalea* isolates from all three subgroups were collected from multiple host genera, none of which were unique to one subgroup, providing support that environment is a more important factor than host in understanding *Porodaedalea* species delimitation. Our subgrouping is somewhat tenuous due to the low sample size, but it provides a basic framework for further studies into population structure and incidence of potentially cryptic species throughout the northern hemisphere. It has been hypothesized that sympatric speciation in North America has led to several, biologically distinct species of *Porodaedalea* that show host specificity (Fischer 1994, 1996). Using the phylogenetic species concept, our analyses only partially support this claim, as we detected unique clades in the southern United States on *Pinus*, and the ranges of these two undescribed species overlap with the range of the Holarctic group. Yet, there appears to be no host specificity when considering all hosts that the *Porodaedalea* Holarctic group, *Porodaedalea* sp. 1 and *Porodaedalea* sp. 2, were collected from, even within the subgenera of *Pinus* (subgenus *Pinus* and subgenus *Strobus*). Therefore, specialization for a particular host does not appear to be a major driver of speciation patterns for *Porodaedalea* in North America (see Thibert-Plante and Hendry 2011). Thus, we are left to conclude that in North America, *Porodaedalea* is not host-specific, contrary to Fischer's hypothesis (Fischer 1996). *Porodaedalea cancriformans* represents an exception, as this species is known only from *Abies* (Larsen et al. 1979).

In the past, phenotypic differences have largely driven species-level identification in *Phellinus* s.l. (see Gilbertson and Ryvarden 1988). While there are phenotypic differences among Holarctic group isolates that could be interpreted as demarcating unique species, these morphotypes were not region- or host-specific. Overall, morphological characters have not always been valuable for species identification in *Phellinus* s.l. (Larsen and Cobb-Poulle 1990). Recently, it was shown that basidiospore, setae and pore width measurements were unable to confidently distinguish European *Porodaedalea* species (Tomšovský et al. 2010). The lack of discriminatory morphological characters in *Phellinus* s.l. has also been discussed by Amalfi et al. (2012), who detected numerous phylogenetic species within the conventionally defined *Fomitiporia robusta* s.l. complex in North America. Amalfi et al. (2012) contend that ecological characters, when coupled with molecular data, may be more informative for species delimitation than relying on morphological characters alone. Phenotypic divergence within a population is often thought to indicate genetic divergence. While the two types of divergence are often linked to one another, numerous examples exist where phenotypic and genetic divergences do not correspond (Kozak et al. 2011), as is the case for the *Porodaedalea* Holarctic group. We believe that a combination of molecular data, macroscopic features and ecological characters (host preference, region of origin and disease etiology) will ultimately delineate *Porodaedalea* into reasonably well-accepted species.

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