1 **Contrasting genetic structuring in the closely related basidiomycetes *Trichaptum***
2 ***abietinum* and *T. fuscoviolaceum* (Hymenochaetales)**
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16 Running title: Contrasting genetic structure in two basidiomycetes

17 **Abstract**

18 *Trichaptum abietinum* and *Trichaptum fuscoviolaceum* (Hymenochaetales, Basidiomycota)
19 are closely related saprotrophic fungi, widely distributed on coniferous wood in temperate
20 regions worldwide. Three intersterility groups have previously been detected in *T. abietinum,*
21 while no prezygotic barriers have been proven within *T. fuscoviolaceum*. The aim of this
22 study was to reveal the phylogeography and genetic relationship between these two closely
23 related species and to explore whether the previously observed intersterility groups in *T.*
24 *abietinum* are reflected in the genetic data. We assembled worldwide fruit body collections of
25 both species (N = 314) and generated DNA sequences from three nuclear (ITS2, LSU, IGS)
26 and one mitochondrial rDNA region (mtLSU). The two species are genetically well separated
27 in all analyses. In correspondence with observations from earlier mating studies, our results
28 revealed that *T. fuscoviolaceum* is genetically more uniform than *T. abietinum*. Multiple
29 genetic sub-groups exist in *T. abietinum* that may correspond to the previously observed
30 intersterility groups. However, there is low consistency across the investigated loci in
31 delimiting the different sub-groups, except for a consistent North American group. As for
32 many other widespread fungi, a complex phylogeographic pattern is found in *T. abietinum*
33 which may have been formed by geographic, as well as multiple genetic intersterility barriers.

34

35 Key words: Basidiomycetes; Hymenochaetales; closely related; phylogeography; *Trichaptum*

36 **Introduction**

37 Biogeographic patterns within fungal species have historically been difficult to study because
38 of their largely hidden lifestyle within soil or substrates (Bisby 1943, Pirozynski 1983).
39 Fungal species have traditionally been described and recognized mainly based on
40 morphological characters. After the inclusion of genetic data in fungal studies in the late part
41 of the last century, it was revealed that fungal morphotaxa defined by fruit body
42 characteristics often included independently evolving phylogenetic lineages with different
43 biogeographic distributions (Thon and Royse 1999). One could recognize distinct populations
44 (Otrosina et al. 1993, Vilgalys and Sun 1994, Koufopanou et al. 2001, Sheedy et al. 2015,
45 Bueker et al. 2016) and species which previously were described as e.g. ecotypes (Hibbett et
46 al. 1995). More recent DNA based phylogeographic studies of fungi show that they largely
47 apply to the same rules as other organism groups (Kohn 2005); their distributions are shaped
48 by biotic and abiotic factors and the current distribution patterns can be traced back to events
49 such as the repeated glaciations during the Pleistocene (Geml et al. 2006, Branco et al. 2015)
50 or human mediated long distance dispersal (Kauserud et al. 2007a, Linzer et al. 2008, Savary
51 et al. 2018). Before the use of molecular tools in mycology, artificial crossings of haploid
52 monospore cultures was a widespread tool to determine species delimitations in fungi (Lange
53 1952, Aschan 1954). In basidiomycetes, the establishment of clamped dikaryotic mycelia was
54 used as an approximation of interfertility (Aanen and Kuyper 1999) although this only
55 represent one early pre-mating stage hindering fruitification and meiosis. In many morphotaxa,
56 two or more intersterility groups with lack of or reduced interfertility (or ability to form
57 dikaryons), were detected using such mating studies (Mounce and Macrae 1938, Anderson et
58 al. 1980). In some species, two sympatric intersterility groups which were partially fertile
59 towards a geographically separated third group were discovered, a so-called A-B-C mating
60 system (Macrae 1967, Kemp 1980). This phenomenon was first described among conspecifics

61 within the morphological defined species *Fomitopsis pinicola* (Sw.) P. Karst. (Polyporales),
62 where partial interfertility of two sympatric intersterility groups towards a third
63 geographically isolated group was demonstrated (Mounce and Macrae 1938). The A-B-C
64 mating system has also been detected within *Amylostereum* Boidin (Boidin and Lanquetin
65 1984). Both the intersterile morphospecies *Amylostereum chailleti* (Pers.) Boidin and
66 *Amylostereum laevigatum* (Fr.) Boidin were interfertile with the geographically isolated
67 *Amylostereum ferreum* (Berk. and M.A. Curtis) Boidin and Lanq. (Boidin and Lanquetin
68 1984). The multiple intersterility groups observed in *Heterobasidion annosum* (Fr.) Bref. s. lat.
69 has later been described as separate biological species (Otrosina and Garbelotto 2010).
70 Dettman et al. (2003) were able to cross *Neurospora crassa* Shear and B.O. Dodge and
71 *Neurospora intermedia* F.L. Tai despite no hybrids between these have been observed in
72 nature. Interspecific crossings from sympatric specimens were less successful compared to
73 specimens obtained from allopatric populations (Dettman et al. 2003). This could indicate the
74 evolution of intersterility barriers in sympatry by reinforcement mechanisms, i.e. natural
75 selection against hybridization to prevent maladaptive offspring (Dobzhansky 1937).
76 Reinforcement would not affect geographically isolated populations, thus making an A-B-C
77 mating system as in *F. pinicola* possible. *Trichaptum* Murrill (Hymenochateales) is a
78 cosmopolitan genus of saprotrophic polypores that causes white rot of both conifers and
79 hardwood. The morphospecies *T. abietinum,* (Pers. Ex J.F. Gmel.) Ryvarden and *T.*
80 *fuscoviolaceum* (Ehrenb.) Ryvarden were previously described as hymenial variants of
81 *Polyporus abietinus* (Pers. Ex J.F. Gmel.) Fr (Donk 1933). Hymenial differences are the only
82 distinct morphological character that separates the species. However, host reoccurrence
83 differs between the species, but artificial studies show that they decompose spruce litter at
84 equal rates (Ræstad 1940, Hakala et al. 2004). Ræstad (1940) concluded that *T. abietinum* and
85 *T. fuscoviolaceum* are subspecies of the same morphospecies. Later, Macrae (1967) conducted

86 an extensive artificial crossing study of mainly North American isolates within the genus
87 *Trichaptum*, where an A-B-C mating system was detected in *T. abietinum* with two sympatric
88 intersterile North American groups partially compatible with a third group from Europe, a
89 pattern indicating reinforcement processes. Isolates belonging to the two North American
90 intersterility groups did not show any geographical structuring within the continent (Macrae
91 1967). As opposed to *T. abietinum*, monokaryotic crossings of *T. fuscoviolaceum* did not
92 indicate any incompatibilities within or between North America and Europe. The same
93 patterns within the two species were described by Magasi (1976) on specimens from Canada.

94 In this study, we analyze the genetic structure within the two morphospecies
95 *T. abietinum* and *T. fuscoviolaceum* and evaluate the results in light of earlier mating
96 experiments conducted by Macrae (1967). We (1) explore the phylogeographic pattern of
97 *Trichaptum abietinum* and *T. fuscoviolaceum* on a worldwide sample with emphasis on North
98 American and European collections, (2) examine if the A-B-C mating system proposed in
99 *T. abietinum* is reflected in the genetic data, and (3) examine how genetically separated the
100 two morphospecies are and whether indications of gene flow between them can be found.
101 Four genetic rDNA regions are analyzed by Sanger sequencing to explore the genetic
102 diversity within and between the species. Based on results from (Macrae 1967) we
103 hypothesize minimum two distinct North American phylogenetic groups within *T. abietinum*
104 in contrary to one North American group within *T. fuscoviolaceum*. Few non-North American
105 isolates were investigated by Macrae (1967) and we expect that additional genetic groups can
106 be detected when sampling on a broader scale.

107

108 **Material and methods**

109

110 *Material*

111 We analysed a total of 166 and 148 herbarium specimens and cultures of *T. abietinum* and *T.*
112 *fuscoviolaceum*, respectively, in this study. For detailed information about the material, see
113 Table S1. All collections were georeferenced based on locality information using Google
114 maps and distribution maps made in R (R Development Core Team 2006. Fig. 1).
115
116 *Molecular analyses*
117 Collections were sampled for molecular analyses from herbarium specimens or cultures.
118 Small pieces of the herbarium specimens (basidiocarps) were cut off using a sterile blade and
119 cultures where opened and scraped off in a sterile bench. The material was frozen to -80 ºC in
120 600 µl cetyltrimethyl ammonium bromide (CTAB). Two sterile tungsten carbide beads were
121 used to crush the material on a Retsch ® MM301 mixer mill (Anders Pihl AS, Dale, Norway)
122 for 4 minutes with the frequency of 20 Hz. DNA extraction was completed following a 2%
123 CTAB protocol (Murray and Thompson 1980) with a few modifications as described in
124 Kauserud et al. (2007b)
125 DNA sequences were obtained from four different regions; ITS (nuclear ribosomal
126 internal transcribed spacer region), LSU (nuclear ribosomal large subunit), IGS (nuclear
127 intergenic spacer), and mtLSU (the mitochondrial ribosomal large subunit). ITS was
128 amplified with the primers ITS4 and ITS5 (White et al. 1990), IGS with the primers
129 CNL12/5SA (Anderson and Stasovski 1992) LSU with LR5/LR0R (Rehner and Samuels
130 1994), mtLSU with ML5/ML6 (White et al 1990) and PCR was performed in 25 µl reactions
131 using puRe*Taq*™ Ready-To-Go™ PCR Beads (GE healthcare, Buckinghamshire, UK) with 5
132 µL of 50X diluted DNA, 2.5 µL of each 5 µM primer and 15 µM sterilized H_2O. For ITS, and
133 nrLSU the PCR program was as follows: denaturation at 94 ºC for 4 min followed by 37
134 cycles of denaturation at 94 ºC for 25 s, annealing at 54 ºC for 30 s and extension at 72 ºC for
135 35 s followed by a final elongation step at 72 ºC for 10 min. The conditions for IGS,and

136 mtLSU were: 94 ºC for 5 min followed by 30 cycles at 94 ºC for 20 s, 54 ºC for 20 s, 72 ºC
137 for 45 s followed by a final step at 72 ºC for 7 min. All PCR products were sequenced in both
138 directions using the ABI BigDye Terminator sequencing buffer and v3.1 Cycle Sequencing
139 kit on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). The
140 5SA primer was not suitable as a sequencing primer, so only CNL12 was used in the
141 sequencing of the nrIGS region. In addition, ITS sequences were obtained from five cultures
142 from the original study by Macrae (1967; Table S1) by direct amplification with Phire Plant
143 Direct PCR Kit (Thermo Scientific, Waltham, USA) using the same PCR program as all ITS
144 sequences. The products were purified with ExpSap-IT (GE healthcare, Waukesha, WI) and
145 sanger sequenced by Eurofins (Constance, Germany).
146 All sequence chromatograms were examined manually in BioEdit (Hall 1999).
147 Alignments were made in MAFFT and manually checked for optimization. All sequences
148 have been accessioned in GenBank (see Table S1 for details).
149
150 *Data analyses*
151 Intraspecific genetic data often hold conflicting signals between DNA sequences due to
152 recombination events. Further, the inclusion of numerous heterozygous sites (mainly
153 dikaryotic tissue was analysed) makes phylogenetic analysis a challenging task. Several of the
154 ITS sequences of *T. abietinum* contained long indels (180-445 bp) in the ITS1 region.
155 Apparently, parts of the ITS1 region are duplicated in some *T. abietinum* specimens (Ko and
156 Jung 2002). Because of the highly complex structure of ITS1, this region was removed from
157 further analyses, and only ITS2 of the ITS was included.
158 Network analyses may be more suitable than conventional tree-based phylogenetic
159 analyses for analysing data with high levels of conflicting signals caused by e.g. intralocus
160 recombination. Thus, alignments for all regions were subjected to a NeighbourNet analyses in

161 SplitsTree 4. In addition, maximum likelihood (ML) trees were made for two alignments: the
162 mtLSU and a concatenated alignment of all specimens with sequences of all three nuclear
163 ribosomal regions (LSU, IGS and ITS2). The ML analyses were performed with the RaxML
164 v8.2.11 (Stamatakis 2006) plug-in in Geneious Prime (Biomatters, Auckland, NZ), using the
165 general time reversible CAT approximation (GTRCAT). Bootstrapping was done with 1000
166 pseudo replicates and one heuristic search per replicate. No outgroups were assigned, but the
167 trees were rooted between species for a more simplistic visual interpretation.
168
169 **Results**
170 *Data characteristics*
171 The geographic distribution of the analyzed specimens are displayed in Fig. 1. The
172 phylogenetic relationship among the *T. abietinum* and *T. fuscoviolaceum* collections was
173 evaluated with four genetic data sets: three nuclear ribosomal regions (ITS2, LSU and IGS)
174 and one mitochondrial region (mtLSU). The nuclear rDNA regions amplified to a higher
175 degree than the mitochondrial regions (see Table S1).
176 All the molecular regions clearly separated *T. abietinum* and *T. fuscoviolaceum*, and
177 100% bootstrap support was obtained in the concatenated nuclear rDNA (Fig. 2a). In the ITS2
178 network analyses, there were also a clear separation between the two species, but a few
179 specimens were placed in intermediate positions (Fig. 3d).
180
181 *Trichaptum abietinum*
182 Phylogenetic network analyses of ITS2 reflect a high genetic diversity in *T. abietinum*,
183 dividing the samples into four main groups, but with some scattered individuals (Fig. 3d):
184 Two European groups, a largely American group and a widespread group with sequences
185 from North America, Europe and Asia. Three of the sequences obtained from isolates used by

Macrae (1967) grouped with the North American group and one sequence grouped with the widespread group (Fig. 3d). Similar groupings were recognized for other genetic regions, e.g. for LSU a widespread, a North American and two European groups were found (Fig. 3c), in mtLSU and IGS the patterns are less clear (Fig. 3 b and c). Noteworthy, the number of individuals included, and which individuals, varies among these four regions (Table S1).

When the three nuclear rDNA regions were combined in the ML phylogeny a widespread, a North American, an Asian and two European groups were recognized (Fig. 2a). An additional North American group was recognized in the mtLSU ML phylogeny, and the Asian group was not supported (Fig. 2b).

Trichaptum fuscoviolaceum

Within *T. fuscoviolaceum* the branch lengths in the phylogenic trees (Fig. 2) are shorter than for *T. abietinum* in all loci analyzed indicating more similarity between the specimens examined. Two groups were recognized in the phylogenetic network analyses of ITS2, one with mainly Eurasian samples and one with a widespread sample from Europe, North America and Asia (Fig. 3d). These same groups were partially recognized in the mtLSU network (Fig. 3b), but not in IGS and LSU (Fig. 3 a and c). From the ML phylogenetic tree, these two groups could be observed in both the nuclear rDNA ML tree and in the mtLSU tree, but were supported in neither (Fig. 2).

Incongruences

Three specimens' sequences clustered with both species for the loci analyzed; one assumed *T. fuscoviolaceum* specimen (Tf6Estonia) was placed within the *T. abietinum* part of the tree in ITS2, but within *T. fuscoviolaceum* in LSU and mtLSU. Furthermore, two specimens

210 (tf152China and tf59Norway) were placed within *T. fuscoviolaceum* based on ITS2, while
211 they were classified as *T. abietinum* in LSU, IGS and mtLSU.
212

213 **Discussion**

214 The main aim of this study was to analyze the genetic structure within *T. abietinum* and *T.*
215 *fuscoviolaceum* on a worldwide sample.
216 Phylogenetic analyses of ITS2, IGS, LSU and mtLSU divided the specimens into two
217 main groups that correspond to the morphospecies *Trichaptum abietinum* (poroid
218 hymenophore) and *T. fuscoviolaceum* (irpicoid hymenophore). Hence, the phylogenetic
219 analyses largely support *T. abietinum* and *T. fuscoviolaceum* as well-separated species.
220 *Trichaptum fuscoviolaceum* holds lower molecular variation and shorter branch lengths and
221 there were no genetically supported sub-groups within *T. fuscoviolaceum* in our data.This
222 corresponds with the crossing tests carried out by Macrae, where no intersterility barriers
223 were detected within *T. fuscoviolaceum* (Macrae 1967) . Despite their similarities in ecology
224 and morphology, there is little evidence of gene flow between the species, although three
225 specimens clustered inconsistently with both species at different loci. These conflicting
226 phylogenetic signals could potentially be a result of introgression, as observed in *Neurospora*
227 (Corcoran et al. 2016), or incomplete lineage sorting, as in *Alternaria alternata* (Fr.) Keissl.
228 (Stewart et al. 2014). Introgression and incomplete lineage sorting cannot easily be
229 distinguished from each other when looking only at a few genes (Skrede et al. 2012, Zhou et
230 al. 2017). In this study, only a few nuclear and mitochondrial rDNA regions were investigated,
231 thus analysis of larger parts of the genome are necessary in order to conclude on the origin of
232 these deviating sequences.
233 In *T. abietinum,* numerous phylogeographic structured sub-groups were detected.
234 Hence, based on the current DNA data we observed a more complex phylogeographic pattern

235 in *T. abietinum* than the three A-B-C groups described by Macrae (1967). The sequences
236 obtained from cultures included in the study by Macrae grouped into two different groups, a
237 geographically restricted group with only North American specimens, and the widespread
238 group with specimens from North America and Eurasia. Monokaryotic isolates from the
239 North American and the widespread group were reproductively isolated groups in the study of
240 Macrae (1967). This indicates that the North American and the widespread groups in our
241 analyses represent the two intersterility groups found in the study by Macrae (1967). The
242 North American specimens in these two groups in our study were collected throughout
243 Canada and USA thus, there is no geographic structure of these two groups in North America.
244 Widespread lineages ranging through the boreonemoral zone of North America and Eurasia
245 have been found in other basidiomycetes: *Serpula himantioides (Fr.) P. Karst.* is comprised of
246 five phylogenetic species with one of them being sampled on all continents except Antarctica
247 (Carlsen et al. 2011). *Peniophorella praetermissa* (P. Karst.) K.H. Larss. s.lat. holds one
248 phylogenetic lineage distributed throughout the Northern Hemisphere while other lineages
249 have a narrower distribution (Hallenberg et al. 2007) In *Gloeoporus taxicola* (Pers.) Gilb. and
250 Ryvarden*,* there are multiple North American and Eurasian lineages, including one
251 widespread lineage extending the taiga (Seierstad et al. 2013). Hence, the existence of
252 circumboreal distributed groups seems to be a common geographic pattern in boreal fungi of
253 the Northern Hemisphere. Different glacial refugia, followed by secondary contact after
254 glaciations can explain the pattern of sympatric populations. However human mediated long
255 distance dispersal, due to global trade, cannot easily be ruled out as a contribution to the
256 widespread groups, as in *S. himantioides* (Kauserud et al. 2006) and *H. annosum* (Linzer et al.
257 2008*)*,since *T. abietinum* is a common fungus that is likely to be brought around within
258 wooden materials and also establish in plantations in non-native areas.
259

260 **Conclusions**

261 The two morphospecies *T. abietinum* and *T. fuscoviolaceum* were consistently well-separated
262 in our multi-locus analyses. We found evidence for genetic sub-groups within *T. abietinum*
263 that likely are reproductively isolated from each other, as well as from *T. fuscoviolaceum*.
264 Although it is hard to conclude based on our material, the A-B-C mating groups proposed by
265 Macrae (1967) is probably reflected in the genetic groups recovered here. However further
266 studies that combine high resolution genetic regions, larger sample size and artificial
267 crossings are necessary to conclude on the status of the incompatibility groups within *T.*
268 *abietinum* and the relationship towards *T. fuscoviolaceum* regarding potential introgression
269 and incomplete lineage sorting.
270

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275

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