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Pronounced ecological separation between two closely related lineages of the polyporous fungus *Gloeoporus taxicola*

Håvard KAUSERUD^{a,*}, Tom Hellik HOFTON^a, Glenn-Peter SÆTRE^b

^aDepartment of Biology, University of Oslo, P.O. Box 1066 Blindern, N-0316 Oslo, Norway

^bCentre for Ecological and Evolutionary Synthesis, Department of Biology, University of Oslo, P.O. Box 1066 Blindern, N-0316 Oslo, Norway

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ABSTRACT

Gloeoporus taxicola is a saprotrophic polypore that produces annual fruit bodies, in Scandinavia exclusively on coniferous wood. In this study, we demonstrate the existence of two ecotypes of *G. taxicola* in Norway; a coastal lowland form fruiting on dead logs and dying branches of standing *Pinus sylvestris*, and a highland form largely fruiting on trunks of *Picea abies* in moist, old-growth forests. Thus, a distinct ecological separation has happened between the two forms both concerning substrate and environmental requirements, leading to a rather distinct difference in distribution. Genetically, there seems to be a more step-wise transition between the two forms, as revealed by sequence analyses of two DNA regions. Genetic variation was significantly higher in the highland form compared with the lowland form, which could be due to disparate immigration histories in Scandinavia. Different evolutionary scenarios may explain the observed pattern, including a former allopatric distribution in isolated glacial refugia, succeeded by immigration into the same region. The partition of the genetic variation among the two forms suggests a recent or ongoing speciation event and possibly some ongoing introgression. Our findings have implications on the conservations status of the taxon, as their susceptibility to modern forestry seems to be different.

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Introduction

New species emerge from ancestral species by divergence through adaptation and genomic change, or by hybridization of ancestral species. Although the emergence of reproductively isolated subpopulations will be associated with the accumulation of genetic differences, they may not always be expressed in terms of morphological divergence in fungi. This is probably mainly because of the relatively simple fruiting structures and considerable developmental plasticity in fungi (Brasier 1987). The literature contains many

examples of fungal morphospecies within which partially or totally reproductively isolated subgroups (cryptic species) have been identified, and recent genetic studies have confirmed that the existence of cryptic species within fungal morphospecies is a highly widespread phenomenon (e.g. O'Donnell *et al.* 2000; Dettman *et al.* 2003a, 2003b; Nilsson *et al.* 2003; Kausserud *et al.* 2006, 2007). The presence of different ecotypes within a morphotaxon, i.e. populations adapted to a particular set of environmental conditions, could be a first indication of the presence of cryptic species.

* Corresponding author.

E-mail address: haavarka@bio.uio.no

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Populations isolated by lack of gene flow may be sympatric (spatially conjunct with overlapping ranges even before speciation), allopatric (disjunct with discrete ranges before and after speciation), or parapatric (ultimately conjunct after speciation). The best evidence for spatial effects on speciation is under conditions in allopatry, which intuitively is the most effective source of prohibiting gene flow. Allopatric speciation seems to be important in fungi, as in other groups of organisms (Kohn 2005). Evidence for allopatric speciation was found among Old and New World species in the genus *Lentinula* (Hibbett 2001). Another example includes the *Heterobasidion annosum* S and F groups, that most likely evolved in allopatry and partially adapted to various host species before secondary contact was established (Korhonen 1978; Johannesson & Stenlid 2003). In the morphospecies *Coniophora puteana*, it was recently shown that three cryptic species co-occur on the North American continent, largely in allopatry (Kausserud et al. 2007). In *C. puteana*, hybridization has apparently occurred among two of the cryptic species where they occur with overlapping ranges in northeastern North America.

The relevancy of parapatric speciation is poorly known in fungi. However, that many fungi have a high dispersal capacity could indicate that parapatric speciation is important. During parapatric speciation, there can be genetic leakage between the subgroups where their ranges overlap, as observed in *C. puteana*. Increase of prezygotic reproductive isolation by natural selection in sympatry is termed reinforcement. The scenario presented by Dobzhansky (1937) began with two species that arose in allopatry, and then met, undergoing some hybridization, producing unfit hybrids. Under this scenario, intraspecific matings would enjoy a fitness advantage, with selection favouring evolution of increased prezygotic isolation. Comparative studies in organisms other than fungi suggest that a pattern of increased prezygotic isolation in sympatry may be relatively common in nature (e.g. Sætre et al. 1997, 2003). In fungi, evidence for reinforcement has been observed in *Heterobasidion annosum*, where sympatric S and F group pairings of isolates were 24 % interfertile, whereas allopatric pairings were about 72 % interfertile (Korhonen et al. 1992). The same relationship was observed among sympatric and allopatric isolates of *Neurospora crassa* (Dettman et al. 2003b). The so-called ABC intersterility systems observed in *Trichaptum abietinum* and *Fomitopsis pinicola*, where two sympatric subgroups are intersterile but both are interfertile with a third allopatric population (Mounce 1930; Macrae 1967; Magasi 1976), could also be explained by reinforcement. Reinforcement may also lead to character displacement due to unfit hybrids, as has been observed in other taxa, e.g. birds (Sætre et al. 1997).

In this study, we present new data from the polypore *Gloeoporus taxicola* (Basidiomycota). *G. taxicola* produces annual reddish fruit bodies mainly on coniferous wood (Ryvarden & Gilbertson 1994). It has a circumboreal distribution in the Northern Hemisphere and has also been recorded in Australia and New Zealand. Its reproductive mode, i.e. whether it is outcrossing (heterothallic) or inbreeding (homothallic), has not yet been investigated. Field observations of *G. taxicola* in Norway during the past few years lead us to suspect that two different forms might exist within this morphospecies, a thought also mentioned by Nitare (2000). Although

its substrates in Norway, *Picea abies* and *Pinus sylvestris*, both occur in lowland and mountainous regions, *G. taxicola* has mainly been observed on *P. sylvestris* in lowland regions and on *Picea abies* in highland regions, indicating the presence of two ecotypes.

The primary aim of the current study was to analyse whether *G. taxicola* includes two ecotypes and also to illuminate the history behind the observed population structure. To pursue these questions, we generated sequences from two different DNA regions, the nu-rDNA ITS region and a part of the translation elongation factor 1a (*efa*) gene, from 46 field collections of *G. taxicola* from Norway. The obtained data enabled us to evaluate the reproductive mode of *G. taxicola* (i.e. whether it is homo- or heterothallic).

Material and methods

Study material

The 46 specimens included in the study are listed in Table 1. Monokaryotic isolates obtained from four fruit bodies were used in mating experiments on 2 % malt extract agar, but these experiments were inconclusive (see below).

DNA analyses

DNA was extracted from all specimens using the 2 % CTAB miniprep method described by Murray & Thompson (1980) with minor modifications: DNA was resuspended in 100 µl distilled water at the final step of extraction, and DNA templates were diluted 50-fold before PCR-amplification. PCR amplification was accomplished using the primers ITS4 and ITS5 (White et al. 1990) for the ITS nu-rDNA region and primers EF595F (CGTGACTTCATCAAGAACATG) and EF1160R (CCGATCTTG TAGACGTCTG) for the translation elongation factor (*efa*) region (Kausserud & Schumacher 2001). The *efa* sequence included two partial and one complete exon and two introns (inferred by comparisons with data of previously published sequences of homologous genes of other fungi). PCR was performed in 30 µl reactions containing 17.5 µl 50× diluted template DNA and 12.3 µl reaction mix (final concentrations: 4 × 250 mM dNTPs, 0.625 mM of each primer, 2 mM MgCl₂ and 1 unit DyNzyme™ II DNA polymerase (Finnzymes Oy, Espoo) on a Biometra thermocycler. The PCR amplification programme was as follows: 4 min at 94 °C, followed by 37 cycles of 30 s at 94 °C, 35 s at 54 °C, 72 °C for 40 s, and a final extension step at 72 °C for 10 min before storage at 4 °C. PCR products were sequenced in both directions by MWG-biotech (Ebersberg).

Sequence chromatograms were controlled and sequences were aligned manually in BioEdit (Hall 1999). Two different sequence datasets were constructed for the *efa* and ITS sequences. In a high proportion of the polymorphic sites, composite heterozygous nucleotides (e.g. C/T = Y) occurred, which is to be expected in dikaryotic (and heterokaryotic) basidiomycete specimens. In two positions in the *efa* alignment, a deletion and a pair of nucleotides ('- /GT') occurred in the same position within some specimens (causing virtually unreadable sequence downstream after this position). This has

Table 1 – Specimens included in the study, their herbarium accession numbers, geographical origin in Norway (municipality), substrate and altitude

Code	Herbarium accession no.	Origin	Substrate	Hasl	efa Genotype	ITS genotype
Lowland form						
GL7	O 165527	Halden	<i>Pinus sylvestris</i>	36	CTGTAGGCC	CACAGCTTCGYACC
GL12	O 66303	Asker	<i>P. sylvestris</i>	30	CTGTAGGCC	CACAGCTTCCTACC
GL13	O 175166	Arendal	<i>P. sylvestris</i>	5	CTGTAGGCC	CACRGCTTCCTACC
GL14	O 67022	Vestby	<i>P. sylvestris</i>	20	CTGTAGGCC	CACAGCTTCCTACC
GL17	O 66527	Nøtterøy	<i>P. sylvestris</i>	50	CTGTAGGCC	CACAGCTTCCTACC
GL18	O 224557	Re	<i>P. sylvestris</i>	120	CTGTAGGCC	CACAGCTTCCTACC
GL19	O 71959	Grimstad	<i>P. sylvestris</i>	20	CTGTAGGCC	CACAGCTTCCTACC
GL21	O 71922	Lillesand	<i>P. sylvestris</i>	150	CTGTAGGCC	YACAGCTTCCTACC
GL23	O 66891	Sogndal	<i>P. sylvestris</i>	?	CTGTAGGCC	CACAGCTTCCTACC
GL24	O 105783	Tingvoll	<i>P. sylvestris</i>	120	CTGTAGGCC	CACAGCTTCCTACC
GL25	O 94043	Drangedal	<i>P. sylvestris</i>	120	CTGTAGGCC	CACAGCTKCCTACC
GL26	O 105480	Tingvoll	<i>P. sylvestris</i>	240	CTGTAGGCC	CACAGCTTCCTACC
GL27	O 64136	Sula	<i>P. sylvestris</i>	200	CTGTAGGMA	CACAGCTTCCTACC
GL28	O 175023	Tønsberg	<i>P. sylvestris</i>	70	CTGTAGGMC	CACAGCTTCCTACC
GL30	O 270160	Ørland	<i>P. mugo</i>	20	YTGTAGGCC	CACAGCTTCCTACC
GL31	O 200312	Andebu	<i>P. sylvestris</i>	150	CTGTAGGCC	CACAGCTTCCTACC
GL33	O 65576	Risør	<i>P. sylvestris</i>	150	CTGTAGGCC	CACAGCTTCCTACC
GL34	O 170140	Risør	<i>P. sylvestris</i>	10	CTGTAGGCC	CACAGCTTCCTRCC
GL35	O 71959	Grimstad	<i>P. sylvestris</i>	20	CTGTAGGCC	CACAGCTTCCTACC
GL37	O 105825	Fredrikstad	<i>P. sylvestris</i>	40	CTGTAGGCC	CACAGCTTCCTACC
GL38	O 201633	Frei	<i>P. sylvestris</i>	?	CTGTAGGCC	CACAGCTTCCTACC
GL45	O 81178	Skodje	<i>P. sylvestris</i>	120	CTGTAGGCC	YACAGCTTCCTACC
GL46	O 146384	Kristiansand	<i>P. sylvestris</i>	?	CTGTAGGCC	CACAGCTTCGYACC
GL47	O 176039	Tingvoll	<i>P. sylvestris</i>	160	CTGTAGGCC	CACAGCTTCCTACC
GL54	Culture	Sigdal	<i>P. sylvestris</i>	230	CTGTAGGCC	CACAGCTTCGYACC
GL55	Culture	Halden	<i>P. sylvestris</i>	100	CTGTAGGCC	CACAGCTTCCTACC
GL56	Culture	Halden	<i>P. sylvestris</i>	100	CTGTAGGCC	CACAGCTTCCTACC
Highland form						
GL8	O 63586	Engerdal	<i>Picea abies</i>	700	CA--ACACC	CATAGCCTTCTACT
GL9	O 63577	Engerdal	<i>P. abies</i>	800	CWXXASRCC ^b	CATAGCTTTCTACT
GL10	O 63595	Engerdal	<i>P. abies</i>	700	CA--ACACC	CATAGCYTTCTACT
GL11	O 250022	Alvdal	<i>Pinus sylvestris</i>	700	CWXXASRCC ^b	CATAGCTTYCTACT
GL15	O 60708	Sigdal	<i>Picea abies</i>	740	CTGTAGGCC ^a	CAYAGCTTYCTAC ^b
GL20	O 105442	Gol	<i>P. abies</i>	520	CA--ACACC	CATAGCTTCYTACT
GL29	O 64165	Nore og Uvdal	<i>P. abies</i>	600	CAXXRSACC	CATAGCTTCTTACT
GL32	O 163673	Sigdal	<i>Pinus sylvestris</i>	800	CTGTAGGCC ^a	CACAGYTTCTTACC ^a
GL36	O 60705	Engerdal	<i>Picea abies</i>	900	CTGTGGGCC	CATAGCTTYCTACT
GL39	O 92432	Trysil	<i>P. abies</i>	730	CAGTGGACC	CATAGCTTYCTAYT
GL40	O 87222	Vågå	<i>Pinus sylvestris</i>	820	CA--ACACC	CATAGCTTYCTAYT
GL41	O 87221	Gausdal	<i>Picea abies</i>	510	CWXXASRCM ^b	CAYAGCTTYCTAC ^b
GL42	O 60451	Gausdal	<i>P. abies</i>	820	CAXXRSACC	CATAGCTTTCTACT
GL43	O 146364	Ringebu	<i>P. abies</i>	800	CTGTAGGCC ^a	CATAGCTTTCTACT
GL44	O 146366	Ringebu	<i>P. abies</i>	880	CA--ACACC	CRTAGCTTTCTACT
GL50	Culture	Engerdal	<i>P. abies</i>	750	CAGTGGACC	CATAGCTTYTACT
GL51	Culture	Ringebu	<i>P. abies</i>	860	CWGTRGRCC ^b	CATAGCTTYTACT
GL52	Culture	Engerdal	<i>P. abies</i>	700	CA--ACACC	CATAGCTTYCTACT
GL53	Culture	Ringebu	<i>P. abies</i>	900	CWXXASRCC ^b	CATAGCTTYCTAYT

In the last two columns, the variable DNA sequence sites observed in the two analysed DNA regions *efa* and ITS are given (Y = C/T, R = A/G, W = A/T, K = G/T, M = A/C, S = C/G, X = -/G or -/T). The variable sites are non-consecutive. a, b Highland form specimens that include (a) or hypothetically might include (b) the predominant highland form genotype (see text).

been indicated as 'X' (see Table 1 and below). The gap ('-') was converted to an ordinary character before the phylogenetic analyses [e.g. '-/T' (= 'X') to 'C/T' (= 'Y')]. The *efa* marker is present in the genome as single-copy gene, whereas the ITS region occurs in multiple copies so that intra-strain variation in the ITS may represent variation between copies in the repeat. However, previous studies of basidiomycete fungi have shown

that multiple ITS types only occur in heterokaryons and, as far as we know, have never been observed in the homokaryotic stage, and that the different ITS variants from a heterokaryon always segregate in the homokaryotic offspring (e.g. Aanen et al. 2001; Kauserud & Schumacher 2003). This implies that the ITS region in heterothallic basidiomycetes effectively behaves as a single locus with Mendelian segregation.

The generated sequences have been accessioned in GenBank with the accession numbers [AM231817–AM231862](#) (efa) and [AM231863–AM231908](#) (ITS).

Statistical analyses

The presence of multiple heterozygous positions (Table 1) made it problematic to conduct phylogenetic analyses. In parsimony analyses, the high level of heterozygous sites in the datasets (leading to a high level of homoplasious characters), resulted in a high number of trees (>100 K) and most branches collapsed in the strict consensus trees. Due to the structure of the datasets, we found it more appropriate to perform a distance-based NJ analyses. In NJ analysis, a diplotype sequence, including heterozygous sites (e.g. 'AYR'), will be placed intermediately between the corresponding homozygous sequences ('ACA' and 'ATG') and one tree topology will be generated instead of many alternatives, as in the parsimony approach. The NJ analyses were performed in PAUP version 4.02b (Swofford 1999) using total character differences and otherwise default settings. BS analyses were conducted in a similar manner using 1 K replicates. *Gloeoporus dichrous* was used experimentally as outgroup in initial phylogenetic analyses but dismissed due to high genetic separation from *G. taxicola*. Instead, mid-point rooting was used for the constructed trees.

In order use the information in the heterozygous sequence sites and thus calculate more accurate estimates of molecular variation, haplotype datasets were constructed for both DNA regions ($n = 92$). For example, in a DNA sequence ('genotype') containing a 'Y' (=C/T) the two resulting sequence haplotypes will include either a 'C' or a 'T'. In sequences with more than one heterozygous site, the heterozygous phase was inferred using a procedure yielding the minimum number of alleles. In short, sequences homozygous at all sites or heterozygous at only one site (known putative haplotypes) are used as templates for inferring a phase of sequences with multiple heterozygous sites. Hence, sequences with two or more heterozygous sites are, whenever possible, assigned to known putative haplotypes found in the sample of sequences. Alternatively, haplotypes requiring a minimum number of mutational steps are inferred. However, in our sample all multi-polymorphic sequences were compatible with the heterozygosity of known putative haplotypes. The procedure may underestimate the number of haplotypes if recombinant genotypes occur. However, descriptive statistics of the level of polymorphism (such as π and k), as well as tests of neutrality (Tajima's D and Fu & Li's F^* and D^* tests) will not be affected as these statistics are based on polymorphic sites *per se* and not haplotypes. Estimates of molecular variation (nucleotide diversity per site and average number of nucleotide differences), deviations from neutrality [Tajima's D (Tajima 1989) and Fu & Li's F^* , D^* and F_s tests (Fu & Li 1993; Fu 1997)] and population differentiation (F_{ST}) were calculated in the program DnaSP 4.0 (Rozas et al. 2003). Haplotype networks were constructed in Arlequin version 2.0 (Schneider et al. 2000) using the inferred haplotypes.

Results

Geographical, ecological and morphological variation

The geographical distribution of the 46 specimens included in the study is shown in Fig 1 and demonstrates that the specimens largely group into two separate forms: a coastal lowland form and an inland highland form. A much divided distribution pattern was observed, both in altitude (Fig 2) and substrate requirements (see Table 1). The 27 lowland specimens were collected below 250 m above sea level (asl) and 26 of the 27 were collected within a few kilometres from the sea. All finds are from areas with rather a mild and oceanic climate. In contrast, the 19 highland specimens were collected above 500 m asl, close to the Scandes mountain range in areas with a continental climate (at least from a Scandinavian perspective) and a long distance from the coast. All lowland specimens were collected from *Pinus sylvestris* except one that was collected on a planted *P. mugo*. On the contrary, 16 out of the 19 highland specimens were collected from *Picea abies* and the

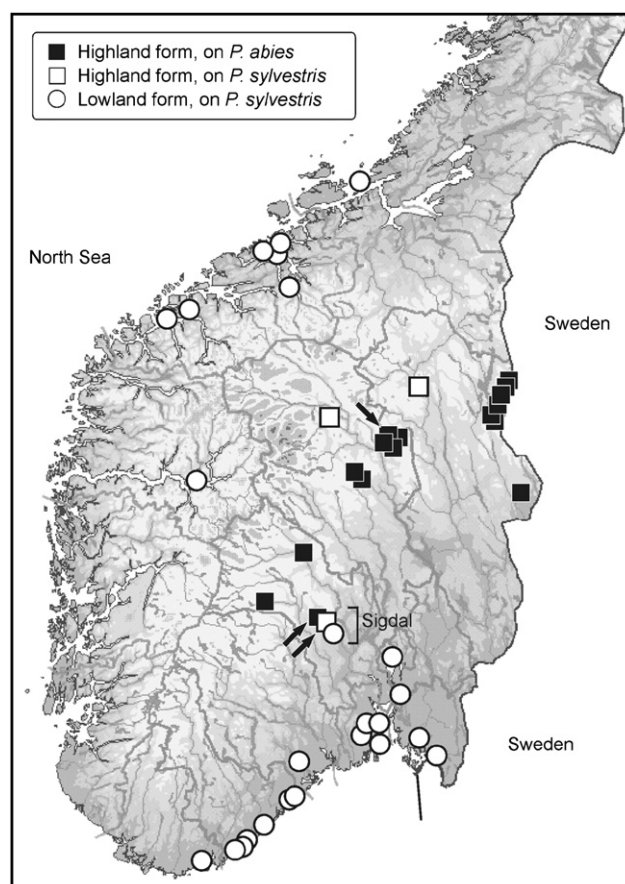


Fig 1 – The geographical distribution in southern Norway of the 46 investigated specimens of *Gloeoporus taxicola*. In the Sigdal region, one lowland specimen (230 m asl) and two highland specimens (> 740 m asl) were found in close proximity. Highland-form specimens possessing typical lowland form genotypes are indicated by arrows (see text for further information).

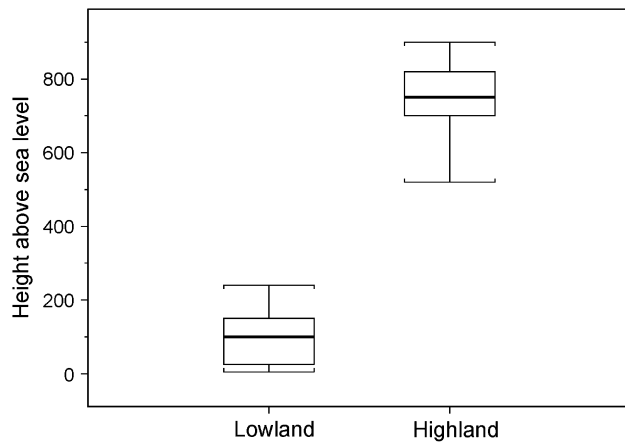


Fig 2 – Boxplots showing the distribution of low- and highland specimens according to altitude. A highly significant difference was observed (t-test, $P < 0.001$).

three remaining from *Pinus sylvestris*. The lowland form was mainly found on dead branches, still attached to living trees, and on dry decorticated logs in sunny and warm localities, whereas the highland form was largely collected on the stem section of fallen logs in humid, old-growth forests.

Preliminary investigations could not reveal any significant micro-morphological differences (e.g. spore size and shape, cystidioles, encrustation) among the two forms. However, macro-morphologically, there seems to be some differences. The highland form is often more deep purplish in colour (especially on drying and with age), whereas the lowland form is more vivid blood-red. Additionally, the highland form seems to more frequently develop large, widely effused, and somewhat fleshier fruiting bodies.

DNA sequence analyses

Among the 46 investigated specimens, nine and 14 polymorphic sites occurred in the *efa* and ITS alignments, respectively. In all polymorphic positions ‘heterozygous’ sites occurred (e.g. C/T = Y), which are to be expected in an outcrossing dikaryotic basidiomycete. All the variable positions are shown in Table 1 and demonstrates that far more genetic variation occurred in the highland form compared with the lowland form, both in *efa* and ITS. Eight variable sites (of which five were singletons) occurred among the 27 lowland specimens and 15 among the 19 highland-form specimens. Ten different multi-locus genotypes occurred in the lowland form, while all the 19 highland-form specimens had unique multi-locus genotypes. None of the polymorphic sites were fixed for different nucleotides in the two forms (cfr Table 1). Eight polymorphic sites that occurred in the lowland form were monomorphic in the highland form and 12 polymorphic sites that occurred in the highland form were monomorphic in the lowland form. At one nucleotide position, a shared polymorphism occurred.

Phylogenetic analyses were undertaken and the resulting phylogenies are shown in Fig 3. In both the *efa* and ITS phylogenies, there were two main groups separated by high BS support values, which corresponded largely to the low- and

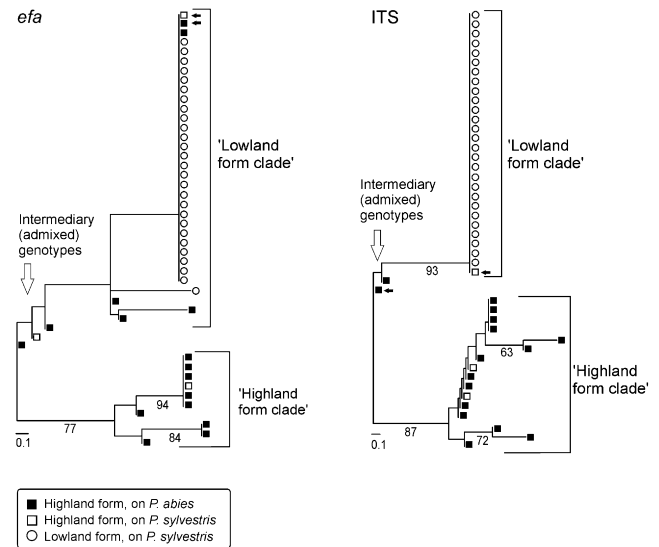


Fig 3 – Phylogenies obtained through NJ analyses of the two investigated DNA regions (*efa* and ITS). BS support values are shown below nodes (1 K replicates). The phylogenies indicate that two genetic main groups occur in *Gloeoporus taxicola*, largely corresponding to the low- and highland ecotypes. Some intermediate genotypes also occur. The black arrows depict two highland-form specimens from the Sigdal region (see text).

highland forms of *Gloeoporus taxicola*. In addition, some intermediate genotypes occurred (Fig 3). The predominant *efa* genotype in the lowland form was found in three highland specimens, suggesting that some gene flow (introgression) may occur between the two forms. It is noteworthy that two of these specimens were collected in the Sigdal region (Fig 1). Hypothetically, the ‘multi-heterozygous’ sequences from additional five highland-form specimens also included the predominant *efa* lowland genotype. Furthermore, one highland specimen (from Sigdal) included the lowland ITS genotype and two other ‘multi-heterozygous’ ITS sequences might, hypothetically, have also included the lowland form ITS type (Fig 1).

In order to analyse the level of molecular variation more thoroughly and use the information in the heterozygous sites, all DNA sequences ($n = 46$) were converted to haplotypes ($n = 92$) assuming minimum number of alleles (see methods). As shown in Table 2, a far higher nucleotide diversity (π)

Table 2 – Molecular variation observed in the low- and highland form, respectively, and between the two forms

	<i>efa</i>		ITS	
	π (s.d.)	k	π (s.d.)	k
Lowland form	0.0004 (0.0003)	0.18	0.0006 (0.0002)	0.33
Highland form	0.0039 (0.0003)	1.86	0.0028 (0.0004)	1.57
Between	0.0045	2.14	0.0051	2.90

π and k denote nucleotide diversity (per site) and average number of nucleotide differences, respectively.

Discussion

The distribution of genetic variation among the two ecotypes indicates a relatively recent split; none of the single sequence sites were fixed. A more or less stepwise transition of genetic variation apparently occurred among the two forms (cfr Figs 3 and 4), indicating that recent gene exchange between the two forms might have occurred. In general, the

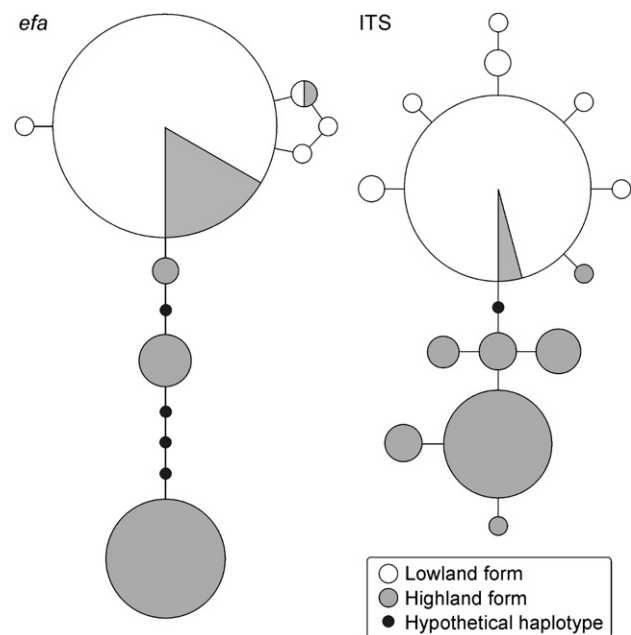


Fig 4 – Haplotype networks constructed from the 92 inferred haplophase sequences. Note that the haplotypes were inferred and not observed, and that the true haplotype diversity may be underestimated (especially concerning the highland form). The sizes of the circles are proportional to the frequencies of the haplotypes.

proportion of loci for which the polymorphisms are shared is expected to change in inverse proportion to the time since genetic isolation began. However, various evolutionary scenarios may explain the observed pattern. In our opinion, the most likely scenario is a former allopatric separation of the two forms, e.g. during the last Weichselian glaciation in two geographically isolated refugia; an eastern and a more southern refugium. According to this scenario, *G. taxicola*, occurring in the eastern refugium, adapted to *P. abies* and co-migrated into Scandinavia with its host tree, and today, represents the highland form. The lowland form had a more southern refugium, and adapted to *Pinus sylvestris*. The geographical distribution of mtDNA *nad1* haplotypes in European *P. sylvestris* is consistent with the hypothesis of a recent common origin of *P. sylvestris* populations in northern Europe from a common glacial refugium in South Europe (Soranzo *et al.* 2000). The outlined scenario coincides somewhat with the hypothesized

	<i>efa</i>				ITS			
	Tajima's D	Fu & Li's D*	Fu & Li's F*	Fu's Fs	Tajima's D	Fu & Li's D*	Fu & Li's F*	Fu's Fs
All	0.088	0.336	0.299	-0.826	-0.955	-1.797	-1.779	-5.677
Lowland	-1.468	-0.434	-0.871	-5.041	-1.883	-2.316	-2.556	-6.506
Highland	1.451	0.204	0.679	1.433	-0.505	-0.029	-0.209	-2.640

Significant deviations from expected ($P < 0.05$) are given in bold.

immigration routes of the S and F sibling species of *Heterobasidion annosum* into Europe (Johannesson & Stenlid 2003). According to these authors, the differentiation between the S and F groups could relate to the migration of the main hosts, *Abies* and *Picea*, into Europe from eastern Asia. One population of *H. annosum*, which followed *A. sibirica* or *P. abies* through Siberia, adapted to attack both species and became the S type. The F type may have originated on the southern migration route of *Abies*.

The genetic variation was significantly higher in the highland form compared with the lowland form, which may relate to the different immigration histories of the two forms. Hypothetically, the highland form could have immigrated on a broad front together with its host *P. abies*, introducing a high amount of genetic variation, whereas the lowland form could have established more in a founder event fashion. The higher genetic variation observed in the highland form coincides with the wider ecological amplitude of this form concerning substrate requirements (the highland form also occurs on *Pinus sylvestris* at a low frequency). It could be that the highland form, due to the higher genetic variation, possesses a more robust enzymatic system that also copes with decay of *P. sylvestris*. According to the two DNA regions analysed, the highland form includes much of the genetic variation observed in the lowland type (cfr Table 1). An alternative hypothesis is that recent gene flow between the two ecotypes is asymmetric and that 'lowland genes' has been incorporated into the highland form at a higher rate than the other way around (see below).

Among the lowland-form specimens, negative values were obtained in tests for deviation from neutral molecular evolution (cfr Table 3), two of the values being significantly different from neutral expectation (the Wright-Fisher model). Several factors may generate and contribute to such negative values, including selection and demographic history (cfr Fu 1997). The observed distribution of polymorphisms in the lowland form is congruent with a scenario of a bottlenecked population expansion common for many species that has expanded from isolated refugia after the last glaciation period (e.g. Ciang et al. 2004; Borge et al. 2005; Brandi et al. 2005). In comparison, the highland form possessed positive values for *efa* and negative for ITS, not significantly different from neutral expectation. It is noteworthy that hybridization may give a similar footprint in the analysis of molecular evolution to selection and demography (Machado et al. 2005), and this factor can therefore not be excluded (see below).

Whether reproductive barriers have originated between the two ecotypes of *G. taxicola* has not yet been elucidated. We performed, experimentally, mating reactions on 2 % malt extract agar between various single spore mycelia (homokaryons). However, the mating reactions, both between homokaryons from the same form and different forms, were inconclusive. It is well-known that it can be difficult to obtain suitable growth conditions to stimulate mating *in vitro* in some fungal taxa (e.g. Kauserud & Schumacher 2001). Different types of semi-natural growth conditions can be tested further to reveal whether mating barriers exist or not, which will be crucial in order to conclude whether introgression occurs between the two forms.

In the *efa* locus, several highland specimens possessed the typical lowland genotype (cfr Table 1) and in ITS, three highland specimens possessed heterozygous ITS genotypes that may include the typical lowland ITS haplotype. The observed sharing of similar haplotypes could be due to allele sharing of ancestral alleles, or alternatively, more recent introgression. One may argue that if the pattern were due to ancestral trans-lineage polymorphisms, there would be clades of alleles shared between the two lineages, but each with its own set of unique alleles, due to the accumulation of novel mutations subsequent to genetic isolation. Conversely, if the pattern is due to recent introgression, alleles of identical sequences will be shared across lineages (Hare & Avise 1998), as observed. However, no typical highland-form genotypes occurred among lowland form specimens. Thus, if introgression occurs, it is largely a unidirectional event from the lowland to the highland form. In the Sigdal region (cfr Fig 1), a putative lowland specimen (collected from *P. sylvestris* 230 m asl) and two highland specimens (collected from *P. sylvestris* and *Picea abies* above 800 m asl) possessed similar genotypes, which is a pattern compatible with introgression. Note that the three highland-form specimens on decaying *Pinus sylvestris* occurred in the outskirts of the distribution range of the highland form (cfr Fig 1), where the possibilities for introgression from the lowland form would be greatest. Several examples of introgression between closely related fungal ecotypes or sister taxa have been published earlier (e.g. Tsai et al. 1994; Newcombe et al. 2000; Brasier & Buck 2001; Kauserud et al. 2007).

Introgression between well-adapted ecotypes may lead to reinforcement of reproductive isolation if the resulting hybrids have reduced fitness. This phenomenon has been observed in *Heterobasidion annosum*, where sympatric S and F group pairings of isolates were 24 % interfertile, whereas allopatric pairings were about 72 % interfertile (Korhonen et al. 1992). The same relationship was observed among sympatric and allopatric isolates of *Neurospora crassa* (Dettman et al. 2003b). Reinforcement may also lead to character displacement due to unfit hybrids, as has been observed in other taxa, e.g. birds (Sætre et al. 1997). The distinct ecological separation, but gradual genetic differentiation, in *G. taxicola* fits well both with ecological diversification in allopatry followed by secondary contact and possibly recent introgression, but also with an ecological character displacement scenario. However, successful mating experiments and more powerful molecular markers (e.g. microsatellites, SNPs or cloning and sequencing of alleles from heterozygous sequences) are necessary in order to analyse these hypotheses more thoroughly.

For the first time, we show in this study that *G. taxicola* has an outcrossing (heterothallic) reproductive mode. The high frequency of heterozygous (segregating) sites is not congruent with a homothallic (selfing) reproductive mode. The observed results may also have implications for the conservation status of *G. taxicola* in Scandinavia. The less frequent highland form has mainly been found in old-growth Norway spruce forests which have not been affected by recent logging activities. This could indicate that this ecotype has a poor capability of maintaining viable populations in areas heavily influenced by logging activity. Preliminary field observations indicate that the highland form shows a patchy distribution, being seemingly non-existent in intensively logged areas, but more

frequent in areas with a high amount of old-growth forest. As a result, it has been used as a 'signal species' for forests with a high conservation value (Nitare 2000). In contrast, the habitat and substrate of the lowland form, namely dying branches and dry logs of *Pinus sylvestris*, is apparently less threatened by human disturbance.

This study presents results that indicate that *G. taxicola* may represent a suitable model to analyse speciation processes in fungi. To expand these results, we intend to perform a phylogeographic study covering the whole distribution range of *G. taxicola* employing more powerful genetic markers.

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