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Analysis of genetic diversity of *Melampsora larici-populina* in Iceland

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Analysis of genetic diversity of *Melampsora larici-populina* in Iceland

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60 ECTS thesis submitted in partial fulfillment of a *Magister Scientiarum* degree in Forest Sciences

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Clarification of contribution

I hereby declare that the writing of the following thesis is my work, done under the supervision and with the assistance of my advisors Dr. Jón Hallsteinn Hallsson and Dr. Halldór Sverrisson. The work has not been presented in any previous applications of a degree.

In the species identification process, I, Dr. Halldór Sverrisson, Rakel Jónsdóttir and, Hrafn Óskarsson, collected leaves infected with *Melampsora larici-populina* but uredinia were selected and collected from leaves by me.

Axelle Andrieux at Institut National de la Recherche Agronomique (INRA), Nancy-Université, France carried out DNA extraction and PCR reactions. Fragment sizing and alleles scoring was done by a private company selected by INRA.

Data analysis was done by me, under the supervision and with the assistance of my advisor Dr. Jón Hallsteinn Hallsson.

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Sigríður Erla Elefsen

Abstract

Black cottonwood, *Populus balsamifera* L. ssp. *trichocarpa* (T. & G.) Brayshaw first grown in Iceland in 1944, has proven to be fast growing and possess the highest capacity for carbon sequestration identified in Icelandic forestry. Black cottonwood in Iceland was almost free of pests until the year 1999 when *Melampsora larici-populina* was first detected in Iceland. The basidiomycete *M. larici-populina* causes foliar rust on *Populus* species from the sections *Aigeiros* and *Tacamahaca*, reducing biomass production and causing economic losses. In the present study, a total of 480 isolates, collected at 15 locations in Iceland were analysed using 25 microsatellite markers. For data analysis, results of 439 isolates analysed with 22 markers were used. Twenty-one of the loci were polymorphic, with an average of 5.25 alleles per locus. The mean observed and expected heterozygosity were 0.350 and 0.375, respectively. According to Principal Coordinates Analysis (PCoA), individuals clustered to two main groups, while samples grouped by locations clustered in three groups. The distribution of genetic diversity was low on a spatial scale, only 9 % between populations by locations and 91 % within populations. Based on Bayesian analyses of population structure the most likely substructuring of the 15 populations by locations was considered three subpopulations with low estimated levels of gene-flow between the subpopulations. If compared to the genetic structure of Icelandic rust collected in 2003, the same population structure was observed over time at Skálholt with evidence of genetic drift. Comparison to data from French *M. larici-populina* population in 2009, showed that the main Icelandic population is genetically different, while this comparison grouped the sample from a single Icelandic sampling location, Lón, among French samples. The population structure observed for the Icelandic rust population is most likely due to isolation and genetic drift as well as repeated events of colonization. In the future, two different dispersal modes can be expected, one by transport of spores between locations within the country, the other by repeated colonization events from abroad. The results reported here underline the importance of closely monitoring the development of fungal diseases in Iceland, as well as the importance of the careful selection of resistance for the Icelandic plant-breeding program.

Ágrip

Ræktun alaskaaspar á Íslandi hófst árið 1944 og hefur hún reynst það tré sem skilar hröðustum vexti hérlendis og mestum afköstum við kolefnisbindingu. Fram til ársin 1999 var alaskaösp nánast laus við skaðvalda en þá greindist asparryð, *Melampsora larici-populina* í fyrsta sinn hérlendis. Kólfsveppurinn *M. larici-populina* veldur blaðryði á aspartegundum sem heyra undir *Aigeiros* og *Tacamahaca.* Þessi sjúkdómsvaldur getur dregið úr framleiðslu lífmassa og valdið efnahagslegu tjóni. Í þessari rannsókn voru 480 sýni sem safnað var á 15 svæðum greind með 25 örtunglum. Við mat á erfðabreytileika var stuðst við niðurstöður af 439 sýnum sem greind voru með 22 þessara örtungla. Eitt örtungl var einbrigða en önnur fjölbrigðin. Fjöldi samsæta fyrir þau var frá þrem til tíu. Meðalfjöldi samsæta í seti var 5,25. Meðal arfblendni var 0,350 samanborið við væntanlega arfblendni sem var 0,375. Meginhnitagreining (PCoA) gaf vísbendingu um að einstaklingar aðgreindust í tvo megin hópa, en PCoA greining á hópum eftir sýnatökustöðum benti til þriggja meginhópa. Af heildar erfðabreytileika var einungis 9 % munur milli hópa eftir söfnunarstöðum á meðan 91% breytileikans fannst innan hópanna. Greining með Bayesian aðferð benti einnig til þess að líklegasta flokkun á hópum eftir söfnunarsvæðum leiddi til skiptingar í þrjá undirhópa, með ummerki um minniháttar flæði erfðaefnis milli þeirra. Einnig var gerður samanburður við íslenskt sýnasafn frá 2003 og franskt gagnasafn frá 2009. Sá samanburður sýndi að ryð í nágrenni Skálholts var samstofna því ryði sem þar var 2003, þó greina megi vísbendingar um genaflökt. Þá benti samanburður við frönsku gögnin til þess að íslenskt ryð væri erfðafræðilega frábrugðið stofnum í Frakklandi fyrir utan ryð frá Lóni. Skiptingu íslenska ryðstofnsins í undirhópa sem fannst hér má hugsanlega bæði rekja til einangrunar og genaflökts auk endurtekins landnáms. Gera má ráð fyrir að ryð geti í framtíðinni borist milli svæða og nýir stofnar geti borist til landsins, hvort sem er af mannavöldum eða með vindi. Niðurstöður rannsóknarinnar sem kynntar eru hér undirstrika mikilvægi þess að fylgjast náið með framvindu ryðsins hér á landi og sömuleiðis að huga vandlega að ryðþoli í íslensku kynbótastarfi á öspum.

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List of abbreviations

AMOVA: Analysis of molecular variance

Ar: Allelic richness

F_{IS}: Within population inbreeding coefficient

F_{ST}: Within subpopulation inbreeding coefficient

IAM*:* Infinite alleles model, a mutation model in Wilcoxon's test

K: Natural genetic group

LD: Linkage disequilibrium

NS: Not significant

N: Number of isolates genotyped

$N_{a:}$ Observed number of alleles

N_e: Effective number of alleles

N_p: Number of private alleles

$H_{O:}$ Observed heterozygosity

H_E: Expected heterozygosity

HWE: Hardy-Weinberg equilibrium

P: Probability

PCoA: Principle coordinates analysis

PCR: Polymerase Chain Reaction

PhiPT (ΦPT): Analysis of molecular variance constructed as percentages of molecular variance

PIC: Polymorphism information content

RAPD: Random amplification of polymorphic DNA

SMM: Stepwise mutation model, a mutation model in Wilcoxon's test

TPM: Two – phase model, a mutation model in Wilcoxon's test

Q-value: Adjusted *P*- value

I. Introduction

1.1. The poplar tree

The genus *Populus*, which includes 32 species, is native to the northern hemisphere and is found growing from the equatorial tropics to the latitudinal and altitudinal limits of tree growth (Dickmann, 2001; Dickmann & Kuzovkina, 2008; Pinon, 1992). Based on leaf and flower characters the genus *Populus* has been divided into six taxonomical sections: *Abaso, Turanga, Leucoides, Aigeiros, Tacamahaca,* and *Populus* (Dickmann & Kuzovkina, 2008).

As many fast growing poplar species have great potential ecological amplitude, they have been introduced in the southern hemisphere (South America, South Africa and Australasia) (Pinon, 1992). The *Aigeiros* and *Tacamahaca* sections of *Populus* have for years been the most important for plantation culture (Dickmann, 2001; Steenackers, Steenackers, Steenackers, & Stevens, 1996). Within *Aigeiros* both the pure species and their hybrids are of great commercial importance (Dickmann, 2001). The most important group of hybrids worldwide is the *P. deltoides* × *P. nigra*, known collectively as *P. canadensis* also referred to as Euramerican hybrids (Dickmann, 2001). Several species of the section Tacamahaca are of economical importance. One of its species, *P. trichocarpa* is a fast growing tree, growing to the largest size of any native poplar or any western hardwood as heights of 50 m and diameters over 1.5 m can be achieved (Dickmann, 2001). Other important groups are the intersectional hybrids of *P. trichocarpa* and *P. deltoides* (*P.* ×*generosa*) or Interamerican poplars (Dickmann, 2001).

European poplar forestry is more or less based on clones of the sections *Aigeiros* (*P. deltoides* and *P. nigra*) and *Tacamahaca* (*P. trichocarpa* and *P. maximowiczii*) (Steenackers et al., 1996). In the mid-20th century, hybrids between *P. deltoides* and *P. nigra* or *P. trichocarpa* were selected for their immunity to rust (Pei, Ruiz, Bayon, Hunter, & Lonsdale, 2005). Cultivation of these so-called 'Unal' clones (e.g. *P. trichocarpa*×*P. deltoides* 'Unal', 'Boelare' and 'Beauprè') was successful for many years (Pei et al., 2005). However in Europe, poplar trees are particularly vulnerable to many pathogens because of their decades long intensive cultivation (Gérard, Husson, Pinon, & Frey, 2006), based on even aged monoclonal stands and limited numbers of cultivars planted in a given region (Gérard et al., 2006; Steenackers et al., 1996). For example, in Northern France up to 80 %

of poplar plantations are of the cultivar *P.*×*generosa* ‘Beauprè’(Miot, Frey, & Pinon, 1999). The low levels of genetic diversity associated with monoculture practice may have contributed to the degradation of poplar health due to biotic aggression by fungi, bacteria and insects (Miot et al., 1999).

1.2. Icelandic forests and forestry

Due to the geographical isolation of Iceland, the number of native trees and shrubs has remained low since the end of the Pleistocene. Pollen studies have shown that the species composition of the Icelandic forest flora has more or less been the same for the last 6000 to 8000 years, indicating *Betula pubenscens*, *Betula nana*, *Sorbus aucuparia*, *Juniperus communis*, and *Salix* ssp. (Eggertsson, 2006).

One species of the genus *Populus* is native in Iceland (Kristinsson, 2010). In the year 1911 individuals of the species *Populus tremula* belonging to the *Populus* section, were found in Iceland, and although no pollen has been found in the stratum it is hypothesized that *P. tremula* may have survived the last ice age in Iceland (Benedikz, 1994). So far, *P. tremula* has been found at seven (Kristinsson, 2010) or eight locations in the north and east site of the country (S. Blöndal, 2002). Globally *P. tremula* is one of the two *Populus* species, surrounding the northern hemisphere. The natural range of *P. tremula* is enormous, reaching through Eurasia and North Africa, as its North American counterpart *P. tremuloides* is the most widely distributed tree species indigenous to North America (Dickmann & Kuzovkina, 2008).

1.3. Populus cultivation in Iceland

The cultivation of black cottonwood, *P. balsamifera* ssp. *trichocarpa* (T. & G.) Brayshaw (formerly known as *P. trichocarpa*), started in Iceland in 1944 (Þorsteinsson, 1990). In the years 1943 to 1950, material was collected at the Kenai Peninsula in Alaska (B. Blöndal & Gunnarsson, 1999) and the first cuttings were cultivated at the nursery Múlakot in southern Iceland (Pálsson, 2000). Soon black cottonwood became a popular garden and ornament tree. However, it was only rarely used in afforestation at that time (Sverrisson, Halldórsson, & Sigurgeirsson, 2006). In an unusual spring weather in 1963 as the temperature dropped suddenly below zero after a long warm period, poplar trees in the capital area and southern Iceland were widely killed or badly damaged (Þorsteinsson, 1990). Following this damage, material for cultivation was collected in more southern and

maritime locations in the Copper River delta and near to the town Yakutat in Alaska (B. Blöndal & Gunnarsson, 1999; Guðmundsson et al., 1977; Njálsson, 1999). A great increase in planting occurred in relation to growing afforestation in Iceland starting in 1990 (Eysteinsson, 2009; Pétursson, 1999). At the same time, experimental forests and clonal trials were established (B. Blöndal & Gunnarsson, 1999; Sigurðsson, 2003; Sverrisson et al., 2006). This increased the proportion of black cottonwood to 2 % of the 75 to 80 million forest tree seedlings planted in Iceland during the period 1945-1998. In 2009, seedlings from black cottonwood were the fifth most common planted tree seedlings, about 282060 or 5.8 % of the total number of seedlings planted in the country (Gunnarsson, 2010).

In the late seventies and early eighties, seeds were for the first time collected from poplar clones that came to Iceland during the period 1944 to 1963 and seed plants were grown (Sigurgeirsson & Kristjánsdóttir, 1995). Later breeding was carried out through crossing of clones and subsequent clonal trials with the aim of adapting the plants to different climatic conditions in Iceland (Sigurgeirsson, 2001; Sverrisson, Sigurgeirsson, & Jónsdóttir, 2011).

Self-seeding can be reasonably quick in damaged wild lands (Kristinsson, 2010) and self-seeding plants of black cottonwood have been found at locations in southern, northern and eastern Iceland (Kristinsson, 2010; Pálsson, 2000). Black cottonwood is now considered an established non-native species in the Icelandic flora (Pálsson, 2000).

In Iceland, the growth of black cottonwood is faster than of any other cultivated species (Snorrason & Sigurgeirsson, 2006; Sverrisson, 2011) and it reaches most height and standing bole volume (B. Blöndal & Gunnarsson, 1999). Where standing bole volume is 6-20 m^3 ha^{-1} yr^{-1} the corresponding carbon sequestration is 8-23 t ha^{-1} yr^{-1} (Snorrason & Sigurgeirsson, 2006). Moreover, it has been shown in a young poplar stand, that carbon sequestration can be duplicated by fertilisation, increasing it to 28.4 t CO_2 ha^{-1} yr^{-1}(Jónsson & Sigurðsson, 2008).

In 2007, hybrids of eastern cottonwood, *P. deltoides* and black cottonwood were created at the Forest Research Station in Mógilsá. These hybrids have shown enhanced growth compared to the Icelandic black cottonwood clones (Sverrisson, 2011). Many of these clones are completely resistant or immune to poplar leaf rust (Sverrisson, 2011).

1.5. The major fungal diseases of *Populus*

A vast number of fungi are able to attack the genus *Populus*, primarily Ascomycetes, but also many Mitosporic Fungi (Fungi imperfecti) and Basidiomycetes (Cellerino, 1999). The fungi causing leaf diseases are usually primary parasites (able to attack healthy plants), whereas those attaching trunks and roots are mostly wound parasites (Cellerino, 1999).

The leaf parasites fungi belonging to the genus *Melampsora* (fam. *Melampsoraceae*, ord. *Uredinales*, class *Teliomycetes*, phylum *Basidiomycota*) are solely responsible for all poplar rust (Cellerino, 1999). Some thirteen species and two hybrids of this genus, have been described on *Populus* (Pei & Shang, 2005), whereof eight species infect poplars in Europe (Cellerino, 1999). Three of them, *M. allii-populina, M. larici-populina,* and *M. medusae*, indigenous in northern Africa to western Asia and south-central Europe, Eurasia, and North America respectively, are pathogenic on the poplar of the sections *Aigeiros* and *Tacamahaca* and their hybrids (Frey, Gérard, Feau, Husson, & Pinon, 2005). Recently, a new species *Melampsora nujiangensis* was found on *Populus yunnanensis* of the section *Tacamahaca*, in China (Liang, Tian, & Kakishima, 2006). Among the *Melampsora* species, *M. larici-populina* causes most severe damage and is responsible for most of economic losses in poplar cultivation (Pinon, 1992; Pinon & Frey, 2005; Steenackers et al., 1996).

1.6 The life cycle of *Melampsora larici-populina*

The rust fungus *M. larici-populina* is an obligate parasite that draws nutrients from live plant tissues in every stage of its life cycle (Cellerino, 1999; Hallgrímsson, 2010). It is heteroecious, macrocyclic, and alternates on *Larix* spp. (Hallgrímsson, 2010). During its lifecycle it produces five different spore stages, urediniospores, teliospores, basidiospores, spermatia, and aeciospores, with its asexual (uredinial) stage on *Populus* spp., and sexual (aecial) stage on *Larix* spp. (Hallgrímsson, 2010).

The rust overwinters in telia (Figure 1) as dominant teliospores on fallen leaves of *Populus.* In spring, karyogamy occurs and a short diploid germ tube or basidie forms where meiosis takes place. Following the germination, windborne, haploid basidiospores are emitted. The basidiospores then infect the alternate host and form haploid spermagonia or pycnia. The role of pycnia is the production of spermatia (pycnospores) and the capture, by means of receptive hyphae, spermatia of the opposite mating type brought by raindrops

and some insects from other pycnia (Cellerino, 1999). Fertilization between spermagonia on larch needles results in formation of the aecia which are long chains of dikaryotic aeciospores, durable airborne spores, capable of reinfecting *Populus* ssp. Afterwards dikaryotic uredinia (Figure 1) are formed on leafs throughout the remaining of the growing season, owing to the production of two or more generations of airborne urediniospores (Cellerino, 1999). The rust can multiply rapidly, producing the next generation of urediniospores within 10 days (Pei, Ruiz, Harris, & Hunter, 2003). Repeated cycling of the dikaryotic urediniospores, which function as diploids, causes rust epidemics on poplars (Pei, Bayon, Ruiz, & Tubby, 2007). In the late summer or autumn, the overwintering telia form and the life cycle is closed (Figure 1).

Figure 1 Different stages of *Melampsora larici-populina*, a) uredinia, b) telia, c) two stages seen on the same black cottonwood plant, with uredinia at the top and telia on the lowest leaf.

Teliospores are of biological significance as the conclusion of the sexual process, i.e. the modifications of the genetic complement that will be transmitted to basidiospores, and perpetuated through next generation (Cellerino, 1999).

It is known that some poplar *Melampsora* species resume production of urediniospores in the next vegetative season without passing to alternate host, either as mycelia close to the dormant buds or in urediniospores that remain attached to the shrunken dry fallen leaves (Cellerino, 1999). It has been pointed out that *M. larici-populina* can overwinter in the uredinial state, at least in relatively warm climates (Smith, 1988). In some areas, where the alternate host (*Larix* sp.) is absent *M. larici-populina* can survive only asexually (Walker, Hartigan, & Bertus, 1974). Recently, genetic hallmarks of asexual reproduction have been demonstrated in *M. larici-populina* in France (Xhaard et al., 2011b).

1.7 Virulences and pathotypes of *Melampsora larici-populina*

Variability within a given *Melampsora* species is mainly defined by the presence of pathotypes or physiological races and aggressiveness (Pinon & Frey, 2005). Van Vloten (1949) was the first to report the existence of physiological races within *Melampsora larici-populina,* although the occurrence of different pathotypes in the field did not become obvious until the 1980s (Frey & Pinon, 1997). So far, eight virulences have been identified with the *M. larici-populina* species (Table 1), with potentially 256 pathotypes (Pinon & Frey, 2005).

Table 1 Virulence known within *M. larici-populina*[a]

Virulence number	Year of description	Initial location	Clones used to detect virulence
1	1982	Belgium, France	'Ogy'
2	1986	France	'Aurora'
3	1949	The Netherlands	'Brabantica'
4	1974	France	'Unal'
5	1982	France, Belgium	'Rap'
6	1994	France	'87B12'
7	1994	Belgium, France	'Beaupré'
8	1997	Belgium, France	'Hoogvorst'

[a] (Pinon & Frey, 2005)

In the years 1974 to 1984 many cultivars previously selected because of their complete resistance to the *M. larici-populina* became severely rusted, due to new virulence strains and new pathotypes (Pinon & Frey, 2005).

The known pathotypes have been organized into five groups, with 'E' meaning Europe (Pinon & Frey, 2005). E1 pathotypes are older pathotypes which have never exhibited virulences 1, 2, 6, 7 or 8, most frequently they have virulence 4 or 3 and 4 (Pinon & Frey, 2005). E2 pathotypes have at least virulence 1, E3 virulence 2, E4 virulence 7 and E5 virulence 8 (Pinon & Frey, 2005). Early in the 1980s the appearance of different rust races, E1, E2 in Belgium (Pinon, van Dam, Genetet, & Dekam, 1987) and E3 in Italy (Pei et al., 2003) were proven. At this time, Unal clones previously selected for immunity to rust became sensitive (Steenackers et al., 1996). During the 1994 growing season, E4 was discovered following resistant clones, including 'Boelare', becoming susceptible to a new pathotype (Steenackers et al., 1996). The last one, rust race E5 was found in 1997 when the

complete resistance of the cultivars ‘Hoogvorst’ and ‘Hazendans’ was broken down (Pinon & Frey, 2005).

Aggressiveness, the second level of variability consists of different latent time, variation in infection described as number of uredinia per unit of leaf area and largeness of uredinia (Pinon & Frey, 2005). All of the three quantitative parameters used to quantify isolate aggressiveness appeared to vary between isolates, including those within the same pathotype (Pinon & Frey, 2005). It is expected, that *M. larici-populina* is able to accumulate virulence, and in that way increase its host range, without losing aggressiveness or fitness (Pinon & Frey, 2005).

Primary factors considered to contribute to rapid spread of newly emerged virulent *M. larici-populina* strains are the compulsory sexual stage on larch which promotes high racial variability, airborne urediniospores, long-lasting multiplications on poplar leaves, and monoclonal poplar stands, which suggests no genetic diversity for resistance and a strong selection pressure on the pathogen (Dowkiw, Husson, Frey, Pinon, & Bastien, 2003). It has been revealed that the pathotypic structure clearly distinguished the cultivated stands with high richness and complexity from the wild stand with low richness and complexity (Gérard et al., 2006). The presence of larch in the vicinity (< 1 km) of cultivated and wild stands has been shown to significantly increase, the number of pathotypes, either for cultivated or for wild stand (Frey et al., 2005), and also to increased the richness of the *M. larici-populina* populations, but it did not increase the complexity (Frey et al., 2005). Although this is not absolute, as other studies have reported only a general but no significant trend toward higher pathotypic richness in the stands with neighbouring larch (Gérard et al., 2006).

In a rust population collected at Skálholt in 2003 and analysed for virulence, virulences 1, 3, 4, 5, 6 and 7 were observed, with types 3 and 4 the most abundant (Barrès et al., 2006). At the same time, six pathotypes were found, with the mean number of virulences per individual as 1.33 (Barrès et al., 2008).

1.8 Distribution of *Melampsora larici-populina*

The rust *M. larici-populina* is native to Eurasia where its distribution range is supposed to encompass that of its natural host *P. nigra* (Barrès et al., 2008). In the past century, the distribution range has expanded worldwide (Barrès et al., 2006; Pei & Shang, 2005). The

most recent areas are North America (Innes, Marchand, Frey, Bourassa, & Hamelin, 2004; Newcombe & Chastagner, 1993; Pinon, Newcombe, & Chastagner, 1994; Steimel, Chen, & Harrington, 2005), East Canada (Innes et al., 2004) and Iceland, where it was first discovered in 1999 (Eyjólfsdóttir, Halldórsson, Oddsdóttir, & Sverrisson, 1999).

During the summer 1999, *M. larici-populina* was only observed in Hveragerði and Selfoss in the southern part of Iceland (Eyjólfsdóttir et al., 1999). In the following years it spread over a larger area, and in 2005, it could be found from Keflavík in the southwest and across the south part of Iceland (Sverrisson, Halldórsson, Kjartansson, & Sigurgeirsson, 2005). Furthermore, it was found in different locations around the country, at Gunnfríðarstaðir in the north, at Hallormsstaður in the east and at Lón in the southeast (Sverrisson et al., 2005). Recently *M. larici-populina* has been detected in two other different locations in northern Iceland, Akureyri (this study) and Vatnsdalur (Oddsdóttir & Sverrisson, 2010).

1. 9 Effects on growth

Heavy infections caused by *M. larici-populina* leads to early leaf drop, delay of the flushing time of the tree in the next growing season and reduction of growth and wood quality (Cellerino, 1999; Steenackers et al., 1996). Following leaf drop, leaf scars are ideal entry points for secondary parasites (Steenackers et al., 1996). In Belgium, before the 1980s rust infections were often in combination with parasites such as the fungi *Dothichiza polulea* and *Marssonina brunnea* (Steenackers et al., 1996). Secondary infections such as *Cytospora chrysosperma* (Pers.) and *Discosporium populeum* Sacc. (Sutton) following *M. larici-populina* epidemics can cause critical economic loss and even plant death (Miot et al., 1999). Worldwide the leaf diseases rust caused by the *Melampsora* species is one of the major poplar diseases (Pei & Shang, 2005; Steenackers et al., 1996) and the rust has been considered as an important external determinant of biomass production (Laureysens, Pellis, Willems, & Ceulemans, 2005).

In Iceland, the rust has been shown to have negative impact on the growth and health of the poplar trees (Albers, Eggertsson, Sverrisson, & Halldórsson, 2006; Halldórsson, Eyjólfsdóttir, Oddsdóttir, Sigurgeirsson, & Sverrisson, 2001). In the study of Albers et al., (2006) a stand of larch right next to poplar trees and high susceptibility to rust explained the strong impact on diameter increment in affected trees compared to almost unaffected trees in the same area, the village Hveragerði.

1.10 Microsatellites

Microsatellites (Schlötterer, 1998), also known as simple sequence repeats (SSRs) (Allendorf & Luikart, 2008), are polymorphic loci that consist of tandem repeats of short repeating units of 1 to 6 nucleotides (Allendorf & Luikart, 2008). The number of repeats at a polymorphic locus can range widely from approximately five to 100 (Allendorf & Luikart, 2008). For amplification by polymerase chain reaction (PCR) primers are designed to hybridize to the conserved DNA sequences flanking the microsatellite region, generally giving PCR products between 75 and 300 base pairs (bp) long (Allendorf & Luikart, 2008).

The main advantage of microsatellites markers is that they are usually highly polymorphic, which they owe to an increased rate of mutation at the microsatellite loci compared to other regions of the genome because of the presence of simple sequences repeats (Allendorf & Luikart, 2008). These high rates of mutation are thought to be caused by mispairing of DNA strands during replications or through imprecise events of recombination (Allendorf & Luikart, 2008).

Although microsatellites have in general proved to be versatile molecular markers, particularly for population analysis, they are not without limits. Substitutions in the primer-annealing site may lead to 'null alleles', where microsatellites fail to amplify in PCR assays (Allendorf & Luikart, 2008). The presence of null alleles results in an excess of homozygotes relative to Hardy-Weinberg proportions (Allendorf & Luikart, 2008).

Microsatellites are widespread in both eukaryotic and prokaryotic genomes (Bhargava & Fuentes, 2010) and due to the high levels of polymorphism, microsatellites have become widely used DNA markers (Allendorf & Luikart, 2008) and have proved to be invaluable in many fields of biology including population genetics (Dutech et al., 2007). Compared to other organisms fungal microsatellites have been harder to isolate and to exhibit lower polymorphism (Dutech et al., 2007). In part at least, this appeared to be due to genomic specificities, such as scarcity and shortness of fungal microsatellite loci (Dutech et al., 2007). However, microsatellites have been useful in some fungal species (Dutech et al., 2007), and recently, species specific microsatellite markers have been developed that can be used to recognize genetic differentiation of *M. larici-populina* (Barrès et al., 2006; Xhaard, Andrieux, Halkett, & Frey, 2009; Xhaard et al., 2011b).

1.11 Genetic structure of *Melampsora larici-populina*

In a study aimed at characterizing the genetic structure of *M. larici-populina* of 13 populations from nine European countries, and one from Canada, using eleven microsatellite markers, it was found that genetic differentiation between populations in continental Europe was low, while the populations from Iceland and Canada were independently distinguishable from continental European populations, suggesting a gene flow on a continental scale (Barrès et al., 2008).

In a recent, study where 25 microsatellite markers were used to genotype 467 individuals collected in several locations in France and one in Italy, it was shown that the individuals clustered in three genetic groups, as groups mainly followed the structure of poplar stands (Xhaard et al., 2011b). The same tendency was observed in the Durance River valley in France (Xhaard et al., 2012). Other methods have been used to test genetic structure of the rust *M. larici-populina*. In 2001, amplified fragment length polymorphism (AFLP) profiles were generated from poplar rust *M. larici-populina* collected at six sites in the UK (Pei et al., 2007). Overall, it was estimated that there was sufficient gene flow among different sites to prevent genetic drift. One population from north-eastern England was differentiated from those from other sites. It was indicated, that sexual reproduction has a major influence on the relative lack of population structure of *M. larici-populina* in the UK. In a study of *M. larici-populina* isolates collected from different ecological loci and host poplars in China were compared to isolates from Britain, France, Germany and Canada (Yu, Liu, & Cao, 2006). In theirs study, intertranslation space of ribosomal DNA (ITS) sequence of isolates from China was more homogeneous with that of isolates from Britain compared with the other isolates. On the other hand, when using intersimple sequence repeat (ISSR) markers the Chinese isolates could be divided into two populations the Western and Northern populations.

2 Aims of study

The overall objective of this study was an increased understanding to gain the population structure of *M. larici-populina* in Iceland, a newly established fungal pathogen of black cottonwood, and its mechanism of introduction to Iceland. The benefit of this project will be increased knowledge and understanding of the genetic characteristics of the fungus, which may benefit breeding projects of black cottonwood and may possibly be exploited to increase crop safety in the future. In addition, the project provides information on the development of a new fungal pathogen during its first years after settlement, possibly serving as an interesting model for population dynamics under an 'island model'. This subject is especially interesting times when transfer of pests from one region to the next is expected to increase.

In the study of Barrès et al., (2008) the Icelandic *M. larici-populina* population appeared to be distinguishable from all other populations analysed at that time and showing signs of founder effect. However as isolations analysed were all collected at the same location, near to Skálholt, the result may not be representative for the entire Icelandic population.

The study consisted of three aims:

(1) To analyse the genetic diversity of the Icelandic *M. larici-populina* population by applying microsatellite markers to determine the total genetic diversity in pooled isolates collected at several different locations.

(2) To examine whether the genetic characteristic of *M. larici-populina* near Skálholt were still the same as in 2003 by comparing genetic properties of isolates collected near Skálholt in 2003 and 2008/2009.

(3) To compare the Icelandic *M. larici-populina* to populations outside Iceland by comparing genetic properties of isolates, collected across France between August and November 2009 to the Icelandic material.

3. Material and methods

3.1 Sample collection

Black cottonwood leaves showing *M. larici-populina* uredinia, were collected from fifteen locations in Iceland in 2008 and 2009 (Figure 2).

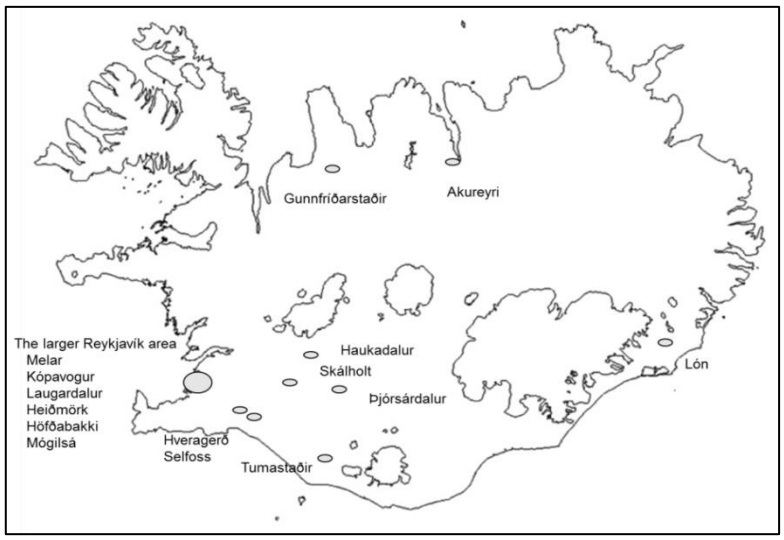

Figure 2 *Melampsora larici-populina* sampling sites in Iceland.

In 2008 samples were collected from the towns Hveragerði (HV) and Selfoss (SE) where *M. larici-populina* was first detected in Iceland (Eyjólfsdóttir et al., 1999), the forest Haukadalur (HA), from a stand of black cottonwood near to Skálholt (SH) and a forest in Þjórsárdalur (TH), all these locations in southern Iceland (SI) (Table 2). In the larger Reykjavík area (HB), the locations were both in population areas, Kópavogur (KO), Höfðabakki (HA), Laugardalur (RL), Melar (RM), and in green areas with forests near the city, Mógilsá (MG) and Heiðmörk (RH) (Table 2). In northern Iceland, leaves were collected from the forest Gunnfríðarstaðir (GU), and in the south-eastern part of the country at a small stand at Lón (LO) (Table 2).

In the year, 2009 infected leaves were collected for the second time in Hveragerði, Selfoss, near to Skálholt, and at Gunnfríðarstaðir. In addition, leaves infected with *M. larici-populina* were collected in Akureyri (AK), a new place of finding in 2009 and at Tumastaðir (TU) in the south of the country since the infected plants in Akureyri were cultivated at a nursery in Tumastaðir (Figure 2, Table 2). Altogether, infected leaves were collected from 380 trees; one to three leaves from each tree.

Table 2 Information on sampling sites [a] for *Melampsora larici-populina* in Iceland

Sampling locations	Location	Year	N_t	N_u	N
Gunnfríðarstaðir (GU)	65°34'N, 20°04'W	2008	28	28	22
		2009	17	27	26
Akureyri (AK)	65°42'N, 18°09'W	2009	20	30	29
Reykjavík, Melar (RM)	64°08'N, 21°57'W	2008	10	19	19
Kópavogur (KO)	64°06'N, 21°53'W	2008	15	30	29
Laugardalur (RL)	64°08'N, 21°52'W	2008	10	20	20
Heiðmörk (RH)	64°03'N, 21°52'W	2008	4	8	7
Höfðabakki (RA)	64°07'N, 21°48'W	2008	10	20	19
Mógilsá (MG)	64°12'N, 21°42'W	2008	33	33	27
Hveragerði (HV)	63°59'N, 21°11'W	2008	25	25	23
		2009	30	30	27
Selfoss (SE)	63°55'N, 20°59'W	2008	27	27	26
		2009	29	30	27
Skálholt (SK)	64°07'N, 20°31'W	2003	…	95	94
Skálholt (SH)	64°09'N, 20°32'W	2008	23	23	22
		2009	25	29	28
Haukadalur (HA)	64°19'N, 20°16'W	2008	27	27	27
Tumastaðir (TU)	63°44'N, 20°03'W	2009	25	30	29
Þjórsárdalur (TH)	64°06'N, 19°57'W	2008	11	22	19
Lón (LO)	64°25'N, 14°53'W	2008	11	22	13
Minimum number			352		
Total number				575	533

[a] For each site; Location = latitude and longitude, Year = year of sampling, N_t = number of trees, N_u = number of uredinia tested, N = number of isolates genotyped (observations accepted), ... = not observed.

In every area *Larix* sp. were found within a distance of 500 meters from sampling sites. At populated areas and at some stands (e.g. Gunnfríðarstaðir and Haukadalur), black cottonwood and *Larix* sp., commonly were planted in a considerable proximity.

An additional collection of 95 isolates from infected poplar leaves collected by Halldór Sverrisson in 2003 near Skálholt (Table 2) (Barrès et al., 2006), was kindly added to the analysis by Dr. P. Frey at Institut National de la Recherche Agronomique.

Moreover, microsatellite data from previous microsatellite analysis of 470 French and Italian *M. larici-populina* samples collected across France and in Italy between August and November 2009 was used for comparison (Xhaard et al., 2011a, 2011b).

3.2 Isolations of urediniospores and sampling of uredinia

Two methods were applied for collecting isolates from infected poplar leaves. At the beginning of the work in 2008, a single uredinium per leaf was grown on fresh leaf discs of

P. balsamifera ssp. *trichocarpa* ‘Keisari’ to increase the material. All together 129 isolates were harvested this way in 2008. However, as there was a risk of contamination from the local rust, infecting the black cottonwood in use, this method was cancelled and a single uredinium representing each isolate were cut separately from poplar leaves. In all cases, uredinia were transferred to Eppendorf tubes one per tube, and kept at -20 °C. In total, 616 isolates were collected from 234 trees in 2008 and 300 isolates from 146 trees in 2009, at average 2.4 uredinia per tree. For gene analyses 480 isolates were selected from the collection in addition to 95 isolates collected in 2003 (Table 2).

3.3 DNA extraction, marker amplification, and fragment detection

All DNA work was carried out in France in 2010. Extraction and polymerase chain reactions (PCR) were carried out at Institut National de la Recherche Agronomique (INRA), while genotyping was done in a private company (business information not available).
Following the BioSprint 96-DNA-protocol (QUIAGEN.com) the DNA was extracted using the BioSprint 96 DNA kit in combination with the BioSprint 96 automated workstation.

The sample collection was analysed using the following twenty-five primer pairs: MLP12 , MLP49, MLP50, MLP54, MLP55, MLP56, MLP57, MLP58, MLP59, MLP66, MLP68, MLP71, MLP73, MLP77, MLP82b, MLP 83, MLP87, MLP91, MLP92, MLP93, MLP94, MLP95, MLP95, MLP97 , and MLP100 (Barrès et al., 2006; Xhaard et al., 2009; Xhaard et al., 2011b). The microsatellite loci were amplified using a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) under the following conditions: An initial denaturing step of 5 min at 95 °C; 32 cycles including 30 s of denaturation at 95 °C, 90 s annealing at 60 °C and 60 s extension at 72 °C; and a final 30 min extension step at 60 °C. PCR was carried out in a 10 µl final reaction volume containing 5 µl of Qiagen® Multiplex PCR kit, 2 µl DNA, 0.2 µM of each reverse and forward primers. Forward primers, were labelled with a fluorescent tag (FAM, NED, VIC, or PET). Three multiplex PCR were run, comprising 9, 7, and 9 loci, respectively (Appendix 1). PCR products from the three multiplex reactions (3 µl PCR1, 4 µl PCR2 and 5 µl PCR3) were pooled and loaded on an ABI 3730 Genetic Analyzer (Applied Biosystems). Fragments were sized with a LIZ-1200 size standard, and alleles were scored using GENEMAPPER 4.0 (Applied Biosystems).

3.4 Data analysis

3.4.1 Microsatellite genetics analysis

For individual microsatellite markers, a standard genetic summary statistics such as observed number of alleles (N_a), allele frequencies, observed (H_O) and expected heterozygosity (H_E),and number of effective alleles (N_e), were calculated for individual microsatellite marker using GenAlEx 6.4.1 (Peakall & Smouse, 2006). The measure, effective numbers of alleles, enable meaningful comparisons of allelic diversity to be made across loci with diverse alleles frequency distribution (Brown & Weir, 1983). For each locus Weir and Cockerham's inbreeding coefficient, F_{IS} (Weir & Cockerham, 1984) and exact test of departure from HWE (probability test, and heterozygote deficiency and excess) was calculated using Genepop on the Web (http://genepop.curtin.edu.au) (Raymond & Rousset, 1995; Rousset, 2008). Estimation of exact *P* value for HWE was done by the Markov chain method (Guo & Thompson, 1992) as Marcov chain parameters for all tests were as followed: Dememorization number as 1000, number of batches 100 and number of interactions per batch 1000.

In orter to test the efficiency of the markers, polymorphism information content (PIC) (Botstein, White, Skolnick, & Davis, 1980) was calculated using PowerMarker 3.25 (Liu & Muse, 2005). PIC refers to the ability of a given marker to detect polymorphism within a population, depending on the number of detectable alleles, and their frequency (Guyomarc'h, Sourdille, Charmet, Edwards, & Bernard, 2002). Loci with many alleles, and a PIC near 1 are most desirable, as locus with PIC value > 0.5 is highly informative, and 0.5 > PIC > 0.25 reasonable informative, whereas locus with PIC < 0.25 is regarded as slightly informative (Botstein et al., 1980).

3.4.2 Population genetics analysis

For examination of populations by location genetic structure summary statistics such as number of private alleles found (N_p) in isolations of separate locations, mean number of different alleles (N_a), mean effective number of alleles (N_e), observed (H_O) and expected hetorozygosity (H_E), were calculated with GenAlEx 6.4.1 (Peakall & Smouse, 2006). Allelic richness (A_r), a measure of allelic diversity that take into account sample size (El Mousadik & Petit, 1996), was calculated for separate populations, using FSTAT 2.9.3.2 (Goudet, 1995).

Inbreeding coefficients (F_{IS}) for each population and pairwise subpopulation inbreeding coefficient F_{ST} were calculated according to Weir and Cockerham, (1984) in FSTAT 2.9.3.2 (Goudet, 1995) and Genepop on the Web (http://genepop.curtin.edu.au) (Raymond & Rousset, 1995; Rousset, 2008) respectively.

An exact test of departure from HWE (probability test, and heterozygote deficiency and excess) and exact test of genotypic differentiation for each population pair (exact G test) were conducted using Genepop on the Web (http://genepop.curtin.edu.au) (Raymond & Rousset, 1995; Rousset, 2008). Estimate of *P*-values for each population pair across all loci was done by Markow chain method using same parameters as before.

3.4.3 Linkage disequilibrium

For genotypic linkage disequilibrium (LD) between loci and between loci in different populations a test was computed using Genepop on the Web (http://genepop.curtin.edu.au) (Raymond & Rousset, 1995; Rousset, 2008), using the log likelihood ratio statistic (G-test). To adjust the *P* value for multiple tests for LD the falls discovery rate (FDR) procedure (Benjamini & Hochberg, 1995), which controls the proportion of significant results (false discovery rate) instead of controlling the chance of making even a single error. The resulting adjusted *P* values ar called Q-values. For these, the procedure implemented in the R package QVALUE (Storey & Tibshirani, 2003) was use.

3.4.4 Test for founder effect

The possibility of a recent reduction in effective population size (founder effect) was tested using the program BOTTLENECK 1.2.02 (Cornuet & Luikart, 1996) which relies on a method based on excess of gene diversity under three models of microsatellite evolution; the infinite-allele model (IAM), the stepwise mutation model (SMM), and the two-phase model of mutation (TPM; with 70% SMM and 30% variation). A Wilcoxon test was used to test for significant excess in gene diversity over expected gene diversity ($H_{obs}>H_{eq}$). The expected distribution of H was found using 2000 iterations. A second method used was a graphical method assuming that bottlenecks cause alleles at low frequency (<0.1) to become less abundant than alleles in one or more intermediate allele frequency class (Luikart, Allendorf, Cornuet, & Sherwin, 1998), giving rise to so-called mode-shift which can be used to discriminate between bottlenecked and stable populations (L-shaped distribution).

3.4.5 Clustering analysis

Principal coordinates analysis (PCoA) using a compilation codominant genotypic distance matrix based on Nei's genetic distance (Nei, 1972) as an input, was carried out using GenAlEx 6.4.1 for population genetic structure analysis. Likewise, an analysis of molecular variance (AMOVA) was conducted in GenAlEx 6.4.1 to examine the distribution of variation and differential connectivity among populations (PhiPT).

BAPS 5.2 (Bayesian analysis of population structure) was used for the grouping of individuals into genetic groups or clusters, and investigating genetic clustering of population at sample locations, for analysis of admixture between populations, and to estimate gene flow between populations (Corander & Marttinen, 2006; Corander, Marttinen, Siren, & Tang, 2008). A clustering model was used to, cluster groups of individuals. Different values of K (number of clusters) 15, 10, 5 and 3 each repeated three times were experimented to find the optimal K. Following populations grouping, using fixed K clustering and runs as 10, an admixture between pre-defined populations was tested and estimate of the gene flow between populations was done. The minimum size of a population taken into account when admixture was tested was set as five. Input number of interactions was set as 100, number of reference individuals from each population was set as 200 and input number of interactions for reference individuals was set equal to ten. This was done to decrease the probability of false results. Afterwards BAPS 5.2 was used to explore the relationships among clusters by drawing a Neighbor-Joining tree based on Nei's distances (averaged over loci) (Saitou & Nei, 1987).

4. Results

4.1 Summary of genetic and genotypic variability of *Melampsora larici-populina* individuals collected in 2008 and 2009

Four hundred and eighty *Melampsora larici-populina* isolates sampled from 380 trees in fifteen locations were genotyped. Of the 480 isolates, 41(8.5 %) isolates were eliminated from further analyses because of high number of loci not amplified (6.0 %) or evidence of contamination (2.5 %). Of the 25 markers genotyped, three markers (MLP59, MLP94, and MLP96) were excluded from the analysis because of a high proportion of missing data. The elimination of samples and markers not showing adequate results were as follows: Fourteen isolates containing more than 40 % missing alleles were eliminated (10 of 25 markers) leaving 466 isolates. Then the three markers MLP59, MLP94, and MLP96 failing in 85/466 (12 %), 90/466 (19 %), and 100/466 (23 %) occasions respectively were omitted. Afterwards sixteen more isolates showing ≥25 % (6/22) missing loci where omitted, leaving 450 isolates, two of them also showing signs of contamination. Finally, eleven more isolates showing signs of contamination were eliminated leading to 439 evaluated qualified.

Of the 439 isolates judged to show sufficient genotyping, 362 (84.5 %) were completely genotyped, 44 (10 %) isolates failed to have 1 locus genotyped, and the remaining 33 (7.5 %) failed to have 2 to 5 loci genotyped. This leads to a different number of isolates genotyped at each locus, ranging from 407 to 439 isolates, the lowest value being for the marker MLP92 (Table 3). On average, 1.6 % of data was missing from the 439 individuals.

Before data analyses, isolates collected in 2008 and 2009 (Table 2) were pooled to one collection. Exact test for genotypic differentiation between these years for each sample pair collected at Gunnfríðarstaðir, Hveragerði, Selfoss and Skálholt was not significant ($P>0.05$).

Of the 22 loci accepted, 21 were polymorphic and one (MLP91) was monomorphic (Table 3). A total of 122 alleles were detected across the 22 loci of which 84/122 (68.9 %) showed low frequency (≤ 0.005) (Table 4). At the polymorphic loci, the observed alleles ranged from three to ten, for MLP77, MLP87, MLP92, MLP93, and MLP95 and MLP100 respectively, giving a mean number of 5.545 alleles per locus (Table 3). In accordance with the high proportion of alleles at low frequency, the number of effective alleles in the

polymorphic loci was relatively low ranging from 1.04 to 2.16, for MLP87, and MLP50 and MLP73 respectively. Mean observed heterozygosity H_O was 0.350 as the mean genetic diversity (i.e. expected heterozygosity, $H_{E)}$ was 0.375. All but two polymorphic loci, MLP55 and MLP68 showed significant deviation from Hardy-Weinberg equilibrium (P <0.05). Thereof eighteen of the twenty-one polymorphic loci showed significant homozygotes deficit (P <0,05), and only two (MLP55 and MLP56) showed significant excess of homozygotes (P <0.05). The locus MLP68 was not significant (P >0.05).

Among the 21 polymorphic loci, most pairs of loci (200/210, 95.2 %) were in significant linkage disequilibrium (Q-value <0.05). This high proportion of pairs of loci in significant linkage disequilibrium, together with widely disturbed heterozygote deficiency suggest some genetic structure (i.e. Wahlund effect (Wahlund, 1928)).

Table 3 Results of *Melampsora larici-populina* microsatellite analysis[a]

Locus	*N*	N_a	N_e	H_O	H_E	F_{IS}	PIC
MLP12	430	7	2.069	0.533	0.517	-0.0296***	0.44
MLP49	424	9	2.059	0.493	0.514	0.0426*	0.45
MLP50	434	7	2.161	0.491	0.537	0.0878***	0.45
MLP54	439	5	1.067	0.043	0.062	0.3079***	0.06
MLP55	439	5	2.122	0.572	0.529	-0.0803	0.42
MLP56	439	5	2.041	0.565	0.510	-0.1065*	0.39
MLP57	438	6	2.024	0.500	0.506	0.0127*	0.39
MLP58	430	5	1.055	0.035	0.053	0.3370***	0.09
MLP66	439	6	2.060	0.485	0.515	0.0584***	0.42
MLP68	416	6	1.100	0.094	0.091	-0.0308	0.18
MLP71	437	5	2.153	0.460	0.536	0.1423***	0.44
MLP73	425	9	2.160	0.520	0.537	0.0327***	0.47
MLP77	409	3	2.083	0.504	0.520	0.0327***	0.49
MLP82	439	5	2.080	0.510	0.519	0.0183***	0.40
MLP83	439	4	2.048	0.501	0.512	0.0220***	0.39
MLP87	433	3	1.040	0.028	0.039	0.2840***	0.06
MLP91	439	1	1.000	0.000	0.000	…	…
MLP92	407	3	1.502	0.039	0.334	0.8827***	0.38
MLP93	439	3	1.051	0.036	0.049	0.2559**	0.05
MLP95	435	10	2.089	0.480	0.521	0.0794***	0.42
MLP97	434	5	1.947	0.447	0.486	0.0822***	0.40
MLP100	436	10	1.594	0.365	0.373	0.0225*	0.34
Mean	431.818	5.545	1.750	0.350	0.375		0.34

[a] For each locus; *N* = number of isolates genotyped, N_a = observed numbers of alleles, N_e = number of effective alleles, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = Inbreeding coefficient within individuals with exact test of departure from Hardy-Weinberg as * = significant at $P < 0.05$, * * = significant at $P < 0.01$, *** = significant at $P < 0.001$ and other values were not significant, PIC = polymorphism information contend, whereas PIC value >0.5 is regarded as highly informative and PIC < 0.25 is regarded as slightly informative, …= not observed.

Table 4 Frequency of alleles for 22 microsatellite markers for *Melampsora larici-populina* in pooled samples collected in 2008 and 2009[a]

Locus	*N*	Allele	Frequency	Locus	*N*	Allele	Frequency
MLP12	430	235	0.002	MLP71	437	356	0.002
	430	241	0.406		437	358	0.493
	430	244	0.024		437	360	0.469
	430	247	0.564		437	362	0.001
	430	250	0.001		437	368	0.034
	430	253	0.001	MLP73	425	419	0.499
	430	269	0.001		425	423	0.004
MLP49	424	344	0.001		425	424	0.002
	424	347	0.002		425	425	0.462
	424	350	0.002		425	427	0.002
	424	353	0.001		425	434	0.001
	424	356	0.432		425	436	0.020
	424	359	0.547		425	439	0.004
	424	362	0.008		425	445	0.006
	424	365	0.005	MLP77	409	432	0.452
	424	379	0.001		409	434	0.524
MLP50	434	267	0.001		409	435	0.023
	434	274	0.001	MLP82	439	172	0.468
	434	277	0.503		439	178	0.511
	434	280	0.032		439	181	0.008
	434	283	0.456		439	184	0.002
	434	286	0.005		439	208	0.010
	434	289	0.001	MLP83	439	148	0.006
MLP54	439	132	0.007		439	154	0.007
	439	135	0.014		439	157	0.510
	439	138	0.968		439	160	0.477
	439	141	0.009	MLP87	433	341	0.006
	439	144	0.002		433	344	0.980
MLP55	439	152	0.484		433	350	0.014
	439	155	0.006	MLP91	440	128	1.000
	439	158	0.486	MLP92	407	327	0.209
	439	161	0.021		407	330	0.789
	439	164	0.003		407	333	0.002
MLP56	439	269	0.502	MLP93	439	131	0.001
	439	281	0.006		439	137	0.975
	439	287	0.001		439	140	0.024
	439	293	0.487	MLP95	435	441	0.479
	439	296	0.003		435	442	0.001
MLP57	438	154	0.455		435	443	0.001
	438	169	0.001		435	450	0.003
	438	172	0.535		435	453	0.002
	438	175	0.005		435	454	0.010
	438	178	0.001		435	456	0.499
	438	181	0.002		435	457	0.001
MLP58	430	241	0.013		435	458	0.001
	430	247	0.007		435	459	0.001

Table 4 Frequency of alleles for 22 microsatellite markers for *Melampsora larici-populina* in pooled samples collected in 2008 and 2009[a] (cont.)

Locus	N	Allele	Frequency	Locus	N	Allele	Frequency
	430	250	0.973	MLP97	434	380	0.007
	430	252	0.005		434	392	0.001
	430	255	0.002		434	396	0.606
MLP66	439	189	0.589		434	400	0.003
	439	192	0.015		434	404	0.382
	439	195	0.023	MLP100	436	187	0.023
	439	198	0.371		436	193	0.001
	439	201	0.001		436	196	0.181
	439	207	0.001		436	199	0.003
MLP68	416	440	0.005		436	205	0.006
	416	444	0.007		436	208	0.771
	416	448	0.006		436	211	0.008
	416	452	0.953		436	214	0.002
	416	456	0.024		436	220	0.003
	416	460	0.005		436	234	0.001

[a] For each locus, *N* = number isolates genotyped

There was no evidence of recent genetic bottlenecks for total population of *M. larici-populina* in Iceland. The Wilcoxon test, performed with Bottleneck assumed all loci to fit IAM, TPM, and SMM, mutation-drift equilibrium as expected in population at Hardy-Weinberg equilibrium. Alternatively, the Mode-Shift analysis showed the typical L-mode shaped indicative of a non-bottlenecked population (Figure 3).

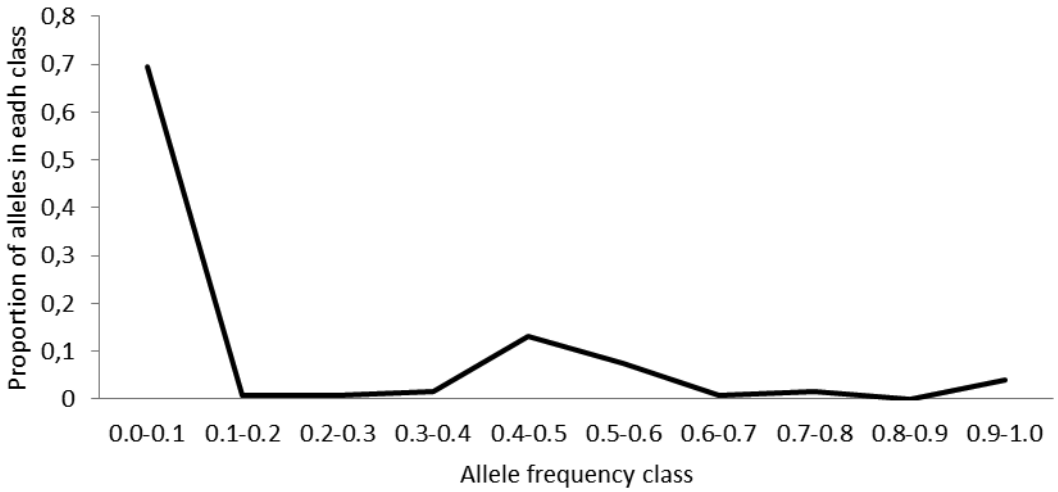

Figure 3 Mode-shift analyses of 22 microsatellites for *Melampsora larici-populina* pooled individuals collected in Iceland during 2008 and 2009 indicating a normal L-shape distribution.

Principal coordinate analysis (PCoA) using genetic distance matrix for input was used to inspect structural pattern of the pooled *Melampsora larici-populina* individuals collected

during 2008 and 2009. The PCoA indicated division of individuals into two main groups, the smaller one including individuals collected at Lón. The second one, including all other individuals analysed (

Figure **4** a, b, c).

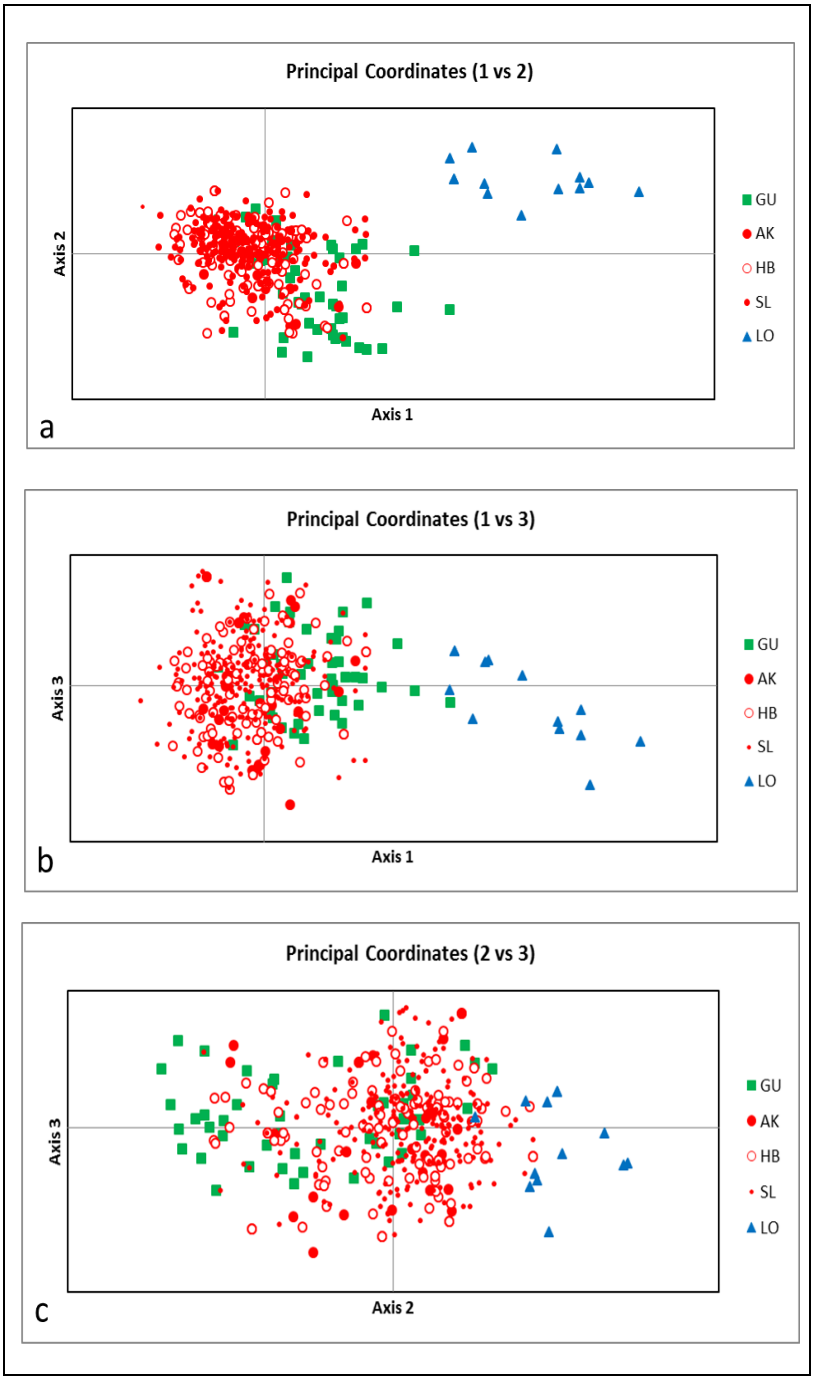

Figure 4 Principal coordinates analysis of *Melampsora larici-populina* microsatellite data from individuals collected in 2008 and 2009. The analysis explains 57.3 % of the variation on the first three axes. Coordinates 1, 2, and 3 explain 22 %, and 19.4 % and 15.8 % respectively demonstrated in a), b) and, c).

4.1.1 Genetic differentiation of 15 spatial populations

Several genetic characteristics of the *Melampsora larici-populina* populations collected in 2008 and 2009 were calculated (Table 5). All populations examined showed relatively high number of polymorphic loci, ranging from 77.3 % to 95.5 %., The number of private alleles was however relatively low, ranging from zero to six, with the exception of Lón (LO) where fifteen private alleles were found (Table 5). As well as, the mean number of different alleles per locus, allelic richness estimated for a minimal sample size of seven, and effective number of alleles were relatively low. In all cases, the highest measures observed, were in Lón (Table 5). Looking at each locus in each population, most were in HWE (Appendix 2) as exact test of HWE was significant at P <0.05 in only one up to four loci per population. Genotyping of the locus MLP92, despite showing significant deviation (P <0.05) from HWE in 13 out of the 15 samples and the F_{IS} values were high in all cases ranging from 0.732 to 1 (individual value not showed). For the RL sample the locus MLP92 was in HWE and in the LO the locus was monomorphic. The within-population inbreeding estimate ranged from - 0.003 to 0.112. Nine samples by locations were found to be in HWE (P >0.05) (Table 5) as the six samples GU, AK, RA, MG, HV and SE showed significant deviation from HWE at P <0.05. Three of the samples GU, SE and LO showed significant heterozygote deficit, whereas none showed significant heterozygote excess.

Among the populations, no pairs of loci were in linkage disequilibrium (Q-value >0.05).

Table 5 Spatial genetic characteristics of the *Melampsora larici-populina* populations, in pooled samples collected in 2008 and 2009[ab]

Population	N^a	Polymorphic loci[a]	$N_p{}^a$	N^b	$N_a{}^b$	$N_{Ar}{}^b$	$N_e{}^b$	$H_O{}^b$	$H_E{}^b$	$F_{IS}{}^b$
Gunnfríðarstaðir	48	20/22	4	46.955	2.409	1.900	1.645	0.305	0.339	0.112***
Akureyri	29	18/22	1	28.773	2.136	1.835	1.660	0.346	0.349	0.026***
Melar	19	17/22	3	18.955	2.091	1.870	1.699	0.344	0.357	0.064
Kópavogur	29	18/22	2	27.500	2.045	1.823	1.692	0.348	0.358	0.045
Laugardalur	20	18/22	2	19.864	2.045	1.826	1.642	0.345	0.339	0.008
Heiðmörk	7	18/22	1	6.955	1.909	1.883	1.600	0.364	0.330	-0.025
Höfðabakki	19	17/22	2	18.955	2.045	1.888	1.692	0.344	0.359	0.067**
Mógilsá	27	17/22	2	26.545	2.182	1.858	1.703	0.358	0.359	0.020***
Hveragerði	51	17/22	2	50.273	2.136	1.775	1.667	0.349	0.345	-0.003***
Selfoss	53	20/22	6	52.000	2.455	1.840	1.689	0.343	0.356	0.046***
Skálholt	50	21/22	4	49.364	2.727	1.948	1.732	0.373	0.365	-0.013
Haukadalur	27	19/22	2	27.000	2.182	1.848	1.657	0.377	0.344	-0.076
Tumastaðir	29	17/22	0	28.227	2.045	1.786	1.646	0.354	0.334	-0.043
Þjórsárdalur	19	17/22	1	18.727	1.955	1.768	1.663	0.352	0.338	-0.015
Lón	13	19/22	15	11.727	2.682	2.505	2.036	0.420	0.436	0.083
Mean		18.02		28.788	2.203	1.890	1.695	0.355	0.354	

For each populate genotyped; N = number of isolates genotyped, N_p = number private alleles.
[b] Mean measures over loci for each population; N^b = mean number of isolates genotyped, $N_a{}^b$ = mean number of different alleles, $N_{Ar}{}^b$ = mean allelic richness, $N_e{}^b$ = mean effective number of alleles, $H_O{}^b$ = mean observed heterozygosity, $H_E{}^b$ = mean expected heterozygosity,
F_{IS}= within-population inbreeding estimate, and departure level and test of Hardy-Weinberg equilibrium as ** = significant at P <0.01 and *** = 0.001.

Pairwise comparisons of F_{ST} estimated with Weir and Cockerham's Φ (1984) and, the genotypic differentiation for each population pair (exact G test) shown in Table 6 indicated evidence for three main clusters of populations. The population at Lón (LO), was highly distinguishable from all other populations with high pairwise F_{ST} values ranging from 0352 to 0.398 and high significant genetic differentiation for all pairs of population ($P < 0.001$). The second one located at Gunnfríðarstaðir (GU) with pairwise F_{ST} values ranging from 0.026 to 0.386 and, the genetic differentiation significant for all pairs of population, with twelve being significant at $P < 0.001$ level. Finally the third cluster including all other populations the pairwise F_{ST} values ranging from - 0.001 to 0.026 and, the genotypic differentiation significant for 21/78 (26.9 %) pairs of population, (11, 5 and 5 pairs being significant at $P < 0.05$, $P < 0.0$ and $P < 0.001$, respectively) (Table 6).

Analysis using the program Bottleneck was carried out on samples from all fifteen sampling sites. Given the small sample size (less than 30) on 12 of 15 of the individual locations and or number of polymorphic loci less than 20 (Table 4) the results were only taken as valid at three locations Gunnfríðarstaðir, Selfoss and Skálholt. These showed no evidence for excess of observed heterozygosity ($H_{obs}>H_{eq}$) in any of the populations regardless of the model assumed (IAM, SMM or TPM; $P<0.005$), suggesting that the populations analysed here have not gone through population bottlenecks recently (data not shown). The mode-shift analysis, showed the typical L-mode shape indicative of non-bottlenecked populations for these three sampling locations (data not shown).

Table 6 Pairwise inbreeding coefficient statistics for *Melampsora larici-populina* populations at fifteen locations as samples collected at same location in 2008 and 2009 were pooled.

Location ID	GU	AK	RM	KO	RL	RH	RA	MG	HV	SE	SH	HA	TU	TH	LO
GU	-	***	***	***	***	*	***	***	***	***	***	***	***	**	***
AK	0.046	-	NS	NS	*	NS	*	**	NS	NS	NS	NS	NS	NS	***
RM	0.042	0.006	-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	***
KO	0.044	0.004	-0.012	-	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	***
RL	0.075	0.018	0.000	0.007	-	NS	**	*	*	NS	*	NS	NS	NS	***
RH	0.047	0.013	0.005	0.021	0.021	-	NS	NS	*	NS	NS	NS	*	NS	***
RA	0.034	0.015	-0.003	0.000	0.027	0.027	-	**	***	***	***	**	**	NS	***
MG	0.055	0.018	0.002	-0.002	0.020	0.018	0.022	-	NS	NS	NS	NS	*	NS	***
HV	0.061	0.002	0.008	0.001	0.013	0.022	0.026	0.001	-	NS	*	NS	NS	NS	***
SE	0.079	0.007	-0.001	-0.005	0.004	0.020	0.024	0.007	0.004	-	***	NS	*	NS	***
SH	0.061	0.005	0.009	0.006	0.015	0.022	0.026	0.002	0.002	0.009	-	NS	NS	***	***
HA	0.082	0.010	0.000	0.003	-0.006	0.020	0.025	0.007	0.000	-0.001	0.005	-	NS	NS	***
TU	0.051	0.004	0.006	0.005	0.008	0.030	0.020	0.011	0.001	0.014	0.004	0.004	-	NS	***
TH	0.026	-0.010	-0.006	-0.001	0.010	0.015	0.016	0.010	-0.002	0.007	0.005	0.010	0.003	-	***
LO	0.386	0.378	0.360	0.374	0.378	0.371	0.352	0.382	0.398	0.396	0.364	0.391	0.380	0.370	-

Above diagonal, an exact G test for genotypic differentiation for each population pair, NS = not significant, * = significant at P <0.05, ** = significant at P <0.01, *** = significant at P < 0.001. Pairwise F_{ST}'s values showed below diagonal.

This was further supported by principal coordinate analysis (PCoA) using a pairwise population matrix of mean population codominant genetic distance for the fifteen sampling sites. It showed that the populations located at Gunnfríðarstaðir and Lón were separated from other populations (Figure 5). The same tendencies were observed in PCoA using a pairwise F_{ST} matrix among the fifteen populations (Figure not shown). However, the percentage of variation explained by the first three axes was lower using F_{ST} matrix, as cumulative value was 87.2 % compared to 94.2 %.

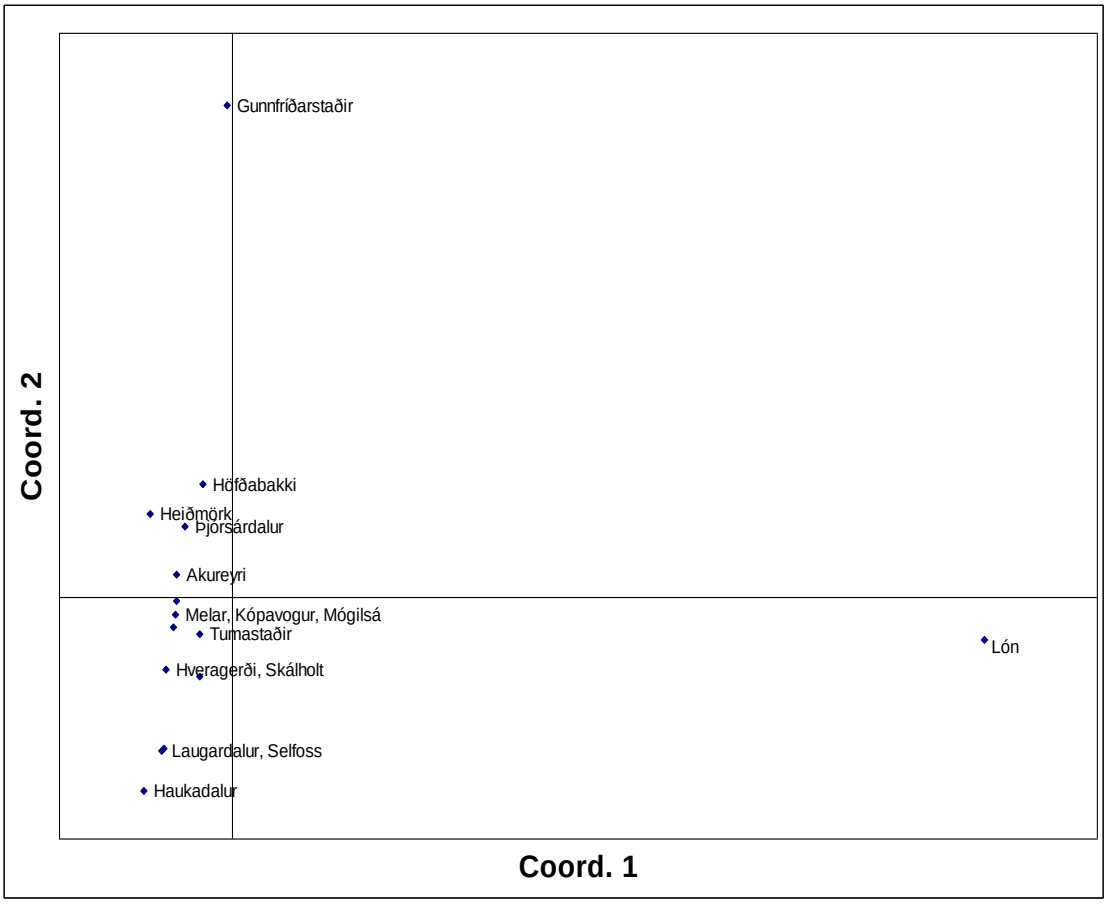

Figure 5 Principal coordinates analysis of *Melampsora larici-populina* microsatellite data from fifteen geographical distinguished populations, explaining 94.2 % of the variation. Coordinates, 1, 2, and 3 explain 82 % 9 % and 3.2 %, respectively.

Analysis of molecular variance (AMOVA) using genetic distance matrix was performed for calculation of PhiPT(Φ_{PT}). The PhiPT(Φ_{PT}) calculated at 999 permutations was 0.094 ($P < 0.001$) indicating that a high percentage of the total genetic diversity of the 15 populations in this study was distributed on a small spatial scale with 91 % the genetic diversity distributed within populations and only 9 % between populations. Following Bayesian analysis of population structure using BAPS 5.4 given 15, 10 and 5 as the upper bound to the number of populations (K) (possibly multiple values), three

runs scored the fifteen populations by locations in 3 clusters or genetic groups (Figure 6). The probabilities for three clusters, were 1. These further analyses of population clustering by locations came to corresponding conclusion and PCoA analysis as populations located at Gunnfríðarstaðir and Lón made two groups when other populations were categorised in one group. Geographical distribution of the clusters are shown in

Figure **7**.

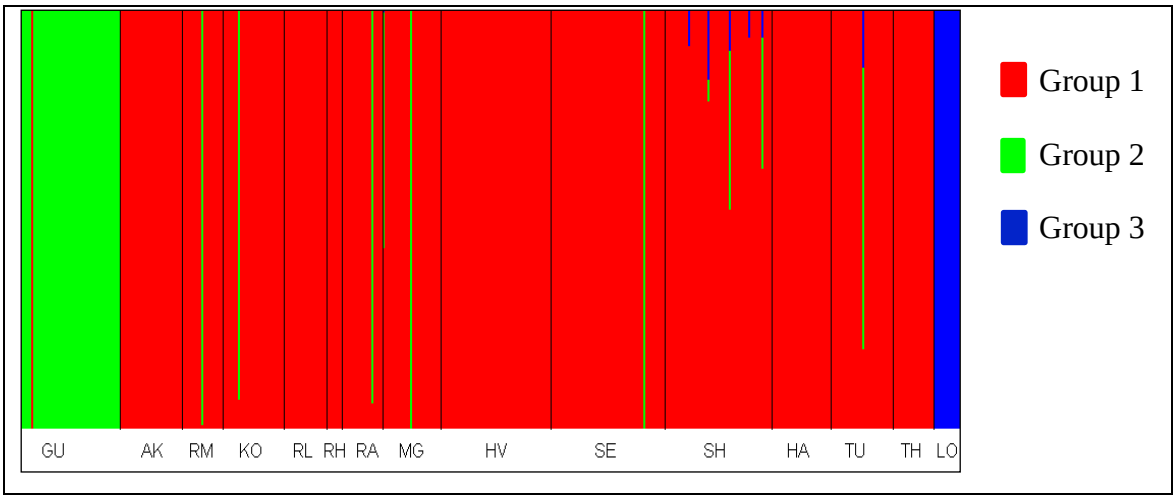

Figure 6 Bayesian analysis of population structure of *Melampsora larici-populina*. Estimate admixture coefficients for populations clustered to three genetic groups. Each column (or vertical line) corresponds to one individual. Each column is coloured with different colours in proportions corresponding to estimated admixture coefficient of the corresponding individuals. The populations are separated by black vertical line and the abbreviation of each location is given below the column.

Eleven individuals appeared to show evidence of genetic admixture from different groups and two individuals in group 1were assigned to group 2 (Figure 6). A plot of gene flow data drawn in BAPS 5.4 with a threshold for the significance of *P*-value of the admixture estimates as 0.05 showed the ancestral admixture of each cluster as 100 % - 98 % (figure not shown). Group 1 comprised of individuals from Akureyri, all locations in the larger Reykjavík area and all locations in southern Iceland. About 2 % of its DNA was introduced via gene flow from groups 2 (Gunnfríðarstaðir) and 3 (Lón), 1.7 % and 0.04 %, respectively. Group 2 (Gunnfríðarstaðir) appeared to have 2.1 % of its DNA from group 1. On the other hand, no DNA introduction via gene flow was observed in group 3 (Lón), probably due to few tests of isolates.

A Neighbor Joining tree, based on Nei's distances indicated very short distance between groups (clusters) 1 and 2 (Figure 8) which was in accordance with the PCoA analyses (

Figure **4**) where individuals belonging to group 1 (GU) and group 2 (AK, HB and SL) clustered together.

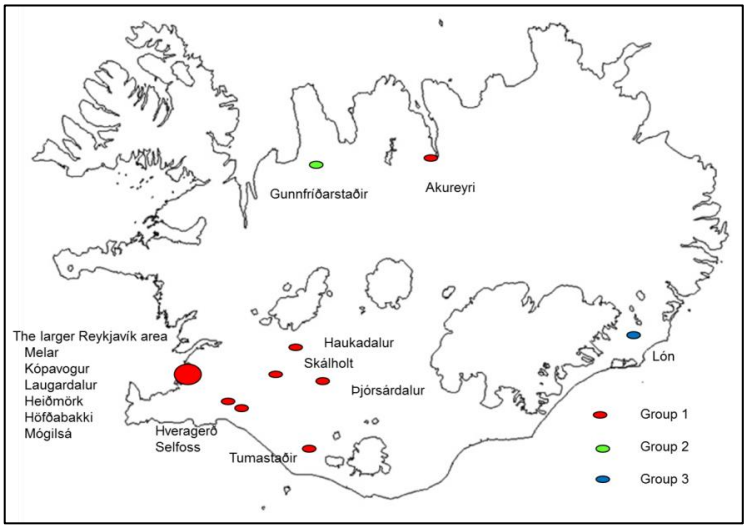

Figure 7 Geographical distribution of the three genetic groups detected by Bayesian analysis of population structure.

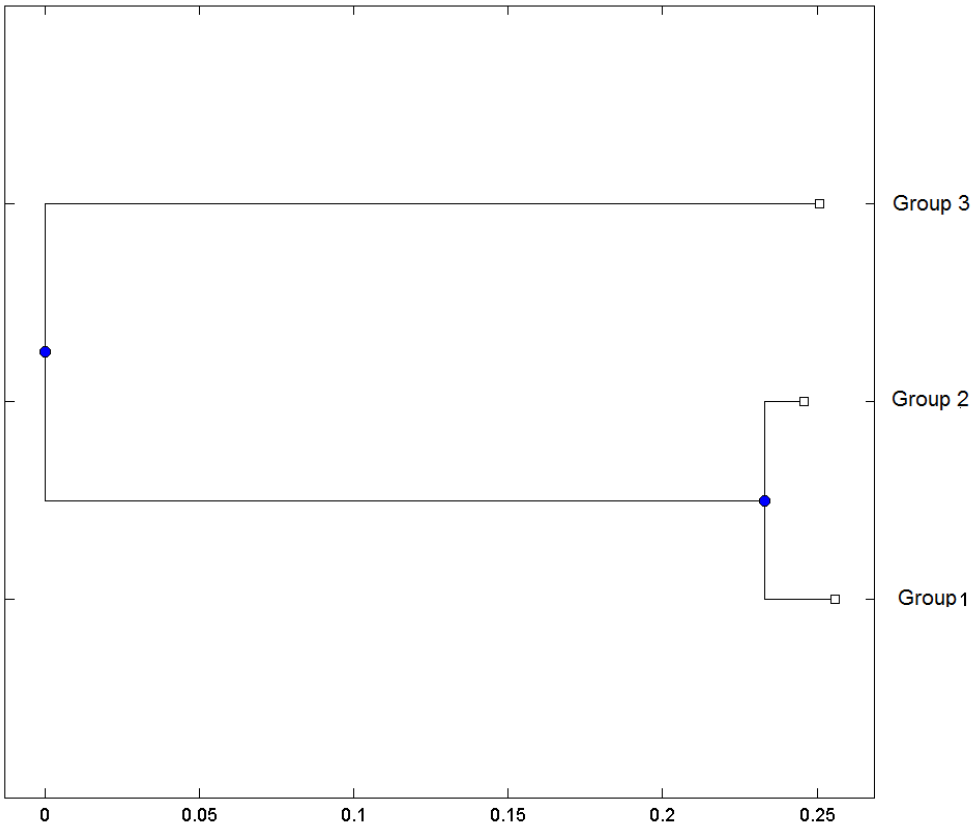

Figure 8 Neighbor-Joining tree based on Nei's distances between the three genetic groups of *M. larici-populina detected in Iceland.* Group1 includes Akureyri (AK), all locations in the larger Reykjavík area (HB), and southern Iceland (SL). Group 2 is Gunnfríðarstaðir and group 3 is Lón.

4.2 Comparison of genetic characteristics of *Melampsora larici-populina* at different time in the same location

A sample collected near Skálholt in 2003 (SK) and samples collected at the same location in 2008 and 2009 (SH) were compared to test for potential change of genetic characteristic of *M. larici-populina* over time. As in analysing the genetic difference of *M. larici-populina* for different locations, the samples collected in 2008 and 2009 were pooled.

Out of the 94 SK isolates tested six failed to have one locus genotyped each, compared to the 50 SH isolates where two and four failed to have five and one locus genotyped, respectively. This reduced the mean number of isolates genotyped (Table 8). Both samples showed relatively high number of polymorphic loci ranging from 86.4 % to 95.5 % and a total of 59 and 60 alleles were detected across the 22 loci tested (Table 8) for SK and SH, respectively. Alleles of very low frequency ≤ 0.005 were only observed in the SK – sample 13/59 (22.0 %) (Table 8), nine of which were private alleles. In the SH population eleven alleles showed frequency 0.01 whereof 9 were private alleles. In both populations, mean numbers of alleles were similar and the mean effective number of alleles was low. Based on minimum sample size of 45 diploid individuals, the mean allelic richness was similar ranging from 2.306 to 2.681. For both samples, the departure levels of Hardy-Weinberg equilibrium were highly significant ($P < 0.001$). Out of the eighteen polymorphic loci observed in 2003, four showed significant homozygote deficit ($P < 0.05$) and five showed significant homozygote excess ($P < 0.05$). Only one of nineteen polymorphic loci observed in 2008/2009 showed significant homozygote deficit ($P < 0.05$) and two showed significant homozygote excess ($P < 0.05$). Two loci, MLP54 and MLP87 were not recognized as polymorphic, due to high frequency of one out of two loci (Table 8).

Pairs of loci exhibiting significant linkage disequilibrium (LD) (Q-value <0.05) were found in both populations. However there was a great difference 57/171 (33.3 %) compared to 1/210 (0.5 %) for SK and SH respectively. Including the numbers of loci showing significant homozygote excess in 2003, significant LD may potentially denote genetic drift. It should however be noted, that different numbers of isolates in the groups were not taken into account.

Table 7 Genetic characteristics of the *Melampsora larici-populina* populations collected at Skálholt in 2003 and 2008/2009, respectively.

Population ID	N_a	Polymorphic loci	N_p	N^b	$N_a{}^b$	$N_{Ar}{}^b$	$N_e{}^b$	$H_O{}^b$	$H_E{}^b$	$F_{IS}{}^b$	LD (%)
SK	94	19/22	13	93.682	2.682	2.306	1.672	0.351	0.349	0.001***	57/171 (33.3)
SH	50	21/22	14	49.364	2.727	2.681	1.732	0.373	0.365	0.013***	1/210 (0.5)
Mean		20/22		71.523	2.705	2.494	1.702	0.362	0.357		

[a] For each populate genotyped; N = number of isolates genotyped, N_p = number private alleles.
[b] Mean measures of loci for each population; N^b = mean number of isolates genotyped, $N_a{}^b$ = mean number of different alleles, $N_e{}^b$ = mean effective number of alleles, $N_{Ar}{}^b$ = mean allelic richness, LD = number of pairs of significant linkage disequilibrium, 171 to 210 comparisons were calculated, depending on the number of monomorphic loci in SK and SH, % = percentage of pairs in significant linkage disequilibrium. $H_O{}^b$ = mean observed heterozygosity, $H_E{}^b$ = mean expected heterozygosity, $F_{IS}{}^b$= mean Fixation index and departure level and test of Hardy-Weinberg equilibrium as ***= 0.001

Table 8 Frequency of alleles for 22 microsatellite markers for *Melampsora larici-populina* at Skálholt[a] in 2003, and in 2008/2009.

		2003		2008/2009	
Locus	Allele	N	Frequency	N	Frequency
MLP12	241	93	0.522	49	0.480
	244	93	0.005	49	0.031
	247	93	0.468	49	0.490
	265	93	0.005	49	…
MLP49	344	94	…	49	0.010
	356	94	0.521	49	0.429
	359	94	0.479	49	0.551
	362	94	…	49	0.010
MLP50	277	94	0.569	49	0.490
	280	94	…	49	0.061
	283	94	0.431	49	0.439
	289	94	…	49	0.010
MLP54	138	94	1.000	50	0.990
	141	94	…	50	0.010
MLP55	152	94	0.463	50	0.440
	158	94	0.521	50	0.550
	161	94	0.016	50	0.010
MLP56	269	94	0.431	50	0.480
	287	94	0.011	50	…
	293	94	0.559	50	0.520
MLP57	154	94	0.590	50	0.430
	172	94	0.410	50	0.560
	178	94	…	50	0.010
MLP528	241	94	0.005	49	…
	247	94	…	49	0.020
	250	94	0.984	49	0.980
	253	94	0.011	49	…
MLP66	189	94	0.452	50	0.600
	192	94	0.005	50	…
	195	94	0.016	50	0.020
	198	94	0.527	50	0.380
MLP68	440	94	…	49	0.020
	452	94	0.995	49	0.929
	456	94	0.005	49	0.051
MLP71	358	94	0.378	50	0.470
	360	94	0.612	50	0.510
	364	94	0.005	50	…
	368	94	0.005	50	0.020
MLP73	419	94	0.590	50	0.450
	425	94	0.404	50	0.470
	427	94	…	50	0.020
	436	94	…	50	0.050
	439	94	…	50	0.010
	447	94	0.005	50	…
MLP77	432	94	0.319	48	0.531

Table 8 Frequency of alleles for 22 microsatellite markers for Melampsora larici-populina at Skálholt[a] in 2003, and inn 2008/2009 (cont.).

Locus	Allele	2003		2008/2009	
		N	Frequency	N	Frequency
	434	94	0.681	48	0.458
	435	94	…	48	0.010
MLP 82	172	94	0.447	50	0.470
	178	94	0.548	50	0.530
	181	94	0.005	50	…
MLP83	148	94	0.005	50	…
	154	94	0.027	50	…
	157	94	0.394	50	0.530
	160	94	0.574	50	0.470
MLP87	344	94	1.000	49	0.990
	350	94	…	49	0.010
MLP91	128	94	1.000	50	1.000
MLP92	327	88	0.182	45	0.067
	330	88	0.807	45	0.933
	336	88	0.011	45	…
MLP93	137	94	0.984	50	0.970
	140	94	0.016	50	0.030
MLP95	441	94	0.314	50	0.490
	453	94	0.005	50	…
	456	94	0.681	50	0.510
MLP97	396	94	0.527	49	0.724
	404	94	0.473	49	0.276
MLP100	187	94	0.011	50	0.080
	196	94	0.250	50	0.270
	208	94	0.729	50	0.630
	211	94	…	50	0.010
	214	94	0.005	50	0.010
	228	94	0.005	50	…

[a] For each locus genotyped; N = number isolates genotyped
... = not observed

Pairwise comparisons of F_{ST} estimated with Weir and Cockerham's Φ (1984) between the two populations was 0.0228 and the genotypic differentiation was calculated as highly significant. Analysis of molecular variance (AMOVA) based on PhiPT values showed that a low percentages of the total genetic diversity of the two groups in the present study was distributed on a small spatial scale with 96 % of the genetic diversity distributed within populations and only 4 % between groups (P <0.01). The Principal coordinate analysis (PCoA) for the two *M. larici-populina* individuals sampled in 2003 and 2008/2009 showed that individuals clustered together (Figure 9). Further, no genetic differentiation was observe between the two groups. In PCoA based on pairwise population matrix of mean population codominant genetic distance, the coordinate 1 explained 100 % of the variation (both groups falling in the same point).

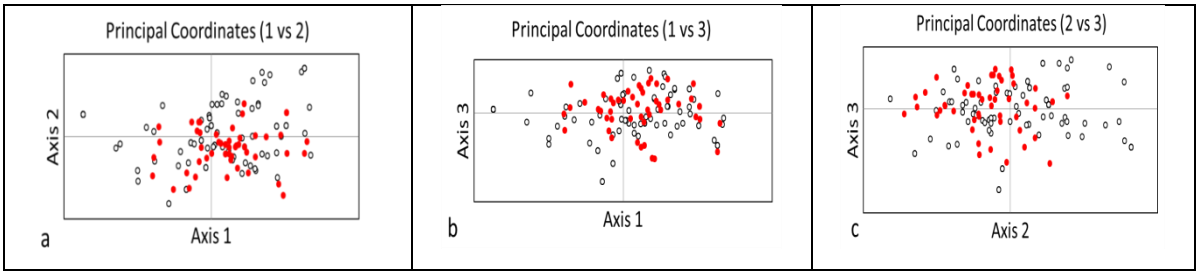

Figure 9 Principal coordinate analysis of *M. larici-populina* microsatellite data from individuals sampled at Skálholt in 2003 and pooled 2008 and 2009 individuals. Open circles and red marks represent the 2003 population and pooled 2008/2009 populations, respectively. This analysis, explaining 62.4 % of the variation on the first three axes. Coordinates 1, 2, and 3, explain 28 %, 19 % and, 15.4 %, respectively demonstrated in a), b) and, c).

According to Bayesian analyses of population structure using BAPS 5.4, samples collected in 2003 and 2008/2009 were grouped to one genetic group.

4.3 Comparison of Icelandic and French *Melampsora larici-populina* populations

Icelandic and French samples of *Melampsora larici-populina* collected in 2008/2009 and 2009, were compared respectively. Microsatellite data from previous microsatellite analysis of 470 French and Italian *M. larici-populina* samples were used for comparison (Xhaard et al., 2011a, 2011b). As three markers (MLP59, MLP94, and MLP96) were excluded from the analysis of the Icelandic data, they were also omitted from the French data.

Pairwise comparisons of F_{ST} estimated with Weir and Cockerham's Φ (1984) between the two populations French versus Icelandic was 0.133 and the genotypic differentiation was calculated as highly significant. Analysis of molecular variance (AMOVA) based on PhiPT values showed that a high percentages of the total genetic diversity of the two groups in the present study was distributed on a relatively small spatial scale with 64 % of the genetic diversity distributed within populations and 36 % between the populations (P <0.01).

Principal coordinate analysis (PCoA) based on a pairwise population matrix of mean population genetic distance between samples from Icelandic and French grouped the sample from Lón with the French samples while underlining the close clustering of remaining Icelandic samples into a single group (Figure 10). The cumulative values for the three first axes were 91.9 %.

Fifteen and 41 private alleles were found within the Icelandic and French populations, respectively. No private allele was found among individuals collected at Lón, the location with the highest number of private alleles when compared only to other Icelandic samples.

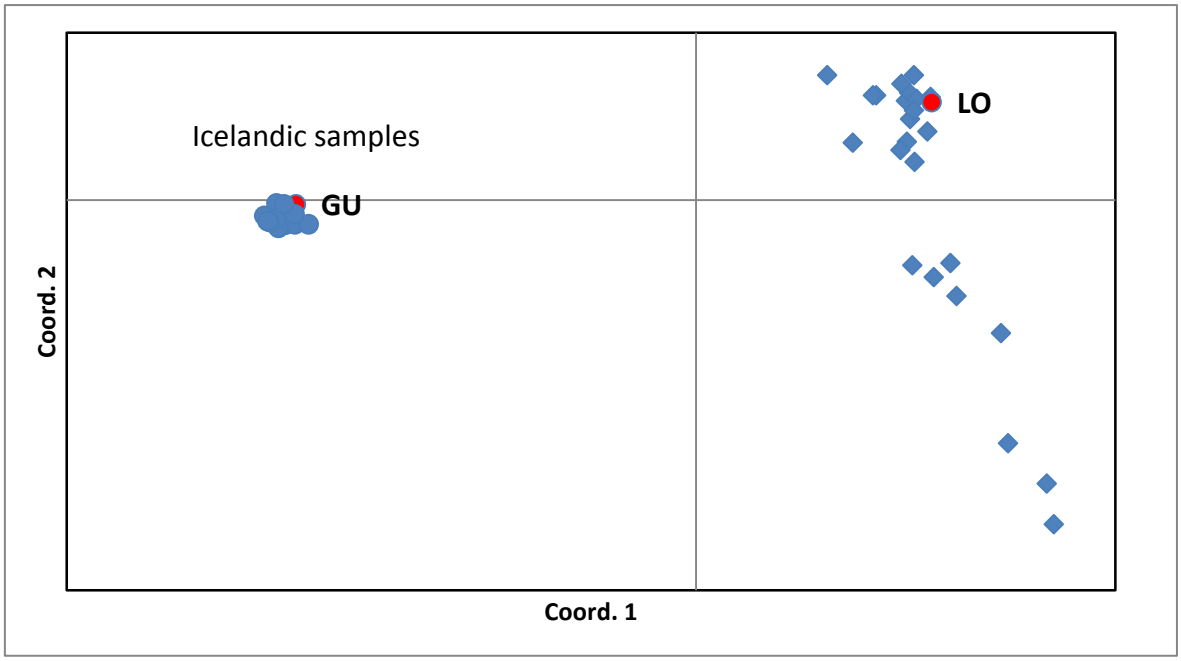

Figure 10 Comparison of *M. larici-populina* populations from Iceland and France based on genetic distance between groups from different sampling sites. Icelandic samples are shown as points and French samples as diamonds, with samples from Lón (LO) and Gunnfríðarstaðir (GU) specially labelled. Principal coordinates analysis explains 91.9 % of the variation on the first three axes. Coordinates 1, 2, and, 3, explain 67.8 %, 17.8 % and, 6.2 %, respectively.

5. Discussion

5.1 Genetic structure of Icelandic poplar rust *Melampsora larici-populina*

In this study, the genetic structure of a plant pathogenic fungus *Melampsora larici-populina* was investigated. This rust fungus has been found in Iceland since 1999, where it alternates on black cottonwood and *Larix* spp.

The analysis was performed using 25 microsatellites, which have previously been demonstrated to work for the analysis of genetic analysis of *M. larici-populina* (Xhaard et al., 2011b). In the present study, three loci were omitted from further analyses due to high and unexplained numbers of missing alleles. Eleven samples were omitted, because of more than two peaks observed for some loci. As germ tubes can fuse together on the poplar leafs in which tetra-nucleate and tri-nucleate can be found (Yu et al., 2009), this was considered as possible source of contamination of an isolate by another isolate. For most loci, polymorphic information content (PIC) was informative based on criteria. The five low PIC values observed were in all cases coinciding with low number of effective alleles observed.

Principal coordinates analysis and Bayesian clustering analysis revealed that *M. Larici-populina* at the fifteen regional populations tested could be clustered into three genetically distinct groups. The populations at the larger Reykjavík area and Southern Iceland plus Akureyri, all clustered in the same group, suggesting that migration may take place in the south-western part of Iceland, while the populations at Gunnfríðarstaðir and Lón belonged to two separate groups in a well-defined geographical area. The clustering of isolates collected at Akureyri in the main group, supports the theory that the rust may have got transmitted with infected plants cultivated in Tumastaðir in Southern Iceland**.** Of interest is the gene flow from the groups located at Gunnfríðarstaðir and Lón, observed in the main group, and the genetic recombination between individuals of different groups. On the other hand, no evidence of DNA introduction via gene flow was observed in Lón. This may possibly be explained by a limited number of investigated isolates located in Lón or even for special geographical isolation.

In no population linkage disequilibrium across loci, was detected. This indicates frequent recombination (Gérard et al., 2006) taking place on *Larix* spp.

5.2 Comparison of genetic characteristics of *Melampsora larici-populina* at different times in the same location

Based on principal coordinates analysis and Bayesian clustering analysis the same genetic group persists in the region since 2003. However, signs of genetic drift over time were observed as high number of private alleles appeared both in the 2003 and the 2008/2009 groups and the genotypic differentiation was calculated as highly significant. Another remarkable variable was the difference of significant linkage disequilibrium between the microsatellite markers, which dropped from 33.3 % to 0.5 % during the period. It is unlikely that the high number in 2003 could be accounted for by asexual reproduction alone, considering that other hallmarks of asexual reproduction, such as a strong heterozygote multilocus genotypes and a heterozygote excess (Halkett, Simon, & Balloux, 2005), were not prevailing. More likely, it may indicated some genetic structure in the 2003 population no longer existing. In a previous study where 22 isolates of the sample collected at Skálholt in 2003 was analysed, a lack of significant linkage disequilibrium was expected to be in accordance with the occurrence of sexual reproduction in Iceland (Barrès et al., 2008). This difference may proceed from different number of investigated isolates (22 vs 94), or even different markers in use. Xhaard et al., (2009) have pointed out, the markers in use at the study of Barrès et al., (2008) were unable to resolve fine-scale population genetic questions, despite of substantial polymorphism.

In the study of Barrès et al., (2008) pathotypes of the Icelandic *M. larici-populina* individuals collected near Skálholt in 2003 were identified. Six pathotypes were found, with the mean number of virulence 1.33 per individual, as virulence 4 was the most common although the rare virulence 7 was one. In the present study, virulence was not tested. However, different virulences could be anticipated and might possibly be linked to different genotype groups denoted. In a recent study, Xhaard et al., (2011) found out that the spatial distribution of virulence 7 rust on *P. nigra* was non-random. Furthermore, in poplar cultivated landscapes in northern France where P. *x generosa* predominates, individuals demonstrated the highest proportion of virulence 7 (Xhaard et al., 2011).

5.3 Comparison of Icelandic *Melampsora larici-populina* samples to French samples

Comparison of Icelandic *M. larici-populina* samples collected in 2008/2009 and French populations collected in 2009 performed by PCoA revealed that the Icelandic *M. larici-populina* populations were with one exception highly different in genetic structure. Only the genetic group observed in Lón, grouped within the range of the genetic diversity of the French populations. Although the comparison between the Icelandic and the French samples does underline a portioning of the Icelandic samples into at least two main groups it is not possible to demonstrate conclusively that the Lón samples originated from France. For this to be possible more samples would be needed from different locations outside of France. In a study of Barrès et al., (2008), PCoA analyses performed on genetic distances, the Icelandic and Canadian populations were separated from the European populations that mostly clustered together (Barrès et al., 2008). The two populations from Iceland and Canada were expected to result from rare long distance dispersal events (LDD), exhibiting signs of strong founder effect. Moreover, Barrès et al., (2008) concluded that the origin of Canadian and Icelandic populations could not be determined from the assignments of these studies, possibly due to the large differentiation in population between European, Canadian and Icelandic populations. The Icelandic isolates tested in the study of Barrès et al., (2008) were a part of the sample collected at Skálholt in 2003 and are compared to samples collected at Skálholt in 2008 and 2009 in this present study. It can therefore be expected that the genetic structure of the Icelandic rust is different from central European rust with the exception of Lón, still leaving the question of the Icelandic rust origin unsolved. Comparison with strains from Scandinavia and even more northern continental European countries would be interesting. Furthermore it might be of interests to compare Icelandic rust to *M. larici-populina* in North England as it has been demonstrated to be different from other UK populations (Pei et al., 2007). It could even be relevant to compare the Icelandic rust to that of more southern locations such as northern Italy. For plant pathogenic fungi, long distance dispersal events can either result from passive transport by wind or from human activity (Linde, Zala, & McDonald, 2009; Viljanen-Rollinson & Cromey, 2002; Walker et al., 1974). In Iceland this may be expected as well. It is known that every year a large number of different Arthropoda come to the country by wind and merchandise (Icelandic Institute of Natural History, 2011), some of them establishing permanently (Icelandic Institute of Natural History, 2012).

6. Conclusions

This study on the genetic structure of *M. larici-populina* in Iceland has revealed that samples from different locations could be clustered into two main genetically distinct groups, where one group consisted of two subgroups. This clustering may possibly result (i) from repeated independent colonization of the pathogen and (ii) genetic drift. Although of low level, these results also revealed active gene-flow from one location to another allowing ancestry admixture.

The results of comparison to French samples underlined the existence of two main genetic groups, but did not conclusively answer the question of the origin of the pathogen. Although the genetic sample group at Lón might possibly origin from France, more samples from different locations would be needed for conclusive identification. Comparison of the Icelandic population with populations from Scandinavia and other northwardly regions would be of most interest.

The results presented here underline the importance of environmental monitoring, as new genetic groups as well as groups of different virulence of the pathogen could be expected to colonize, and spread to different locations. These results should also apply to new pathogens and are relevant for all recently introduced plant species. A rational next step in the analysis of the diversity of the Icelandic *M. larici-populina* is to analyse whether the diversity measured here with neutral markers translates into functional differences in pathogenicity.

7. References

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Appendix 1

Morphology characteristics of the microsatellite loci tested[ab]

Locus[a]	Repeat unit	Allele range	Multiplex PCR No.[b]
MLP12	complex (AAG)n	235-269	2
MLP49	(GAT)18	344-379	3
MLP50	(TCT)16	267-289	2
MLP54	(ATG)14	132-144	1
MLP55	(ATC)15	152-164	1
MLP56	(AAC)7	269-296	2
MLP57	(CAA)13	154-181	1
MLP58	(AAG)12	241-255	2
MLP59	(ATC)13	305-335	2
MLP66	(GAT)12	189-207	1
MLP68	(TTGA)12	440-460	3
MLP71	(AG)14	356-368	2
MLP73	(AC)14	419-445	3
MLP77	(AT)10	432-435	3
MLP82	(TAC)10	172-208	1
MLP83	(ATG)10	148-160	1
MLP87	(TGT)8	341-350	3
MLP91	(GTT)10	128	1
MLP92	(TTG)11	327-333	2
MLP93	(GTT)11	131-140	1
MLP94	(TTC)10	452-477	3
MLP95	(ACA)9	441-459	3
MLP96	(TGG)9	403-413	3
MLP97	(GATT)9	380-404	3
MLP100	(ACA)18	187-234	1

[a] Three loci MLP59, MLP95 and MLP66 were eliminated in data processing

[b] For each loci; multiplex PCR No., as microsatellites loci were amplified in three multiplex PCR

Appendix 2

Test of Hardy-Weinberg equilibrium in *Melampsora larici-populina* populations, as populations collected at same location during 2008 and 2009 were pooled

Locus	GU	AK	RM	KO	RL	RH	RA	MG	HV	SE	SH	HA	TU	TH	LO
MLP12	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
MLP49	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
MLP50	NS	**	NS	*	NS	**	NS	NS	NS	NS	NS	NS	NS	NS	NS
MLP54	NS	NS	...	NS	...	NS	...	...	...	NS	NS	NS	...	...	*
MLP55	NS	NS	NS	NS	NS	NS	NS	NS	**	NS	NS	NS	NS	NS	NS
MLP56	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS
MLP57	NS	NS	NS	NS	NS	NS	**	NS	NS	**	NS	NS	NS	*	...
MLP58	...	...	NS	...	NS	NS	...	...	...	NS	NS	NS	...	...	*
MLP66	NS	NS	NS	NS	*	NS	*	NS	NS	NS	NS	NS	NS	NS	NS
MLP68	NS	NS	...	NS	...	...	NS	NS	NS	NS	NS	NS	NS	NS	NS
MLP71	*	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS
MLP73	NS	NS	NS	*	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS
MLP77	*	NS	NS	NS	NS	NS	NS	***	NS	NS	NS	NS	NS	NS	NS
MLP82	NS	NS	NS	NS	NS	NS	NS	NS	NS	**	NS	NS	NS	NS	NS
MLP83	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS
MLP87	NS	...	...	...	...	...	...	...	...	...	NS	...	...	...	NS
MLP91	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
MLP92	***	***	***	***	NS	*	***	***	***	***	***	***	*	***	...
MLP93	NS	...	...	...	NS	...	...	...	...	NS	NS	...	...	...	NS
MLP95	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS
MLP97	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
MLP100	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*

[a] For each locus, NS = not significant, * = significant at $P < 0.05$, ** = significant at $P < 0.01$, and *** = significant at $P < 0.001$.
… = monomorphic.

Molecular analysis of Icelandic populations of the poplar fungal pathogen *Melampsora larici-populina* shows evidence of repeated colonization events

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ABSTRAC

The basidiomycete *Melampsora larici-populina* causes foliar rust on *Populus* species from the sections *Aigeiros* and *Tacamahaca*, causing reduction in biomass production and economic losses. In the present study, samples of Icelandic *M. larici-populina* were collected for analysis of genetic diversity and population structure. A total of 439 isolates, collected at fifteen locations, and analysed using 22 microsatellite markers were compared to data from French *M. larici-populina* populations. Twenty-one of the loci analysed were polymorphic, with an average of 3.4 alleles per locus. The mean observed and expected heterozygosities for all populations were 0.35 and 0.38. Evidence was found for a substructure within the Icelandic population with three subpopulations being the most likely scenario with low levels of gene-flow. The population structure seen here is most likely shaped by both isolation and genetic drift as well as repeated events of colonization. In the future it can therefore be expected that regional poplar rust genotypes in Iceland change by two different modes; on one hand by transport of spores within the country and on the other hand by repeated colonization events. The results reported here underline the importance of closely monitoring the development of fungal diseases in Iceland, and to carefully select resistance for Icelandic plant breeding programs.

Keywords poplar leaf rust, colonization, population structure, microsatellites, linkage

Introduction

Species of the genus *Populus* are native to the northern hemisphere and occur from the equatorial tropics to the geographical limits of tree growth. The *Aigeiros* and *Tacamahaca* sections of *Populus* have for years dominated plantation culture and both the pure species and their hybrids are commercially important. Several species of the *Tacamahaca* section are of economic value, of which *P. trichocarpa* (synonym: *P. balsamifera* ssp. *trichocarpa*) reaches the largest size of any western hardwood with heights of 50 m and diameters of over 1.5 m (Dickman, Isebrands, Eckenwalder, & Richardson, 2001).

Plant pathogens can seriously affect production by reducing both quality and yield. This is especially important in monoculture where production relies on relatively few species and few cultivars of each species. The problem further accentuates when pathogen populations have great evolutionary potential with high levels of genetic diversity, large effective population size, a mixed reproduction system, high mutation rates, and rapid gene flow between regions (McDonald & Linde, 2002). European forestry, which is more or less based on clones of the sections *Aigeiros* (*P. deltoides* and *P. nigra*) and *Tacamahaca* (*P. trichocarpa* and *P. maximowiczii*), has often been hard hit by disease outbreaks (Steenackers, Steenackers, Steenackers, & Stevens, 1996). In the mid 20 century, hybrids between *P. deltoides* and *P. nigra* or *P. trichocarpa* were selected for their immunity to rust, and the cultivation of these so-called 'Unal' clones (e.g. *P. trichocarpa*×*P. deltoides* 'Unal', 'Boelare' and 'Beauprè') was successful for many years (Pei, Ruiz, Bayon, Hunter, & Lonsdale, 2005). However, poplar trees in Europe are particularly vulnerable to many pathogens because of their decades long intensive cultivation, even aged monoclonal stands, and limited numbers of cultivars planted in a given region (Gérard, Husson, Pinon, & Frey, 2006; Steenackers et al., 1996). For example, in Northern France up to 80% of poplar plantations belong to the cultivar *P.*×*generosa* 'Beauprè' and the low levels of genetic diversity associated with monoculture may have contributed to the degradation of poplar health because of fungi, bacteria, and insects infestations (Miot, Frey, & Pinon, 1999).

Due to the geographical isolation of Iceland, the number of native trees and shrubs has remained low since the end of the Pleistocene. Pollen studies have shown that the composition of the Icelandic forest flora has been more or less the same for the last 6000 to 8000 years (Eggertsson, 2006). Black cottonwood, *P. balsamifera* ssp. *trichocarpa* (T. & G.) Brayshaw (formerly known as *P. trichocarpa*), was first cultivated in Iceland in 1944 (Þorsteinsson, 1990) with material collected at the Kenai Peninsula in Alaska (Blöndal & Gunnarsson, 1999). Black cottonwood soon became a popular garden tree, although it was only rarely used in afforestation at that time (Sverrisson, Halldórsson, & Sigurgeirsson, 2006). Increased afforestation in 1990 lead to extensive plantings of this species (Eysteinsson, 2012) and at the same time experimental forests and clonal trials were established which saw an increase in black cottonwood planting (Petursson, 1999). Self-seeded plants have been found and black cottonwood now belongs to naturalized plants in Iceland (Palsson, 2000).

A large number of fungi attack species of the genus *Populus*, primarily *Ascomycetes* but also many mitosporic fungi (Fungi imperfecti) and *Basidiomycetes*. The fungi causing leaf diseases

are usually primary parasites whereas those attacking trunks and roots are mostly wound parasites (Cellerino, 1999). The leaf fungi belonging to the genus *Melampsora* are responsible for all poplar rust (Cellerino, 1999), but around thirteen species and two hybrids of this genus have been described on *Poplulus* (Pei et al., 2005) eight of which infect poplars in Europe (Cellerino, 1999). Three of them, *M. allii-populina, M. larici-populina,* and *M. medusae*, are found distributed from Northern Africa to Western Asia as well as in South-Central Europe, Eurasia, and North America (Frey, Gérard, Feau, Husson, & Pinon, 2005).

Among the *Melampsora* species, the rust fungus *M. larici-populina* causes the most damage and is responsible for most of the economic losses in poplar cultivation (Pinon & Frey, 2005; Steenackers et al., 1996). *M. larici-populina* is an obligate parasite that draws nutrients from live plant tissues at every stage of its life cycle. It is heteroecious, macrocyclic, and alternates on *Larix* spp. During its lifecycle it produces five different spore stages, urediniospores, teliospores, basidiospores, spermatia, and aeciospores, with its asexual (uredinial) stage on *Populus* spp., and sexual (aecial) stage on *Larix* spp. (Cellerino, 1999). The rust overwinters on fallen leaves of *Populus.* In spring, karyogamy occurs and a short diploid germ tube is formed where meiosis takes place. Following germination, windborne haploid basidiospores are emitted which then infect the alternate host and form haploid spermagonia or pycnia. The role of pycnia is to produce spermatia (pycnospores) and to capture the opposite mating type, brought by raindrops or insects from other pycnia (Cellerino, 1999). Fertilization between spermagonia on larch needles results in formation of the aecia which are long chains of dikaryotic aeciospores, durable airborne spores, capable of reinfecting *Populus* ssp. Afterwards dikaryotic uredinia are formed on leaves for the remainder of the growing season, owing to the production of two or more generations of airborne urediniospores (Cellerino, 1999). Rust epidemics on poplars are caused by repeated cycling of the dikaryotic urediniospores which function as diploids (Pei, Bayon, Ruiz, & Tubby, 2007). In late summer or autumn, the overwintering telia form and the life cycle is closed. Teliospores are of biological significance, since they represent the conclusion of the sexual process (Cellerino, 1999). It is known that some poplar *Melampsorae* resume their production of uredinispores in the next vegetative season without passing to alternate host (Cellerino, 1999). It has been assumed that *M. larici-populina* can overwinter in the uredinial state, at least in relatively warm climate (Smith, Dunez, Phillips, Lelliott, & Archer, 1988), and recently a genetic group of *M. larici-populina* in S-France was found to display hallmarks of strict asexually reproduction, (Xhaard et al., 2011a).

Variability within the *Melampsora* species is mainly defined by the presence of pathotypes or physiological races (Pinon & Frey, 2005). So far, eight virulences have been seen within the *M. larici-populina* species, with potentially 256 pathotypes (Pinon & Frey, 2005). The known pathotypes have been organized into five E groups (E1-E5) with rust race E5 first identified in 1997 (Pinon & Frey, 2005). The main factors that are thought to contribute to rapid spread of emerging virulent *M. larici-populina* strains are the compulsory sexual stage on larch, which favors high racial variability, airborne urediniospores, long-lasting multiplications on poplar leaves, monoclonal poplar stands, and a strong selection pressure on the pathogen (Dowkiw, Husson, Frey, Pinon, & Bastien,

2003). The presence of larch in the vicinity (<1 km) has been shown to significantly increase the number of pathotypes (Frey et al., 2005).

Heavy infections caused by *M. larici-populina* lead to early leaf drop, delayed flushing time of the tree in the next growing season, and reduced growth and wood quality (Cellerino, 1999; Steenackers et al., 1996). Following leaf drop, leaf scars are ideal entry points for secondary parasites such as *Dothichiza polulea* (Steenackers et al., 1996). Secondary infections following *M. larici-populina* epidemics can cause critical economic loss and even plant death (Miot et al., 1999). Worldwide the leaf rust caused by the *Melampsora* species is one of the major poplar diseases (Pei et al., 2005; Steenackers et al., 1996), and the rust has been considered as an important external determinant of biomass production (Laureysens, Pellis, Willems, & Ceulemans, 2005).

M. larici-populina is native to Eurasia where its distribution range overlaps with that of its natural host *P. nigra* (Barrès et al., 2008). In the past century the distribution range has expanded worldwide (Barrès et al., 2006; Pei et al., 2005) with North America (Newcombe & Chastagner, 1993; Steimel, Chen, & Harrington, 2005), East Canada (Innes, Marchand, Frey, Bourassa, & Hamelin, 2004) and Iceland, where it was first discovered in 1999 (Eyjólfsdóttir, Halldórsson, Oddsdóttir, & Sverrisson, 1999), being among the most recent places of identification.

During the summer 1999, *M. larici-populina* was only seen in Hveragerði and Selfoss in the south part of Iceland (Eyjólfsdóttir et al., 1999). In the next few years the rust spread and in 2005 it could be found from Keflavík in the southwest and across the southern part (Sverrisson, Halldórsson, Kjartansson, & Sigurgeirsson, 2005). Moreover, it was found at Gunnfríðarstaðir in the north, at Hallormsstaður in the east, and at Lón in the southeast part of the country (Sverrisson et al., 2005). More recently *M. larici-populina* has been found at two locations in northern Iceland, in Akureyri (this study) and Vatnsdalur (Oddsdóttir & Sverrisson, 2010). In Iceland as elsewhere, rust has been shown to negatively affect the growth and health of poplar trees (Albers, Eggertsson, Sverrisson, & Halldórsson, 2006; Halldórsson, Eyjólfsdóttir, Oddsdóttir, Sigurgeirsson, & Sverrisson, 2001).

Compared to other organisms, fungal microsatellites have proved harder to isolate and have been shown to exhibit lower polymorphism (Dutech et al., 2007). Despite this, microsatellite markers have successfully been developed for *M. larici-populina* (Barrès et al., 2006; Steimel et al., 2005; Xhaard et al., 2011a; Xhaard, Andrieux, Halkett, & Frey, 2009). In a genetic study on thirteen populations of *M. larici-populina* from nine European countries and one population from Canada using eleven microsatellite markers, it was found that genetic differentiation between populations in continental Europe was low, while the two populations in Iceland and Canada were independently distinguishable suggesting effective gene-flow on the European continent (Barrès et al., 2008). In a recent study, where 25 microsatellite markers were used to genotype 467 samples collected in several locations in France and one in Italy, it was shown that the individuals clustered in three genetic groups (Xhaard et al., 2011a).

It is necessary to study the genetic structure of pathogen populations as it can reveal evolutionary potential of populations. This is particularly important when it comes to choosing the right breeding strategy for durable disease resistance. *M. larici-populina* populations from all over

Europe have therefore been extensively studied using different methods of analysis revealing high levels of population variation (e.g. Barrès et al., 2008; Xhaard et al., 2011a). In a population study on *M. larici-populina* the Icelandic population analysed appeared to be distinguishable from all other populations and to show signs of a founder effect (Barrès et al., 2008). However, as samples analysed were all collected at the same location, near to Skálholt, the result may not be representative for the whole Icelandic population. The objectives of the present study were: (1) to add to the on-going analysis of global diversity of poplar pathogens by analysing the genetic diversity of Icelandic populations of *M. larici-populina* and to compare the results to other European populations and (2) to strengthen future breeding projects for disease resistance in Icelandic poplar by evaluating the evolutionary potential of Icelandic *M. larici-populina* populations.

The results will increase the knowledge of the genetic characteristics of the *M. larici-populina* fungus, and thus enhance breeding projects of black cottonwood. Additionally, the study will provide information on the development of a fungal pathogen during its first years after settlement, serving as a potential model for population dynamics under an 'island model'. This subject is especially interesting in times when the distribution of both plants and pests is expected to shift due to climate change and the transfer of pests from one region to another is likely to increase.

Materials and methods

Sample collection and DNA isolation

Infected poplar leaves were collected at fifteen locations in Iceland in the summers of 2008 and 2009. One to three leaves were collected from each of 380 trees. In addition, 95 samples from leaves collected in 2003 near Skálholt (SH) were re-analysed (Table 1) (Barrès et al., 2006). Leaves were collected in Hveragerdi (HV), Selfoss (SE), the forest in Haukadalur (HA), a stand of black cottonwood near Skálholt (SH), a forest in Thjórsárdalur (TH), at Gunnfríðarstaðir (GU) in northern Iceland, at a small poplar stand at Lón (LO) in the south-eastern part of Iceland, in Akureyri (AK), where infections were first identified in 2009, and at Tumastaðir (TU), a nursery in the south, from where the infected plants in Akureyri originated. Leaves were also collected at six locations in the Reykjavík area (LR): Kópavogur (KO), Höfðabakki (RA), Laugardalur (RL), Melar (RM), and the forested areas Mógilsá (MG), and Heiðmörk (RH) (Figure 1 and Table 1). In many locations, black cottonwood and *Larix* sp. had been planted fairly close together and at every sampling location *Larix* sp. were found within a distance of 500 meters from sampling sites.

Two methods were used for collecting *M. larici-populina* from leaves. A total of 129 isolates harvested in 2008 were isolated by growing a single uredinium per leaf on fresh leaf discs of *Populus trichocarpa* 'Keisari'. However, to minimize the risk of contamination from local rust this method was changed and instead a single uredinium for each isolate was cut directly from leaves. In all cases, single uredinia were transferred to Eppendorf tubes and kept at -20°C. A total of 616 isolates were collected from 234 trees in 2008 and 300 isolates from 146 trees in 2009. A total of 480 isolates were selected from the collection for genetic analysis (Table 1).

All DNA work, including DNA extraction and polymerase chain reaction (PCR), was carried out at the Institut National de la Recherche Agronomique (INRA), with genotyping outsourced under a contract with INRA. DNA was extracted using the BioSprint 96 DNA kit in combination with the BioSprint 96 automated workstation following the BioSprint 96 DNA protocol as recommended (www.qiagen.com).

Marker amplification and fragment detection

Samples were analysed using the following twenty-five microsatellite markers: MPL12, MLP49, MLP50, MLP54, MLP55, MLP56, MLP57, MLP58, MLP59, MLP66, MLP68, MLP71, MLP73, MLP77, MLP82, MLP83, MLP87, MLP91, MLP92, MLP93, MLP94, MLP95, MLP96, MLP97, and MLP100 (Barrès et al., 2006; Xhaard et al., 2011a, 2009). The microsatellite markers were amplified using a GeneAmp 9700 thermocycler (www.appliedbiosystems.com) under the following conditions: An initial denaturing step of 5 min at 95°C; 32 cycles including 30 s of denaturation at 95°C, 90 s annealing at 60°C, a 60 s extension step at 72°C; and a final 30 min extension step at 60°C. The PCR was carried out in a 10 µL final reaction volume containing 5 µL of Qiagen® Multiplex PCR kit, 2 µL DNA, 0.2 µM of each reverse and forward primers. Forward primers, were labeled with a fluorescent tag (FAM, NED, VIC, or PET). Three PCR multiplexes were run with nine, seven, and nine markers. PCR products from the multiplex reactions (3 µL of PCR1, 4 µL of PCR2, and 5 µL of PCR3) were pooled and analyzed using the ABI 3730 Genetic Analyzer (Applied Biosystems). Fragments were sized with a LIZ-1200 size standard and alleles were scored using GENEMAPPER 4.0 (Applied Biosystems).

Analysis of molecular data

For individual microsatellite markers summary statistics such as observed number of alleles (ON_A), effective number of alleles (EN_A), observed (H_O) and expected hetorozygosity (H_E) were calculated using GenAlEx 6.4.1 (Peakall & Smouse, 2006). For examination of population genetic structure summary statistics such as number of private alleles (NPA), observed number of alleles (ON_A), effective number of alleles (EN_A), observed (H_O) and expected hetorozygosity (H_E) were calculated for individual sampling locations using GenAlEx 6.4.1 (Peakall & Smouse, 2006). Mean allelic richness (A_r), a measure of allelic diversity that takes into account sample size (El Mousadik & Petit, 1996), was calculated using FSTAT 2.9.3.2 (Goudet, 1995). Principal coordinate analysis (PCoA) was carried out based on 'Nei genetic distance' matrix as implemented in GenAlEx 6.4.1 (Peakall & Smouse, 2006). Analysis of molecular variance (AMOVA), based on the PhiPT (Φ_{PT}) distance between populations, using 'Codom-genotypic distance' with 999 permutations was carried out using GenAlEx 6.4.1 (Peakall & Smouse, 2006).

For each locus Weir and Cockerham's inbreeding coefficient, F_{IS} (Weir & Cockerham, 1984) and an exact test for the departure from HWE was calculated using Genepop on the Web (Raymond & Rousset, 1995; Rousset, 2008). Estimation of exact P-value was by way of Markov chain method (Guo & Thompson, 1992) with the parameters as follows: Dememorization 1000, batches 100, and iterations per batch 1000.

BAPS 5.2 was used for the grouping of individual samples to populations, grouping of pre-defined populations using fixed K-clustering, for the analysis of admixture between populations, and to estimate gene flow between populations. For clustering of groups of individuals the upper limit to the number of populations was put equal to twenty. For admixture estimates based on pre-defined populations the last population in the dataset was used to define one more population for admixture analysis, and the maximum population size taken into account was set as five. Number of iterations and number of reference individuals from each population were set equal to fifty, with the number of iterations for reference individuals set equal to ten. Fixed K-clustering of pre-defined populations was done for K=2-15, fifteen being equal to the number of sampling sites, and gene flow between populations was calculated using BAPS 5.2 with a p-value set as 0.05 (Corander & Marttinen, 2006; Corander, Waldmann, Marttinen, & Sillanpaa, 2004). Nei et al.'s D_A distance (Nei, Tajima, & Tateno, 1983) and Cavalli-Sforza chord distance (Cavalli-Sforza & Edwards, 1967) between populations were calculated using PowerMarker 3.25 (Liu & Muse, 2005). Bootstrapping was carried out with 1000 replications and the corresponding Neighbor-Joining trees displayed using MEGA 5 (Kumar, Nei, Dudley, & Tamura, 2008).

The possibility of a recent reduction in effective population size was tested using the program BOTTLENECK 1.2.02 (Cornuet & Luikart, 1996). This relies on a method based on excess of observed heterozygosity over expected heterozygosity under three models of microsatellite evolution; the infinite-allele model (IAM), the stepwise mutation model (SMM), and the two-phase model of mutation (TPM; with 70% SMM and 30% variation). A Wilcoxon test was used to test for significant excess in observed heterozygosity over expected heterozygosity ($H_{obs}>H_{eq}$). The expected distribution of H was found based on 1000 iterations.

Multilocus 1.3 (Agapow & Burt, 2001) was used to test the significance of gametic disequilibrium among pairs of loci for *M. larici-populina* by calculating both the Index of Association (I_A) and , a version of I_A modified to remove the dependency on the number of loci (Maynard Smith, Smith, O'Rourke, & Spratt, 1993). Also, genotypic linkage disequilibrium was tested using Genepop on the Web (http://genepop.curtin.edu.au) using the log likelihood ratio statistic (G-test).

Results

Genetic diversity of *M. larici-populina* in Iceland

Four hundred and eighty *M. larici-populina* isolates sampled from 380 trees in fifteen locations were genotyped, of which 41 isolates were eliminated from further analyses because of high number of loci not amplified or evidence of contamination (Table 1 and Figure 1). First, fourteen samples containing more than 40% missing alleles (10 of 25 markers or more) were eliminated from the data set leaving 466 isolates. Secondly, three markers of the 25 markers genotyped were excluded from the analysis because of a high proportion of missing data. The markers omitted were MPL59, MLP94, and MPL96 failing in 85 (12%), 90 (19%), and 100 (23%) samples, respectively. Subsequently sixteen more samples showing ≥25% (6/22) missing loci where omitted. Finally, eleven more samples showing signs of contamination were eliminated leaving 439 samples for further analysis. Of the samples used for analysis, 362 (84%) were genotyped with all markers, 44

(10%) samples failed with one marker, and 33 (6%) samples failed with two to five markers. This leads to different numbers of samples genotyped at each locus, ranging from 439 to 407 samples, the lowest value being for marker MLP92 (Table 2).

Of the 22 loci used for analysis all were polymorphic with the exception of MLP91 (Table 2). Of the total of 122 alleles detected, 46.6% of alleles had a frequency ≤0.05 (data not shown). For polymorphic loci the number of observed alleles ranged from three to ten, with a mean number of 5.55 alleles per locus (Table 2). As expected, considering the high proportion of low frequency alleles, the effective number of alleles (EN_A) was relatively low ranging from 1.04 (marker MLP87) to 2.16 (markers MLP50 and MLP73). The mean genetic diversity (expected heterozygosity, H_E) was 0.38 and the observed heterozygosity (H_O) 0.35. Only marker MLP68 was in Hardy-Weinberg equilibrium (HWE). Among the polymorphic loci, 174 pairs out of 210 were in significant linkage disequilibrium, suggesting sub-structuring and/or the lack of sexual stages in the fungal lifecycle (data not shown).

The number of private alleles was generally low, ranging from zero to fifteen alleles for different sampling locations (Tables 3 and 4). Further, the mean number of alleles, allelic richness, and effective number of alleles were all relatively low. In all cases, the highest values were seen in Lón (LO). Samples at all locations displayed significant LD (Table 5). Looking at each locus in each population, most markers were in HWE (non-significant at $P>0.05$) with only one to four loci per population out of equilibrium (data not shown).

Analysis of molecular variance (AMOVA) based on PhiPT values shows that a low percentage of the total genetic diversity of the 15 populations in this study was distributed on a small spatial scale with 91% of the genetic diversity distributed within populations and only 9% between populations ($P<0.001$). The results of principal coordinate analysis (PCoA) based on genetic distance between individuals suggest that samples could be grouped into two, or possibly three, clusters depending on sampling location (data not shown). PCoA using genetic distance between groups based on sampling location showed that the samples from Lón (LO) are clearly separate from all others, with samples from Gunnfríðarstaðir (GU) also separating from the rest. The remaining thirteen sampling locations cluster together (Figure 1A).

Results of Bayesian analysis of population structure using BAPS 5.2, based on sampling locations, underlines the close clustering of most of the Icelandic sampling sites (Figure 2B). The most likely number of clusters is identified as K=3 with the samples from Gunnfríðarstaðir (GU) and Lón (LO) forming distinct populations. Little admixture between these three sub-populations seem to have taken place, with only thirteen individuals showing evidence for genetic admixture between different clusters (data not shown). Analysis of gene-flow showed the ancestral admixture of each cluster as 98%-100% (data not shown). The genetic distance between samples from different sampling locations was analysed using Nei et al.'s D_A distance and Cavalli-Sforza chord distance, and the results visualized as Neighbor-Joining trees (Figure 2C and data not shown). The two different distances yielded topologically identical (isomorphic) trees supporting the results of the Bayesian analysis of population structure revealing a clustering of Lón (LO) and Gunnfríðarstaðir (GU) on one hand and the rest on the other hand.

Results from the molecular analysis of 474 French *M. larici-populina* samples (Xhaard et al., 2011a, 2011b) were used for comparison to the Icelandic samples. This comparison grouped the samples from Lón (LO) with the French samples while underlining the close clustering of the remaining Icelandic samples into a single group (Figure 3).

Analysis using the program Bottleneck carried out on samples from all fifteen sampling sites as well as the pooled data showed no evidence for excess of observed heterozygosity ($H_{obs}>H_{eq}$) in any of the populations regardless of the model assumed (IAM, SMM or TPM; $P<0.005$). This suggests that the populations analysed here have not recently gone through population bottlenecks (data not shown).

Discussion

Analysis of genetic structure within the Icelandic *M. larici-populina* population identifies three clusters as the most likely scenario. A comparison to French samples underlines a separation into at least two groups, with close ties between the French samples and the samples originating from Lón (LO). The analysis predicts low levels of gene-flow between these sub-populations. Although the comparison between the Icelandic and French samples does underline a portioning of the Icelandic samples into at least two main groups, it cannot be conclusively shown that the Lón (LO) samples originate from France. More samples would be needed from different locations to confirm this. The analysis presented here also shows evidence of a possible bottleneck in the populations from Lón (LO), supporting the possibility of a recent colonization event in the southern part of Iceland, although a larger sample is needed to conclusively demonstrate that.

Analysis of linkage equilibrium suggests that none of the populations studied have fully reached linkage equilibrium. Although it is difficult to identify the cause, it at least suggests that genetic exchange and recombination is not sufficient to break up the observed linkage between makers. Whether this can be explained by sub-structures within sampling locations cannot be demonstrated with the current data set.

For breeding programs aimed at reducing the negative effects of fungal pathogens the evolutionary potential of populations is of prime importance, irrespective of the plant species. For the risk assessment process, fungal populations are considered to have high evolutionary potential when they have a mixed reproduction system, moderate gene or genotype flow, as well as a large effective population size (McDonald & Linde, 2002). The relatively high levels of genetic diversity, considering the recent colonization in Iceland, and the evidence for repeated colonization events, show that the *M. larici-populina* population should be able to respond relatively rapidly to selection exerted by changes in resistance. This underlines the importance of carefully selecting poplar clones for future breeding purposes and not relying on a narrow genetic base in the selection of genetic diversity.

The results presented here underline the importance of careful characterization of pathogen populations prior to initiating resistance breeding projects and underlines the importance of monitoring the genetic diversity of pathogenic populations. A rational next step in the analysis of the diversity of the Icelandic *M. larici-populina* is to analyse whether the diversity measured here with neutral molecular markers translates into functional differences in pathogenicity. Further, it

would be interesting to compare the Icelandic populations to populations from Scandinavia and the British Isles, as these are two plausible points of origin for the Icelandic *M. larici-populina* populations. Knowing the origin could be used to predict the genotypes of future fungal colonization events and possibly to focus future breeding effects towards pathotypes most likely to colonize Iceland in the future.

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Figures

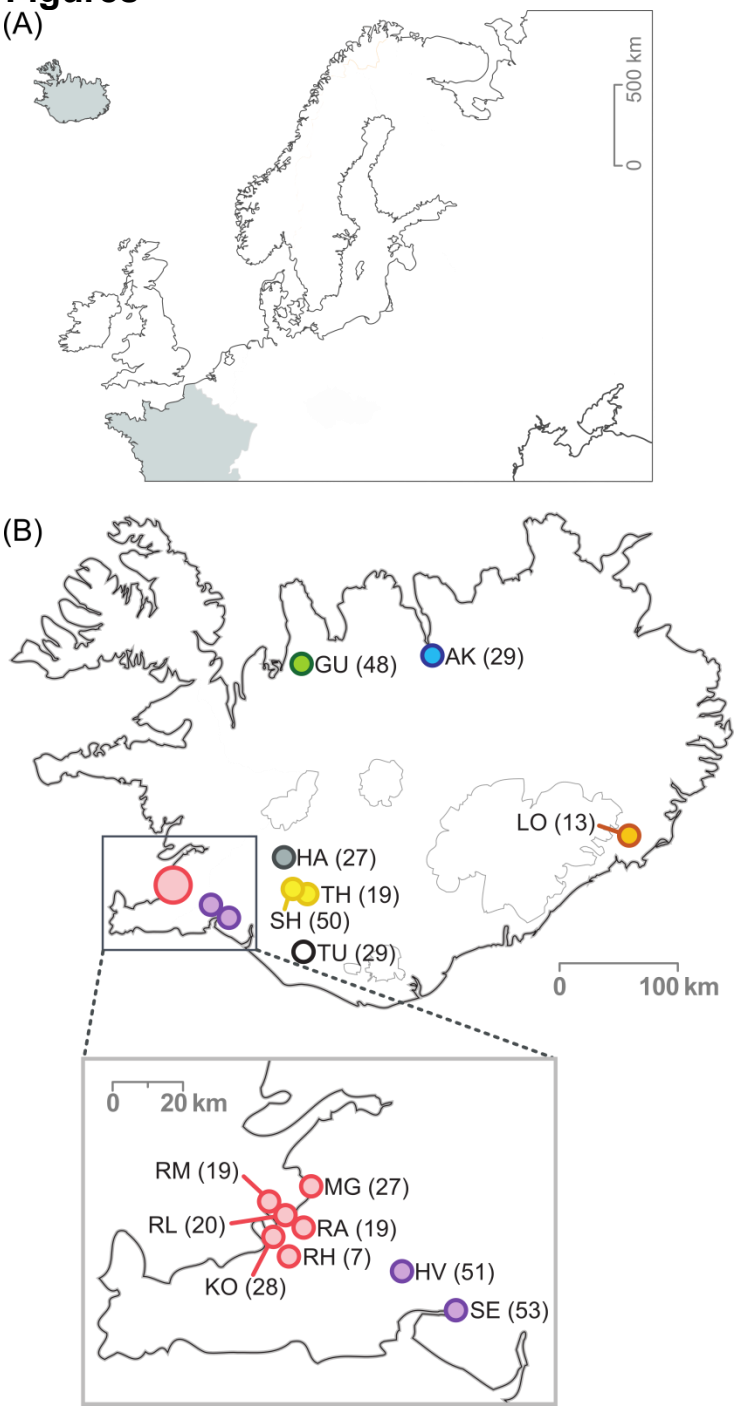

Fig. 1 Locations of *Melampsora larici-populina* sampling sites in Iceland. (A) A regional perspective for sampling sites, with Iceland and France shown in gray. (B) Fifteen sampling locations in Iceland: Akureyri (AK), Gunnfríðarstaðir (GU), Lón (LO), Haukadalur (HA), Skálholt (SH), Thjórsárdalur (TH), Tumastaðir (TU), Hverageröi (HV), Selfoss (SE), and the six sampling sites in the Reykjavík area, Mógilsá (MG), Höfðabakki (RA), Melar (RM), Laugardalur (RL), Kópavogur (KO), and Heiðmörk (RH) (see inset for detailed locations). The number of samples collected at each site are shown in parenthesis.

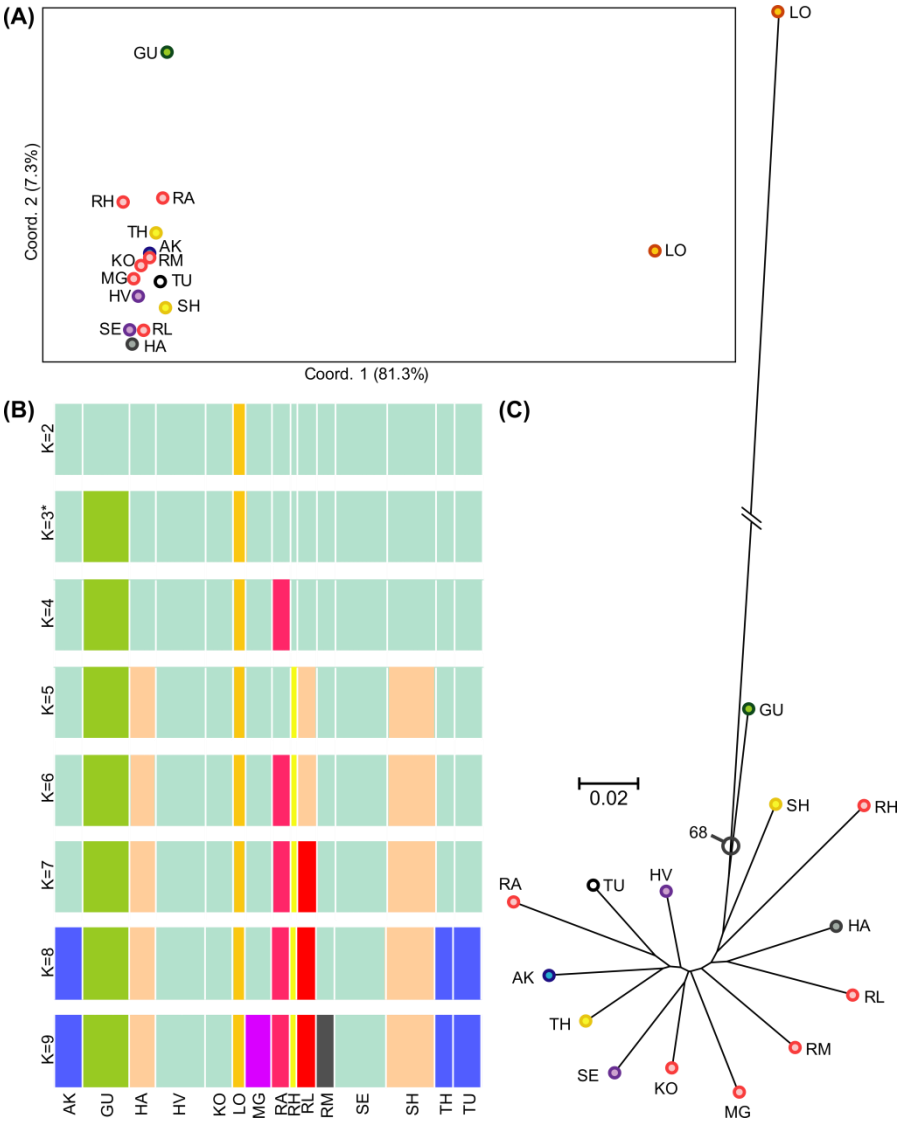

Fig. 2 Analysis of population structure for Icelandic *Melampsora larici-populina* samples from fifteen sampling sites. (A) Principle Coordinates Analysis (PCoA) based on 'Nei's genetic distance' as calculated in GenAlEx 6.4.1, resolving a total of 88.6% of genetic variation on the first two axes. (B) Bayesian analysis of population structure (BAPS) with fixed K-clustering from K=2 to K=9, identified K=3 as the most likely number of clusters (marked with an asterisk). (C) A Neighbor-Joining tree showing Nei's et al. (1994) genetic distance between groups based on sampling location. Colour coding as in (A) with bootstrapping values above 50% shown, based on 1000 replicates. The Lón (LO) branch is broken for clarity.

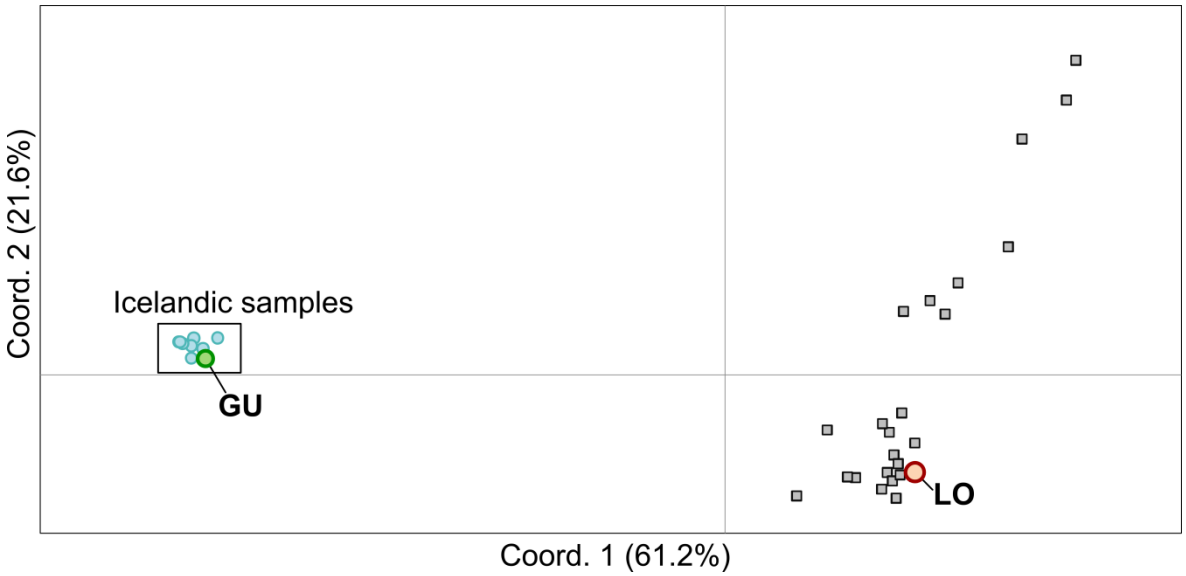

Fig. 3 Comparison of *M. larici-populina* populations from Iceland and France based on genetic distance between groups from different sampling sites. French samples are shown as boxes and Icelandic samples as circles, with samples from Lón (LO) and Gunnfríðarstaðir (GU) labelled specially.

Tables

Table 1 Information on *Melampsora larici-populina* sampling in Iceland

Sampling site	Location	Year of sampling	nt[a]	nu[b]	ni[c]
Akureyri (AK)	65°42'N, 18°09'W	2009	20	30	29
Gunnfríðarstaðir (GU)	65°34'N, 20°04'W	2008	28	28	22
		2009	17	27	26
Haukadalur (HA)	64°19'N, 20°16'W	2008	27	27	27
Hverageröi (HV)	63°59'N, 21°11'W	2008	25	25	23
		2009	30	30	27
Kópavogur (KO)	64°06'N, 21°53'W	2008	15	30	29
Lón (LO)	64°25'N, 14°53'W	2009	11	22	13
Mógilsá (MG)	64°12'N, 21°42'W	2008	33	33	27
Reykjavík Höfðabakki (RA)	64°07'N, 21°48'W	2008	10	20	19
Reykjavík Heiðmörk (RH)	64°03'N, 21°52'W	2008	4	8	7
Reykjavík Laugardalur (RL)	64°08'N, 21°52'W	2008	10	20	20
Reykjavík Melar (RM)	64°08'N, 21°57'W	2008	10	19	19
Selfoss (SE)	63°55'N, 20°59'W	2008	27	27	26
		2009	29	30	27
Skálholt (SK)	64°07'N, 20°31'W	2003	NA	95	94
Skálholt (SH)	64°09'N, 20°32'W	2008	23	23	22
		2009	25	29	28
Thjórsárdalur (TH)	64°06'N, 19°57'W	2008	11	22	19
Tumastaðir (TU)	63°44'N, 20°03'W	2009	25	30	29
TOTAL			380	575	533

[a] number of trees

[b] uredinia tested

[c] isolates genotyped

Table 2 Results of *Melampsora larici-populina* microsatellite analysis for individual microsatellites

Locus	N[a]	ON_A[b]	EN_A[c]	A_r[d]	H_O[e]	H_E[f]
MLP12	430	7	2.07	6.84	0.53	0.52
MLP49	424	9	2.06	8.88	0.49	0.51
MLP50	434	7	2.16	6.81	0.49	0.54
MLP54	439	5	1.07	5.00	0.04	0.06
MLP55	439	5	2.12	5.00	0.57	0.53
MLP56	439	5	2.04	4.93	0.57	0.54
MLP57	438	6	2.02	5.85	0.50	0.54
MLP58	430	5	1.06	5.00	0.04	0.05
MLP66	439	6	2.06	5.85	0.49	0.52
MLP68	416	6	1.10	6.00	0.09	0.09
MLP71	437	5	2.15	4.93	0.46	0.54
MLP73	425	9	2.16	8.95	0.52	0.54
MLP77	409	3	2.08	3.00	0.50	0.52
MLP82	439	5	2.08	5.00	0.51	0.52
MLP83	439	4	2.05	4.00	0.50	0.51
MLP87	433	3	1.04	3.00	0.03	0.04
MLP91	439	1	1.00	1.00	0.00	0.00
MLP92	407	3	1.50	3.00	0.04	0.33
MLP93	439	3	1.05	2.93	0.04	0.05
MLP95	435	10	2.9	9.67	0.48	0.52
MLP97	434	5	1.95	4.94	0.45	0.49
MLP100	436	10	1.59	9.86	0.37	0.37
Mean	431.8 (2.09)	5.55 (0.51)	1.75 (0.10)	5.47	0.35 (0.05)	0.38 (0.05)

[a] number of samples genotypes

[b] number of observed alleles

[c] mean allelic richness

[d] effective number of alleles

[e] observed heterozygosity

[f] expected heterozygosity

Table 3 Results of microsatellite analysis by sampling sites.

Site	N[a]	ON_A[b]	EN_A[c]	NPA[d]	A_r[e]	H_O[f]	H_E[g]	F_{IS}[h]
AK	29	2.14	1.66	1	1.84	0.35	0.35	0.026
GU	48	2.41	1.65	4	1.90	0.30	0.34	0.112
HA	27	2.18	1.66	2	1.85	0.38	0.34	-0.076
HV	51	2.14	1.67	2	1.78	0.35	0.34	-0.003
KO	28	2.05	1.69	2	1.82	0.35	0.36	0.045
LO	13	2.68	2.04	15	2.51	0.42	0.44	0.083
MG	27	2.18	1.70	2	1.86	0.36	0.36	0.020
RA	19	2.05	1.69	2	1.89	0.34	0.36	0.067
RH	7	1.91	1.60	1	1.88	0.36	0.33	-0.025
RL	20	2.05	1.64	2	1.83	0.34	0.34	0.008
RM	19	2.09	1.70	3	1.87	0.34	0.36	0.064
SE	53	2.45	1.69	6	1.84	0.34	0.36	0.046
SH	50	2.73	1.73	4	1.95	0.37	0.36	-0.013
TH	19	1.95	1.66	1	1.77	0.35	0.34	-0.015
TU	29	2.05	1.65	0	1.79	0.35	0.33	-0.043
Average[i]		2.20	1.69		1.89	0.35	0.35	0.000

[a] number of samples analyzed from a given sampling site

[b] average number of observed alleles

[c] effective number of alleles

[d] number of private alleles

[e] mean allelic richness

[f] observed heterozygosity

[g] expected heterozygosity

[h] within-population inbreeding estimate

[i] average based on 439 samples genotyped with 22 markers

Table 4 Private alleles at different sampling sites

	Allele[a]	Freq (%)[b]		Allele[a]	Freq (%)[b]
AK	Mlp57 (169)	1.7	KO	Mlp95 (442)	1.8
GU	Mlp50 (267)	1.0	KO	Mlp54 (144)	3.6
GU	Mlp57 (175)	4.2	MG	Mlp95 (443)	1.9
GU	Mlp82b (184)	2.1	MG	Mlp12 (253)	1.9
GU	Mlp97 (392)	1.1	RA	Mlp95 (459)	2.6
HA	Mlp12 (269)	1.9	RA	Mlp73 (424)	5.3
HA	Mlp58 (255)	3.7	RH	Mlp100 (199)	21.4
HV	Mlp66 (207)	1.0	RL	Mlp49 (379)	2.5
HV	Mlp49 (353)	1.0	RL	Mlp56 (287)	2.5
LO	Mlp83 (148)	19.2	RM	Mlp95 (457)	2.8
LO	Mlp83 (154)	23.1	RM	Mlp12 (235)	5.3
LO	Mlp71 (362)	3.8	RM	Mlp12 (250)	2.6
LO	Mlp95 (450)	12.5	SE	Mlp100 (234)	0.9
LO	Mlp95 (453)	8.3	SE	Mlp50 (274)	1.0
LO	Mlp95 (458)	4.2	SE	Mlp66 (201)	0.9
LO	Mlp54 (132)	23.1	SE	Mlp57 (181)	1.9
LO	Mlp58 252 ()	15.4	SE	Mlp49 (350)	2.0
LO	Mlp49 (347)	10.0	SE	Mlp93 (131)	0.9
LO	Mlp73 (434)	6.3	SH	Mlp50 (289)	1.0
LO	Mlp73 (445)	31.3	SH	Mlp57 (178)	1.0
LO	Mlp82b (181)	26.9	SH	Mlp49 (344)	1.0
LO	Mlp82b (208)	34.6	SH	Mlp73 (427)	2.0
LO	Mlp56 (281)	19.2	TU	Mlp100 (193)	1.7
LO	Mlp87 (341)	19.2			

[a] name of marker with allele size in parenthesis

[b] frequency of each private allele

Table 5 Results for the analysis of index of association for three identified clusters within the Icelandic *M.-l. populina* population

	I_A[a]	[a]
Lón (LO)	0.631**	0.037**
Gunnfríðarstaðir (GU)	0.251**	0.014**
Rest	-0.586**	-0.035**
IS-ALL	**-0.041*** **	**-0.002*** **

*** P≤0.001; ** P≤0.005; * P≤0.01; ns non-significant

[a] index of multilocus gametic disequilibrium

[b] index of multilocus gametic disequilibrium