FPS COST Action FP1202

Strengthening conservation:
a key issue for adaptation of marginal/peripheral populations
of forest trees to climate change in Europe (MaP-FGR)

COMPARISON OF GENETIC VARIABILITY AMONG MAP OF BIRCH IN ITS SOUTHERNMOST BOUNDARY ON THE APPENINE AND BALKAN REGION

Title

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Topic: Comparison of Genetic Variability among MaP of birch in its southernmost boundary on the Appenine and Balkan region

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Introduction

During the last glacial age, tree populations moved to the southernmost latitudes in Europe (Stewart et al., 2010). By the end of glacial age, tree populations started to migrate to higher latitudes, releasing some populations in the rear. These nuclei constitute the Marginal/Peripheral (MaP) populations (Hampe and Petit, 2005).
The persistance of these individuals is threatened by climate change, because they have to cope with longer dry spells and higher temperatures (advance in the timing of the onset of summer drought or increasing winter temperatures). Under these circumstances, these populations have to move northwards and at high elevations to avoid extinction, but species ranges are limited by biotic and abiotic constrains, that can exacerbate the isolation of the MaP populations (Higgins et al., 2003).
Consequently, MaP populations risk to lose genetic diversity and future adaptive potential due to decreased gene flow among populations, reduction of recombination as an effect of partially asexual reproduction, elevated levels of inbreeding, fixation of deleterious alleles, and higher occurrence of genetic drift.

One of the species facing this kind of threat is silver birch (*Betula pendula* Roth). The distribution of silver birch covers almost all Europe from the Mediterranean to Northern Europe where the species is abundant and more continuous. In the western and southern parts of the distribution range, like in the Iberian peninsula, Greece and Italy, its presence is patchy (Theodoropoulos et al., 2003), (Vakkari, 2009).
The genetic pattern of silver birch in the main distribution area is characterized by high levels of adaptively neutral genetic variability within populations and the differentiation among populations is very low (Palmé et al., 2003; Palmé et al., 2004; Maliouchenko et al., 2007; Jadwiszczak, 2012). On the other hand, very little is known about the amount and organization of genetic variation in the southern marginal areas. Area of refugia, as those cited above, are expected to show reduced within-population genetic diversity and, on the other hand, higher genetic differentiation compared with surrounding recolonized regions (Hampe and Petit, 2005).

The general aim of the study is to investigate the patterns of genetic variation in two European areas of refugia for silver birch, in Greece and Italy, looking at how much random genetic drift has decreased within-population variation, searching for increased inbreeding and increased between-population variation. Under the RGV/FAO, a programm funded by the Italian Ministry of Agriculture Food and Forestry Policies (Mipaaf), I have sampled and analyzed seven MaP birch populations in Italy. The STSM aimed to extend the latitudinal and longitudinal sampling gradient, collecting samples in the Southern Balkans and running a first comparison of results.
The presented results are only partial and need further analysis of samples, due to the different protocol and sequencer used in the Italian and Greek laboratories.

Materials and Methods

Site description

1) Greek populations
Two sites were selected as marginal populations in Greece:

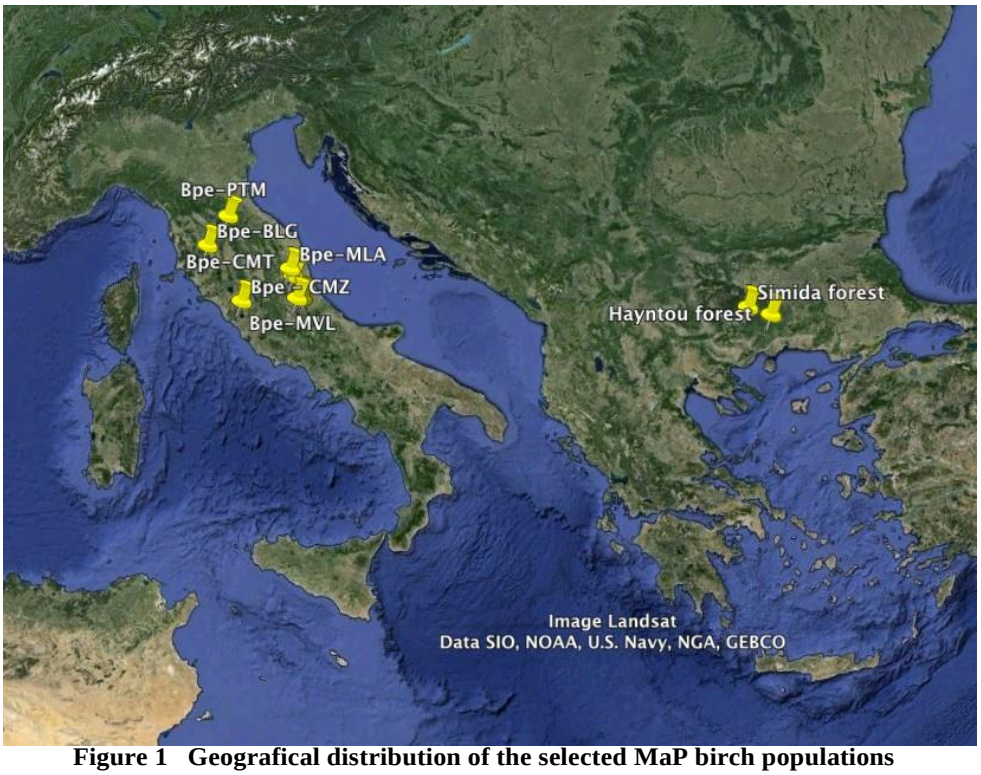

Figure 1 Geografical distribution of the selected MaP birch populations

- The Simida Forest

The main forest cover at the Simida (=birch in Greek) Forest is made up by Scotch pine (*Pinus sylvestris*) and *B. pendula*, together with European black pine (*P. nigra*) and beech (*Fagus syvatica*), sporadic Norway spruce (*Picea abies*). Birch trees vegetate in the dominant layer of this forest, frequently forming vast non-mixed stands. The altitude varies around 1.000 m. Birch trees are vigouros with diameters 18-40 cm and height up to 25 m. Most of the birch individuals originated from seed. The presence of young plants is elevated.

- The Haidou Forest

The Haidou Forest is a beech forest with Scotch pine, birch and Macedonian fir (*Abies × borisii-regis*). Birch trees are scattered in the forest floor, not rarely forming more dense nuclei. Birch also forms stands at altitudinal limit, in the remnant of pastures which have been used for hundreds of years by the nomad animal farmers, where it competes with Scotch pine. The mean altitude is about 1000 m.

2) Italian populations

Birch populations in Italy are more scattered than in Greece. They are located on the Appenine and usually cover small surface. They can be found in mixed forests with beech, chestnut and Turkey oak (*Quercus cerris*) or as pioner species at the altitudinal limit on screes. A peculiar site is Caldara di Manziana (CMZ) where a pure birch stand vegetates close to a warm water spring rich on sulphuric emissions. Further, BLG stand is also distinctive for its location on a peat bog at low elevation.

The geographic distribution and main characteristics of the sampled populations are shown in figure 1 and table 1.

Table 1 Geographic location of *Betula pendula* populations of this study

Population name	Label	Coord N	Coord E	alt (m)	collected genotypes
Pratomagno	PTM	43.6116	11.71139	1070	27
Riserva Naturale di Stato del Belagaio	BLG	43.07906	11.19843	460	23
Monti della Laga - Amatrice	MLA	42.67171	13.31173	1330	5
Lago di Campotosto	CMT	42.53909	13.39772	1380	10
Monte Sirente	MSR	42.15613	13.6205	1500	31
Monte Velino	MVL	42.14387	13.4907	1550	25
Caldara di Manziana	CMZ	42.0893	12.09738	260	26
Simida Forest	ELS	41.47387	24.15583	1000	30
Haidou Forest	ELH	41.29319	24.66515	950	20

Field sampling consisted in the collection of leaf or floral/leaf buds, kept in fresh environment till the time of DNA extraction. Plants were spatialized by about 50 m.

DNA extraction

DNA was extracted by manually pulverizing samples with liquid nitrogen in a mortar. The Greek samples were then treated according to the CTAB method. The DNA amount was quantified by a UV spectrophotometer. Samples were then diluted to a 20 ng μl^{-1} working solution.
The primers were selected based on the paper by (Kulju et al., 2004). The selected primers are listed in Table 2.

Table 2 List of primers used in this study

accession number	primer sequences 5-3'	locus	bp
AF310856 F	ACGCTTTCTTGATGTCAGCC	L1.10*	168-209
AF310856 R	TCACCAAGTTCCTGGTGGAT		
AF310846 F	AGACCATGCCTGGGCCTT	L2.2	132-155
AF310846 R	CGCAACAAAACACGATGAGA		
AF310851 F	CTCCTTAGCTGGCACGGAC	L3.1	219-241
AF310851 R	CCCTTCTTCATAAAACCCTCAA		
AF310858 F	TTGAGATAGACGATAGAGGTAAAGCA	L4.4	261-295
AF310858 R	AGGCATTTCTCCAATTTTCTT		
AF310862 F	AAGGGCACCTGCAGATTAGA	L5.4	230-262
AF310862 R	AAAATTGCAACAAAACGTGC		
AF310863 F	GAGGAAGTCTCAGCTGACGTG	L5.5	121-146
AF310863 R	TCCTTTTCAGTTTTCTGATTTCTG		
AF310854 F	GTTTTGGGTTTCCACTTCCA	L7.1a	146-152
AF310854 R	ACTGGTAATACCTTTACCAAGCC		
AF310864 F	GGGGATCCAGTAAGCGGTAT	L7.3	178-226
AF310864 R	CACACGAGAGATAGAGTAACGGAA		
AF310866 F	GGCCAACAGATATAAAACGACG	L7.8	295-307
AF310866 R	TTTTAAATGCCCACCTTCCC		

Polymerase chain reaction (PCR) amplifications were conducted on the extracted DNA in multiplex, according the Table 3.

Table 3 Combination of primers and dyes

	MIX1	MIX2	MIX3
red	AF310854-PET		AF310863-PET
green	AF310866-6-FAM	AF310846-VIC	AF310864-VIC
blu		AF310856-6-FAM	AF310858-6-FAM
yellow	AF310851-NED	AF310862-NED	

- Comparison of the genetic diversity in the populations studied

Standard genetic diversity parameters for the different populations were calculated, as the average number of allele per locus and the observed frequency of heterozygous (Ho) samples averaged over loci, that can provide evidence of inbreeding.

Within-population diversity was estimated by the expected heterozygosity (He), which is the frequency of heterozygous individuals expected under Hardy-Weinberg equilibrium. Since populations in natural environment usually do not meet the conditions required by the Hardy-Weinberg equilibrium, their allele frequencies change from one generation to the next. The comparison of observed versus expected outcomes offers an estimation of how far the population deviates from Hardy-Weinberg equilibrium. The F_{IS} measures the deviations of genotype frequencies from Hardy-Weinberg proportions in the subpopulation, and F_{ST} measures the degree of genetic differentiation among populations. F_{IS} and F_{ST} range from zero to one with F_{IS} that may become negative in the case of an excess of heterozygotes, while F_{ST} is always positive and values close to one indicate a great genetic differentiation. The PhiPT approach was used to partitioning the variation into within- population and among-population components. PhiPT is a F_{ST} analogue that is calculated as the proportion of the variance among populations, relative to the total variance; in other words it represents the correlation between individuals within a population, relative to the total.

Causes of differentiation are random drift, selection, founder effects, and bottleneck, opposed to high migration rate between populations which can prevent or slow down differentiation. Thus, the partitioning of genetic variation within or between populations can provide insights into the populations' dynamic.

Measurements of genetic distance and multivariate analysis of genetic data (PCoA) was run.

At this stage all data analyses were performed by GeneAlEx 6.502 (Peakall and Smouse, 2006).

Results

In this paragraph some preliminary results are reported. As mentioned before, during the STMS in Thessalloki, I was able to use protocols and sequener for analyses different from those I used for analyzing Italian samples, which caused a not straight interpretation of results so far.

All the primers amplified polymorphic products.

For SSR data, a total of 144 alleles were observed across the 9 loci studied. The mean number of alleles per loci ranged between 4 and 8.

The frequency of private alleles was extremely low, ranging from 1 to null values.

Departure from the Hardy–Weinberg equilibrium was not particularly evident, as demonstrated by the low Fixation Index (F), very close to zero, expected under random mating.

Table 4 Mean allelic patterns across populations (value±*s.e.*). Na = Number of different alleles; Na (Freq >= 5%) = number of different alleles with a frequency >= 5%; Ne = number of effective alleles; I = Shannon's information Index; No. Private Alleles = number of alleles unique to a single population; No. LComm Alleles (<=25%) = number of locally common alleles (Freq. >= 5%) found in 25% or fewer populations; No. LComm Alleles (<=50%) = number of locally common alleles (Freq. >= 5%) found in 50% or fewer Populations; Ho = Observed Heterozygosity; He = expected heterozygosity; F = Fixation Index = (He - Ho) / He = 1 - (Ho / He)

Population	BLG	CMT	CMZ	MLA	MSR	MVL	PTM	ELS	ELH
N	22.444	9.556	25.889	4	28.444	23.111	24.667	3.889	4.667
	0.242	*0.242*	*0.111*	*0*	*0.689*	*0.484*	*0.687*	*0.309*	*0.167*
Na	7.333	7.778	5.556	3.778	7.222	4.778	7.333	4.333	4.333
	1.054	*0.954*	*0.973*	*0.324*	*0.940*	*0.969*	*1.179*	*0.441*	*0.289*
Na Freq. >= 5%	5.222	7.778	4.222	3.778	5.222	3.222	4.444	4.333	4.333
	0.494	*0.954*	*0.795*	*0.324*	*0.465*	*0.521*	*0.475*	*0.441*	*0.289*
Ne	4.456	5.158	3.441	2.963	4.345	2.937	4.605	3.787	3.672
	0.768	*0.803*	*0.714*	*0.248*	*0.673*	*0.478*	*0.667*	*0.451*	*0.344*
I	1.585	1.721	1.256	1.166	1.571	1.108	1.586	1.341	1.342
	0.163	*0.166*	*0.183*	*0.081*	*0.147*	*0.192*	*0.156*	*0.114*	*0.082*
No. Private Alleles	0.889	0.444	0.333	0.222	0.889	0.000	1.000	1.000	0.889
	0.455	*0.242*	*0.167*	*0.222*	*0.351*	*0.000*	*0.289*	*0.236*	*0.261*
No. LComm Alleles (<=25%)	1.333	1.889	1.111	0.333	1.444	1.000	1.333	1.556	1.444
	0.373	*0.455*	*0.389*	*0.167*	*0.475*	*0.333*	*0.527*	*0.294*	*0.338*
No. LComm Alleles (<=50%)	3.111	4.444	2.889	1.444	3.000	2.222	2.889	2.222	2.333
	0.633	*1.069*	*0.873*	*0.294*	*0.799*	*0.778*	*0.949*	*0.324*	*0.236*
Ho	0.631	0.701	0.541	0.694	0.684	0.573	0.773	0.696	0.678
	0.068	*0.059*	*0.053*	*0.069*	*0.058*	*0.093*	*0.028*	*0.102*	*0.12*
He	0.728	0.802	0.635	0.738	0.736	0.588	0.760	0.811	0.793
	0.050	*0.043*	*0.060*	*0.029*	*0.042*	*0.080*	*0.036*	*0.044*	*0.032*
F	0.086	0.076	0.107	-0.08	0.04	-0.023	-0.055	-0.004	0.016
	0.095	*0.067*	*0.077*	*0.103*	*0.075*	*0.101*	*0.056*	*0.153*	*0.18*

Table 5 Analysis of Molecular Variance (AMOVA) based on G-Statistics by locus

	L1.10*	L2.2	L3.1	L4.4	L5.4	L5.5	L7.1a	L7.3	L7.8	**Tot**
Fis	-0.083	-0.189	-0.145	-0.095	-0.047	0.022	0.580	0.146	-0.040	0.035
Fst	0.244	0.269	0.205	0.298	0.152	0.206	0.142	0.122	0.124	0.194
Probabilities for G-Statistics by Locus										
Fis	0.999	1.000	1.000	1.000	0.964	1.000	0.132	0.962	0.942	1.000
Fst	0.001	0.001	0.001	0.001	0.001	0.001	0.009	0.001	0.005	0.001

The variance among population relative to the total variance was significant (PhiPT= 0.25; P<0.001) and indicated a moderate genetic diversity among populations. Most of the genetic variability was attributable to differences among individuals within the same population: 75% of the diversity was expressed within population, while 25% of the variation was found among populations.

Table 6 Analysis of molecular variance (AMOVA) calculated using PhiPT

Source	df	SS	MS	Est. Var.	%
Among Pops	8	136.007	17.001	0.879	25%
Within Pops	145	380.519	2.624	2.624	75%
Total	153	516.526		3.504	100%

Stat	Value	P(rand >= data)
PhiPT	0.251	0.001

Greek and Italians populations showed an evident separation. In Italy, the population from the sulphuric spring (CMZ) is far from the others. The three central populations in the Lazio-Abruzzo regions (MVL-MLA-MSR) are close to each others, except the one from Campotosto (CMT). Tuscan populations (PTM and BLG) are close to each other.
Greek populations formed a unique group.

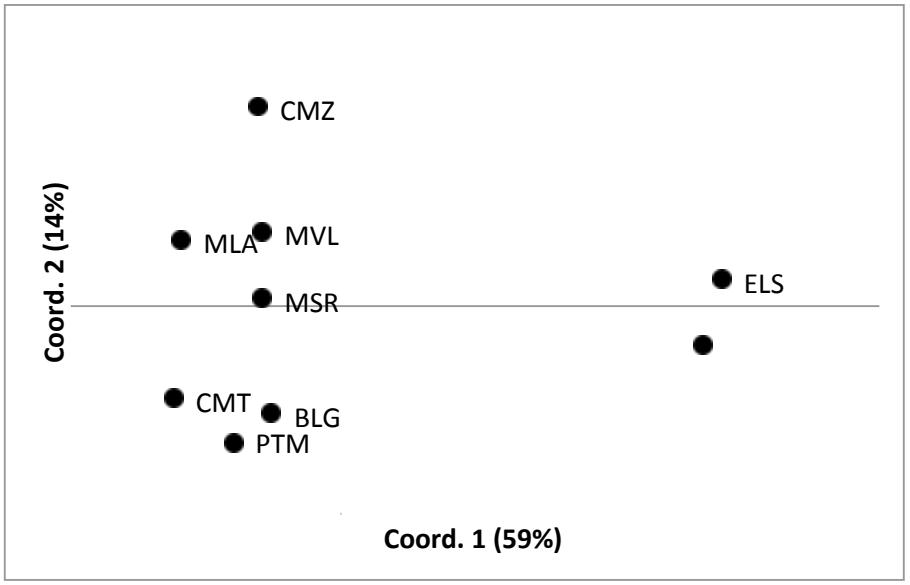

Figure 2 PCoA of the first and second principal coordinates based on the distances calculated from allelic data

This study served as base case for submitting a poster to the IUFRO conference "Genomics and forest tree genetics", that will be held in June 2016, with the following title "Population genetics of *Betula pendula* Roth marginal populations in their Southern European limits", by de Dato G, Teani A, Mattioni C, Monteverdi MC, Avramidou E, Ganopoulos I, Malliarou E, Villani F, Aravanopoulos F, Ducci F. It has been accepted. Further investigation will be run soon, adding missing samples, conforming methods and using more advanced softwares (STRUCTURE, GENEPOP, Arlequin, etc), in order to have more reliable results and data interpretation.
The analyses of the genetic structure of these populations can point to find the existance of different or low variable gene pools and consequently can help in determining in the long run if structural trait diversity can be related to an environmental complexity (soil, nutrients, water availability, radiation, temperature variations between sites), rather than to a genetic differentiation. Results will also point out to the potential peculiarities of marginal birch populations compared to core population diversity (available from the literature).

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