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Addressing Biodiversity Conservation Methods with *Fagus sylvatica* Genetic Indicators

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Abstract

Species biological history revealed by genetic indicators can provide guidelines for long-term biodiversity conservation in Natura 2000 network. *Fagus sylvatica* is the keystone species which regulates in the Mediterranean Eco-Region ecosystem structure, function and composition. Six hundred fifty nine *F. sylvatica* individuals have been sampled across 20 sites of European interest in Southern Italy and analyzed at 5 microsatellite loci. For sites marked by both maximum heterozygosity (*Ho*) and minimum heterozygote deficit (*Fis*) (IT9210210, ITA070099, IT9210205 and IT9220075) it is suggested to avoid impacts by adopting very conservative measures. Promoting migration processes (pollen flow and seed flow) would be appropriate where it has been monitored low heterozygosity and high genetic disequilibrium. Margin effect due to dryness should be buffered with appropriate belts of thermophilus broad leaved tree species.

Keywords

***Fagus sylvatica*, Genetic Diversity, Indicator, Natura 2000**

1. Introduction

Natural, semi-natural habitats and species of interest under the E.U. directives (Habitats and Birds) should be protected either in the European Special Areas of Conservation (SAC) or Special Protection Areas (SPA), both integrated in the living space of Natura 2000 network [1] [2].

The montane belt of the Mediterranean Eco-Region is targeted by several Natura 2000 "sites", each bearing woods dominated by *Fagus sylvatica* L. (European beech). Beech is the keystone long-living species which

regulates ecosystem structure, function and composition [3] [4]. The favourable conservation status of each protected site is a pre-requisite to maintain the ecologic and economic benefits. Thus, the regional-based implementation of conservation measures, biodiversity monitoring and governance are mandatory [1].

F. sylvatica sub-population genetic integrity in terms of composition (allelic richness and effective population size), structure (genetic diversity and genealogical structure) and functions (fitness and gene flow) [5] is necessary to gain the whole set of associated ecosystem benefits (e.g. water capture, soil protection, flora and fauna conservation) [6]. Complex relationships among living organisms [7] are linked to the genetic integrity of the dominant species (*F. sylvatica* in this case study) [4]. Mature beech wood, assessed as number of effectives (*Ne*), guarantees sufficient environmental humidity, dead wood, efficient seed production and pollination, richness of invertebrates (e.g. insects), vertebrates (e.g. amphibians), focal likens, bryophytes and fungi, food and nesting opportunity for birds, focal flora and resilience to environmental change [4].

It is suggested that it would be prudent to protect, above all, the keystone long-living species (*F. sylvatica*) because it plays its major role in shaping the optimal ecological context [8]. After protecting and preventing impacts on beech wood, planning different species-specific protection measures would be realistic and, probably, effective.

Species communities focal to beech woods, in the southern-most latitude, are isolated on the mountain slopes or on high plains. Population fragmentation and geographic isolation on the mountain peaks will increase in the future because of global warming. Trends forecast tree migration of about 500 - 600 km northward by an increase of 2˚C - 3˚C as well as migration on mountain tops [9]-[11]. *F. sylvatica* shows an efficient pollen-flow (wind pollinated) and very limited seed dispersal [12].

In South Italy the genetic structure of the native *F. sylvatica* is linked to the glacial niches and appears finely regulated by both the post-glacial migration processes across the landscape and site-specific impacts [8].

Beech *in situ* conservation is necessary to maintain or increase the species fitness in the coming centuries. Heterozygosis which is linked to plant resilience and adaptation can be maintained by applying conservation biology methods [3] [13].

Pedologic substrate and ground flora are currently used to differentiate beech forests: at high altitude on clay soils the herbaceous indicator is *Asyneuma trichocalicinum*; on brown soil is *Melica uniflora* and *Pulmonaria vallarsiae* and, on acid humus are *Luzula* and *Milium effusum* [14]. Phytosociological patterns being strictly related to relatively short-term environmental changes and even more to the complex biological knowledge of the experts [15] can be hardly associated to *F. sylvatica* genetic diversity which is the outcome of evolution.

Thus, genetic indicators of the species biological history would be appropriate to safeguard *in situ* sub-populations of long-living species [16].

Genetic diversity distribution at a regional spatial scale can easily be assessed with microsatellite loci (nuclear and cytoplasmic). The interpretation of basic genetic indicators, such as allelic richness, heterozygosis and deviation from genetic equilibrium, can consistently improve conservation guidelines.

In this study, genetic diversity at five nuclear and two chloroplastic micro-satellite loci has been assessed in different *F. sylvatica* sub-populations targeting 20 areas of European interest (South Italy) with the goal to fix a hierarchically first level management criterion for homogeneous habitats.

2. Material and Method

2.1. Sampling Design

F. sylvatica has been sampled across 20 sites scattered over the mountains of the Lucanian Apennine, Cilento, including Etna, Vulture and Foresta Umbra (**Table 1**). Leaves or buds for DNA isolation were harvested from aged beech individuals sampled spaced of 150 - 200 linear meters to avoid the sampling of close relatives. The whole population is represented by 659 individuals and each sub-population average census size is *No.* = 34. Each individual plant has been in field geo-referenced and for each site, soil type, exposition, altitude, site name, forest composition and structure were recorded.

2.2. Molecular Analyses

Total genomic DNA was purified using the Trans-Prep chemistry and ABI PRISM 6100 Nucleic-Acid prep Station (Applied Biosystems). DNA concentration and quality was assessed either by gel electrophoresis or spectrophotometer. Five microsatellite loci (three nuclear and two chloroplastic), were analyzed (**Table 2**). Three out of 5 nuclear microsatellites, (FS1-03, FS3-04 and FS4-46) are mapped markers located on chromosomes LG1-F,

Table 1. Natura 2000 sites sampled to assess *Fagus sylvatica* genetic indicators.

E.U. Code	Mount	Site-specific "Standard Data Forms" can be accessed on:
IT9210210	Vulture	http://natura2000.eea.europa.eu/natura2000/SDF.aspx?site=IT9210210
ITA070099	Etna	http://natura2000.eea.europa.eu/natura2000/SDF.aspx?site=ITA070009
IT9210205	Volturino (Raimondo)	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210205
IT9220075	Lago Duglia C. T.	http://www.natura2000basilicata.it/it9220075-lago-duglia-casino-toscano-e-piana-di-s-francesco
IT9210190	Paratiello	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210190
IT8050030	Gelbison	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT8050030
IT9210110	Faggeta Moliterno	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210110
IT9210195	Raparo	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210195
IT9210215	Li Foi	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210215
IT8050046	Cervati	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT8050046
IT9210200	Sirino	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210200
IT9210240	Serra Calvello	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210240
IT9210115	Pierfaone	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210115
IT9210170	Caldarosa	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210170
IT8050053	Alburni	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT8050053
IT9210180	Madonna Viggiano	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210180
IT8050034	Maddalena	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT8050034
IT9210165	Alpi	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210165
IT9210185	La Spina	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9210185
IT9110004	Foresta Umbra	http://natura2000.eea.europa.eu/Natura2000/SDF.aspx?site=IT9110004

Table 2. *Fagus sylvatica* microsatellite loci, amplification primers, allele types and reference.

Locus	Primer 5'-3'	Alleles MW range (bp)	Alleles (No)	Ref.
FS4-46	GCAGTCCTCCACCATTACTATACAACAGCAGGCTATCCAT	197 - 400	14	[18]
FS1-03	Ned-CACAGCTTGACACATTCCAACT GGTAAAGCACTTTTTCCCACT	96 - 136	22	[18]
FS115	Vic-TCAAACCCAGTAAAATTTCTCAGC CTCAATGAACTCAAAAAC	107 - 139	18	[18]
FS3-04	Fam-AGATGCACCACTTCAAATTCTCTCCTCAGCAACATACCTC	209 - 220	5	[18]
MFC5	Fam-ACTGGGACAAAAAAACAAAAGAAGGACCAAGGCACATAAA	281 - 386	30	[19]
Cmcs3	Fam-AGAGTAAGGTTTTATTAGTATAGACTCGATAGTATTTGTCGAT	181 - 182 - 183	3	[20]
Cmcs12	Vic-ATATTGGTAAAACGGCAACTTTTATGGCATGAAAACAACTC	246 - 247 - 248	3	[20]

LG3-M e LG11-M respectively [17]. To carry out capillary electrophoresis the forward microsatellite primer was labelled in 5' position with different dyes (6-Fam, Vic and Ned). At 5' end of the reverse primer a tail bearing the GTGTCTT sequence was added in order to reduce the plus-A effect. PCR reactions for nuclear loci were carried out in 25 ul, containing 20 - 30 ng of target DNA, buffer 1X (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 2 mM of MgCl2, 0.2 mM of dNTPs (Invitrogen), 0.4 uM of each primer (Applied Biosystem), 1 U of Taq DNA polymerase (Invitrogen). Thermocycler was programmed as it follows: 5 minutes of DNA denaturation (95˚C, 30 cycles at 1 minute of DNA denaturation (95˚C), 1 minute of annealing at 60˚C, 1 minute of extension (72˚C) with a final extension of 8 minutes (72˚C). The DNA at the two chloroplast loci (Cmcs3 and Cmcs12) was amplified with little modifications (0.2 uM of each primer, 35 cycles, annealing temperatures at 52˚C and a final extension for 5 minutes at 72˚C) of the original protocol [24]. Capillary electrophoresis has been achieved using the 3130 sequencer (Applied Biosystems) adopting the standard running conditions: 1 - 1.5 ul of PCR products were diluted in 10 ul of formamide; DNA denaturation occurred at 95˚C for 3 - 5 minutes, cooled on ice for 5 - 10 minutes and than loaded on microplates before starting the run. With the software Gene-Mapper 3.7 (Applied

Biosystems) each peak has been converted to molecular weight by applying the options microsatellite default full range and local Southern.

2.3. Statistical Analyses

Data analysis adopted a Bayesian approach [21]. Expected heterozygosity (*He*), observed heterozygosity (*Ho*), fixation index (*Fis*) and allelic richness (*A*) were computed using Genetic Data Analysis software [22]. Allelic richness has been also standardized using the rarefaction method [23]. The ranking of sub-populations by the increasing F_{is} allowed the identification of beech stands close to the Hardy-Weinberg equilibrium.

3. Results

The average number of alleles per locus is $A = 9.7$ (**Table 3**). The number of nuclear alleles per site is evenly distributed while the chloroplast alleles are more localized. On average, the observed heterozygosis is lower than the expected ($H_o = 0.56$ vs $H_e = 0.78$) and the heterozygote deficit measured by the fixation index is $F_{is} = 0.28$ (**Table 3**).

After ranking Natura 2000 sites by the increasing F_{is} it has been possible to identify three groups of sites. The first including beech stands close to the Hardy-Weinberg equilibrium (Vulture, Etna, Volturino, Lago Duglia with low fixation index); the second with intermediate fixation index (Paratiello, Gelbison, Moliterno, Raparo, Li Foy, Cervati, Sirino, S. Calvello, Pierfaone, Caldarosa) and the third group with high fixation index bearing sub-populations in strong disequilibrium (Alburni, M. Viggiano, Maddalena, Alpi, La Spina, Foresta Umbra). Overall, as expected, sites with higher observed heterozygosity revealed a lower fixation index and vice-versa ($r = -0.91$ $P < 0.05$) (**Table 3** and **Figure 1**).

Chloroplastic markers detected height *F. sylvatica* haplotypes across the whole set of sites. The maximum of six different haplotypes is on Etna volcano; it follows Cervati (four haplotypes) and Vulture volcano (three haplotypes) (**Table 3**). The latter three sites, because of maternal marker richness are likely the most close to the pre-glacial beech locations (**Table 3**). Cervati should be the beech wood close to a glacial niche because of its richness of maternal haplotypes although as consequence of historical impacts the last old growth generation has a higher *Fis*. Sites with just one haplotype indicate recent colonization.

4. Discussion

Heterozygote deficit at microsatellite loci (F_{is}) is an indirect estimate of the deviation from equilibrium due to non-random mating. The average fixation index is higher (*Fis* = 0.28) than what reported using isozymes for the European assessed beech population (*Fis* = 0.115) [24], or with microsatellites for the Italian (*Fis* = 0.117) [25], or European populations (*Fis* = 0.192) [26].

Several sub-populations (group two and three) with high heterozygote deficit affect the average *Fis* average value. High *Fis* values are generated by the pollination among close relatives very likely in sub-populations with low *Ne* (**Figure 2**), often reproductively isolated by distance (or phenology). Natural (e.g. valleys) and human generated (e.g. coppicing, clear-cutting and land use) ecological fragmentation has contributed to geographic isolation and, in parallel, to the reduction in sizes of the effective number of trees (data not shown). It is currently argued that, even if the demographic census size is high, a dense tree composition of each forest could perform as a sort of barrier for pollen migration. Nonetheless this hypothesis has never been validated with data. In addition, forest composition with different tree species (mixed woods) or pure stands with heterogeneous patterns can favour pollination efficiency. Etna, Vulture, Lago Duglia and Paratiello (high *Ho* and low *Fis*) have all beech stands irregularly patterned and often (Etna and Vulture) mixed with different perennial species (data not shown).

Etna and Vulture are challenging sites from the conservation point of view because, despite their geographic isolation and low demographic densities, they show high heterozygosity and genetic equilibrium.

From the analysis of the genetic indicators in this paper it is inferred that:

1) High allelic richness for chloroplast haplotypes demonstrates either proximity of the actual populations (Etna and Vulture) to the pre-glacial sites and/or appropriate *Ne*.

2) Natural selection in these sites acted a pressure over a long time-scale (interglacial).

3) The actual phenotypes express highest fitness to present and future ecological conditions.

Table 3. Genetic diversity of *Fagus sylvatica* sub-populations measured with the following indicators: *Ho*, *He*, *Fis*, *A* and chloroplast haplotype number. Sites are sorted by *Fis.*

E.U. Code	Mount	Type of beech wood	Geology	Altimetry (*m* a.s.l.)	Sample size (*n.*)	Expected heterozygosity (*He*)	Observed heterozygosity (*Ho*)	Fixation index (*Fis*)	Alleles/locus (*A*)	Std alleles/locus (*A* [6])	Chloroplastic Haplotype (*n.*)
IT9210210	Vulture	Mixed with *Q. cerris* > 10%; *Abies* present	Volcanic	590 -1100	16	0.727	0.675	0.072	7.8	3.01	H1(12); H2(3); H4(1)
ITA070099	Etna	Mixed with *Castanea sativa*, *Pinus nigra* and *Genista aetnensis*	Volcanic	> 1500	11	0.747	0.675	0.096	5.4	2.94	H2(1); H3(2); H6(4); H7(2); H8(2)
IT9210205	Volturino (Raimondo)	Dominant with termophile shrubs present and rare *Acer* spp.	Siliceous-carbonate	1046 - 1290	18	0.803	0.700	0.128	9.6	3.11	H1(18)
IT9220075	Lago Duglia C. T.	*Abies alba* < 10%	Carbonate-siliceus	1070-1680	18	0.788	0.673	0.147	9.8	3.20	H3(18)
IT9210190	Paratiello;	Mixed with *Q. cerris* < 10%	Carbonate	890 - 1250	46	0.807	0.630	0.219	10.4	3.40	H1(23); H2(24)
IT8050030	Gelbison;	Dominant with several broadleaf spp.	Arenaceus-carbonate	1250-1693	16	0.785	0.525	0.223	8.4	3.38	H2(13); H4(3)
IT9210110	Faggeta Moliterno	Dominant with presence of *Ilex aquifolium*, *Corylus avellanae*, *Carpinus* spp and *Cornus mas*	Carbonate	> 900	25	0.751	0.56	0.254	8.8	3.03	H2(2); H4(23)
IT9210195	Raparo	Pure stand	Carbonate	1320- 1600	18	0.767	0.567	0.261	8.2	3.11	H2(18)
IT9210215	Li Foi	Pure stands + patches of *Q. cerris* and *I. aquifolium*	Carbonate-siliceus	1055 -1314	44	0.805	0.593	0.263	10.6	3.33	H2(44)
IT8050046	Cervati	Dominant with *Acer* spp. plus *C. sativa*	Carbonate	1335 - 1795	46	0.782	0.574	0.266	11.4	3.18	H1(5); H2(1); H3(7); H5(33)
IT9210200	Sirino	Pure stand	Carbonate	1279 - 1650	11	0.697	0.509	0.270	4.8	2.59	H2(11)
IT9210240	Serra Calvello	Dominant with *Q. cerris* < 10%; *A. alba* present.	Carbonate	1159 - 1390	114	0.795	0.578	0.273	13.2	3.20	H1(114)
IT9210115	Pierfaone	Dominant with *Q. cerris* < 10%; *A. alba* present.	Carbonate-siliceus	1288 - 1570	60	0.783	0.565	0.278	11.2	3.21	H1(60)
IT9210170	Caldarosa	Dominant with *Q. cerris* < 10%; *A. alba* present.	Siliceus-arenaceus	1278 - 1447	43	0.828	0.591	0.286	11.8	3.37	H2(43)
IT8050053	Alburni	Pure stand	Carbonate	980 - 1500	47	0.782	0.536	0.315	11.4	3.20	H1(10); H2(37)
IT9210180	Madonna Viggiano	Pure stand	Arenaceus-carbonate	1250 - 1727	53	0.812	0.551	0.321	12	3.34	H1(36); H2(17)
IT8050034	Maddalena	Dominant with *Populus tremula*, *C. sativa* and *C. avellana*	Carbonate	1000 - 1310	23	0.784	0.461	0.361	8.2	3.21	H1(23)
IT9210165	Alpi	Pure stand	Carbonate	1030 - 1615	17	0.743	0.462	0.377	6.2	2.93	H1(5); H2(12)

Continued

IT9210185	La Spina	Dominant with P. nigra plus Alnus, Fraxinus and termophilus shrubs	Carbonate	930 - 1395	23	0.794	0.470	0.408	8.0	3.16	H1(2); H2(21)
IT9110004	Foresta Umbra	Dominant with *I. aquifolium* and *Hedera elix*	Carbonate	600 - 870	21	0.805	0.476	0.409	9.4	3.17	H2 (21)
		Average			34	0.784	0.564	0.279	9.7	-	

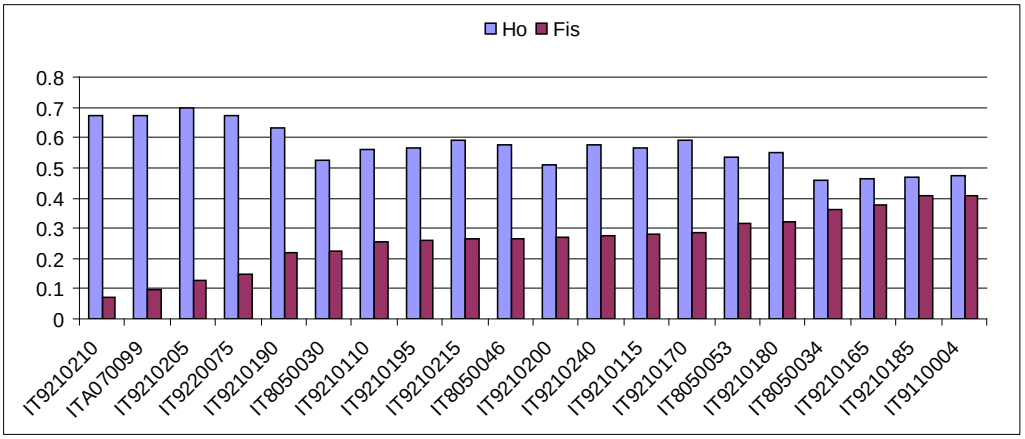

Figure 1. Relationships between *Ho* and *Fis* for each sub-population of *Fagus sylvatica* labeled with its E.U. code.

On the opposite extreme is Foresta Umbra which shows low heterozygosity and the highest *Fis*. Here, only the most common cytoplasmatic haplotype is present; geographic isolation is evident given that the site is outside the Apennine corridor and *Ne* is lower because of maternal common ancestry.

5. Management Perspective

Forest genetic diversity is one of the most important resources to be transmitted to the next human generation. Especially beech genetic diversity across the Mediterranean zone should be considered strategic for ecosystems, landscape quality and human services (e.g. mountains as "water towers") [27] [28]. An appropriate *in situ* conservation of *F. sylvatica*—at least in Natura 2000 sites—by applying the basics of conservation biology rather than usual silviculture (**Figure 3**) is crucial for habitats and ecosystems [3] [13]. The most hierarchically appropriate conservation action would be the preservation of *Ne* being able to generate the fittest next generation from seeds within each beech wood. Shortly, it follows the management guideline based on both *Ho* and *Fis* indicators: where *Ho* is maximum and *Fis* minimum (Vulture, Cervati, Paratiello, Volturino e Lago Duglia), it is necessary to avoid impacts by adopting very conservative measures. It is good to consolidate the historical management by maintaining and increasing the actual effective population size. Wind protecting tree layers on the south, south-east faced slopes would be beneficial for beech in reducing the margin effect due to dryness [8] [10]. Such vegetation buffer layer can be realized with broad leaved thermophile species where appropriate (e.g. *Quercus cerris*, *Malus sylvestris*, *Pyrus pyraster*, *Acer pseudoplatanus*, *A. platanoides* and *A. campestre*, *Fraxinus angustifolia*, *F. ornus* or *Sorbus domestica*, *S. aucuparia* and *S. aria*). In the remaining sites where it has been monitored a significant deviation from the genetic equilibrium (high *Fis*) it is necessary to favour gene flow (pollen flow and seed flow). The introduction of genotypes (artificial gene-flow) from the sites with maximum diversity (*Ho* and *A*) can increase site specific genetic diversity (*Ho*). Site selection for seed sampling can be further improved using as proxies the ecologic factors: pedogenetic substrate, soil type, altimetry, exposition, community structure and composition. Especially on the south and south-east exposed slopes beech cutting and thinning should be avoided. Spring frost and spring-summer dryness are fatal for shoots and coppiced plants. To

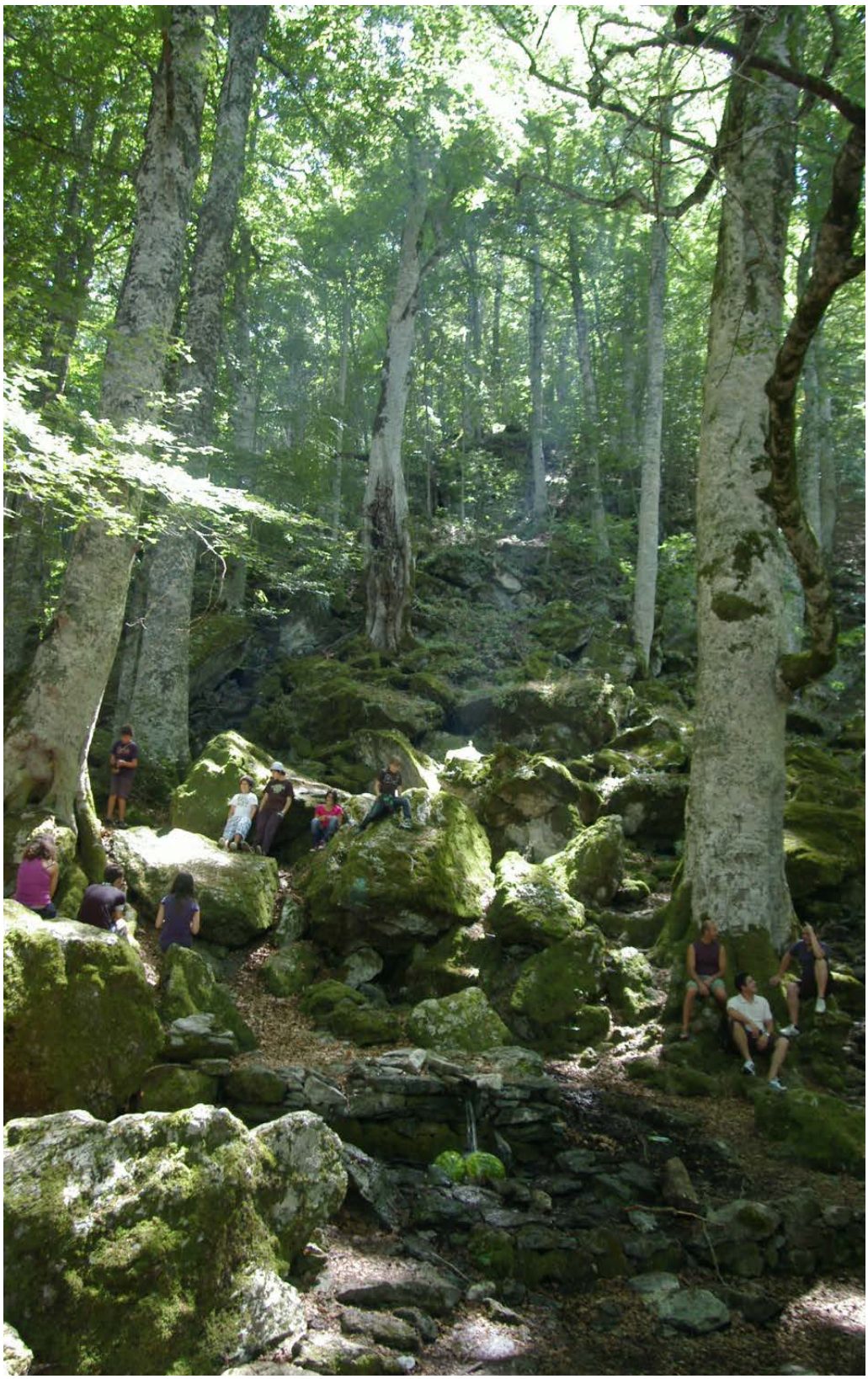

Figure 2. *Fagus sylvatica* family of effective individuals escaped from the usual thinning/coppicing in Pollino National Park—Italy (site: La Catusa).

Figure 3. Direct and indirect impacts caused by systematic usual silvicultural methods in Li Foi site (IT9210215).

achieve *in situ* conservation [29] seeds should be sampled according to specific guidelines [30] [31].

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