Genetic Differentiation of Pinus sylvestris L. and Pinus mugo aggr. Populations in Switzerland

By C. NEET-SARQUEDA[1])

Institut de Botanique Systématique et de Géobotanique,
Bâtiment de Biologie, University of Lausanne,
CH-1015 Lausanne, Switzerland

(Received 5th October 1993)

Summary

Electrophoretic allozyme differentiation at 11 loci was studied in *Pinus sylvestris* and *Pinus mugo* aggr. populations, as well as in 3 populations containing individuals with intermediate morphology between *P. sylvestris* and *P. uncinata*. The 14 populations sampled from different geographical areas of Switzerland are clustered in 2 main groups, a *P. sylvestris* and a *P. uncinata* group, which confirms the taxonomic status of these 2 species. Morphologically intermediate populations are grouped with *P. sylvestris* and there is no evidence for an introgressive hybrid zone hypothesis. A comparative analysis of discriminant scores obtained for intermediate form individuals using both morphological and genetic data clearly confirms the presence of both species in the intermediate form populations which are thus mixed stands of *P. sylvestris* and *P. uncinata*.

Key words: Biosystematics, *Pinus sylvestris*, *Pinus mugo*, *Pinus uncinata*, populations genetics, electrophoresis, hybrid zones, Switzerland.

FDC: 165.3; 165.5; 165.71; 174.7 *Pinus*; (494).

Introduction

For many years, introgressive hybridization has been implicated as one of the primary factors leading to evolutionary change in plant groups (ANDERSON, 1949; GRANT, 1981; RIESEBERG et al. 1988). More recently, hybridization patterns have even received wider attention and the evolutionary importance of hybrid zones has been demonstrated by theoretical and empirical studies, e. g. BARTON and HEWITT (1985, 1989), HEWITT (1988) and HARRISON (1990). However, although there is nowadays clear evidence that hybrid zones occur in many taxa, some of the classical examples of introgression that were first evidenced using morphological data have not been confirmed as cases of introgressive hybridization by molecular data (RIESEBERG et al. 1988). In this paper I examine the case of 2 pine species, *Pinus sylvestris* L. and *Pinus uncinata* DC, that have so far been considered as most likely to produce introgressive hybrids, e.g. by MARCET (1967) and NEET-SARQUEDA et al. (1988).

In Switzerland, the *Pinus mugo* aggr. (GREUTER et al., 1984) is represented by the species *P. mugo mugo* TURRA and *P. uncinata* DC. which are usually not found in sympatry with the widely distributed *P. sylvestris*. The general ecology and geographical distribution of these pine species are described by MIROV (1967) and FARJON (1984). Over the last 100 years, several authors have shown the existence of morphologically intermediate forms between *P. uncinata* and *P. sylvestris* throughout Europe, e.g. in France (GAUSSEN, 1931, 1960; FLOUS, 1933; PROBST, 1983), in Poland (STASZKIEWICZ, and TYSZKIEWICZ, 1969, 1972) in Czechoslovakia and Denmark (PETERSEN, 1903) and in Spain (VIGO, 1974) (for further references see also MIROV, 1967; CHRISTENSEN, 1987) In Switzerland, intermediate forms have been found by several authors (for references see NEET-SARQUEDA et al., 1988), in particular by MARCET (1967), who has analyzed morphological data that confirm the intermediate status; MARCET reaches the conclusion that there is natural hybridization between *P. uncinata* and *P. sylvestris*.

It should be underlined that the experimental hybridization of *P. sylvestris* and *P. mugo* aggr. has been investigated by several authors (see WRIGHT and GABRIEL, 1958; and KOSINSKI, 1991, for reviews). The results do not show a clear-cut pattern of compatibility between the 2 taxa but leave little doubt that hybridization is possible. LIESE (1927), DENGLER (1942) and SCHÜTT and HATTEMER (1959) have artificially produced hybrids, as well as JOHNSON (1939) and REHDER (1940), who obtained hybrids with both species either as male or female parent. However, JOHNSON and HEIMBURGER (1946) failed to obtain hybrids while KORMUTAK and LANAKOVA (1988), who have carried out extensive hybridological studies, consider *P. sylvestris* and *P. mugo* as a poorly crossable combination, actually bordering on incompatibility.

In order to quantify the degree of genetic differentiation between these two species and to assess to what extent natural hybridization occurs, I have undertaken an electrophoretic analysis of populations sampled from various parts of Switzerland and in particular from parapatric contact populations where morphologically intermediate forms are found.

Materials and Methods

I studied 14 populations, including 3 *P. sylvestris* populations, 7 of *P. uncinata*, 1 of *P. mugo mugo* and 3 populations consisting of intermediate forms between *P. uncinata* and *P. sylvestris* (*Table 1;* in the case of population no 3, groups of individuals sampled at different altitudes were pooled). These populations were taken from 2 main geographical regions of Switzerland: the Jura mountains and the Alps (Valais and Grisons). The Grisons populations number 11, 12 and 14 are the same as those studied by MARCET (1967). In each area a *P. sylvestris*, a *P. uncinata* and an intermediate form population were sampled (*Fig. 1*). Additionally, in the Jura mountains, 4 *P. uncinata* populations were taken from peat-bogs. Since these bogs are relatively small and isolated, these four populations may be considered as spatially limited populations.

Bud samples were collected from 25 individuals in each population and preserved at —70 °C. To provide enzyme samples, the buds were mashed in a Tris-glycin buffer and were then analyzed by starch gel electrophoresis (ASHTON

[1]) Résidences du Nozon 3, CH-1317 Orny, Switzerland

Table 1. — Populations included in the analysis (Coordinates are given in Swiss national map units).

Population	No	Locality	Coordinates	Altitude	Slope	Exposition
VALAIS						
P. sylvestris	1	Chamoson	583.96/117.61	820	39°	S
P. uncinata	2	Solalex	577.20/126.73	1525	11°	SE
Intermediate 1	3	Devin	576.42/098.72	1200	31°	SE
Intermediate 2	3	Devin	575.80/097.60	1525	31°	SE
JURA						
P. sylvestris	4	Pompaples	528.63/169.25	540	7°	SE
P. uncinata	5	Creux du Van	546.28/199.01	1040	37.5°	NW
Intermediate	6	Treymont	550.18/200.70	880	42°	NW
JURA - Peatbog populations						
P. uncinata	7	Pré Rodet	503.03/158.03	1040	0°	-
P. uncinata	8	Les Rousses (F)	496.05/150.55	1059	0°	-
P. uncinata	9	Les Enfers	569.75/237.25	960	0°	-
P. uncinata	10	Chaux/Breuleux	570.50/230.50	975	0°	-
GRISONS						
P. sylvestris	11	Schlarigna	786.85/153.85	1740	0°	-
P. uncinata	12	Wolfgang/Davos	784.65/189.95	1665	7.5°	SW
Intermediate	14	Punt Präspöl	808.30/171.35	1750	7.5°	NE
P. mugo mugo	13	Flüela	797.90/181.50	1880	35°	S

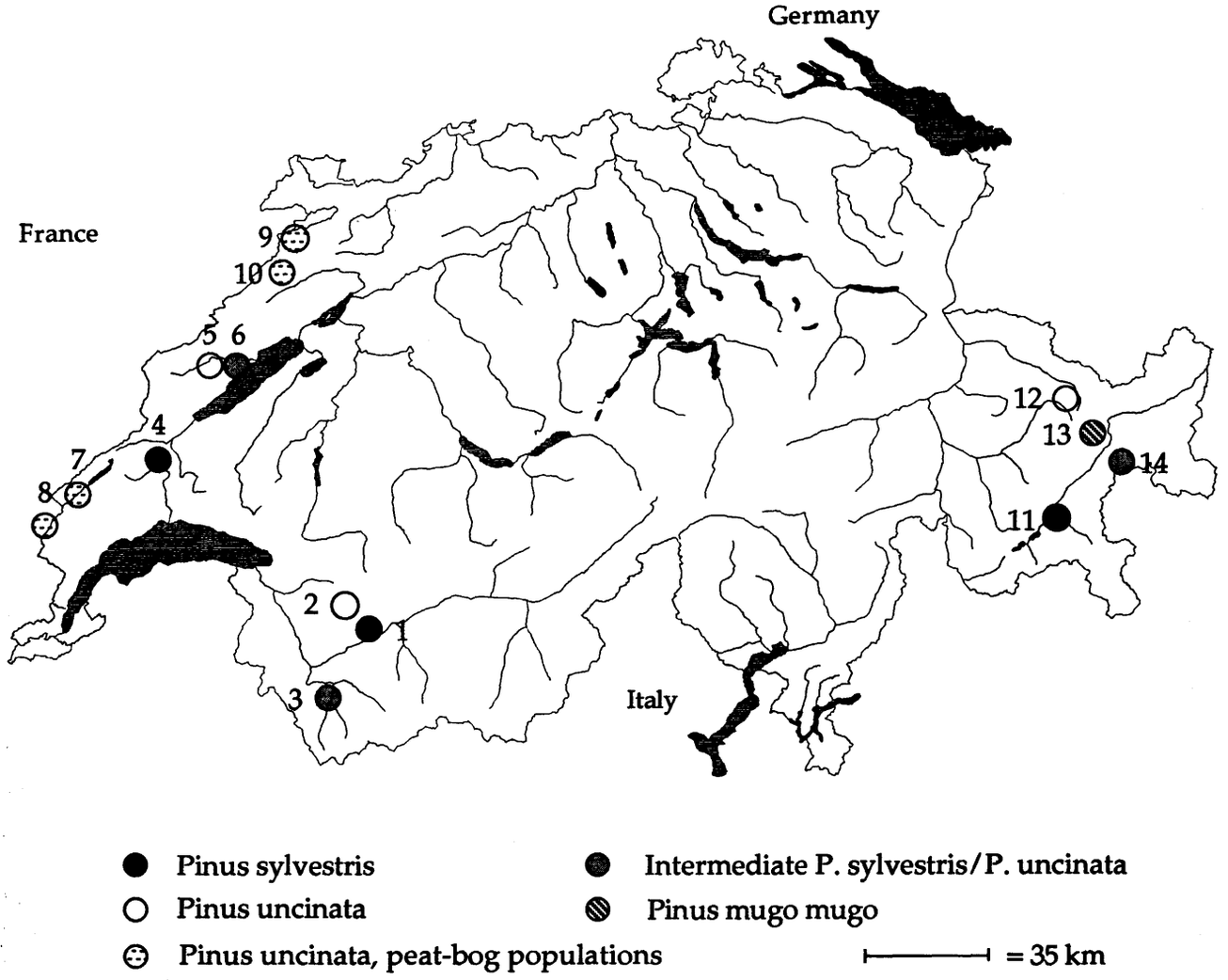

Figure 1. — Map of Switzerland with the position of the 14 populations studied.

and BRADEN, 1961; RUDIN and RASMUSON, 1973). Enzymes assayed were leucine amino-peptidase (LAP) (see RUDIN, 1977 for description in Scots pine), glutamate-oxaloacetate-transaminase (GOT) (RUDIN and EKBERG, 1978), fluorescent esterase (F-EST) (YAZDANI and RUDIN, 1982), glutamic dehydrogenase (DGH) (RUDIN, pers. comm.), malate dehydrogenase (MDH) (RUDIN and EKBERG, 1978) and shikimate dehydrogenase (ShDH) (SZMIDT and YAZDANI, 1984). These systems were used to allow analysis at 11 allozyme loci. Allele frequencies, expected heterozygosities, NEI's (1978) standard genetic distance measure, UPGMA cluster analyses (SNEATH and SOKAL, 1973) were calculated using mainly a Fortran program by ROYCHOUDHURY and TATENO (GRAF, 1980), BIOSYS 1.7 (SWOFFORD and SELANDER, 1989) and the SYSTAT package for statistics (Systat Inc. Evanston, IL, USA). KRUSKAL-WALLIS one-way analysis of variance and MANN-WHITNEY U tests (SIEGEL and CASTELLAN, 1988) were performed to compare population genetic parameters between populations. G-tests for independence (SOKAL and ROHLF, 1981) using raw allelic data between randomly selected pairs of populations were used for comparing allelic frequencies between populations.

In order to examine individual variation, I also undertook a comparative analysis of morphological and genetic data. This was done using for both data types a multivariate discriminant function analysis. For each geographical area (Jura, Valais and Grisons), a discriminant function was computed for both morphological and genetic data using the SPSS-X computer package (SPSS Inc., Chicago, IL, USA). The morphological data included 5

Table 2. — Allelic frequencies for the 11 enzymatic systems analysed (for population numbers see *Table 1*).

Populations:		1	2	3	4	5	6	7
Locus	Alleles							
GOT A	1	0.00	0.00	0.04	0.04	0.00	0.02	0.00
	2	1.00	1.00	0.96	0.96	1.00	0.98	1.00
GOT B	1	0.00	0.13	0.06	0.02	0.09	0.00	0.04
	2	0.36	0.45	0.36	0.46	0.46	0.33	0.26
	3	0.64	0.42	0.58	0.50	0.45	0.67	0.70
	4	0.00	0.00	0.00	0.02	0.00	0.00	0.00
F-EST	0	0.00	0.00	0.00	0.02	0.00	0.00	0.00
	1	0.84	0.93	0.80	0.73	0.84	0.81	0.84
	2	0.02	0.07	0.14	0.10	0.11	0.15	0.16
	3	0.14	0.00	0.06	0.15	0.05	0.04	0.00
GDH	0	0.02	0.00	0.00	0.00	0.00	0.00	0.00
	1	0.32	0.60	0.36	0.33	0.48	0.44	0.68
	2	0.66	0.38	0.58	0.67	0.43	0.54	0.30
	3	0.00	0.02	0.06	0.00	0.09	0.02	0.02
MDH A	1	0.21	0.03	0.04	0.13	0.09	0.02	0.00
	2	0.79	0.87	0.88	0.87	0.80	0.85	1.00
	3	0.00	0.10	0.08	0.00	0.11	0.13	0.00
MDH B	1	0.64	0.35	0.70	0.75	0.09	0.63	0.64
	2	0.36	0.65	0.30	0.25	0.91	0.37	0.36
MDH	1	0.91	0.58	0.96	0.94	0.80	0.78	0.71
A 1/2	2	0.09	0.42	0.04	0.06	0.20	0.22	0.29
ShDH	1	0.00	0.00	0.04	0.02	0.05	0.02	0.00
A	2	0.77	0.40	0.72	0.83	0.52	0.67	0.21
	3	0.16	0.60	0.24	0.13	0.39	0.28	0.79
	4	0.07	0.00	0.00	0.02	0.04	0.03	0.00
	5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ShDH	2	0.11	0.00	0.08	0.02	0.00	0.02	0.00
B	1	0.00	0.05	0.04	0.00	0.00	0.00	0.00
	3	0.89	0.95	0.88	0.98	0.98	0.98	1.00
	4	0.00	0.00	0.00	0.00	0.02	0.00	0.00
LAP A	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	2	1.00	0.97	1.00	0.97	0.89	1.00	0.84
	3	0.00	0.03	0.00	0.03	0.11	0.00	0.16
	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LAP B	1	0.00	0.00	0.00	0.07	0.00	0.00	0.00
	2	0.95	0.97	1.00	0.93	0.89	0.94	0.90
	3	0.05	0.03	0.00	0.00	0.11	0.06	0.10
	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Populations:		8	9	10	11	12	13	14
Locus	Alleles							
GOT A	1	0.00	0.12	0.00	0.00	0.00	0.02	0.02
	2	1.00	0.88	1.00	1.00	1.00	0.98	0.98
GOT B	1	0.00	0.00	0.04	0.02	0.00	0.04	0.00
	2	0.25	0.29	0.14	0.26	0.20	0.17	0.35
	3	0.75	0.71	0.82	0.72	0.80	0.79	0.65
	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F-EST	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1	0.78	0.83	0.84	0.74	0.88	0.85	0.64
	2	0.11	0.07	0.00	0.18	0.10	0.15	0.29
	3	0.11	0.10	0.16	0.08	0.02	0.00	0.07
GDH	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1	0.44	0.27	0.32	0.34	0.46	0.27	0.23
	2	0.52	0.60	0.68	0.66	0.48	0.71	0.71
	3	0.04	0.13	0.00	0.00	0.06	0.02	0.06
MDH A	1	0.07	0.02	0.00	0.06	0.04	0.12	0.00
	2	0.93	0.98	1.00	0.90	0.92	0.81	0.89
	3	0.00	0.00	0.00	0.04	0.04	0.07	0.11
MDH B	1	0.37	0.02	0.13	0.74	0.20	0.15	0.33
	2	0.63	0.98	0.87	0.26	0.80	0.85	0.67
MDH A 1/2	1	0.73	0.88	0.62	0.96	0.64	0.90	0.83
	2	0.27	0.12	0.38	0.04	0.36	0.10	0.17
ShDH A	1	0.00	0.02	0.04	0.04	0.02	0.06	0.00
	2	0.47	0.48	0.50	0.62	0.68	0.33	0.81
	3	0.53	0.48	0.46	0.02	0.24	0.50	0.17
	4	0.00	0.02	0.00	0.12	0.06	0.11	0.02
	5	0.00	0.00	0.00	0.02	0.00	0.00	0.00
ShDH B	2	0.00	0.00	0.00	0.16	0.00	0.00	0.04
	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3	0.97	1.00	1.00	0.82	1.00	0.89	0.92
	4	0.03	0.00	0.00	0.02	0.00	0.11	0.04
LAP A	1	0.00	0.00	0.00	0.04	0.00	0.02	0.00
	2	0.98	0.88	0.92	0.94	0.94	0.94	0.96
	3	0.02	0.12	0.08	0.02	0.06	0.02	0.02
	4	0.00	0.00	0.00	0.00	0.00	0.02	0.02
LAP B	1	0.00	0.00	0.00	0.00	0.06	0.02	0.00
	2	0.91	0.94	0.96	0.84	0.88	0.96	0.89
	3	0.09	0.06	0.04	0.16	0.06	0.00	0.11
	4	0.00	0.00	0.00	0.00	0.00	0.02	0.00

variables measured on the needles of all the individuals of the *P. sylvestris, P. uncinata* and intermediate form populations sampled (see NEET-SARQUEDA et al., 1988, for details on the methods). The genetic data were the actual raw data used to compute allelic frequencies. They were coded as follows in order to use each enzymatic system as a variable: a value of 1 was given for individuals homozygous at the most frequent allele, a value of 0.5 for heterozygous individuals and a value of 0 for individuals homozygous for other alleles. The 2 discriminant functions obtained for each geographic area were used to separate the *P. sylvestris* and *P. uncinata* populations and to classify the intermediate form individuals into either species according to the discriminant scores obtained with morphological and genetic data.

Results

The basic data for the 14 populations are given in *tables 2* and *3*. As shown in *table 2*, among the 11 studied loci, all were polymorphic and none contained diagnostic alleles characterizing 1 of the *Pinus species* under study. Systematic differences in allelic frequencies between *P. sylvestris* and *P. uncinata* include the frequencies of alleles 1 and 2 in MDH-B, allele 1 having a relatively high frequency in *P. sylvestris* and allele 2 2a relatively low frequency while *P. uncinata* has the reverse pattern. Such systematic differences are also found, to a lesser degree, in GDH. In the case of MDH-B, the intermediate form populations of the Jura and Valais show a pattern of frequencies similar to those of *P. sylvestris*, while the intermediate form popula-

Table 3. — Average heterozygosity (unbiased measure: H), number of polymorphic loci (p) and mean number of alleles per locus (a) of the populations included in the analysis.

Population	No	H	p	a
VALAIS				
P. sylvestris	1	0.2624	9	2.1
P. uncinata	2	0.2887	10	2.2
Intermediate 1+2	3	0.2624	9	2.4
JURA				
P. sylvestris	4	0.2533	11	2.5
P. uncinata	5	0.3047	10	2.5
Intermediate	6	0.2800	10	2.4
JURA - Peatbog populations				
P. uncinata	7	0.2611	8	1.9
P. uncinata	8	0.2744	10	2.1
P. uncinata	9	0.2439	10	2.3
P. uncinata	10	0.2290	8	1.9
GRISONS				
P. sylvestris	11	0.2974	10	2.6
P. uncinata	12	0.2623	9	2.4
Intermediate	14	0.2868	11	2.5
P. mugo mugo	13	0.2629	11	2.7

Table 4. — Standard genetic distance D (Nei, 1978) between the 14 populations studied (for population numbers see *Table 1*):

Populations:	1	2	3	4	5	6	7
1							
2	0.0584						
3	0.0000	0.0521					
4	0.0000	0.0721	0.0000				
5	0.0535	0.0129	0.0557	0.0711			
6	0.0034	0.0282	0.0000	0.0082	0.0386		
7	0.0734	0.0227	0.0576	0.0825	0.0628	0.0361	
8	0.0328	0.0164	0.0334	0.0499	0.0198	0.0147	0.0256
9	0.0643	0.0418	0.0672	0.0866	0.0086	0.0534	0.0757
10	0.0587	0.0310	0.0675	0.0848	0.0219	0.0417	0.0614
11	0.0042	0.0855	0.0044	0.0084	0.0808	0.0099	0.0763
12	0.0403	0.0260	0.0480	0.0620	0.0133	0.0226	0.0598
13	0.0520	0.0427	0.0560	0.0799	0.0199	0.0434	0.0653
14	0.0187	0.0541	0.0208	0.0259	0.0269	0.0144	0.0887

Populations:	8	9	10	11	12	13
9	0.0214					
10	0.0122	0.0091				
11	0.0465	0.0868	0.0802			
12	0.0122	0.0178	0.0063	0.0575		
13	0.0142	0.0073	0.0141	0.0671	0.0235	
14	0.0239	0.0287	0.0309	0.0266	0.0179	0.0306

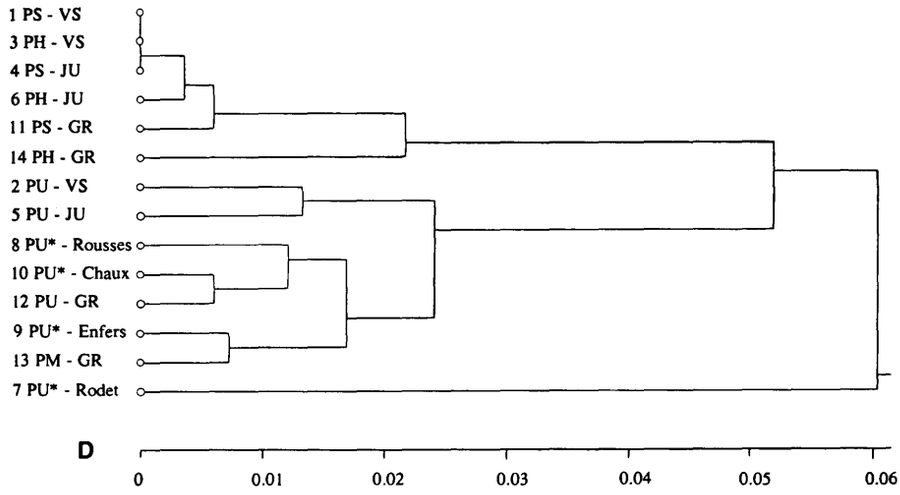

Figure 2. — UPGMA average clustering analysis performed with Nei's (1978) standard genetic distance D, for the 14 populations studied (PS = *Pinus sylvestris*, PU = *Pinus uncinata*, PH = Intermediate form populations, PM = *Pinus mugo mugo;* VS = Valais, JU = Jura, GR = Grisons, * = Jura peat-bog populations).

tion of the Grisons and the *P. mugo mugo* population show a frequency pattern similar to those of *P. uncinata.*

In *table 3,* population genetic parameters are given for each population studied. A Kruskal-Wallis one-way analysis of variance taking the 3 *P. sylvestris,* the 3 *P. uncinata,* the 4 peat-bog *P. uncinata,* the 3 intermediate form populations and the *P. mugo mugo* population as 5 separate groups shows that there is no significant heterogeneity in the values of mean heterozygosity (KW = 4.68, p = 0.32), number of polymorphic loci (KW = 3.92, p = 0.42) or mean number of alleles per locus (KW = 7.87, p = 0.10).

However, when analyzing the 3 *P. uncinata* populations vs the 4 peat-bog *P. uncinata* populations by means of a Mann-Whitney U test, it appears that for the mean heterozygosities (U = 1, $p < 0.05$) and mean numbers of alleles per locus (U = 1, $p < 0.05$) there are significantly lower values in the peat-bog populations.

Given the geographical distribution of the populations analyzed (*Fig. 1*), the genetic distance results (*Tab. 4*) and in particular the UPGMA clustering results (*Fig. 2*), the distinctness of the *P. sylvestris* and *P. uncinata* populations throughout the Swiss geographical range is clearly demonstrated. The fact that the 2 species form well defined clusters, although distances between conspecific populations can be around 250 km to 300 km (*Fig. 1*) underlines this point. The intermediate form populations are closely related to and grouped with the *P. sylvestris* populations. The *P. mugo mugo* population is grouped with *P. uncinata* populations. One population, the *P. uncinata* peat-bog population of Pré-Rodet is isolated from all other groups.

I would strongly suggest that *figure 2* shall be globally interpreted as evidence for the existence of 2 main groups: a *P. sylvestris* group and a *P. uncinata* group, without evidence for an intermediate form group that can be identified as containing only hybrid individuals characterized by introgressive hybridization.

To support this hypothesis, I have drawn 2 random samples of 10 pairs of populations, 1 including within-group pairs and the other including between-group pairs of populations. These pairs have then been compared by G-tests of independence applied to raw allelic data (absolute numbers rather than allelic frequencies). The results show that most within-group pairs (80%) do not differ significantly in terms of allelic data, while most between-group pairs (70 %) do differ significantly (*Table 5*). In a second step, I have analyzed individual rather than population data, which enables a more detailed insight into the composition of the intermediate form populations. This was done using the scores of intermediate individuals on discriminant functions calculated only with the reference populations of each geographical area. Since the functions calculated are highly discriminant (*Table 6*), one may expect hybrid individuals, if any, to be intermediate on both functions. The comparison of the discriminant scores obtained by each intermediate form individual showed that only 1 or 2 individuals in each intermediate population had rather intermediate discriminant scores on both genetic and morphological functions, i.e. values close to the cut-off limit of 0. As shown in *table 6,* according to the intermediate population, 28 % to 37.5 % (mean 34 %, n = 3) of the individuals analysed are not classified in the same species by both discriminant functions, while all other individuals are clearly classified into one or the other species (note that all intermediate individuals could not be analysed with the genetic discriminant function because electrophoretic results were not obtained for every individual at every locus).

Discussion

Previous work on the population genetics of *P. sylvestris* and *P. mugo* aggr. in Czechoslovakia has found allelic frequencies similar to those found to be systematically characteristic (MDH-B and GDH) for the 2 species (Filppula et al., 1992). Furthermore, although *P. sylvestris* and *P. mugo* aggr. are very closely related (Prus-Glowacki et al., 1985), the results presented here confirm their taxonomic status and are consistent with the conclusions of other authors on this point (Prus-Glowacki and Szweykowski, 1983; Filppula et al., 1992). There is also an indication, on the basis of a single population of *P. mugo mugo,* that *P. uncinata* and *P. mugo mugo* could be conspecific.

Other studies have also found reduced heterozygosity in spatially limited Scotch pine populations (Petrova et al., 1990), as in the case of the differences shown here between the peat-bog populations and other populations

Table 5. — G-tests for independence using raw allelic data between randomly selected pairs of populations analysed (significance level at 0.01). Within-group and between-group sets of populations are based on the "sylvestris" and "uncinata" groups derived from *fig. 2* (see text).

Population pairs	G	df	p
Within-group pairs of populations			
5 and 12	19.53	14	0.159 NS
7 and 8	26.78	18	0.104 NS
6 and 14	19.38	15	0.207 NS
3 and 14	28.87	16	0.032 NS
4 and 11	21.55	18	0.287 NS
2 and 13	44.31	17	<0.001
8 and 13	23.72	17	0.134 NS
8 and 12	19.78	16	0.245 NS
1 and 14	18.00	16	0.343 NS
5 and 10	45.29	16	<0.001
Between-group pairs of populations			
1 and 2	52.31	18	<0.001
12 and 14	26.16	18	0.109 NS
1 and 13	45.19	19	0.001
11 and 10	64.94	16	<0.001
11 and 12	57.92	19	<0.001
2 and 11	41.00	16	<0.001
3 and 9	19.24	14	0.162 NS
6 and 10	69.32	16	<0.001
3 and 12	40.79	17	0.002
8 and 11	25.66	16	0.071 NS

of *P. uncinata.* Thus, although 1 of the peat-bog populations of *P. uncinata* is isolated from the 2 taxonomic groups identified here (*Fig. 2*), I may suggest that this may be due to genetic drift in a spatially isolated population. Alternative hypotheses for this pattern of heterozygosity are strong selective pressures under extreme conditions or the possible consequences of recent colonisation or even plantations in peat-bogs. The first alternative corroborates evidence showing that flowering phenology can be influenced by extreme peat-bog origin in *P. sylvestris* (HAGMANN, 1972). The second alternative is suggested by palynological studies that demonstrate that pine woodlands in peat-bogs are, in some cases, of recent origin rather than relict populations of native *Pinus* (O'CONNELL, 1990).

With respect to the status of the morphologically intermediate populations and at the light of the results presented here, it can be excluded that the individuals of the intermediate populations are members of a hybrid zone (BARTON and HEWITT 1985, 1989; HEWITT, 1988; HARRISON, 1990) or of a zone of introgressive hybridization (ANDERSON,

Table 6. — General results of the genetic (GEN) and morphological (MOR) discriminant functions and of the classification of the individuals of the intermediate form populations (see text). The means given are the mean discriminant scores for *P. sylvestris* (PS) and *P. uncinata* (PU) populations. Numbers in italics give the population numbers (see *Tabl. 1* and *Fig. 2*).

	Jura		Valais		Grisons	
	GEN	MOR	GEN	MOR	GEN	MOR
Means:	PS*4*: 1.40	PS*4*: 2.61	PS*1*: 1.56	PS*1*: 3.40	PS*11*: 1.58	PS*11*: 3.55
	PU*5*: -2.34	PU*5*: - 2.61	PU*2*: - 1.66	PU*2*: -3.40	PU*12*:-1.58	PU*12*:-3.55
Wilk's λ :	0.22	0.12	0.27	0.08	0.28	0.07
χ^2 :	25.84	91.02	37.64	116.80	55.07	120.37
p :	0.004	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
% correct classification:	95.83	97.92	91.43	100	94.00	100
Intermediate form populations classified:	Pop. *6*		Pop. *3*		Pop. *14*	
2 x as *P. sylvestris*	4		10		3	
2 x as *P. uncinata*	1		1		13	
1 x in each species	3		6		7	

1949; GRANT, 1981). In contrast to studies like the one of BOUSQUET et al. (1990) on 2 *Alnus* species, where morphologically intermediate form populations are also genetically intermediate, the results of this study do not support the hypothesis of introgressive hybridization. This is consistent with the results of FILPPULA et al. (1992) who reject the hypothesis of hybridization between *P. sylvestris* and *P. mugo* aggr. on the grounds of electrophoretic and cpDNA analyses. It is however known that hybrid zones are complex and variable (HEWITT, 1988; BARTON and HEWITT, 1989) and therefore I have attempted to examine in detail individual variation by the means of a comparison between the results of morphological and genetic discriminant function analyses. This approach has enabled to show that for around 34 % of individuals, morphological and isozyme classifications differ. This may indicate that a few hybrid individuals are found in the intermediate populations. It should however not be overlooked that the discriminant function approach has a degree of uncertainty quantified by the rate of correct classification of the discriminant functions (*Table 6*) and that, as consequence, one would expect a certain degree of disagreement between independent discriminant classifications. Moreover, only 1 or 2 individuals in each intermediate form population were found to have rather intermediate values on both genetic and morphological discriminant functions. This finding of an absence of introgressive hybridization pattern is important because several studies have so far given various morphological and biochemical indications supporting the introgressive hybridization hypothesis (PROBST, 1983; SANDOZ, 1987; NEET-SARQUEDA et al., 1988; PLUMETTAZ CLOT, 1988; LAURANSON, 1989). However, these authors have all underlined that they had no strict evidence available to confirm the hypothesis of genetic introgression (see in particular SANDOZ, 1987; PLUMETTAZ CLOT, 1988; and LAURANSON, 1989).

Alternative hypotheses for the status of the morphologically intermediate populations are:

1. The intermediate populations are mixed samples including both species;
2. The intermediate forms are in fact *P. sylvestris* individuals that converge towards a *P. uncinata* morphology for ecological reasons. The results presented here do in fact also show that Hypothesis 2 is not correct since the discriminant function approach confirms that there are individuals of both species in the intermediate form populations. Thus, Hypothesis 1 best describes the status of the intermediate form populations, which thus consist of either *P. sylvestris* or *P. uncinata* individuals with hybrids, if any, being in very limited numbers. Data from previous work (e.g. NEET-SARQUEDA et al., 1988), and in particular data from LAURANSON (1989) are compatible with this result since a close look at the facts presented by these studies shows that intermediate individuals are usually grouped either with one or with the other species and that obviously intermediate individuals are not at all frequent. In short, the results presented here confirm the taxonomic status of *P. sylvestris* and *P. uncinata* and demonstrate that in the populations described as intermediate there is no introgressive hybridization as previously thought on the grounds of the analysis of morphological data.

Acknowledgements

This work is part of a PhD thesis in preparation at the University of Lausanne. We thank Prof. O. SAVOLAINEN and her team of the University of Oulu for support, technical advice and help during data analysis, Dr. D. RUDIN, Dr. A. SZMIDT and INGABRITT CARLSSON of the University of Umea for technical guidance, Dr. N. BARTON, Dr. R. HARRISON, Dr. J. SZYMURA for very useful comments during the hybrid zone meeting in Lausanne in 1991, Dr. FELDMEYER-CHRISTE for help finding peat-bog material and Dr. C. NEET for statistical advice and comments on previous drafts of the manuscript. This work was also kindly supported by Prof. N. GALLAND, Prof. H. CLÉMENÇON and Prof. P. HAINARD of the University of Lausanne.

Literature Cited

ANDERSON, E.: Introgressive Hybridization. Wiley and Sons, New York (1949). — ASHTON, C. and BRADEN, A.: Serum β-globulin polymorphism in mice. Austr. J. Biol. Sci. **14**: 248—253 (1961). — BARTON, N. H. and HEWITT, G. M.: Analysis of hybrid zones. Ann. Rev. Ecol. Syst. **16**: 113—148 (1985). — BARTON, N. H. and HEWITT, G. M.: Adaptation, speciation and hybrid zones. Nature **341**: 497—503 (1989). — BOUSQUET, J., CHELIAK, W. M., WANG, J. and LALONDE, M.: Genetic divergence and introgressive hybridization between *Alnus sinuata* and *A. crispa* (Betulaceae). Pl. Syst. Evol. **170**: 107—124 (1990). — CHRISTENSEN, K. I.: Taxonomic revision of the *Pinus mugo* complex and *P. x rhetica* (*P. mugo* x *sylvestris*) (Pinaceae). Nord. J. Bot. **7**: 383—408 (1987). — DENGLER, A.: Herkufts- und Kreuzungsversuche im Versuchsgarten des Waldbauinstituts Eberswalde. Mitt. Deutsch. Dendrol. Ges. **55**: 157—169 (1942). — FARJON, A.: Pines. Drawings and description of the genus *Pinus*. Brill and Backhuys, Leiden (1984). — FILPPULA, S., SZMIDT, A. E. and SAVOLAINEN, O.: Genetic comparison between *Pinus sylvestris* and *P. mugo* using isozymes and chloroplast DNA. Nord. J. Bot. **12**: 381—386 (1992). — FLOUS, F.: Les pins montagnards et subalpins des Pyrénées. Bull. Soc. Hist. Nat. Toulouse **65**: 299—308 (1933). — GAUSSEN, H.: Les forêts de la vallée de l'Aure. Trav. du Lab. Forest. de Toulouse **3** (9): 1—10 (1931). — GAUSSEN, H.: Les gymnospermes actuelles et fossiles, Genre *Pinus*. Trav. du Lab. Forest. de Toulouse **2**: 6 (11): 1—272 (1960). — GRAF, J.-D.: Génétique biochimique, taxonomie et évolution des Arvicolidae (Mammalia, Rodentia). PhD thesis, University of Lausanne (1980). — GRANT, V.: Plant speciation. Second Ed. Columbia University Press, New York (1981). — GREUTER, W., BURDET, H. M. et al. (eds):Med-Checklist 1. Pteridophyta, Gymnospermae, Dicotyledones, Genève (1984). — HAGMANN, M.: On some factors influencing the yield from seed orchards of *Pinus sylvestris* L. and their interclonal and intraclonal variation. Forest Tree Improvement **4**: 67—83 (1972). — HARRISON, R. G.: Hybrid zones: windows on evolutionary process. In: D. FUTUYMA and J. ANTONOVICS (Eds.). Oxford surveys in Evolutionary Biology **7**: 69—128 (1990). — HEWITT, G. M.: Hybrid zones — Natural laboratories for evolutionary studies. Tree **3**: 158—167 (1988). — JOHNSON, L. P. V.: A descriptive list of natural and artificial interspecific hybrids in North American forest-tree genera. Canad. Jour. Res., C **17**: 411—444 (1939). — JOHNSON, L. P. V. and HEIMBURGER, C.: Preliminary report on interspecific hybridization in forest trees. Canad. Jour. Res. C **24**: 308—312 (1946). — KORMUTAK, A. and LANAKOVA, M.: Biochemistry of reproductive organs and hybridological relationships of selected Pine species (*Pinus sp.*). Acta dendrobiologica, Bratislava, 119 pp. (1988). — KOSINSKI, G.: Hybridization. In: M. GIERTYCH and C. MATYAS (Eds): Genetics of Scots Pine. Developments in Plant Genetics and Breeding, 3. Elsevier, Amsterdam, pp. 183—189 (1991). — LAURANSON, J.: Exploration de la diversité biochimique chez les conifères: contribution à l'étude de l'hybridation *Pinus uncinata* RAM. X Pinus sylvestris L. et à la connaissance du complexe spécifique *Pinus nigra* ARN. PhD thesis, University Claude Bernard Lyon 1 (1989). — LIESE, J.: Zur Frage der Bastardbildung zwischen *Pinus sylvestris* und *montana*. Forstarchiv **3**: 202—205 (1927). — MARCET, E.: Über den Nachweis spontaner Hybriden von *Pinus mugo* TURRA und *Pinus sylvestris* L. aufgrund von Nadelmerkmalen. Ber. Schweiz. Bot. Ges. **77**: 314 361 (1967). — MIROV, N. T.: The genus *Pinus*. The Ronald Press Company, New York (1967). — NEET-SARQUEDA, C., PLUMETTAZ CLOT, A.-C. and BÉCHOLEY, I.: Mise en évidence de l'hybridation introgressive entre *Pinus sylvestris* L. et. *Pinus uncinata* DC. en Valais (Suisse) par deux méthodes multivariées Bot. Helvetica **98**: 161—169 (1988). — NEI, M.: Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics **89**: 582—590 (1978). — O'CONNELL, C.: Ecology and history of a bog pine woodland at Derryclure, County Offaly, Ireland. British Ecol. Soc. Bull. **21**: 116—121 (1990). — PETERSEN, O. G.: Tiddskrift for Skovvaesen 15 B (1903). — PETROVA, IV., SANNIKOV, S. N., RYABONKON, S. M., DUKHAREV, V. A. and SANNIKOVA, N. S. 1990: Genetic differentiation of bog and dry-valley populations of *Pinus sylvestris* in Western Siberia. Sov. J. Ecol. **20**: 350—354 (1990). —

PLUMETTAZ CLOT, A.-C.: Phyto-écologie des pinèdes valaisannes et contribution à la taxonomie du genre *Pinus*. PhD thesis, University of Lausanne (1988). — PROBST, A.: Variation intraspécifique et introgression entre *Pinus uncinata* RAM. et *Pinus sylvestris* L. en forêt d'Osséja (Pyrénées-Orientales). Thèse de doctorat de 3e cycle, Université Paul Sabatier, Toulouse (1983). — PRUS-GLOWACKI, W. and SZWEYKOWSKI, J.: Studies on isoenzyme variability in populations of *Pinus sylvestris* L., *Pinus mugo* TURRA, *Pinus uliginosa* NEUMANN and individuals from a hybrid swarm population. Bull. soc. sc. et lettres de Poznan D. **22**: 107—122 (1983). — PRUS-GLOWACKI, W., SZWEYKOWSKI, J. and NOWAK, R.: Serotaxonomical investigation of the European Pine species. Silvae Genet. **34**: 4—5 (1985). — REHDER, A.: Manual of cultivated trees and shrubs. MacMillan, New York. 996 pp. (1940). — RIESEBERG, L. H., SOLTIS, D. E. and PALMER, J. D.: A molecular reexamination of introgression between *Helianthus annuus* and *H. bolanderi* (Compositae). Evolution **42**: 227—238 (1988). — RUDIN, D.: Leucine-aminopeptidase (LAP) from needles and macrogametophytes of *P. sylvestris* L. Inheritance of allozymes. Hereditas **85**: 219—226 (1977). — RUDIN, D. and EKBERG, I.: Linkage studies *in Pinus sylvestris* L. using macrogametophyte allozymes. Silvae Genet. **27**: 1—12 (1978). — RUDIN, D. and RASMUSON, B.: Genetic variation in esterases from needles of *Pinus sylvestris* L. Hereditas **73**: 89—98 (1973). — SANDOZ, H.: Recherches taxonomiques biogéographiques et phytoécologiques sur les principaux conifères subalpins des Alpes: Mélèze d'Europe, Pin cembro, Pin à crochets et Pin mugho. Thèse, Université d'Aix-Marseille III, 650 pp. (1987). — SCHÜTT, P. and HATTEMER, H. H.: Die Eignung von Merkmalen des Nadelquerschnittes für die Kiefern-Bastarddiagnose. Silvae Genetica **8**: 93—99 (1959). — SIEGEL, S. and CASTELLAN, N. J.: Nonparametric statistics for the behavioral sciences. MacGraw-Hill, New York (1988). — SNEATH, P. H. and SOKAL, R. R.: Numerical taxonomy. Freeman & Co., San Francisco (1973). — SOKAL, R. R. and ROHLF, F. J.: Biometry. The principles and practice of stitistics in biological research. 2nd edition. Freeman and Co., San Francisco (1981). — STASZKIEWICZ, J. and TYSZKIEWICZ, M.: Naturalne mieszance *Pinus mugo* TURRA x *Pinus sylvestris* L. w. Kollinie Nowotarskiej. Fragm. Florist. Geobot. **15**: 187—212 (1969). — STASZKIEWICZ, J. and TYSZKIEWICZ, M.: Variability of natural hybrids of *Pinus sylvestris* L. x *Pinus mugo* TURRA (=*P. rotunda* LINK) in south-western Poland and in some selected localities of Bohemia and Moravia. Fragm. Florist. Geobot. **18**: 173—191 (1972). — SWOFFORD, D. L. and SELANDER, R. B.: BIOSYS — 1. A computer program for the analysis of allelic variation in population genetics and biochemical systematics. Release 1.7. Illinois Natural History Survey, Champaign, Illinois (1989). — SZMIDT, A. and YAZDANI, R.: Electrophoretic studies of genetic polymorphism of shikimate and 6-phosphogluconate dehydrogenase in Scots pine (*Pinus sylvestris* L.). Arbor. Kornickie **29**: 63—72 (1984). — VIGO, J.: A propos des forêts de cônifères calcicoles des Pyrénées orientales. Documents phytosoc. **7—8**: 51—54 (1974). — WRIGHT, J. W. and GABRIEL, W. J.: Species hybridization in the hard pines, series Sylvestres. Silvae Genetica **7**: 109—115 (1958). — YAZDANI, R. and RUDIN, D.: Inheritance of fluorescent esterase and α-galactosidase in haploid and diploid tissues of *Pinus sylvestris* L. Hereditas **96**: 191—194 (1982).

Micropropagation of Platanus acerifolia in vitro

By V. GJULEVA[1]) and A. ATANASOV[2])

(Received 8th November 1993)

Summary

The possibility for micropropagation from vegetative buds of *Platanus acerifolia* WILLD. collected from 4-years and 50 years plants was tested. The buds of the 4 years old plants showed significantly higher morphogenic activity. The induction processes occured best under the condition of GD (1972) medium supplemented with 0.3 mg/l BAP. The best multiplication rate was achieved at 0.5 mg/l BAP. The rooting of cultures on 1/2 GD with 0.5 mg/l IBA was 100% and adaptation in vivo was 70% after a month. This simple system for micropropagation is suitable for propagation of elite trees.

Key words: Platanus acerifolia, axial buds, in vitro propagation.
FDC: 165.442; 176.1 *Platanus acerifolia.*

Introduction

Platanus acerifolia is a valuable, fast growing woody species and the most popular tree in the cities and urban areas in Bulgaria, due in part to its resistance to air polution. Vegetative propagation methods have been described by DELKOV (1977) and VLACHOV (1982), however results from these experiments with cuttings and seedlings are variable. They demonstrate both main obstacles for its intensive propagation: the limited possibility for production of a sufficient number of seedlings, the distinct dependence of the rooting capability on humidity, the temperature during the conservation in coldhouse and the type of cutting — summer or winter.

A method for overcoming these problems is in vitro micropropagation wich can be used all year round. In addition, induction of the axillary buds is a useful method for mass clonal micropropagation especialy of decidious species which are known with their episodic growth (AHUJA, 1982, 1983, 1984; CHALUPA, 1983; EVERS, 1987; VIEITEZ, 1982, 1983, 1985, 1991). As a result of those investigations many protocols exist but results with *Platanus* species are limited (EVERS 1988).

In these study the effect of different culture conditions and plant hormones on the morphogenic response were investigated as well as the in vitro rooting of *Platanus acerifolia.*

Materials and Methods

Plant material

Cuttings of 4- and 50-years old plants of *Platanus acerifolia* grown outdoors in the Lulin nursery, Sofia, were taken and stored at 40 °C for several days. After sterilization treatment with 5% Ca-hypochlorite for 18 min and 3-fold rinsing with sterile distiled water for 15 min, 15mm microcuttings were established weekly in 3 different periods (15.9 to 20.10; 15.1 to 30·1; 15.5 to 30.6)

Culture conditions

Modifications and standard media for proliferation in vitro were used. These included the basal media MS (1962), SH (1972), GD (1972) and WPM (1981) supplemented with BAP from 0 mg/l to 3 mg/l with or without IBA from 0.001 mg/l up to 0.01 mg/l.

For rooting in vitro both liquid with sterile perlite and solidified with 7 g/l agar variants were used with the following media: halfstreng basal macroelements of Ms (1962) and GD (1972 supplemented with IBA, IAA or NAA from 0.0 up to 2.0 mg/l.

[1]) Department of Genetics, Tissue culture Lab., Forest Research Institute, 1080 Sofia-C, Box 15, Bulgaria
[2]) Institute of Genetic Engeneering, 2232-Kostinbrod-2, Bulgaria