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Genetic diversity patterns in *Phragmites australis* at the population, regional and continental scales

Carla Lambertini [a,b,*], Mats H.G. Gustafsson [a], Jane Frydenberg [a], Maria Speranza [b], Hans Brix [a]

[a] *Department of Biological Sciences, Aarhus University, Denmark*
[b] *Department of Agro-Environmental Sciences and Technologies, University of Bologna, Italy*

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Abstract

Genetic diversity, population structure and interrelationships were investigated in eight populations of the common reed, *Phragmites australis*, in the Po Plain, Italy, by means of amplified fragments length polymorphisms (AFLPs) and random amplified polymorphic DNAs (RAPDs). Patterns of genetic diversity were analysed in relation to size, age and degree of human impact in the wetlands and compared with that of a distant population in Romania. Genetic distances between Po Plain clones and geographically distant clones were measured to determine the geographical extent of the gene pool.

Nearly all populations studied are polyclonal and little correlation was found between genetic diversity and size, age and degree of human impact on the wetlands. One large (86 ha) monoclonal stand occurred in an old wetland with rather stable environmental conditions over a long time period, whereas polyclonal stands were younger and characterized by disturbance. On the interpopulation level it was not possible to differentiate between Po Plain populations and the Romanian population, indicating that a very extensive gene pool exists in Europe, to which both Po Plain and Romanian populations belong. There is however a certain degree of genetic structure among the populations that is not correlated with geographic distance, but is most likely related to *P. australis* colonization dynamics. A significant ''stepwise'' increase in average genetic distances was observed between clones >500 and >1500 km distant suggesting some kind of genetic pattern on a very large scale. Based on these results, *P. australis* populations in Europe could be considered members of a single meta-population.

Keywords: *Phragmites australis*; AFLP; RAPD; Genetic diversity; Population structure; Genetic relationship; Genetic distance; Meta-population; Common reed; Wetland

1. Introduction

Phragmites australis (Cav.) Trin ex Steud., the common reed, is a perennial emergent aquatic plant with a nearly worldwide distribution. Throughout most of its range, it typically forms closed, monodominant stands in the littoral zone of lakes, along rivers and in marshes of various kinds (Brix, 1999; Brix and Cizkova, 2001). The annual stems develop from an underground perennial rhizome system, which is responsible for the rapid vegetative expansion of the species. Sexual reproduction occurs but is pollen limited and affected by partial self-incompatibility (Ishii and Kadono, 2002).

Varying degrees of clonal diversity have been found in *P. australis* populations, ranging from monoclonal to polyclonal (Neuhaus et al., 1993; Koppitz et al., 1997). The colonization of a wetland by *P. australis* typically begins on the shores where numerous seeds germinate. Colonization then continues with the vegetative expansion of the seedlings, and as spaces between plants are progressively filled in, the clones start to compete for space. This process may result in a complex spatial distribution, where different clones intermingle. Clonal diversity is still high at the ''propagation and establishment stage'', while it decreases during the subsequent ''stationary stage'', in which a small number of clones well adapted to the local environmental conditions prevails (Koppitz and Kühl, 2000). Low clonal diversity and monoclonal populations could be the result of such a selection process and be an indication that stands have grown under stable conditions for a long time (Watkinson and Powell, 1993). Seedling recruitment is

* Corresponding author at: Aarhus University, Department of Biological Sciences, Plant Biology, Ole Worms Allé, Bld. 115, Denmark.
Tel.: +45 8942 4713; fax: +45 8942 4747.
E-mail address: carla.lambertini@biology.au.dk (C. Lambertini).

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generally considered to occur only during ''windows of opportunity'' such as after a physical disturbance (Eriksson, 1997; Clevering, 1999). Favourable conditions for seed germination and seedling growth have been observed to occur after clearing of existing vegetation on bare riverbanks and after episodic drawdowns (Weisner et al., 1993). Seedling establishment is not very frequent in mature *P. australis* populations (Barrett et al., 1993). This and the clonal growth described above limit opportunities for gene flow within populations of *P. australis* and decrease genetic diversity.

Little is known about the extent and mechanics of gene flow via seeds and pollen between populations of *P. australis*. New wetlands are often rapidly colonized by *P. australis* even in areas far from reproducing stands. Long-distance gene flow may thus be an important factor in shaping genetic variation patterns.

In the Po Plain, northern Italy, numerous *P. australis*-dominated wetlands occur that are connected by a complex network of natural rivers and channels with regulated flow. Some are very old and represent the remnants of the extensive reed-dominated marshes that once covered the Po floodplain. Today most of the plain is drained and intensively cultivated. In the 1990s a number of wetlands were restored in compliance with European Community policies, which provided financing for transformation of croplands into wetlands. Today these wetlands are primarily maintained as hunting reserves. The management, mostly in the form of harvesting, may affect the genetic structure and diversity of *P. australis* populations as it indirectly creates opportunities for the seedling establishment, modifies the clonal architecture of populations, and affects the mating and dispersal opportunities (Charpentier, 2002). We expect to find high clonal variability in this area as a consequence of intra- and interpopulation gene flow. We also hypothesize that stands will consist of a mosaic of clones due to dispersal of rhizomes in connection with the harvest.

In a recent phylogeographic study of *Phragmites*, based on 238 collections spanning most of the vast distribution area, Lambertini et al. (2006) identified a large, fairly well supported, but poorly resolved ''core group'' within *P. australis*. Most European clones, including the ones from the Po Plain, belong to this group. The aims of the present study were (i) to assess the genetic diversity within and between *P. australis* populations in the Po Plain area and relate the diversity to wetland size, age and degree of human impact, and (ii) to compare the variation patterns in the Po Plain with geographically distant populations.

2. Materials and methods

2.1. Genetic variation at the population scale

Eight *P. australis* populations were sampled in the Po Plain, each representing a distinct wetland differing in size, age as well as past and present uses (Table 1). The geographic distance between the two most distant populations is 25 km. In each wetland a total of 15 specimens (shoots) were collected from 10 to 15 *P. australis*-dominated patches of vegetation, visibly separated from other such patches. In seven out of eight populations, two or three individuals were collected in at least one patch. The random amplified polymorphic DNA (RAPD) technique was used to identify genotypes and detect the presence of individual clones in populations. Four to seven different clones from each population, as identified by the RAPD analyses, were subsequently amplified for fragments length polymorphism (AFLP). In the Bentivoglia population, which appeared to be monoclonal based on RAPDs, two additional specimens were collected at a distance >100 m from the previously sampled patches and AFLP fingerprinted in order to get an idea of the limits of this apparently very extensive clone. In total 46 clones from eight Po Plain populations were analysed by AFLP.

Information about the history and management of the wetlands was acquired from owners and employees of the wetlands and from ''Bonifica Renana'', the board in charge of water regulation and distribution in the Po Plain area.

2.2. Genetic variation at the regional scale

The genetic variation pattern found among Po Plain populations was compared with that of the mixed cytotype population of Lake Razim in the Danube Delta, Romania (Clevering and Lissner, 1999). The eleven samples from Lake Razim have different ploidy levels (4*x*, 6*x*, 8*x* and 12*x*) and some of the specimens are known to be very closely related, as they originated from seeds of the same inflorescence in the greenhouses of the Netherlands Institute of Ecology in Heteren (Clevering, personal communication, 1999). Specimens 654RO and 655RO are from the same inflorescence, and 658RO, 659RO and 660RO from a panicle of another clone (Table 2). The mother panicles were collected along a 500 m transect on the land-side of the shore of Lake Razim (Clevering, 1999).

2.3. Genetic variation at a continental scale

The genetic distances of the Po Plain clones to increasingly more distant *P. australis* clones were evaluated by comparison with clones from Europe and adjacent parts of Africa and Asia. All belong in the *P. australis* ''core group'' retrieved in the phylogeographic study by Lambertini et al. (2006), based on the same AFLP markers. The R Package version R 3.02 (Legendre and Vaudor, 1991) was used to calculate distances of the European clones to the Boscosa wetland of the Po Plain. The European clones were divided into five groups according to their geographical distance from the Po Plain (Table 2). Group 1 included clones at a km range between 25 and 500 km, group 2 clones 500–1000 km away, group 3 clones 1000–1500 km away, and group 4 clones from 1500 to 2000 km distance. Group 5 included clones more than 2000 km away (maximum 2530 km). For each group, minimum, maximum and average genetic distances with Po Plain clones were calculated.

2.4. RAPD

Genomic DNA was extracted from dry leaves with 2× CTAB buffer (Rogers and Bendich, 1985) and Proteinase K.

Table 1
Po Plain wetlands from which *Phragmites australis* populations were sampled

Wetland	GPS coordinates		Size (ha)	Age	Previous management	Current management	Hydrographic basin	Inlet water
Bentivoglia	44 31 04 N	11 42 05 E	86	Known since early 1900	Water reserve for ricefields and used later for hunting and fishing activity	Hunting	Canale Medicina	[a]Canale Medicina + Po River
Boschetti	44 37 14 N	11 43 00 E	7	1967	Water reserve for fields	Hunting	Scolo Durazzo	[b]Precipitation
Boscosa	44 33 27 N	11 37 44 E	88	Known since early 1900	Hunting and fishing activity. Enlarged in 1992	Hunting	Po River	[c]Po River
Comune	44 43 00 N	11 31 60 E	140	Known since early 1900	Water reserve for ricefields	Hunting	Canale Navile	[d]Canale Navile + Po River
Ercolana	44 41 24 N	11 30 05 E	50	1993	Crops	Hunting	Canale Navile	[d]Canale Navile + Po River
Fracassata	44 31 13 N	11 38 48 E	33	Known since early 1900	River levee and water reserve for ricefields	Hunting	Canale Medicina	[a]Canale Medicina + Po River
Quadrone	44 31 04 N	11 42 05 E	37	Known since early 1900	River levee and water reserve for ricefields	Natural Reserve since 1997	Canale Medicina	[a]Canale Medicina + Po River
Tombe	44 41 58 N	11 28 29 E	55	1975	Ricefield till 1950. Crops to 1975. Enlarged in 1992	Hunting	Canale Navile	[d]Canale Navile + Po River

[a] Quadrone, Bentivoglia and Fracassata wetlands are in the basin of Canale Medicina and are supplied by Medicina water course during the winter. During the summer they receive water from Po river.
[b] Boschetti is supplied only by precipitation and is subject to occasional draught during the summer.
[c] Boscosa is supplied by Po river all year.
[d] Comune, Tombe and Ercolana wetlands are located in the basin of Canale Navile and are supplied by Canale Navile during the winter. During summer the wetlands receive water from Po river.

DNA was isolated with chloroform/isoamylalcohol (24:1), precipitated with isopropanol, washed with 70% ethanol and resuspended in 1× TE buffer. DNA concentration was measured with a spectrophotometer at 260 nm. DNA was diluted to a final concentration of 25 ng μL^{-1}.

PCR was performed as described by Koppitz et al. (1997), with some modifications. Two primers were used for amplification, and the procedure was repeated several times on a subset of samples to ensure reproducibility. For primer M13 (5′-GAGGGTGGCGGTTCT-3′), 2 μL of DNA solution was added to 25.5 μL sterile distilled water, 5 μL 10× polymerase buffer, 8 μL dNTP (1.25 mM), 3 μL Mg acetate (3 mM), 6 μL M13 primer (10 ng μL^{-1}), and 0.5 μL Taq (5 units μL^{-1}). The PCR amplification reaction was performed in a MJ Research-Peltier Thermal Cycler PTC-200 programmed for 42 cycles of 20 s at 93 °C, 60 s at 50 °C, 40 s at 72 °C, followed by a final termination step of 6 min at 72 °C. For primer GACA (5′-GACAGACAGACAGACA-3)′ 3 μL of DNA solution was added to 16 μL sterile distilled water, 5 μL 10× polymerase buffer, 8 μL dNTP (1.25 mM), 1.5 μL Mg acetate (3 mM), 16 μL GACA primer (10 ng μL^{-1}), and 0.5 μL Taq (5 units μL^{-1}). The PCR amplification reaction was performed with an initial step of 3 min at 94 °C, then 40 repeats of 30 s at 93 °C, 60 s at 50 °C, 40 s at 72 °C, followed by a final termination step of 6 min at 72 °C. Amplified products were separated on 1.4% agarose gels in 1× TAE buffer, which included two lanes of 100 bp ladder plus (MBI, Fermenta).

One gel was run for each primer and for each of the eight Po Plain populations. The two primers, M13 and GACA, detected a total of 37 RAPD fragments (respectively 18 and 19 fragments) that were scored visually by means of Cross Checker version 2.91 software (Buntjer, 2000).

2.5. AFLP

The protocols for DNA extraction, PCR reaction and electrophoresis are described in Lambertini et al. (2006). Three primer combinations E-ACTcy (5′-AGACTGCGTACCAATTCACT-3′) + M-CTT (5′-GATGAGTCCTGAGTAACTT-3′), E-CAGcy (5′-GACTGCGTACCAATTCCAG-3′) + M-ATG (5′-GATGAGTCCTGAGTAACAG-3′) and E-CGTcy (5′-GACTGCGTACCAATTCCGT-3′) + M-CAG (5′-GATGAGTCCTGAGTAACAG-3′) were used. In total 128 AFLP polymorphic fragments were scored.

2.6. Data analysis

The genetic diversity within and among Po Plain and Razim populations was evaluated using Popgene ver. 1.32 (Yeh et al., 1997) to calculate Nei's gene diversity (h) (Nei, 1973), Shannon's information index (I) (Lewontin, 1972) and the number and percentage of polymorphic fragments (AFLP) within and among populations. For the Po Plain populations these genetic diversity parameters were related to the size and age of the wetlands in a multiple regression analysis

Table 2
Populations of *Phragmites australis* core group (Lambertini et al., 2006) within a range of 2500 km from Po Plain

P. australis populations	No. of samples per population	State	Locality	km from Boscosa
(1) 25–500 km from Po Plain				
75IT	1	Italy	Gorgona Island	186
207IT	1	Italy	Albano S. Alessandro, Bergamo	191
79SL, 170SL	2	Slovenia	Lake Cerkniško	270
171SL, 172SL	2	Slovenia	Ljubljana	280
64DE	1	Germany	Chiemsee	374
620CZ	1	Czech Republic	Rozmberk	500
(2) 500–1000 km from Po Plain				
77HU, 663HU, 664HU, 668HU	4	Hungary	Lake Ferto	537
70FR	1	France	Campignol, Narbonne	707
640DE, 641DE	2	Germany	Lusatia mining area, Lake Plessa	778
639DE	1	Germany	Lusatia mining area, Lake Schlabendorf	824
300ES	1	Spain	Mallorca, Alcudia	872
67BE, 146BE	2	Belgium	Schelde	914
163NL	1	Holland	Verdronken Land van Saeftinghe	935
78PL	1	Poland	Krakow	946
52ES	1	Spain	el Garxal (Ebro)	998
(3) 1000–1500 km from Po Plain				
66NL, 602NL	2	Holland	Slotermeer	1002
72ES, 74ES, 95ES	3	Spain	Gallocanta	1063
209GB	1	Great Britain	Thamesmead	1150
174TN	1	Tunisia	Chenini (Gabés)	1195
609DK, 610DK	2	Denmark	Vejlerne	1249
49DK	1	Denmark	Norsminde Fjord	1278
670GB	1	Great Britain	Blacktoft Sands	1288
50DK	1	Denmark	Knebel Vig	1299
81RO, 84RO, 624RO, 625RO	4	Romania	Lake Obretinu-Mare/Oborny	1308
643RO, 650RO, 651RO, 652RO, 654RO, 655RO, 656RO, 657RO, 658RO, 659RO, 66ORO	11	Romania	Lake Razim	1366
85LI	1	Lithuania	Silute	1388
57GR	1	Greece	Creete	1495
(4) 1500–2000 km from Po Plain				
615SE	1	Sweden	Tåkern	1562
58IE	1	Ireland	Kilcock	1574
165IE	1	Ireland	Hazelhatch	1594
164IE	1	Ireland	Lake Ree	1615
83EE	1	Estonia	Lake Vortsjarv	1807
54FI, 160FI	2	Finland	Åland	1816
217FI	1	Finland	Raisionlahti, Turku	1903
159EE	1	Estonia	Lake Peipsi	1942
(5) 2000–2530 km from Po Plain				
169RU	1	Russia	St. Petersbourg	2110
141SE	1	Sweden	Luleå	2423
H13IL	1	Israel	Lake Huleh, Hulata	2426
637SE	1	Sweden	Gammelstaden	2426
90IL, 91IL	2	Israel	Yerokham, Negev Highlands	2528

Populations are ordered according to increasing geographical distance from Boscosa wetland (Po Plain) and divided in five kilometric ranges.

(StatGraphics Plus 4.1, Manugistics Inc., Rockville, MD, USA). Populations that were known since the early 1900s were assigned an age of 100 years.

The genetic structure was evaluated using AMOVA based on the distance matrix of pairwise differences with Arlequin ver. 2.000 (Schneider et al., 2000). AMOVA was calculated within and among the eight Po Plain populations, within and among all nine populations studied, including Razim, and within and among two groups, one consisting of the eight Po Plain populations and the other of the Razim population. The fixation index (F_{ST}), calculated with the θ algorithm of Weir and Cockerham (1984), indicates the extent of genetic differentiation among populations (Michalakis and Excoffier, 1996). It was calculated also for each of the AFLP fragments amplified with the aim of differentiating the Po Plain populations from the Razim population, based on the presence/absence of exclusive AFLP markers or with high F_{ST} value. The significance of the fixation indices associated with the different levels of genetic

Table 3
Genetic diversity of *Phragmites australis* in Po Plain and Razim populations

Populations	No. of clones fingerprinted by AFLP	Nei's gene diversity (*h*)	Shannon's information index (*I*)	No. of polymorphic fragments	Polymorphic fragments (%)
Within populations					
Bentivoglia	3	0.148	0.213	44	34.4
Boschetti	5	0.071	0.107	25	19.5
Boscosa	4	0.091	0.134	29	22.7
Comune	7	0.138	0.211	52	40.6
Ercolana	7	0.133	0.202	50	39.1
Fracassata	5	0.081	0.120	27	21.1
Quadrone	6	0.178	0.261	57	44.5
Tombe	6	0.139	0.208	48	37.5
Razim (RO)	11	0.157	0.241	62	48.4
Among populations					
Po Plain	43	0.169	0.272	93	72.7
Po Plain and Razim	54	0.175	0.283	99	77.3

structure was tested using non-parametric permutation procedures (1000 permutations) (Excoffier et al., 1992). The genetic structure of the investigated populations was compared with that reported in other studies by calculating the genetic structure among populations with the G_{ST} algorithm of Nei (1987) using Popgene ver. 1.32.

The correlation between population pairwise F_{ST} and geographic pairwise distances was tested in a Mantel test (Mantel, 1967; Smouse et al., 1986) using Arlequin ver. 2 based on 1000 permutations.

Neighbour-joining analysis (NJ), using PAUP* 4.0b10 (Phylogenetic Analysis Using Parsimony; Swofford, 1998) was carried out to evaluate the relationships among the Po Plain clones. The analysis was based on restriction-site distances (Nei and Li, 1979) and a *P. vallatoria* (Plunk. ex L.) Veldk. specimen was included as outgroup. The data were subject to jack-knife analysis with 37% character deletion (Farris et al., 1996), "emulate jac resampling" and 1000 replicates. Pairwise genetic distances (Nei and Li, 1979) between clones were also calculated with PAUP.

Differences in pairwise genetic distances between the increasingly geographically distant groups of *P. australis* clones from the Po Plain were tested in an ANOVA with Statgraphics Plus 4.1. Bonferroni multiple range test was applied at the 99% confidence level. Significance was also tested with a non-parametric Kruskal–Wallis test at the 95% confidence level with the same program.

3. Results

3.1. Genetic diversity

Among the 15 specimens analysed in each Po Plain population, the number of distinct genotypes ranged from 12 to 15, except for the Bentivoglia population, which was found to be monoclonal (excluding the two clones, from patches M and N, subsequently collected at the edge of the area close to the inlet river). In the other populations at least one genotype was found in each patch. In Tombe and Quadrone more than one clone was found in the same patch in two cases. No genotype was present in more than one population.

On the intrapopulation level, Nei's gene diversity (*h*) of the Po Plain populations ranged between 0.07 and 0.18, Shannon's information index (*I*) ranged between 0.11 and 0.26, the number of polymorphic fragments ranged between 25 and 57, and the percentage of polymorphic fragments ranged between 19.5 and 44.5% (Table 3). No correlation was found between these genetic parameters and size or age of the wetlands in the Po Plain. The genetic diversity of the Romanian Razim population was within the range of the Po Plain population ($h = 0.16$; $I = 0.24$) in spite of the different ploidy levels and the higher number of polymorphic fragments.

On the interpopulation level, the genetic diversity and percentage of polymorphic fragments was higher than that observed within populations. Nei's gene diversity (0.17) was within the range seen within populations. When the Razim population was included in the interpopulation analysis, all diversity measures increased (Table 3).

3.2. Genetic structure

Most of the genetic variation in the Po Plain populations occurs within (85.3%) rather than among (14.7%) populations (Table 4). The fixation index (F_{ST}) among the Po Plain populations (0.15) indicates that there is a certain degree of genetic subdivision among the eight populations, but by including the Romanian population in the analyses the F_{ST} increased only slightly. When testing the Po Plain populations against the Romanian population, the fixation index among groups of populations decreased to 0.04. Pairwise F_{ST} in the Po Plain populations ranged from 0.02 to 0.32. The Boschetti population showed, with one exception, the highest pairwise F_{ST} with the other Po Plain populations indicating some genetic separation of this population from the other Po Plain populations. Pairwise F_{ST} between the Romanian population and the eight Po Plain populations ranged between 0.10 and 0.21 and the Romanian population was found to be genetically less distinct than Boschetti from the other Po Plain populations.

Table 4
Phragmites australis: results of AMOVA and G_{ST}

Source of variation	df	Sum of squares	Variance components	% of variation
(A) Po Plain populations				
Among populations	7	129.74	1.605 Va	14.7
Within populations	38	354.92	9.340 Vb	85.3
Total	45	484.65	10.945	
Fixation index				
F_{ST}	0.147			
P (Va and F_{ST})	<0.01			
G_{ST}	0.2786			
(B) Po Plain populations + Razim population (without grouping)				
Among populations	8	162.24	1.696 Va	14.9
Within populations	48	463.46	9.655 Vb	85.01
Total	56	625.70	11.352	
Fixation index				
F_{ST}	0.149			
P (Va and F_{ST})	<0.01			
G_{ST}	0.2741			
(C) Two groups: Po Plain populations and Razim population				
Among groups	1	32.50	0.412 Va	3.6
Among populations within groups	7	129.74	1.550 Vb	13.3
Within populations	48	463.46	9.655 Vc	83.1
Total	56	625.70	11.618	
Fixation indexes				
F_{CT}	0.035	P (Va and F_{CT}) > 0.05		
F_{SC}	0.138	P (Vb and F_{SC}) < 0.01		
F_{ST}	0.169	P (Vc and F_{ST}) < 0.01		
G_{ST}	0.0795			

(A) Within and among Po Plain populations; (B) within and among nine populations (Po Plain and Razim populations); (C) within and among two groups: Po Plain populations (group 1) and Razim population (group 2). P values are based on 1023 permutations.

No correlation was detected between the F_{ST} value and the geographic distance between populations.

None of the detected AFLP fragments were unique for individual populations or groups of populations. Fragments that initially appeared exclusive to the Bentivoglia population were found to be present also in Romania, and fragments that were exclusive to octoploids and dodecaploids of the Razim population were found also in the Po Plain clones.

3.3. Genetic interrelationships at the population scale

The genetic relationships among the Po Plain clones can be seen from the NJ tree in Fig. 1. Only a few groups were well supported (jack-knife values from 96 to 100%) and these were all small: two pairs of clones from Comune, one pair of clones from Boschetti, one pair of clones from Tombe, and one pair of clones from Quadrone. The remaining supported groups were small (two to four clones) with weaker support (jack-knife values from 53 to 77), except for a large group comprising 36 Po Plain clones representing virtually all wetlands, drainage areas and irrigation regimes.

To understand the nature of the relationships among clones of the Po Plain populations, pairwise genetic distances between clones were calculated. Genetic distances among clones of different populations, tentatively considered as unrelated, range between 0.011 and 0.072. Shorter distances were found inside the populations (Table 5A). The genetic similarities between related clones group around two levels of variation. Very short distances were found between clones of the same patch at Quadrone (0.002) and at Tombe (0.004) and between clones of two neighbouring patches at Comune (0.003) and Boschetti (0.004). The pairs of clones from Quadrone, Tombe and Comune differed in two DNA fragments, and the two clones from Boschetti differed in three fragments. Genetic distances, of the same level as between unrelated clones, were found within the population of Comune (0.010). The two clones differed in six DNA fragments. The clones of different populations that were considered as unrelated and with a genetic distance between 0.011 and 0.072 differed in 7–45 DNA fragments. The Romanian population generally showed a similar genetic distance range as the Po Plain populations (Table 5A). The sibling specimens 654RO and 655RO had a genetic distance of 0.027 and differed in 21 DNA fragments, while the siblings 658RO, 659RO and 660RO had genetic distances between 0.020 and 0.029 and differed in 16–21 DNA fragments.

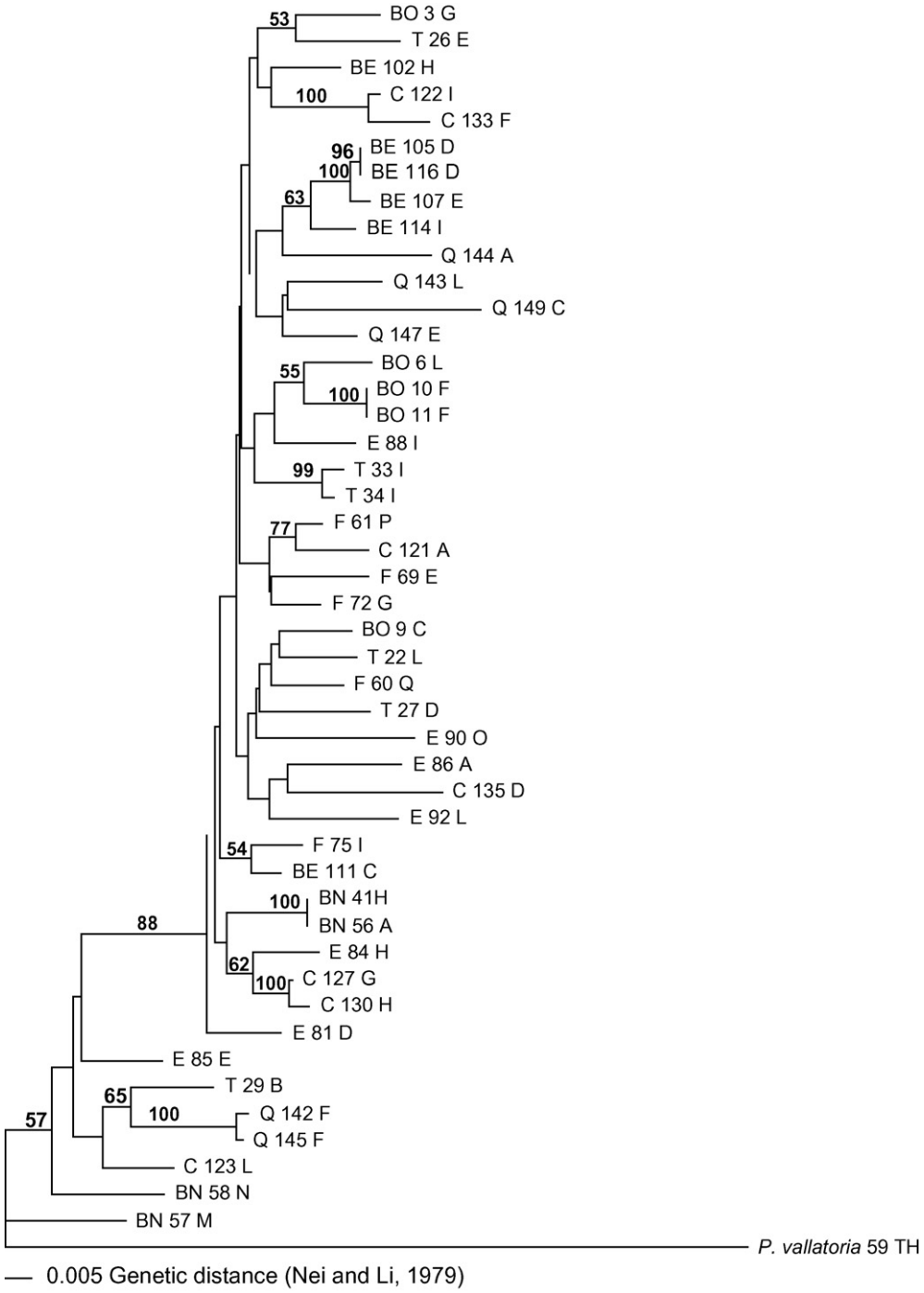

Fig. 1. Neighbour-joining tree of Po Plain *Phragmites australis* clones. Terminals are labelled with population abbreviation (BE: Boschetti, BN: Bentivoglia, BO: Boscosa, C: Comune, E: Ercolana, F: Fracassata, Q: Quadrone, T: Tombe), sample number and patch letter. Consecutive patch letters indicate neighbouring patches. When the same patch letter is associated with different sample numbers of a single population, more than one shoot was collected in that patch. BN58N and BN57M are the clones collected from Bentivoglia after RAPD analysis, close to the inlet river.

3.4. Genetic relationships at a continental scale

Irrespective of the geographical scale considered, the Po Plain clones showed a wide range of variation in genetic distance to other clones (Table 5B). Ranges of genetic distance largely overlapped between geographical distance ranges considered. As the Po Plain clones were compared to geographically successively more distant clones, the maximum genetic distances between these and the Po Plain clones remained roughly the same. In contrast, minimum genetic distance did show a considerable increase as the range of geographical distances exceeded 2000 km. Only in the >2000 km range did minimum and maximum genetic distances correspond to the shortest and longest geographical distances.

Table 5
Range of genetic distance between *Phragmites australis* populations (min., max., average and standard deviation)

Geographic distance range	No. pairwise calculations	Min. genetic distance	Clones with min. genetic distance		Km distance between clones	Max genetic distance	Clones with max. genetic distance		Km distance between clones	Average genetic distance	Standard deviation	ANOVA
(A) Within population												
Bentivoglia	3	0.030	BN57M-IT	BN58N-IT	<1	0.049	BN56A-IT	BN57M-IT	<1	0.041	0.010	
Boschetti	10	0.004	BE105E-IT	BE107D-IT	<1	0.024	BE107E-IT	BE111C-IT	<1	0.018	0.007	
Boscosa	6	0.016	BO6L-IT	BO10F-IT	<1	0.033	BO9C-IT	BO10F-IT	<1	0.026	0.007	
Comune	21	0.003	C127G-IT	C130H-IT	<1	0.059	C123L-IT	C133F-IT	<1	0.034	0.014	
Ercolana	21	0.017	E81D-IT	E84H-IT	<1	0.043	E85E-IT	E92L-IT	<1	0.032	0.008	
Fracassata	10	0.015	F61P-IT	F72G-IT	<1	0.030	F60Q-IT	F69E-IT	<1	0.020	0.006	
Quadrone	15	0.002	Q142F-IT	Q145F-IT	<1	0.064	Q142F-IT	Q144A-IT	<1	0.042	0.017	
Tombe	15	0.004	T33I-IT	T34I-IT	<1	0.048	T27D-IT	T29B-IT	<1	0.033	0.011	
Razim (RO)	56	0.019	650RO	654RO		0.047	658RO	643RO		0.032	0.007	
(B) km range from Po Plain												
0–25	899	0.011	BE111C-IT	F75I-IT	9	0.072	T29B-IT	IT35C	4	0.035	0.012	a
25–500	343	0.016	F72G-1T	620CZ	500	0.064	BN57M-IT	171SL	280	0.035	0.009	a
500–1000	635	0.014	BE111C-IT	146BE	914	0.068	BN57M-IT	70FR	707	0.038	0.010	b
1000–1500	1247	0.013	F75I-IT	670GB	1288	0.067	BN58N-IT	656RO	1366	0.038	0.010	b
1500–2000	387	0.019	BO10F-IT	164IE	1615	0.079	BN57M-IT	615SE	1562	0.042	0.010	c
2000–2530	257	0.044	F72G-1T	169RU	2110	0.074	BN57M-IT	90IL	2530	0.044	0.011	c

(A) Within Po Plain and Razim populations. Clones are labelled with population abbreviation (BE: Boschetti, BN: Bentivoglia, BO: Boscosa, C: Comune, E: Ercolana, F: Fracassata, Q: Quadrone, T: Tombe), sample number, patch letter and country acronym; (B) between Po Plain clones and geographically distant clones. Clones are labelled with sample reference and country acronym. ANOVA results are based on Bonferroni multiple range test at the 99% confidence level.

The average genetic distance increased slightly with increasing geographical distance. Standard deviation remained nearly constant between geographical ranges. Analysis of variance revealed highly significant differences in the genetic distances under and over 500 km, and under and over 1500 km of range.

4. Discussion

4.1. Genetic diversity

Seven of the eight *P. australis* populations studied in the Po Plain were polyclonal, but one fairly large wetland (86 ha) at Bentivoglia was found to be monoclonal. This wetland has probably never been drained. The patches from which the specimens were collected are in the oldest part of the wetland, which is managed exclusively for hunting purposes. These patches have been frequently cut, but rhizomes have not been removed in the last 50 years, at least, and presumably never. Two additional clones were found close to the inlet river where disturbance and opportunity for seedling establishment is higher. The low genetic variability in the Bentivoglia population can probably be explained by the rather stable environmental conditions over a long time period, as has also been observed for other monodominant clonal species (Watkinson and Powell, 1993). In the polyclonal populations clonal variability is high, and every *P. australis* patch consists of at least one distinct genotype. In two out of seven cases there were two different genotypes in the same patch. The polyclonal wetlands are comparatively young, often restored from rice fields or other croplands within the last 30 years, and disturbance occurs very frequently. The genotypes found in the eight Po Plain wetlands are exclusive to each *P. australis* population and were not found in other patches.

Within the Po Plain populations Quadrone, a river levee that has been a nature reserve since 1997, has the most diverse population. The management of this wetland is aimed at keeping channels and ponds free of vegetation, allowing high flow of water at any time of the year. The expansion of *P. australis* stands is therefore strictly controlled. In recent years part of the wetland has been drained, which may have created opportunities for the recruitment and establishment of new clones. Boschetti, on the other hand, is the least genetically diverse population, which is in accordance with its small size and lower level of human disturbance. No correlation however was found between age and/or size of wetland and genetic diversity of the *P. australis* populations, although it should be noted that sample size is small and wetland age in some cases probably underestimated. Hence, it is probably the human impact associated with the management of the wetlands that are of importance for the genetic diversity in the Po Plain populations.

The Romanian Razim population has a genetic diversity at the same level as that of the Po Plain populations. Lake Razim is located in the Danube Delta and has been a UNESCO biosphere reserve since 1999. Regrettably, very little information is available on present and past disturbance in the study area. It is surprising to note that populations so geographically distant and so different in terms of climate and environmental conditions in general as Lake Razim and the Po Plain, have the same extent of genetic diversity. Higher genetic diversity (Nei's gene diversity between 0.08 and 0.30) has been found by Guo et al. (2003) in 15 *P. australis* populations in the Yellow River Delta in China. A diversity-generating factor in the delta is the advancement of the shoreline, which creates suitable habitats for the establishment of *P. australis* clones.

The interspecific variation in the percentage of polymorphic loci (Ps) in plants is largely explained by the breeding systems and seed dispersal mechanisms (Hamrick and Godt, 1996). Mean levels of Ps for outcrossing and wind-dispersed plant species are 62.4, and 62.7 for Poaceae species. Lower Ps levels were observed within the Po Plain population (from 19.5 to 44.5) and the Razim populations (48.4), while higher values were registered on the interpopulation level (72.7 among the Po Plain populations; 77.3 including the Razim population). It is interesting to note how each population contributes a number of different loci (and different genotypes) to the gene pool of *P. australis* in the Po Plain.

4.2. Genetic structure

The analysis of molecular variance shows that most of the genetic variation is within populations, i.e. among the clones of each population, which is typical of long-lived, outcrossing and late successional taxa (Nybom and Bartish, 2000). The F_{ST} values at the interpopulation level are relatively high compared to various plant groups and categories described by Hamrick and Godt (1996). G_{ST} values are close to the average for Poaceae species (0.28) but higher than the average level of genetic variation among populations of outcrossing and wind-dispersed plant species (0.10), outcrossing monocots (0.16), outcrossing and widespread plant species (0.17), and perennial outcrossing plant species (0.09). A certain degree of genetic structure seems therefore to be present among the *P. australis* populations investigated in this study, which seems to have little to do with the geographical distribution of the populations. For comparison, differentiation among populations of another clonal, emergent aquatic plant, *Typha latifolia*, was one order of magnitude lower ($F_{ST} = 0.013$) in populations of different hydrographic basins in the Flanders (Lamote et al., 2005). The least genetically diverse population of Boschetti is also the most genetically isolated population as indicated by the pairwise F_{ST} among populations. It can be speculated that the genetic isolation is a result of the hydrographic isolation of this wetland from the network of wetlands to which the other investigated populations in the Po Plain belong. Boscosa, which like all other investigated populations in the Po Plain, is supplied by the Po river, also showed relatively high F_{ST} values with the other populations and, like Boschetti, was not exceptional with respect to the extent of genetic diversity within populations (Table 3). At the regional level the genetic structure observed on the interpopulation level collapsed. Fixation indices decreased radically and geographically very distant populations were found to be genetically very similar, indicating possible gene flow between the populations. This pattern can be explained by

the lack of gene flow within and between populations of the same gene pool after the ''propagation and establishment stage'' (Koppitz and Kühl, 2000). With time, the different contingents of genotypes present in each population, and different selection pressure, differentiate populations. The competitive exclusion of clones can affect the genetic diversity by reducing the number of genotypes. This could explain why the least genetically diverse populations are also the most isolated ones. Weak gene flow at the population level was documented also by Guo et al. (2003) in the populations of Yellow River Delta. By contrast, in the clonal species *Cirsium arvense* (Asteraceae) the amount of population differentiation was similar between founder and established populations indicating that selection acts mostly in the very early stage of succession (Solè et al., 2004). A pattern more similar to that of *P. australis* in the Po Plain in terms of genetic diversity and distribution of genetic variation was found in the riparian perennial *Silene tatarica* in northern Finland (Tero et al., 2003).

4.3. Genetic interrelationships at the population scale

The genetic differences detected among the Po Plain clones show that clones within one population are very different. A few close relationships are suggested by the NJ tree (mostly pairs) and these are characterized by very short genetic distances. The clones showing the shortest genetic distances were collected either from the same patch (Q142F–Q145F and T33I–T34I) or from two neighbouring patches (BE105D–BE107E and C127G–C130H) 10 m apart, separated by an open area with short wetland vegetation. The two patches may have been connected by rhizomes. The specimens differ for just two and three AFLP fragments out of 128, have different RAPD patterns and DNA was extracted twice (for RAPD and AFLP analyses). A possible explanation for these minimal genetic differences could be somatic mutations, which are known to occur in several clonal species (Klekowski, 1997), including *P. australis* (Connor et al., 1998). Another possibility which has support from preliminary microsatellites results of cross-pollination experiments (Lambertini et al., unpublished), could be self-pollination followed by establishment of selfed seeds.

An interesting case of an intermediate level of difference between a pair of clones within a population is seen at Comune. The two clones (C122I–C133F) were collected in distant and evidently disconnected patches and their genetic distance of 0.010 is of the same order of magnitude as between clones of different Po Plain populations, but in contrast to these, the relationship between the two clones from Comune has 100% jack-knife support. The genetic distance is more than twice that found in the aforementioned pairs of clones where somatic mutation or self-pollination were suspected. The nature of the relationship remains to be determined, but it could perhaps represent a descent or sibling relationship. The known cases of sibling relationships in the Razim population show, however, greater genetic differences. This may not be so surprising, as the degree of similarity between siblings obviously varies as a function of the genetic similarity between the parents and stochastically through recombination.

4.4. Genetic relationships at a continental scale

Samples analysed in the present study were included in an AFLP-based phylogeographic study of the genus *Phragmites* (Lambertini et al., 2006). Samples from the Po Plain and Razim Lake formed part of a large group within *P. australis* in which relationships among clones were largely uncertain. This group comprised all European clones belonging to *P. australis*, along with several clones from other continents. Apart from some minor clusters of Po Plain clones (consisting of pairs of clones with very short genetic distances), most of these appeared to have their closest relatives in other parts of Europe rather than in the same or nearby wetlands. Also Razim Lake sibling clones showed more genetic similarities with clones from other regions or continents than between themselves.

The present study shows that the genetic distances between the Po Plain clones and geographically distant clones increase significantly with clones over 500 km away. Additional significant increase in genetic distance is observed when clones over 1500 km were considered. Variation is, however, considerable, and even among clones more than 1500 km from the Po Plain, some have genetic distances shorter than between neighbouring clones in the Po Plain. This indicates that gene flow does occur within these distance ranges, but with a lower frequency. The ''stepwise'' increase in genetic distances with increasing geographical distance may indicate the presence of some kind of genetic pattern at a very large geographical scale.

Possible explanations for an apparently continent-wide gene pool would include the possibility of long-distance dispersal by seeds or gene flow by pollen. The reproductive biology of *P. australis* is still not fully resolved (Ishii and Kadono, 2002), but it is clear that pollen can be carried long distances by air. Seeds can be dispersed by air or water (Coops and Van der Velde, 1995) or stick to the feather of migratory birds. New wetlands are often colonized very quickly by *P. australis*, indicating that many seeds are transported either by air or water. Additional indications of the efficiency of the dispersal of *P. australis* are its virtually cosmopolitan distribution, its occurrence in isolated patches of suitable habitat such as oceanic islands (Saltonstall, 2002), and its presence in nearly every lake, pond, river, and marsh in the northern temperate zone. The frequency of such long-distance dispersal and pollination events remains to be determined, but our data suggest that all *P. australis* populations continent wide in Europe are one single meta-population. Deliberate introduction and unintentional dispersal by humans could explain some of the remarkable trans-continental affinities between clones, and could be expected to partly disrupt geographical variation patterns. Nevertheless, we find support for the existence of a weak geographical pattern, but on a very large scale only.

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