Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology

Official Journal of the Societa Botanica Italiana

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tplb20

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To cite this article: Lucia Conte , Daniele Dallai , Andrea Modica , Giovanna Pezzi , Christian Rebecchi & Fabrizio Buldrini (2020): A littoral grass growing inland: genetic diversity of *Puccinellia fasciculata* around mud volcanoes in Italy, Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, DOI: 10.1080/11263504.2020.1845847

To link to this article: https://doi.org/10.1080/11263504.2020.1845847

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https://doi.org/10.1080/11263504.2020.1845847

A littoral grass growing inland: genetic diversity of *Puccinellia fasciculata* around mud volcanoes in Italy

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ABSTRACT

Puccinellia fasciculata is a littoral miohalophyte with a Mediterranean-Atlantic distribution, uncommonly found in inland sites. The main Italian inland population is located in the Salse di Nirano Reserve, an area internationally renowned for the phenomenon of mud volcanoes. In this study, molecular markers are used to characterize this population and its possible relationships with the nearest littoral conspecific population. A survey of ISSR markers revealed low levels of genetic diversity ($He = 0.081$ in the inland population, 0.105 in the littoral one) and a weak genetic differentiation ($Gst = 0.24$ within the inland population, 0.28 among this and the littoral one). A sequence analysis of three non-coding regions of chloroplast DNA found no genetic differentiation both within the inland population and between the two populations, and revealed a common origin for the two dating back to the middle Holocene. The apparent incongruence between the results from the two approaches may be explained by differences between ISSR and non-coding cpDNA markers in capturing signatures related to gene flow; their integrated information implies a mixed reproductive strategy and a common evolutionary history for the two examined populations. Effective conservation strategies are recommended for the inland population of *P. fasciculata* and its habitat.

ARTICLE HISTORY
Received 23 May 2020
Accepted 29 October 2020

KEYWORDS
Gene flow; genetic drift; ISSR; miohalophyte; MRCA; *rpL*16 intron; *rpo*B-*trn*C; *trn*C-*trn*D

Introduction

Halophytic plant communities are found in two major types of halophytic habitats: the inland (continental) and coastal (maritime). The inland halophytic vegetation develops in response to local soil and hydrological conditions, and is characterized by high landscape-scale patchiness and rapid shifts among vegetation types due to uneven salt accumulation in the soil and water supply, various tolerance responses of plant species to salt stress and different human disturbances. European inland saline habitats mainly belong to the Pannonian biogeographical region, characterized by solonetz soils. Recent studies on the inland halophytic vegetation of central and southern Europe are focused on alkaline wetlands and salt marshes, dry alkaline grasslands, saline meadows and the management of various types of vegetation of salt-affected soils (Stevanović et al. 2019 and authors therein).

The review by Weising and Freitag (2007), summarizing phylogeographic work on halophytes growing on both inland and coastal salt sites in Europe, already revealed a striking diversity about their phylogeographic patterns. Studies addressing issues like genetic diversity, gene flow and genetic structure of coastal and inland halophyte populations by means of molecular markers are in line with their conclusions. Lambracht et al. (2007) recognized two major genetic groups in the analyzed populations of the perennial herb *Triglochin maritima* L., concluding that the extant populations of this species must have reached the North and Baltic Sea coasts from inland areas. A clear separation was detected also among populations of *Spergularia media* (L.) C.Presl from inland and coastal salt sites of Germany, the Netherlands, Denmark, Austria, France and Italy by Prinz et al. (2010), and explained by the isolated nature of suitable inland salt habitats with concomitant reduction of gene flow to and among these sites. Conversely, lack of a sharp genetic structure was reported in populations of *Suaeda maritima* (L.) Dumort. growing in central Germany and along the coasts of the North Sea and the Baltic Sea by Prinz et al. (2009), suggesting that the investigated populations and regions are connected by considerable gene flow, perhaps through long-distance seed dispersal by water flow.

In the Italian North Adriatic coastal regions (Friuli-Venezia Giulia, Veneto and Emilia-Romagna) there is an extensive floristic and vegetational mosaic typical of lagoon systems in which it is possible to distinguish six prevalent plant communities, including the saline and brackish meadows developing on soils from wet to periodically flooded and

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Supplemental data for this article is available online at https://doi.org/10.1080/11263504.2020.1845847.

Published online 07 Dec 2020

dominated by hemicryptophytes, such as *Puccinellia fasciculata* (Torr.) E.P.Bicknell (Blasi and Biondi 2017).

Puccinellia fasciculata (Borrer's saltmarsh-grass) is a perennial clonal miohalophyte (Miselli et al. 1991), specialized in colonizing habitats characterized by water flows and soil salinity variations, therefore, it is found along coastal wetlands that are flooded and drained by salt water brought in by the tides; however, it is able to occupy also inland brackish or saline areas, where NaCl content has strong seasonal variations related to different rainfall amounts. Its native range spans from western Europe to western and central Mediterranean areas, with extensions into the Black Sea region (van der Maarel and van der Maarel-Versluys 1996), although it has been introduced in southern Africa, southern Australia, New Zealand, the eastern coast of North America and south-west of United States.

In Italy it is discontinuously distributed on the northern-Adriatic coasts from Friuli-Venezia Giulia to Abruzzo and on the coastal areas of Tuscany, Sicily and Sardinia (Pignatti et al. 2017). The region Emilia-Romagna hosts the only three inland populations of *P. fasciculata* today known in Italy; they characterize halophytic grasslands occurring in natural brackish soils, two of which ascribed to the priority habitat 1340* - Inland salt meadows (European Commission 2013). All three populations are close to mud volcanoes, geological structures formed by the ascent to the earth's surface of connate saline waters and called "salse", since the extrusive mud formations have a medium size and lack solid blocks in the argillaceous emissions (Ranjbaran and Sotohian 2015). The largest one of the three inland populations is found in the Salse di Nirano Regional Natural Reserve, located in the low hill of the northern Apennines, about 20 kilometres from Modena city centre, already protected as "Geosite" and included in the Site of Community Importance IT4040007 (Council Directive 92/43/EEC 1992).

P. fasciculata is the flag species of the Reserve, growing as a pioneer in four spatially distinct subpopulations around as many different extrusive cones shaped by a more intense venting activity, that is marked by intermittent emission of cold liquid mud mixed with clay, methane, minor oil scum and gas bubbles escaping from central craters and running down the sides of the cones themselves (Castaldini et al. 2011; Castaldini and Conventi 2017; De Nardo 2019). At regional level *P. fasciculata* is listed among the species of European interest for the conservation of floristic diversity and categorized as vulnerable (Ambiente Regione Emilia-Romagna 2017) according to IUCN guidelines (IUCN 2012), since its restricted and fragmented distribution might still decrease, as a result of possible fluctuations in the area of occupancy due to natural or anthropogenic disturbance (i.e., submersion of the plants by mud flows, adverse trampling impact in non-protected areas of the Reserve).

Small and isolated populations are expected to suffer from genetic erosion and increasing genetic divergence, through the effects of random genetic drift, high levels of inbreeding and decreased gene flow, potentially leading to reduced possibilities of recovery in the future (Jacquemyn et al. 2007). Over time, also the *P. fasciculata* population located in the Reserve might undergo genetic depauperation and limit its ability to adapt to environmental changes, eventually becoming more vulnerable to local extinction events under novel selection pressures. Therefore, it is of primary importance to evaluate the genetic variation in this population both in its overall amount and distribution among subpopulations. Moreover, the existence itself of the Reserve population of *P. fasciculata* is an intriguing topic: little is known about its possible origin and time of colonization of this inland salt site by the species. Undoubtedly, until the middle of the last century, the species was more widespread, at least in northern Italy, and was detected in various inland sites, consisting of saline springs, sub-saline meadows or brackish marshes, situated in the Po valley from Piedmont to Veneto, but also at higher altitude, on the hills closing the valley on its west side, on the slopes of the Euganean hills (Veneto) and on the pre-Apennine hills adjacent to the valley (Bertolani Marchetti 1954). Man-induced modifications of environmental conditions, such as increased urbanization and agricultural practices, construction of thermal complexes or reclamation works draining marshes and saline meadows, have reduced habitat suitability for this miohalophyte, no longer found in most of the aforementioned inland sites; nevertheless, a common evolutionary background may not be excluded for the Reserve population and other still extant stands of *P. fasciculata*.

Mud volcanoes are found almost everywhere on Earth, most of all in the Mediterranean and Tethyan regions (Kopf 2002), and some attention has been directed towards the natural vegetation associated with them: vegetation and species diversity patterns have been studied in the mud volcanoes of the Sakhalin island, Crimean Peninsula and Azerbaijan (Korznikov 2018 and authors therein; Isayeva 2019); also, several coastal plant species were found in the natural vegetation surrounding mud volcanoes in south Trinidad (Comeau 1993-1994), and mud volcano plant species with coastal affinity were noted around two mud volcanoes in north-east Borneo (Ting and Poulsen 2009). However, to our knowledge, no studies have so far exploited molecular markers to focus on issues of genetic variation, gene flow and biogeographic patterns of species associated to mud volcanoes.

Here *P. fasciculata* growing in the Salse di Nirano Regional Natural Reserve is used as a model species to assess population genetic parameters and gene flow extent of a miohalophyte surrounding mud volcanoes craters; moreover, this inland population has been compared to the nearest littoral conspecific population, located at a distance of 130 km, in an attempt to gain information about their biogeographic evolution through time.

Two different molecular markers were employed in the analyses: the nuclear markers ISSR (inter-simple sequence repeats), allowing the estimation of neutral genetic variation, and its partition, as a result of the balance between pollen/seed-mediated gene flow and genetic drift, and the sequencing of non-coding regions of chloroplast DNA (NC-cpDNA), that capture signatures from exclusively seed-mediated gene flow, because of the chloroplast cytoplasmic inheritance

typical of most angiosperms. Although the ISSR technique has several limitations, such as dominance and uncertain locus homology, it has an advantage of surveying the entire genome, providing high level of allelic variability, so it is particularly helpful in evaluating genetic polymorphisms among closely related individuals; also, it indirectly gives insight into reproductive modes influencing the variation pattern (Puglia et al. 2018). By contrast, chloroplast DNA is characterized by low mutation rate and is only affected by the process of seed dispersal; therefore, analyses based on cpDNA sequences are indicative of population range shifts and allow to make inferences about populations history through the application of phylogeographic analyses (Kohrn et al. 2017). Such an integrate approach produces both contemporary and historical information about the patterns of population genetic diversity, related to geographic and environmental features, and has been successfully applied in studying species of phylogeographic, taxonomic or conservational interest (Li et al. 2008; Qiu et al. 2009; Ferreira et al. 2015; Vyšniauskienė et al. 2015; Puglia et al. 2018). Moreover, the basis of species conservation is indeed the maintenance of genetic variation in populations; thus, knowing the natural genetic diversity of a species of interest would be a crucial step toward the development of conservation strategies (Oliveira et al. 2012).

We expected to find low levels of genetic diversity in the populations of *P. fasciculata*, due to the habitat fragmentation and reduction experienced by the species in the Po valley; moreover, since the very scattered occurrence of the species today may be interpreted as a remnant of a once much wider distribution range in northern Italy, inland and coastal populations could still retain traces of ancient interactions. Therefore, in the current study, we focused on the following objectives: to describe the spatial pattern of the *P. fasciculata* population located in the Salse di Nirano Regional Natural Reserve; to assess the amount of genetic diversity maintained and distributed in this inland population; to compare diversity estimates from the Reserve population with those concerning a small sample from a littoral stand of *P. fasciculata*; to infer indirect information about reproductive system and phylogeographic history of the two examined populations; to provide basic knowledge to support conservation measures needed to protect the species in the Reserve.

Materials and methods

Study species

P. fasciculata (Supplemental online material 1) is a caespitose and rhizomatous hemicryptophyte, with a compact tuft (Hughes 1976; Alonso et al. 2010). Its erect or geniculate ascending culms, spanning from 6 to 60 cm, are enveloped at the base by desiccated leaf sheaths. Leaves are planate, up to 16 cm with an eciliate, membranous ligule (Clayton et al. 2006 onwards; Pignatti et al. 2017). The secondary inflorescence is a panicle, 2.5-16 cm long, with sterile apical spikelets and basal fertile 6-8 flowered spikelets, disarticulating at maturity. Flowers are hermaphrodite, with 3 anthers (0.3-1 mm long) and a glabrous ovary, which develops an ellipsoid caryopsis 1.5 mm long (POWO 2019). Pollen grains, oval to spheroidal, measure 24.70-32.90 μm in diameter (Julià and Villodre 1994). The species is tetraploid ($2n = 4x = 28$, Jones and Newton 1970) and shows various reproductive strategies, other than allogamy mediated by anemophilous pollination during the flowering period (from June to August). Jones and Newton (1970) reported that, during the anthesis, the anthers may become dehiscent without completely protruding from the glumes, thus making possible self-pollination events within the same floret or spikelet. Moreover, its rhizomes, acting as a mean of vegetative reproduction, allow a quick propagation (Alonso et al. 2010). Hydrochory and anemochory have been reported as the preferential mode for seed dispersion (Dausse et al. 2008; Invasive.Org 2018). Some evidence for ornithochory comes from data on stomach contents of dabbling ducks in Europe, suggesting that seed of this species might be indeed bird-disseminated (Green et al. 2016).

Study sites

The study focused on the inland population of *P. fasciculata* located in the Salse di Nirano Regional Natural Reserve (hereafter SN), and included four sampling sites corresponding to the four subpopulations growing around the main venting cones (Figure 1, Table 1). To obtain a picture of the distribution of *P. fasciculata* in the Reserve, an evaluation of the spatial extent of the SN subpopulations was made in the years 2015-2017, using a Topcon GTS-303 Total Station. The areas with *P. fasciculata* were visually distinguished and their perimeters traced through 1295 geo-referenced points taken in the field. Such points were used to build a polygon shapefile in Quantum Geographic Information System software (http://qgis.osgeo.org). In 2015, *P. fasciculata* occupied an area of 3233 m^2 and the extent of the subpopulations varied between 322 and 1481 m^2; in 2016, the species occupied an area of 1770 m^2 and the extent of the subpopulations varied between 222 and 724 m^2; in 2017, it occupied an area of 4170 m^2 and the extent of the subpopulations varied between 494 and 1728 m^2 (Supplemental online material 2). Maps indicating the spatial distribution of the subpopulations of *P. fasciculata* around the four venting cones are reported in Supplemental online material 3. The high salinity of the soil, and the unpredictable direction of mud flowing from the craters, makes the areas immediately around the craters an extreme environment, where only isolated individuals of *P. fasciculata* may be found. Beyond this mostly aphytoic area, the soil salinity, fluctuating from 2 to 6 g/l for the periodical rainfall, is still too high for non-halophilous species and too variable for strict halophytes: here, the shifting salinity and the absence of competitors allow the growth of more numerous plants of *P. fasciculata*, scattered and irregularly dispersed in a roughly circular area around each cone. A more external zone, where soil salinity is lower than in the former one, is covered by other less salt-tolerant species, principally *Elymus athericus* (Link) Kerguélen, along with *Atriplex patula* L., *Lotus tenuis* Waldst. & Kit. ex Willd. and *Bupleurum tenuissimum* L. Out of this third zone, the

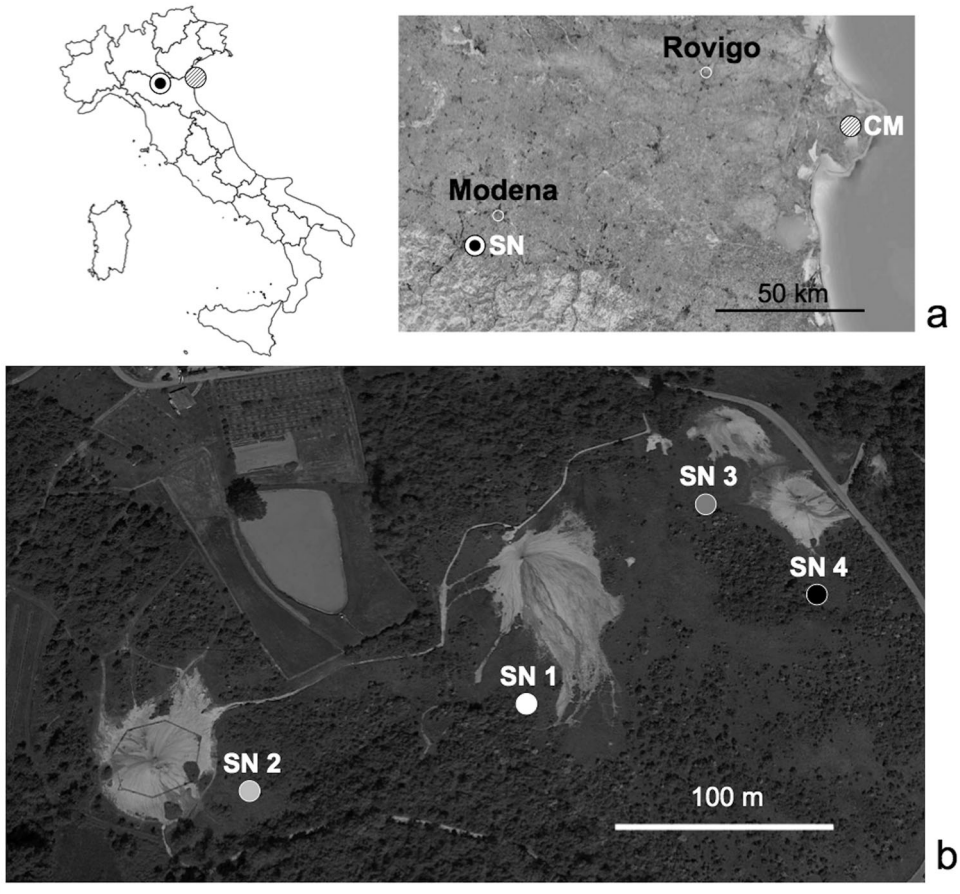

Figure 1. *Puccinellia fasciculata*. (a) Geographical location of the inland (SN) and littoral (CM) populations considered in this study. (b) SN sampled subpopulations (from SN1 to SN4) identified by codes given in Table 1.

Table 1. Details of populations and subpopulations of *Puccinellia fasciculata* sampled for the ISSR and the NC cpDNA analyses.

Population	Subpopulation code	Province, Region	Latitude N	Longitude E	N*	N**
Salse di Nirano Regional	SN1	Modena, Emilia-Romagna	44°30′49"	10°49′25"	20	5
Natural Reserve (SN)	SN2	Modena, Emilia-Romagna	44°30′46"	10°49′17"	20	5
	SN3	Modena, Emilia-Romagna	44°30′52"	10°49′30"	20	5
	SN4	Modena, Emilia-Romagna	44°30′50"	10°49′32"	20	5
Ca′ Mello (CM)		Rovigo, Veneto	44°53′28"	12°23′58"	20	5

N*: sample size for the ISSR analysis; N**: sample size for the NC-cpDNA analysis.

vegetation is represented by a semi-ruderal poliphytic arid grassland (Dallai et al. 2016).

A second focus was upon a population chosen as the coastal stand of *P. fasciculata* currently most contiguous to SN, located at a distance of 130 km from it, within the Regional Park of the Po Delta (locality Ca' Mello, about 50 kilometres from Rovigo, Veneto region), hereafter indicated as CM (Figure 1, Table 1). This fifth sampling site consists of brackish, depressed (-3 m a.s.l.) land-reclamation terrains, situated at about 80 m from the coastline of the Adriatic Sea, and affected by seepage of saline water. Here, *P. fasciculata* grows in sparse individuals together with *Puccinellia festuciformis* (Host) Parl., *Salicornia* spp. and *Atriplex patula*, around a small lake of ca. 30 × 100 m.

Sampling design and DNA extraction

Leaves were collected from 20 individuals for each sampling site in the SN area, for a total amount of 80 samples; individuals were chosen in the portions common to the areas covered by the species over the 3-year period; non-destructive sampling was conducted in two subsequent years (2018, 2019) during the vegetative season of the species, from October to February, in order to have fresh leaf tissue, more effective for DNA extraction.

Twenty individuals sampled in a single site from the CM area were included in the survey (Table 1); therefore, for the purpose of this study, the terms "site" and "area" are interchangeable for the CM sampling location. A complete voucher specimen was collected per population and stored at the Herbarium of Bologna (BOLO).

Leaves were collected from plants located at a distance of at least 2 m, to reduce the likelihood of sampling clonal individuals, and placed in silica gel. After lyophilisation, total genomic DNA was extracted from dried leaf tissue using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. DNA quality and quantity were assessed by spectrophotometry (absorbance at 260 nm; BioPhotometer,

Table 2. ISSR and NC-cpDNA primers used to analyse, respectively, 100 and 25 individuals of *Puccinellia fasciculata*, with relative sequence and annealing temperature.

primer name	cpDNA region	Sequence (5′-3′)	Annealing temperature
UBC-840 [a]		$(GA)_8YT$	50 °C
UBC-845 [a]		$(CT)_8RG$	45 °C
UBC-856 [a]		$(AC)_8YA$	53 °C
UBC-868 [a]		$(GAA)_6$	48 °C
F71	*rpL*16 intron [b]	5′-GCTATGCTTAGTGTGTGACTCGTTG-3′	58.5 °C
R1516		5′-CCCTTCATTCTTCCTCTATGTTG-3′	
$trnC^{GCA}$	*trn*C-*trn*D [c]	5′-CCAGTTCAAATCTGGGTGTC-3′	58.5 °C
$trnD^{GCU}$		5′-GGGATTGTAGTTCAATTGGT-3′	
rpoB	*rpo*B-*trn*C [d]	5′-CKACAAAAYCCYTCRAATTG-3′	58.5 °C
$trnC^{GCA}$		5′-CACCCRGATTYGAACTGGGG-3′	

[a]Set no. 9, Biotechnology Laboratory, UBC.
[b]Small et al. (1998).
[c]Demesure et al. (1995).
[d]Shaw et al. (2005).

Eppendorf). All 100 samples were surveyed for ISSR, while a subset of 25 individuals (5 for each sampling site) was sequenced at three NC-cpDNA regions.

ISSR fingerprint and data analysis

The ISSR analysis followed standard procedures. The following 12 different ISSRs primers from the UBC set no. 9 (Biotechnology Laboratory, University of British Columbia) were tested on one individual from each sample site: 813 [$(CT)_8T$], 814 [$(CT)_8A$], 823 [$(TC)_8C$], 827 [$(AC)_8G$], 836 [$(AG)_8YA$], 840 [$(GA)_8YT$], 845 [$(CT)_8RG$], 848 [$(CA)_8RG$], 852 [$(TC)_8RA$], 856 [$(AC)_8YA$], 858 [$(TG)_8RT$] and 868 [$(GAA)_6$]; four of them, showing the most informative, readable, and repeatable profiles, were selected for the entire analysis (Table 2). 30 ng of template DNA and 40 pM of the required primer were added to each dried reaction mixture purchased by "PuRe Taq Ready-To-Go PCR Beads" kit (GE Healthcare, Life Sciences), along with distilled water, up to a final volume of 25 µl. Amplification reactions were carried out in an MJ Mini thermal cycler (Bio-Rad) with the following PCR profile: initial denaturation (7 min, 94 °C), 45 cycles of denaturation (1 min, 94 °C), annealing (1 min, 45 °C for 845, 48 °C for 868, 50 °C for 840, 53 °C for 856) and extension (2 min, 72 °C), plus a final extension step (7 min, 72 °C). Afterwards, PCR products were separated at 90 V for 2 h 45 min on 1% agarose gel (1x TAE buffer) and stained with SYBR Safe (Invitrogen), using the standard 1 kb DNA ladder (Jena Bioscience) as a size reference. Each gel was then photographed under ultraviolet light (Ultra lum Inc.) obtaining a permanent scanned record available for subsequent automated scoring. Band analysis was carried out using the image analysis software GelAnalyzer 19.1 (http://www.gelanalyzer.com); only non-overlapping and highly reproducible bands were considered as detectable fragments, scored as either present (1) or absent (0), and entered into a binary matrix.

Analysis of the ISSR binary profiles was performed by using software packages able to handle both co-dominant and dominant markers or specifically developed for dominant markers. To estimate genetic diversity, percentages of both private bands (*pb*%) and polymorphic fragments (*P*%), Nei's gene diversity (or expected heterozygosity, *He*), and Shannon information index (*I*) were calculated with GenAlEx 6.5 (https://biology-assets.anu.edu.au/GenAlEx/Welcome.html); these parameters, together with the average number of bands (*Nb*), were measured both for each SN sampling site (subpopulation level) and on the whole data set from the two areas SN and CM (population level). The coefficient of genetic differentiation (*Gst*), based on Nei's gene diversity, and the amount of gene flow [$Nm = (1 - Gst)/4Gst$] were also estimated for both the inland area and between the two areas. Estimates of hierarchical genetic structure were obtained with the same software, through the analysis of molecular variance AMOVA at three different levels (within sampling sites, among sampling sites, and among areas), by computing 999 random replicates. Mantel regression of the pairwise relationship between genetic and geographical distances, implemented in the same software, was repeated both for the two sampled areas and for the four SN sampling sites, with significance of the autocorrelation coefficient tested by 999 resamplings. In addition, to visualize the genetic relationships among individuals sampled from both the areas, according to their molecular multilocus ISSR profiles, two different approaches were used. At first, samples were grouped, without prior knowledge of their source location, applying an UPGMA clustering analysis based on Dice similarity matrix (Dice 1945), with 999 bootstraps, using PAST 4.02 (https://folk.uio.no/ohammer/past/). Then, a Bayesian admixture analysis, implemented in STRUCTURE 2.3.4 (https://web.stanford.edu/group/pritchardlab/structure.html), was carried out to infer the most likely number of clusters (K) of genetically similar individuals and the individual percentages of membership assigned to them. Probabilities for a range of K starting from 1 to the number of sampled sites plus 3 (K = 1-8), were examined selecting an initial burn-in period of 10,000 iterations (followed by 100,000 further MCMC generations replicated 10 times) and choosing default settings for the remaining parameters (admixture ancestry model, correlated allele frequencies). The optimal value for K was determined using Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/), and the ΔK statistic method as described in Evanno et al. (2005).

NC-cpDNA sequencing and data analysis

For this study, 7 primer pairs were preliminarily tested on a single specimen of *P. fasciculata* for the following NC-cpDNA

Table 3. Genetic diversity in *Puccinellia fasciculata* determined by ISSR markers at SN sites (subpopulation level, based on 124 loci) and at SN and CM areas (population level, based on 160 loci). Site and area codes follow Table 1 and Fig. 1. N: sample size for each site/area; *Nb*: number of bands; *pb*%: percentage of private bands; *P*%: percentage of polymorphic loci; *I*: Shannon diversity index; *He*: Nei's gene diversity; *Gst*: coefficient of genetic differentiation; *Nm*: gene flow.

Site/Area	N	*Nb*	*pb*%	*P*%	*I*	*He*	*Gst*	*Nm*
SN1	20	25	10.53	37.90	0.144	0.090	0.24	0.79
SN2	20	28	24.64	46.77	0.175	0.107		
SN3	20	27	28.75	60.48	0.220	0.134		
SN4	20	25	11.32	36.29	0.143	0.090		
average	*20*	**26**	**18.81**	**45.36**	**0.171**	**0.105**		
SN	80	26	15.22	35.16	0.132	0.081	0.28	0.65
CM	20	27	46.15	43.75	0.170	0.105		

regions: *trn*D-*trn*T, *trn*T-*trn*F, *trn*C-*trn*D, *psa*A-*trn*S, *rpL*32-*trn*L, *rpo*B-*trn*C and *rpL*16 intron. The first fours are well known in the literature for a number of angiosperm taxa (Taberlet et al. 1991; Demesure et al. 1995; Shaw et al. 2005), while the remaining threes are known to be polymorphic in *P. fasciculata* or in other congeneric species (Consaul et al. 2010; Birch et al. 2014) Eventually, primer pairs giving one clear and replicable electrophoretic band were chosen for subsequent analyses; in particular, the three primer pairs used to amplify the *rpL*16 intron and the intergenic spacers *trn*C-*trn*D and *rpo*B-*trn*C (Table 2) were as reported in Small et al. (1998), Demesure et al. (1995) and Shaw et al. (2005), respectively. Amplification of the three NC-cpDNA regions was performed on 5 specimens for each sampling site as previously described for the ISSR markers, using PCR procedures optimized for these fragments (25 µl reaction volume containing 100 ng DNA template and 20 pmol of each primer). The amplification cycles were as following: initial denaturation (4 min, 94 °C), 30 cycles of denaturation (45 sec, 94 °C), annealing (45 sec, 58.5 °C) and extension (3 min, 72 °C), plus a final extension step (10 min, 72 °C). Amplified DNA products were checked by electrophoresis in 1% agarose gel (1x TAE buffer at 90 V for 2 h), visualized by SYBR Safe staining (Invitrogen), together with 1 kb DNA ladder (Jena Bioscience), and photographed under ultraviolet light (Ultra lum Inc.). Their purification and bidirectional sequencing were done on a capillary ABI 3730 DNA Sequencer (Seqgen), at Bio-Fab Research (Rome, Italy).

Complementary sequences were assembled for each individual using the online tool HVDR (http://hvdr.bioinf.wits.ac.za/fmt/), compared to sequences of *Puccinellia nuttalliana* (Schult.) Hitchc. within the BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), with data deposited in NCBI Genbank (https://www.ncbi.nlm.nih.gov/genbank/). The accession numbers are reported in the Supplemental online material 4. Multiple sequence alignment was carried out with ALIVIEW 1.26 (https://ormbunkar.se/aliview/#top) and low quality ends were removed. *rpL*16, *trn*C-*trn*D and *rpo*B-*trn*C belonging to the same individual were concatenated using Mesquite 3.61 (https://www.mesquiteproject.org) and the combined sequences were analyzed with PopArt 1.7.2 software (http://popart.otago.ac.nz/index.shtml) to calculate the number of *P. fasciculata* haplotypes, in addition to the following statistics: number of segregating sites, number of parsimony-informative sites and nucleotide diversity. Relationships among the identified haplotypes were displayed as a statistical parsimony network, generated by the TCS method implemented in the software. The UPGMA clustering method, included in PAST 4.02, was then performed, to identify genetic clusters according to the Tajima-Nei index (Tajima and Nei 1984), on the NC-cpDNA sequence data, with 999 bootstraps. A Bayesian analysis, based on a strict-clock model, implemented in BEAST2 (http://www.beast2.org), was performed both to represent possible relationships among *P. fasciculata* haplotypes and to estimate their divergence times, together with the age of the most recent common ancestor (MRCA); XML input files were prepared using BEAUti (provided as a part of the BEAST2 package). Since neither fossil records nor specific cpDNA substitution rates of *Puccinellia* spp. were available, a mean of 2.0×10^{-9} s/s/y, with a SD of 6.080×10^{-10} s/s/y, relying on the averaged values known for most Angiosperms (Zhao et al. 2019), was assumed to calibrate the clock rate. The HKY model of nucleotide mutation and the Coalescent Bayesian Skyline model for population dynamics were set in the analysis. Two independent MCMC analyses of 10 million generations (sampling once every 1000 generations) were run. The analyses output parameters were checked for convergence in Tracer 1.7.1 (https://beast.community/tracer), summarized in the maximum clade credibility tree, after a burn-in percentage of 10%, with TreeAnnotator 2.3.1 (wrapped up in BEAST2) and visualized as a chronogram in FigTree 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/), with mean values and credible intervals (95% higher posterior densities: HPDs) of node age estimates.

Results

ISSR analysis results

Examples of ISSR banding patterns generated by the four primers (840, 845, 856, 868) selected for the analysis are available in Supplemental online material 5. For the 100 samples of *P. fasciculata* taken in the two areas SN and CM, the four selected primers generated a total of 160 bands (Supplemental online material 6), all of which were polymorphic, and distinguished all individuals as separate molecular phenotypes. Amplification products had a variable length between 100 and 2000 bp. A subtotal of 124 bands could be referred to the 80 samples taken in the SN area (Supplemental online material 7).

Table 3 shows the number of bands at subpopulation and population level (drawn from the two matrices 80 x 124 and 100 x 160, respectively), and the genetic diversity measures for the four SN sampling sites based on 124 loci, together with their averaged estimates; measures for the SN area as a

whole and for the single site from CM area, based on 160 loci, are also indicated.

The average number of bands (*Nb*) per SN subpopulation ranged from 25 (SN1) to 28 (SN2) and the average number of bands for the two populations SN and CM was, respectively, 26 and 27. The four SN subpopulations exhibited the same pattern of low genetic diversity: the mean percentage of polymorphic loci (*P*%) was 45.36. Values of the Shannon index (*I*) and Nei's gene diversity (*He*) were respectively $I = 0.171$ and $He = 0.105$, averaged over the four groups; the mean frequency of private bands (*pb*%), unique to a single group, was 18.81. All individuals from SN area had lower percentages of private bands (*pb*% = 15.22) and polymorphic loci (*P*% = 35.16), compared to those from CM area (*pb*% = 46.15 and *P*% = 43.75 respectively), as well as lower estimates of Shannon index ($I = 0.132$ vs. 0.170) and Nei's gene diversity ($He = 0.081$ vs. 0.105). A moderate level of genetic differentiation was detected among the four SN subpopulations ($Gst = 0.24$); the same estimate referred to the two areas SN and CM was slightly higher ($Gst = 0.28$). The amount of gene flow, inferred from *Gst*, seems to be quite limited, not only between SN and CM areas ($Nm = 0.65$), but even among the contiguous subpopulations sampled in the SN area ($Nm = 0.79$).

Congruently with the above estimates, the AMOVA, partitioned into three hierarchical levels, showed that most of the variance arose from the highest levels (33% from the area level plus 21% from the site level), while 46% of the total diversity occurred among individuals, at the intra-sampling site level (Table 4); in line with previous results, in the UPGMA dendrogram (Figure 2), supported by a cophenetic correlation coefficient of 0.934, individuals from CM area were gathered together and split from the remaining samples (at a similarity level of 0.25, with a statistical support of 100% bootstrap value), whereas samples from SN area clumped in 4 subclusters, mostly corresponding to the SN subpopulations, within a second and larger cluster.

In the admixture analysis implemented in STRUCTURE, the highest likelihood of the data identified by Structure Harvester was obtained when samples were clustered into only two genetic groups ($K = 2$), clearly coincident with the geographical location of the two populations, whereas genetic substructure within SN was not so evident as in previous analyses. The resulting plot (Figure 3), representing the estimated membership for each individual to each cluster, indicates a very low level of admixture between SN and CM areas, with only 4 individuals from SN cluster showing minimal signs of genetic admixture with the CM individuals.

Significant, but extremely low correlation between geographic and genetic distances, was found by the Mantel test performed both between the two areas and within SN population (Supplemental online material 8): when all 100 samples were included, the correlation coefficient *Rxy* was 0.09 ($P = 0.001$) and geographic distance accounted only for 0.8% of the variation in genetic distance ($R^2 = 0.008$); comparable results were obtained when the test was restricted across the 80 samples from the four SN subpopulations: the correlation coefficient was still lower ($Rxy = 0.06$, $P = 0.001$) and geographic distance accounted for 0.3% of the variation in genetic distance ($R^2 = 0.003$).

NC-cpDNA analysis results

After trimming low quality ends, the *rpL*16 intron, plus the *trn*C-*trn*D and *rpo*B-*trn*C intergenic regions surveyed across the 25 individuals of *P. fasciculata* were 851 bp, 1705 bp and 830 bp respectively. They were combined along a total length of 3386 bp. Total alignment is shown in Supplemental online material 9.

Relying on PopArt 1.7.2 output, 22 different haplotypes (summarized in Supplemental online material 10) were inferred from the NC-cpDNA dataset, exhibiting 40 segregating (polymorphic/variable) sites and 23 parsimony-informative sites. The value of nucleotide diversity was low ($\pi = 0.005$), suggesting small differences between haplotypes. Unlike ISSR-based results, the haplotype TCS network showed a complex topology (Figure 4) with no obvious relation with sampling locations. The 22 identified haplotypes, connected to one another by 7 or fewer mutations, mostly corresponded to as many single individuals; only three pairs of SN samples shared the same haplotype and no dominant haplotype was detected. Also in the UPGMA dendrogram based on the comparison of NC-cpDNA sequences, supported by a cophenetic correlation coefficient of 0.713 (Supplemental online material 11), individuals from all sampling sites exhibited an extensive intermingling, and no clear geographic-based separation among clusters was recognizable.

The chronogram obtained from a molecular clock applied on a Bayesian analysis of the combined cpDNA dataset, implemented in BEAST2 (Figure 5), was broadly consistent with the hypothesis of no spatial structure pattern for the examined groups; no geographic trend was evident in the clustering of the *P. fasciculata* samples, however it has been possible to estimate the age of the most recent common ancestor (MRCA) of all individuals as 6500 yr BP (95% HPD interval: 5100–7900 yr BP); this period corresponds to the early-middle Holocene.

Discussion

ISSR-based genetic variation and differentiation

Based on ISSR fingerprint, moderate levels of polymorphism and low levels of ISSR genetic diversity (*P*% = 45.36, $I = 0.171$, $He = 0.105$) were encountered on average in the four subpopulations of *P. fasciculata* from the Reserve area SN; likewise, genetic variation was quite low when referred to the two areas ($I = 0.132$, $He = 0.081$ for SN area; $I = 0.170$, $He = 0.105$ for CM area). Higher estimates have been found in other Poaceae already screened with the same markers: *P*% = 48, $I = 0.249$, $He = 0.164$ in *Dendrocalamus membranaceus* Munro (Yang et al. 2012); *P*% = 89.28, $I = 0.471$, $He = 0.311$ in *Panicum virgatum* L. (Zhang et al. 2016); *P*% = 77.78, $I = 0.41$, $He = 0.28$ in *Eleusine coracana* (L.) Gaertn. (Brhane et al. 2017); *P*% = 91.13, $I = 0.45$, $He = 0.30$ in

Table 4. Hierarchical analysis of molecular variance (AMOVA), among and within 5 sampling sites of *Puccinellia fasciculata* from SN and CM areas, based on ISSR markers. Data include degrees of freedom (d.f.), sum of squares (SSD), mean squared deviation (MSD), variance component estimates, percentage of total variance contributed by each component and significance of variance (*P* value after 999 random permutations).

Source of variation	d.f.	SSD	MSD	Variance component	% Total variation	*P* value*
Among areas	1	293.495	293.495	6.301	33	< 0,001
Among sampling sites	3	275.625	91.875	4.144	21	< 0,001
Within sampling sites	95	855.250	9.003	9.003	46	< 0,001
Total	99	1.424.370		19.447	100	

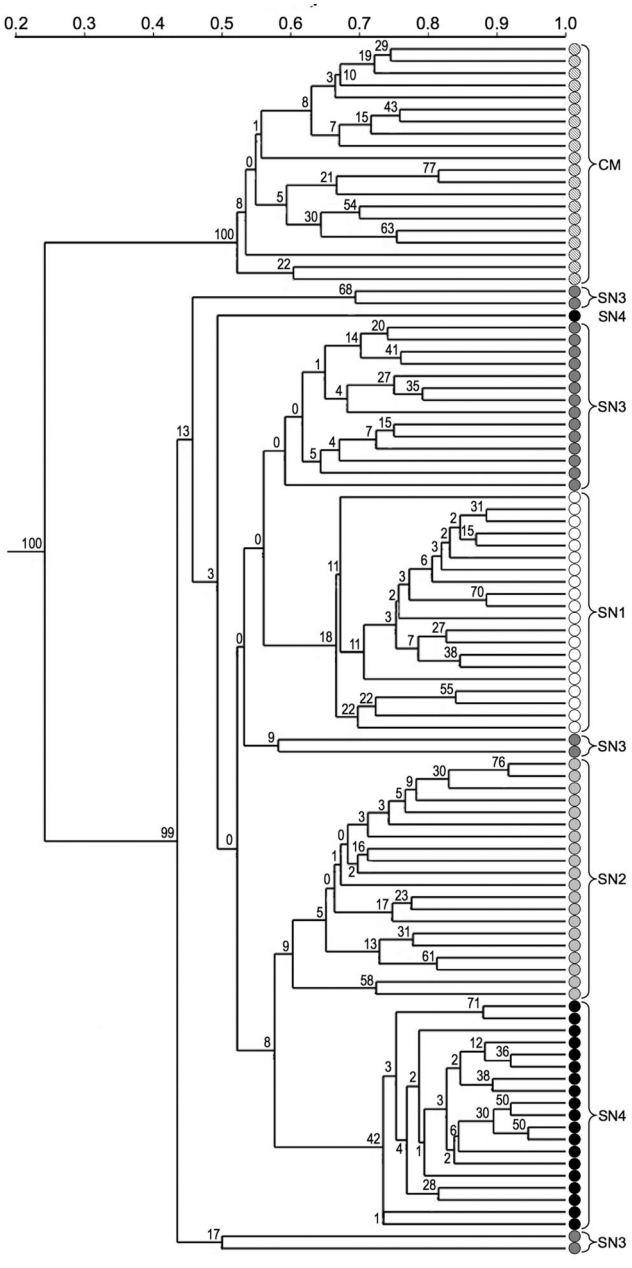

Figure 2. UPGMA dendrogram for 100 specimens from 5 sampling sites of *Puccinellia fasciculata* inferred from ISSR markers, constructed according to the Dice coefficient of genetic similarity. The axis at the top of the figure shows the scale of the applied similarity coefficient. The numbers at the branches represent values of bootstrap obtained after 999 replications. Cophenetic correlation coefficient was 0.934. Codes and symbols of sampling sites follow Figure 1 and Table 1.

Saccharum spp. (Oliveira et al. 2017); *P*% = 90.47, *I* = 0.40-0.46, *He* = 0.26-0.30 in *Triticum boeoticum* Boiss. (Pour-Aboughadareh et al. 2017); *P*% = 67.25, *He* = 0.27 in *Anomochloa marantoidea* Brongn. (Vieira et al. 2020); *P*% = 86.69, *I* = 0.438, *He* = 0.440 in *Festuca arundinacea* Schreb. (Shahabzadeh et al. 2020). These comparisons suggest that, in general, the genetic variation of *P. fasciculata* is somewhat impoverished and confirm the hypothesis that endangered species with fragmented habitat experience genetic depauperation (Stojanova et al. 2020 and authors therein).

The distribution of genetic variation within the SN area (*Gst* = 0.24), and between the 2 areas (*Gst* = 0.28), shows a weak structure in both cases; also the estimates of genetic differentiation based on AMOVA (33% between SN and CM areas, 21% within SN area) supported a certain degree of genetic differentiation, as well as the topography of the UPGMA dendrogram, showing a complete segregation of CM area individuals and a general separation of SN area individuals (largely grouped by sampled subpopulations). This pattern of genetic structure, however, is only partially confirmed by the output from STRUCTURE analysis, detecting just two genetic clusters, corresponding to specimens from the two areas, with a very limited mutual mixture.

The close relationship between levels of genetic variation or genetic structure and plant reproductive traits has been documented in many studies demonstrating that long-lived, allogamous taxa retain most of their variation within groups, whereas autogamous and/or clonal taxa allocate more variation among groups (Nybom et al. 2014; Wu et al. 2015; Gallego-Tévar et al. 2019), often displaying a marked genetic structure. The values of genetic diversity estimated across the *P. fasciculata* individuals are noticeably lower compared to the value found in allogamous species (*He* = 0.260), resulting closer to that reported for autogamous species (*He* = 0.091, Csergö et al. 2009); it is also similar to the average referred to species reproducing both sexually and clonally (*He* = 0.123, Dev et al. 2010). The genetic differentiation, measured as *Gst*, was lower than the average referred to other angiosperms (*Gst* = 0.637, Petit et al. 2005) and close to the expected average for species with mixed mating systems (*Gst* = 0.2, Nybom 2004). Then, contrary to the general expectations, both low levels of genetic variation and low or no structure among *P. fasciculata* sampled stands were recognized by ISSR fingerprinting, especially in those representing the four SN subpopulations. This pattern may be explained by different and coexisting life-history and reproductive traits.

P. fasciculata is described in literature as perennial, although it can behave as annual (Jones and Newton 1970). The species is well adapted for wind-pollination (Hughes 1976), but cross-pollination is not the rule for the species: self fertilization has also been reported as a consequence of intra-floral proximity between anthers and stigma, with the

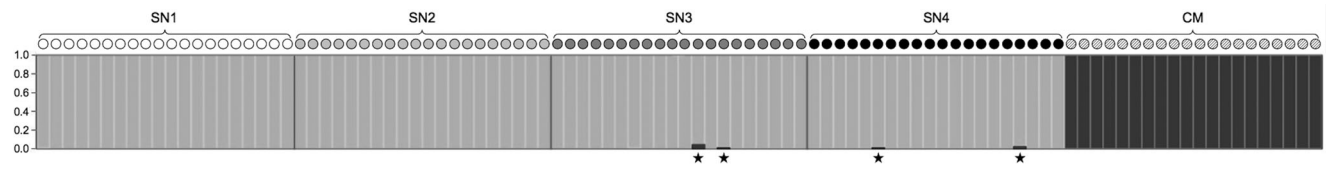

Figure 3. STRUCTURE bar plot for 100 specimens of *Puccinellia fasciculata*, based on ISSR markers, inferred for K = 2. Each bar corresponds to a single individual, with the dark or light grey segments representing the membership proportions to each of the two clusters. The stars indicate the possible admixed individuals. Specimens are grouped by sampling areas (SN and CM). Codes and symbols of sampling sites follow Figure 1 and Table 1.

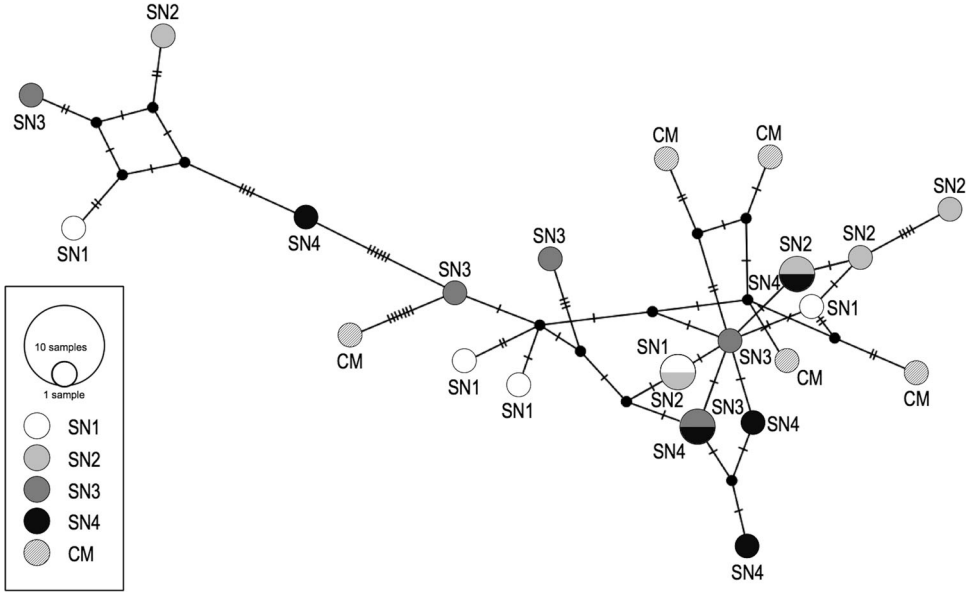

Figure 4. TCS haplotype network based on NC-cpDNA markers from 25 specimens of *Puccinellia fasciculata*. Each circle represents a unique haplotype; the size of circles is proportional to the number of individuals sharing the same haplotype. Hatch marks along branches represent the number of mutations between nodes. Codes and symbols of sampling sites follow Figure 1 and Table 1.

pollen dropping on to the stigmas of the same floret or other florets in the same spikelet (Jones and Newton 1970; Edgar 1996). Small caryopses (1.5 × 0.6 mm, Alonso et al. 2010), with no hairs or appendages on the surface, are produced in abundance (Jones and Newton 1970; Hughes 1976) and wind-blown, although localized hydrochory has been reported for *P. fasciculata* and other species characterizing the vegetation of a polder from north-western France (Dausse et al. 2008; Invasive.Org 2018). Furthermore, the species propagates by underground rhizomes acting as a mean of extensive vegetative reproduction (Alonso et al. 2010); the private bands detected in the sampled groups (*pb*% = 18.81 averaged over the four SN subpopulations and *pb*% = 46.15 for the CM population) may indeed be attributable to fixation of specific alleles through autogamy or asexual reproduction.

It is known that anemophily (Friedman and Barrett 2009) and anemochory (Geyner et al. 2018) both enhance gene flow and are associated with high levels of genetic diversity and low genetic differentiation; conversely, autogamy (Bryan et al. 2017) and clonal recruitment (Kumar and Agrawal 2019) can result in erosion of genetic heterogeneity and incidence of genetic drift, nevertheless they are expected to be favourable when populations are small, allowing even a single individual to be sufficient for colonization (Harmon-Threatt et al. 2009).

Estimates of gene flow lower than the critical threshold of 1.0 are generally linked to strong differentiation among groups of conspecific individuals (Khan et al. 2010); the low level of gene flow (*Nm* = 0.79), found among the contiguous groups of SN area, may be explained by genetic drift effects, counteracting the homogenizing action of gene flow (Ueno et al. 2015); this interpretation is supported by the Mantel test result, showing that geographic distance accounted only for 0.3% of the variation in genotypic distance within the Reserve SN area. The low degree of gene flow (*Nm* = 0.65) estimated between the SN and CM areas appears to be compatible with the distance of more than 100 kilometres separating them; however, also in this case the geographic discontinuity is unlikely to have impacted on the genetic structure pattern, since, according to the Mantel test output, geographic distance accounted only for 0.8% of the variation in genotypic distance between the areas.

Based on the data reported in this study, *P. fasciculata* populations maintain a mixed reproductive strategy in which gene flow and genetic drift act as alternative and complementary evolutionary mechanisms, allowing the species to colonize and persist in harsh environments characterized by soil salinity variations, like the Salse di Nirano or coastal wetlands. Similar results were also reported in *Sedum hispanicum* L. (Pezzi et al. 2017) growing in another extreme environment, such as the gypsum outcrops of northern Apennines.

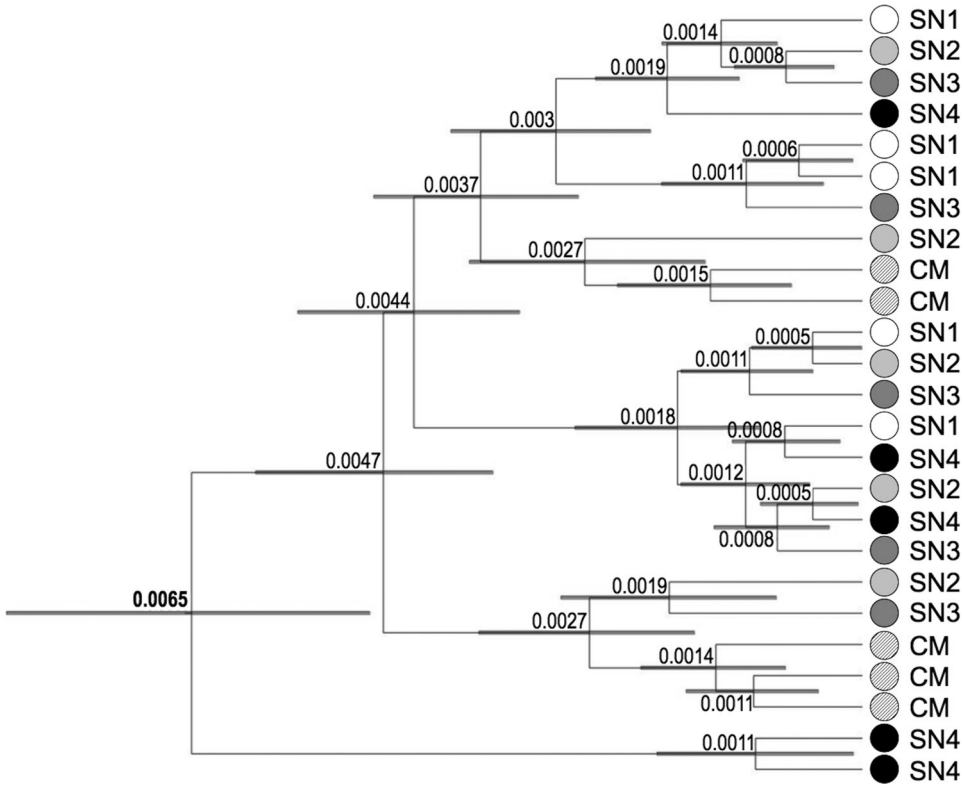

Figure 5. Bayesian chronogram showing the relationships and the divergence times of 25 specimens of *Puccinellia fasciculata* estimated in BEAST2, based on on NC-cpDNA markers. Node ages set at average divergence time (expressed in million years); grey bars at the nodes represent 95% highest posterior density for the estimated mean dates. Codes and symbols of sampling sites follow Figure 1 and Table 1.

NC-cpDNA-based diversity pattern and molecular dating

CpDNA-based analyses show results partly congruent with those obtained from the ISSR fingerprinting. A pattern of low genetic variation is also highlighted by the low nucleotide diversity recovered ($\pi = 0.005$), but the genetic discontinuity, more or less marked, indicated by the nuclear markers, is not confirmed by the plastid ones. The TCS network, the UPGMA dendrogram and the Bayesian chronogram all agree in assessing no structure both within the SN area and between SN and CM areas: the four Reserve subpopulations were not discriminated, nor SN and CM populations recognized as distinct groups.

The observed discrepancy may be explained by the different gene flow signatures captured by nuclear and cpDNA markers; the latter, being maternally inherited, allow to separate seed dispersal from pollen flow, therefore results derived from cpDNA-based analyses are seed-depending and must be referred only to seed dispersal. It has been already pointed out that movement of seeds can be more important than pollen flow in determining the spatial dynamics of plant populations (Epperson 2007), and, according with Ouborg et al. (1999), wind pollination stands for a capacity for long distance gene flow, but it is the rate of seed dispersal that determines migration patterns and species' range width; still, long-term persistent seed banks often play a crucial role in spatial and temporal dynamics of species surviving hypersaline conditions (Deák et al. 2014).

These arguments might support the conclusion that also the genetic connection found for the examined stands of *P. fasciculata* in the two regions depends on a seed-mediated gene flow acting as a homogenizing force that prevents differentiation among groups of plants; however, given the distance of over 100 kilometres between the inland and coastal populations, the action of seed dispersal over this large area must have been more effective in the past, when suitable sub-saline habitats were common and widespread.

Evidence in support of this hypothesis comes from the molecular dating analysis, according to which the initial diversification of lineages leading to the array of haplotypes here examined began in the middle Holocene, with the most recent common ancestor (MRCA) placed at 6500 yr BP, and has developed regardless of their geographic location. Studies about the evolution of the Po plain indicate that its coastal line was heavily affected by successive sea level fluctuation in the Holocene, with two transgressive pulses developed, at about 8000 and 6000 yr BP; as a consequence of sea rising, marine environments graded landward into large low brackish lagoons and coastal marshes (Stefani and Vincenzi 2005); in historic times, brackish waters have been rising westwards invading freshwater swamps and even reaching emerged areas (Bondesan et al. 1995). A system of large marsh areas, called Valle Padusa, still existed in the low Middle Ages, and extended along the Po river from the coast to the inside for more than a hundred of kilometres (Soriani 1834). Of course, these large areas, reclaimed over time and subjected to intense human activities, no longer exist, although persistent inland sub-saline islands have harboured *P. fasciculata* until a few decades ago; in the 1950s the species still survived in several sites throughout the Po valley

and on the adjacent hills towards west and north-east, as well as in the Apennine "salse" (Bertolani Marchetti 1954).

The homogeneous distribution of genetic diversity and the divergence time estimations here referred, together with inferences about the historical range dynamics of *P. fasciculata*, are in agreement with the hypotheses depicting a common evolutionary history of the stands examined in this study and a past larger and more continuous range of the species, compatible with distances of seed dispersal and extending over the inland brackish wetlands of the Po valley, till reaching the surrounding hillsides. Under these assumptions, the analyzed stands represent the remnants of past continuous populations, historically experiencing effective genetic interchange, whose reduction and isolation has not lasted enough to induce a spatial pattern of genetic structure. Nevertheless, ongoing events of long range dispersal may not be excluded: it may be useful to cite a study on *Puccinellia distans* (Jacq.) Parl., a species very similar to *P. fasciculata*, according to which genetic affinity found in individuals from different Eurasian countries was attributed to anemochory, as well as to antropochory (Bar et al. 2015). If the same dispersal mechanisms occurred in *P. fasciculata*, its seed could cover some tens of kilometres, making a connection between the Po valley and the highest sites, like the Salse di Nirano, repeatedly possible even nowadays.

Conclusions and conservation implications

This study represents the first genetic assessment of a species growing on the extreme environment represented by the extrusive formations of mud volcanoes. The subpopulations of *P. fasciculata*, growing around the venting cones in the Salse di Nirano Regional Natural Reserve, exhibited notable fluctuations in the areas occupied from 2015 to 2017, shrinking by one-third in the second year. Such demographic instability may be expected in a species colonizing an extreme and rapidly changing environment like the "salse", where eruption events make the mud substrate rather unstable, with highly fluctuating physical and chemical conditions. The freshly vented mud, very saline, flows easily, burying plants standing along its way, and tends to form thick deposits; this dynamic process may be more or less stressed depending on irradiance intensity or periodic rainfall.

Nuclear and plastid markers entail low genetic diversity both in the Reserve population and in the coastal one, possibly resulting from different co-occurring reproductive and dispersal strategies. *P. fasciculata* might stand chance of surviving in sub-saline soils through a mixed reproductive strategy involving both pollen/seed mediated gene flow and genetic drift.

CpDNA-based results provide evidence for current seed flow not only over short distances, but also over longer ranges. Significant historical and environmental events might be involved in the homogeneous pattern revealed by the cpDNA markers; the examined stands of *P. fasciculata* possibly represent remnants of lineages starting to diverge as early as in the Holocene and allowed to spread over once wide and contiguous areas, that have become successively reduced and fragmented; over time the species, more widespread in the Po valley up to a few decades ago, may have experienced number/size reductions as a consequence of habitat loss.

Conclusive hypotheses about diversity patterns in *P. fasciculata* warrant further investigation on the basis of additional markers and a wider sampling, however this preliminary attempt could serve as a basis for forthcoming investigations on this and other mud volcanoes species, even outside our territory.

From a conservation perspective, these results confirm that the inland populations of *P. fasciculata* need to be prioritised for the maintenance of genetic variation they still harbour. On a larger scale, management goals should include both the species and the inland habitat where it is known to occur; the importance of the habitat 1340* has been already pointed out, as designated prioritary at European level (European Commission 2013), nevertheless it is more and more often found only in a degraded state (Fehér 2007). Although this fragile environment is carefully protected in the Reserve, further specific projects should take account of the strong fluctuations in the spatial extent of the population, focusing on control of mud flows and artificial enlargements of the area covered by the habitat 1340*. *In situ* preservation should address special attention to the optimization of low-impact tourist paths, planning them far enough away from the sub-saline environment around each venting cone and taking into account that mud flows can change direction suddenly and unpredictably, while *ex situ* conservation could integrate the maintenance of living collection of plants inside Botanical gardens and the preservation of seed collections in germplasm banks for medium or long-term reintroduction programmes.

Acknowledgments

The authors are grateful to: Marzia Conventi, for granting scientific collection permits in the Salse di Nirano Regional Natural Reserve; Luciano Callegari, Mauro Pellizzari and Roberto Rizzieri Masin, who accompanied us in the field; Lucilla Comparsi, for her technical software assistance; Giovanni Cristofolini, who kindly reviewed a preliminary version of this manuscript.

Disclosure statement

The authors declare that they have no conflict of interest.

Funding

"Salse di Nirano" Regional Natural Reserve - Multiannual and Multidisciplinary ResearchThis work was supported by the Research Agreement among the Municipality of Fiorano Modenese, the Emilia Centrale Parks Authority, the University of Parma, the University of Modena and Reggio Emilia and the University of Bologna about Dynamics of halophilous vegetation for the conservation of the 1340* priority habitat in the Salse di Nirano Regional Natural Reserve - Multiannual and Multidisciplinary Research, under Grant No. 1254 (28/6/2017).

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