

Genetic drift versus natural selection affecting the evolution of spectral and functional traits of two key macrophytes: *Phragmites australis* and *Nuphar lutea*

Maria Beatrice Castellani¹ | Alice Dalla Vecchia^{2,3}  | Rossano Bolpagni^{2,3} | Roberto Natale¹ | Erika Piaser³ | Lorenzo Lastrucci⁴ | Andrea Coppi¹  | Paolo Villa³ 

¹Department of Biology, University of Florence, Florence, Italy

²Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parma, Italy

³Institute for Electromagnetic Sensing of the Environment (IREA), National Research Council of Italy (CNR), Milan, Italy

⁴Natural History Museum, Botanical Collections, University of Florence, Florence, Italy

Correspondence

Alice Dalla Vecchia, Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parma, Italy.
Email: alice.dallavecchia@unipr.it

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Abstract

- Both genetic and phenotypic intraspecific diversity play a crucial role in the ecological and evolutionary dynamics of organisms. Several studies have compared phenotypic divergence (P_{st}) and differentiation of neutral loci (F_{st}) to infer the relative roles of genetic drift and natural selection in population differentiation (P_{st} - F_{st} comparison). For the first time, we have assessed and compared the genetic variation and differentiation at the leaf trait level in two key macrophytes, *Phragmites australis* and *Nuphar lutea*.
- To this aim, we quantified and described the genetic structure and phenotypic diversity of both species in five lake systems in north-central Italy. We then investigated the relative roles of genetic drift and natural selection on leaf trait differentiation (P_{st} - F_{st}), assuming that F_{st} reflects divergence caused only by genetic drift while P_{st} also incorporates the effects of selective dynamics on the phenotype.
- In terms of genetic structure, the results for *P. australis* were in line with those observed for other Italian and European conspecific populations. Conversely, *N. lutea* showed a more complex genetic structure than expected at the site level, probably due to the combined effect of genetic isolation and its mixed mating system. Both species exhibited high variability in leaf functional traits within and among sites, highlighting a high degree of phenotypic plasticity. P_{st} - F_{st} comparisons showed a general tendency towards directional selection in *P. australis* and a more complex pattern in *N. lutea*. Indeed, the drivers of phenotypic differentiation in *N. lutea* showed a variable mix of stabilising and directional selection or neutral divergence at most sites.
- The prevalence of vegetative over generative reproduction leads *P. australis* populations to be dominated by a few clones that are well adapted to local conditions, including phenotypes that respond plastically to the environment. In contrast,

Andrea Coppi and Paolo Villa should be considered joint senior authors.

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in *N. lutea*, the interaction of a mixed mating system and geographical isolation among distant sites tends to reduce the effect of outbreeding depression and provides the genetic basis for adaptive capacity.

5. The first joint analysis of the genetic structure of these two key macrophytes allowed a better understanding of the relative roles of genetic drift and natural selection in the diversification of phenotypic traits within habitats dominated by *P. australis* and *N. lutea*.

KEYWORDS

aquatic plants, common reed, leaf reflectance, P_{st} - F_{st} comparison, yellow water-lily

1 | INTRODUCTION

In recent decades, several studies on evolutionary processes inferred the relative role of genetic drift and natural selection on species diversification (Andrews et al., 2016; Orsini et al., 2013). However, the extent to which both evolutionary forces affect populations depends largely on the ecological features of species. It is well known that the degree of individual specialisation varies widely among plant species, mirroring a multitude of physiological, behavioural, and ecological mechanisms (Bolnick et al., 2003) directly involved in driving the species' response to both biotic and abiotic factors (Bolnick et al., 2011; Eller et al., 2017; Hughes et al., 2008) and their ecological resilience (Moran & Ormond, 2015). Therefore, a better understanding of patterns and processes of genetic and phenotypic diversity at intraspecific level is crucial for ecological, evolutionary and conservation studies (Chave, 2013; Mimura et al., 2017).

To determine how the degree of genotypic and phenotypic differentiation is caused by selective vs. neutral processes, several studies—as far as we know, none strictly on aquatic plants—have compared phenotypic divergence (P_{st}) and differentiation of neutral loci (F_{st}), i.e. P_{st} - F_{st} comparisons (Brommer, 2011; Leinonen et al., 2013). If phenotypic features evolved neutrally, the proportion of their variation among populations should be comparable to that of variation in allele frequencies at neutral loci ($P_{st}=F_{st}$). By contrast, if P_{st} is higher or lower than F_{st} , the differentiation of phenotypic features is more likely to be shaped by natural selection (directional or divergent), or stabilising selection, respectively (Brommer, 2011; Chapuis et al., 2008; Leinonen et al., 2008, 2013; Marin et al., 2020; Martin et al., 2008; Merilä & Crnokrak, 2001; Seymour et al., 2019; Whitlock, 2008). According to Leinonen et al. (2006) and Brommer (2011), P_{st} is a proxy of the quantitative genetic differentiation index (Q_{st}) and it is used when additive variance cannot be readily easily quantified (i.e., in field studies; Brommer, 2011). The problems of using P_{st} as an approximation of Q_{st} are well known in the literature. However, the estimation of Q_{st} requires individuals from different populations to be grown together in a common garden (Leinonen et al., 2008), so the P_{st} index is commonly used when the populations under study are located in different areas and may present locally adapted forms.

Regarding the interconnection between genetic differentiation and phenotypic plasticity, a quantitative focus can be provided by measurement of variation in plant functional traits (FTs), especially in relation to those pertaining to the leaf economics spectrum (Dalla Vecchia et al., 2020; Dalle Fratte, Bolpagni, et al., 2019; Díaz et al., 2016; Pierce et al., 2012). FTs such as leaf total area (LA, including petioles), specific LA (SLA) or pigments content (chlorophyll-a, Chl-a; and the ratio between chlorophyll-a and -b, Chl-a/Chl-b) help to quantify the responses of species and communities to abiotic factors. Moreover, new windows on plant functional ecology were opened by the expansion of remote sensing-based applications, enabling high-throughput investigation of plant variability in spatial and temporal dimensions (Wang & Gamon, 2019), recently extended to account for specific spectral features of aquatic plant species (Villa et al., 2021). Joining functional and spectral-based approaches for characterising plant structure and physiology across sites and ecosystems can offer a cross-feedback for a better understanding of ecosystem functioning and consequently providing effective management strategies (Castellani et al., 2023; Villa et al., 2017).

Here, we studied two key freshwater macrophytes, the helophyte *Phragmites australis* (Cav.) Trin. Ex Steud. (or common reed) and the floating hydrophyte *Nuphar lutea* (L.) Sm. (or yellow water-lily), as models to infer the relative role of genetic drift and natural selection on the diversification of leaf traits. Both target species are often dominant and widespread macrophytes and as such can act as ecosystem engineers, shaping the colonised habitats through their physiology and physical structure (Thomaz, 2021). Common reed is a sub-cosmopolitan helophyte, dominating the riparian vegetation of most freshwaters and brackish wetlands globally. This species may also colonise disturbed wetlands, artificial ditches, mined areas, and landfill, thus proving a broad ecological amplitude. These characteristics make *P. australis* a keystone species in freshwater ecosystems and support several habitats and non-habitat ecosystem services (Kiviat, 2013). *P. australis* plays an essential role as a wind and wave breaker (Karstens et al., 2016; Takeda & Kurihara, 1988; Vymazal, 2011) and its functional traits seem to be determined by trophic conditions (Eid et al., 2021). Yellow water-lily is a floating-leaved macrophyte distributed across lower latitudes of Europe, northwest Africa (locally

known for Algeria), and eastwards to central and southwest Asia (Padgett, 2007). Its robust petiole and the large leaf area foster a low hydrodynamic environment mitigating the wave movement and promoting sedimentation (Puijalon et al., 2011; Schoelynck et al., 2014). Its leaf traits appear to be regulated by water depth and sediment features in hyper-eutrophic environments (Dalla Vecchia & Bolpagni, 2022).

The main aim of this work was to assess and compare the genetic variation and leaf trait differentiation of the two target macrophytes, *P. australis* and *N. lutea*. We first quantified and described the genetic structure and phenotypic diversity—estimated from leaf functional traits directly measured and inferred from foliar reflectance—in different populations sampled across lake systems in northern and central Italy. We then investigated the correspondence between the degree of population differentiation at neutral genetic markers and quantitative FTs, respectively expressed by F_{st} and P_{st} indices, to infer the forces (i.e., stabilising selection, directional/divergent selection, or neutral divergence) shaping the population differentiation of each trait. Comparing F_{st} and P_{st} , we assume that F_{st} , determined by neutral markers, reflects divergence caused only by genetic drift (Reynolds et al., 1983). Thus, F_{st} provides a null expectation and allows estimation of what would happen to populations in the absence of selection (Merilä & Crnokrak, 2001; Noguerales et al., 2016; but see Edelaar et al., 2011). By contrast, P_{st} also incorporates the effects of selective dynamics on the phenotype. Consequently, the $P_{st}-F_{st}$ comparison involves three possibilities in deciphering the evolution of sampled populations of *P. australis* and *N. lutea*: (1) $P_{st} > F_{st}$ indicates that traits divergence overcome that attributable to genetic drift alone, suggesting that directional or divergent selection (DS) is leading to population differentiation; (2) $P_{st} = F_{st}$, indicates that neutral divergence cannot be excluded as a possible cause of phenotypic differentiation (NS); and (3) $P_{st} < F_{st}$ indicates that phenotypic differentiation is less than expected based on genetic drift alone, suggesting that trait divergence is most likely to be the product of stabilising selection (SS) (Leinonen et al., 2008; Merilä & Crnokrak, 2001).

2 | MATERIALS AND METHODS

2.1 | Study area and sampling design

The study area included five lake ecosystems (sites hereafter) in both central and northern Italy (Figure 1): Lake Chiusi (CH; 43°03'22" N, 11°57'56" E); Lake Massaciuccoli (MA, 43°50'0" N, 10°19'30" E); Mantua lakes system (MN, 45°09'36" N, 10°47'48" E); Lake Iseo, including the Torbiere del Sebino wetland (IS, 45°43'00" N, 10°05'00" E); and lakes Pusiano and Annone (PA, 45°48'40" N, 9°18'45" E). They are phytogeographically comparable sites covering a wide environmental gradient, in terms of ecological conditions (i.e., trophic status), geographical features (i.e., size and catchment land cover), and plant community structure (i.e., macrophyte community types, or growth forms). The five lake systems differ in areal size, ranging from c. 3.8 to 65.3 km². In terms of trophic conditions, the lakes range from

oligo-mesotrophic (IS) and mesotrophic (Pusiano), up to eutrophic (Annone), and even hypertrophic conditions (CH, MA, and MN).

Depending on the lake area and the relative coverage of the target plant communities (*P. australis* and *N. lutea*), 10 × 10 m plots were sampled in each site to be representative of the local ecological heterogeneity (*N. lutea* was not present in MA; Table S1). Out of the total 78 sampled plots, 50 were dominated by *P. australis* and 28 by *N. lutea*. Eight leaves were randomly taken from each plot for the functional, spectral, or genetic analyses. When it was not possible to use the very same leaf for two or more analysis types (e.g., leaf traits and spectral reflectance), leaves from the same culms (for *P. australis*) or same rosettes (for *N. lutea*) were collected as matches.

2.2 | Spectral data

Reflectance in the visible to shortwave infrared spectrum range (350–2,500 nm) was measured from all the fresh leaves within seconds (maximum 1 min) after cutting them from the plants. Measurements were done using a portable high resolution spectroradiometer (SR-3500, Spectral Evolution, Lawrence, U.S.A.), with a spectral resolution of 3 nm for wavelengths under 1,000 nm, and <8 nm up to 2,500 nm. To minimise disturbance due to background reflection of transmitted light, leaves were placed on a black neoprene plate (reflectance factor <5%) during spectra measurements. Leaf reflected radiance was measured with a contact probe with an internal light source (5 W) as an average of 10 scans, and finally calibrated to reflectance using as reference the contact probe readings taken over a Spectralon panel (Labsphere, North Sutton, U.S.A.; reflectance factor >95%).

2.3 | Functional traits

Five of the eight intact, well-developed leaves of *N. lutea* and *P. australis* were used to measure structural traits (LA, mm²; SLA, mm²/mg), while three were used to measure biochemical traits (Chl-a, µg/g; Chl-a/Chl-b). After gently cleaning water-saturated leaves of debris and epiphytes, they were scanned for measuring LA. Leaves were subsequently dried at 50°C until constant weight to quantify the dry weight (mg). LA was determined analysing scanned images with the software ImageJ (Rasband, 2018), whereas SLA was calculated as the ratio between LA and dry weight. High values of SLA indicate an acquisitive behaviour, implying lower investments in structural tissues for photosynthetic organs and a higher photosynthetic capacity per mass unit (Dalle Fratte, Brusa, et al., 2019). Chlorophyll content was determined spectrophotometrically after 24-hr extraction in 80% acetone (Wellburn, 1994).

Additional leaf traits were derived from the inversion of PROSPECT-D model starting from leaf reflectance. PROSPECT-D is a physical model based on radiative transfer theory that simulates leaf optical properties (reflectance and transmittance) in the spectrum domain ranging from 400 to 2,500 nm, based on

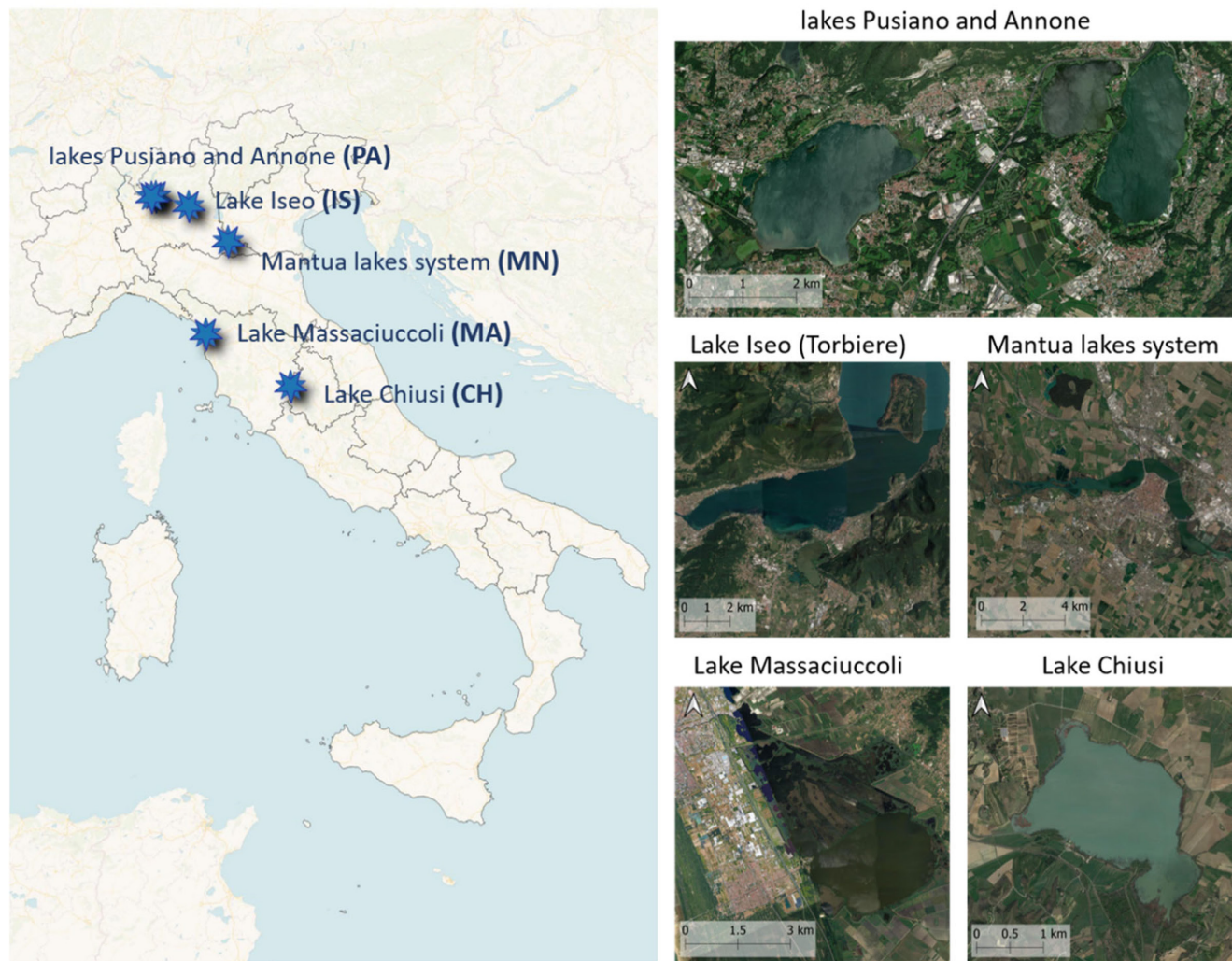


FIGURE 1 Geographic location of the study area with distribution of the sampling sites. Satellite images of each site (at different scales), taken from QGIS' Google Satellite Basemap, are shown on the right.

leaf biochemical constituents (chlorophylls, carotenoids, anthocyanins, water, and dry matter) and an anatomical structural parameter, termed *N* (Féret et al., 2017). PROSPECT-D was inverted through iterative optimisation, minimising the RMSE between measured and simulated reflectance with optimal spectral domain and configuration for each individual trait (Spafford et al., 2021), using the R package *prospect* (Féret & de Boissieu, 2022; R Core Team, 2019). The four traits derived from PROSPECT-D model inversion output are: total chlorophylls (Chl_{ab}); dry matter content on area basis, or leaf mass per area (LMA); dry matter content on weight basis or leaf dry matter content (LDMC); and the mesophyll structure parameter, as a proxy for mesophyll complexity (Nmesophyll). As PROSPECT was designed and calibrated on terrestrial plant species, to increase the accuracy of prediction over aquatic plants, species-specific correction factors were applied to raw model inversion output for Chl_{ab}, LMA and LDMC, separately for *P. australis* and *N. lutea* samples, based on actual trait scores measured over a subset of sampled leaves ($N=238\text{--}394$, varying with trait). Such recalibration reduced the relative error

of modelled traits by 1%–10% depending on the trait, with nRMSE ranging from 6% (LDMC) to 12% (Chl_{ab}).

2.4 | DNA extraction and amplified fragment length polymorphism protocol

A total of 400 and 206 samples (from five to eight leaves per plot) were analysed for *P. australis* and *N. lutea*, respectively. Each leaf tissue sample was ground in a mortar with sterile sand. DNA extraction was carried out using the 2× cetyltrimethylammonium bromide protocol (Doyle & Doyle, 1990). Quality and quantity control of extracted DNA were performed using Bio-Photometer (Eppendorf, Germany). Amplified fragment length polymorphism (AFLP) analysis was performed with minor modifications of previous studies using molecular tools (see Coppi et al., 2014 and references therein). Two combinations of primers were selected for analyses in both species: hex_{EcoRI}-CTA/MseI-ATG and fam_{EcoRI}-TAC/MseI-ATG for *P. australis* and hex_{EcoRI}-ACG/MseI-TTA and fam_{EcoRI}-CTA/

Msel-CTC for *N. lutea*. AFLP profiles obtained by capillary electrophoresis were analysed using GeneMarker v1.5 (SoftGenetics LLC, State College, PA, United States).

2.5 | Analyses of genetic variation at sampling plot and site level

The average genetic diversity within a sampling plot (AGD) was computed as the probability that two homologous sites are different (Nei, 1987) by using Arlequin v2.000 (Schneider et al., 2000). The percentage composition of polymorphic bands was calculated as $[(nfrag/ntotal) \times 100]$, where nfrag was AFLP loci detected for each sampling plot or site and ntotal the number of total detected bands for the primer pair.

As for the genetic structure, molecular variance analysis (AMOVA, Excoffier et al., 1992) was performed using Arlequin v2.000 (Schneider et al., 2000) to determine the distribution of total genetic variation at different hierarchical levels: (1) within and among sampling plot; and (2) within and among sites. The analyses were performed separately for the two hierarchical levels. Tests of the variance components and the percentage of total expressed variation were conducted to assess the statistical support for the different groups. Genetic distances between populations and sites were estimated by computing Slatkin's linearised pairwise F_{st} values (Slatkin, 1995). Following Yang et al. (2016), loci under selection (outliers) were detected using BayeScan v2.01 to remove them from the calculation of neutral genetic differentiation. Outliers are loci that fall over a threshold value set on the logarithm of posterior odds values (LogPO), determined as in Foll & Gaggiotti (2008). The number of pilot runs was kept at 20, with a length of 10,000 iterations each (Coppi et al., 2018).

2.6 | P_{st} - F_{st} comparison

Phenotypic variance, estimated from functional leaf traits, were compared to F_{st} values using *Pstat* package (Da Silva & Da Silva, 2018) in R environment. F_{st} values for both species were extrapolated from the molecular variance analysis (see Tables S2 and S3). For each site, F_{st} values at population level were averaged and bootstrapped 95% confidence intervals were calculated using R. P_{st} values were determined with the bootstrap method (1,000x) under a confidence level of 95% using *Pstat* package (Da Silva & Da Silva, 2018; see Table S2).

P_{st} was calculated from between population (σ_B^2) and within population (σ_W^2) components of variance for each trait following Brommer's (2011) expression:

$$P_{st} = \frac{c/h^2\sigma_B^2}{c/h^2\sigma_B^2 + 2\sigma_W^2} \quad (1)$$

where c is the proportion of the total variance attributed to additive genetic effects between populations and h^2 is the heritability *stricto sensu*. In this index, the c/h^2 ratio quantifies the proportion

of phenotypic differences observed between populations that can be attributed to additive genetic variance (Brommer, 2011; Leinonen et al., 2008). The problem of using P_{st} as an approximation of Q_{st} is mainly because values of c/h^2 are unknown in natural populations (Pujol et al., 2008). Consequently, the starting point is the null assumption $c/h^2=1$ (i.e., $c=h^2$). However, natural populations may be subject to genotype-environmental interactions and low values of c/h^2 (i.e., $c < h^2$) assumes a more important role of environmental factors in determining between-population variance than within-population variance. Therefore, the lower the critical c/h^2 ratio is ($c/h^2 < 1$) when P_{st} exceeds F_{st} , the more likely it is that the trait is being shaped by selection (Brommer, 2011). Dealing with this issue, we tested three different c/h^2 (0.5, 0.63, 1; Table S2) and we eventually assessed the strength of P_{st} - F_{st} comparisons for the more conservative case ($c/h^2=0.5$).

Cohen's d was used to calculate the effect size for P_{st} - F_{st} differences:

$$\text{Cohen's } d = \frac{\mu(P_{st}) - \mu(F_{st})}{\sigma(P_{st}, F_{st})} \quad (2)$$

where $\mu(P_{st})$ is the P_{st} value computed from *Pstat*, $\mu(F_{st})$ is the average F_{st} score, and $\sigma(P_{st}, F_{st})$ is the pooled standard deviation derived as the sum of $\sigma(P_{st})$ and $\sigma(F_{st})$. to derive pooled standard deviation $\sigma(P_{st})$ is estimated using the lower bound of 95% confidence intervals computed from *Pstat* as $\frac{2(P_{st} - \text{lowCI}_{P_{st}})}{3.92}$, and $\sigma(F_{st})$ is estimated using the 95% confidence intervals of F_{st} average as $\sqrt{N \frac{(95\%_{\text{upCI}} F_{st} - 95\%_{\text{lowCI}} F_{st})}{3.92}}$.

As a first interpretation, we categorised the continuous range of Cohen's d scores into three situations, following the scale proposed by Sawilowsky (2009), denoting dominant mechanisms underlying intra-specific diversity in terms of P_{st} - F_{st} , such as: SS when $d \leq -0.8$; DS, when $d \geq 0.8$; and an intermediate situation indicating NS, when $-0.8 < d < 0.8$.

To investigate whether natural selection dominance (DS) is driven by directional or divergent evolution patterns, the distribution of trait values and AFLP mismatches were assessed in both species and within each site (see Figures S2 and S5, respectively). Mismatch distributions (i.e., the distribution of pairwise differences among haplotypes; Rogers & Harpending, 1992) were calculated using Arlequin v2.000 software (Schneider et al., 2000). Unimodal distribution was interpreted as the effect of directional selection, while bimodal to multimodal distribution of pairwise differences or measured traits was interpreted as the effect of divergent selection (Choudhuri, 2014).

To determine whether P_{st} - F_{st} comparisons are biased by the choice of markers (Edelaar et al., 2011), the relationship between P_{st} and F_{st} difference and average genetic diversity over loci at site level was investigated and tested using a linear regression (see Figure S1).

3 | RESULTS

3.1 | Functional traits

Overall, 250 and 130 leaves of *P. australis* and *N. lutea*, respectively, were analysed for structural traits, and 150 and 84 leaves for biochemical traits. In *P. australis*, the median values of traits ranged as

follows: LA from 6,525.4 (IS) to 10,536.0 mm² (MN), SLA from 12.1 (MN) to 13.9 mm²/mg (MA), Chl-a from 2,021.4 (CH) to 3,069.2 µg/g (MN), and Chl-a/Chl-b from 4.0 (CH) to 4.7 µg/g (PA). As for *N. lutea*, LA ranged from 56,748.9 (IS) to 80,657.0 mm² (PA), SLA from 6.1 (PA) to 9.1 mm²/mg (CH), Chl-a from 693.3 (PA) to 934.9 µg/g (IS), and Chl-a/Chl-b from 3.4 (CH) to 4.4 µg/g (IS; Figure S2).

Concerning the PROSPECT-D derived traits, 400 and 224 leaves were analysed for *P. australis* and *N. lutea*, respectively. In *P. australis*, the median values of traits ranged as follows: Chl_ab from 51.8 (CH) to 56.2 µg/cm² (PA) and LMA from 72.9 g/m² (MA) to 81.5 g/m² (IS), LDMC ranged from 0.40 (MA) to 0.45 g/g (IS), and Nmesophyll from 1.41 (MA) to 1.57 (PA). As for *N. lutea*, Chl_ab ranged from 33.1 (CH) to 40.8 µg/cm² (PA) and LMA from 76.8 (CH) to 108.2 g/m² (PA), LDMC ranged from 0.16 (CH) to 0.19 g/g (PA), and Nmesophyll from 1.57 (CH) to 2.15 (PA; Figure S2).

The intraspecific leaf reflectance variability and the comparison between leaf traits measured and estimated from PROSPECT-D inversion are shown in (Figures S3 and S4, respectively).

3.2 | Amplified fragment length polymorphism analysis

3.2.1 | Phragmites australis

The AFLP analysis was successfully performed on 395 samples. The selected combinations of primers produced a total of 341 loci, 130 for the combination hex_EcoRI-CTA/MseI-ATG and 211 for that of fam_EcoRI-TAC/MseI-ATG. Within sampling plots, the percentage of polymorphic loci (PPL) ranged from a maximum of 87.7% (MN14) down to a minimum of 29.6% (CH08; Table S1). Within each site, the average percentage of polymorphic loci varied from 97.4% in MN to 76.8% in CH. The AGD levels varied from 0.086 (CH02) to 0.353 (MA08) within sampling plot and from 0.176 to 0.321 (CH and MN respectively) at site level. Regarding AMOVA, the greatest percentage of the total genetic variation occurred within sampling plots

(66.9%), rather than among sampling plots (33.1%; Table 1a). The same can be observed across sites, where genetic differentiation within sites (79.3%) was higher than among sites (20.7%; Table 1b). The BayeScan analysis identified one outlier locus that had a posterior probability >0.78 (at a threshold of log₁₀ PO >0.5).

3.2.2 | Nuphar lutea

The AFLP analysis was successfully performed on 203 samples. The selected combinations of primers produced a total of 191 loci, 94 for the combination hex_EcoRI-ACG/MseI-TTA and 97 for that of fam_EcoRI-CTA/MseI-CTC. Within sampling plots, the PPL ranged from a maximum of 92.7% (ISO6) down to a minimum of 35.6% (CH17; Table S1). Within each site, the average percentage of polymorphic loci varied from 86.4% for Iseo to 77.5% for PA. The AGD levels varied from 0.098 (CH05) to 0.287 (MN33) within sampling plots and from 0.212 to 0.267 (Chiusi and Iseo, respectively) at the site level. Regarding AMOVA, the percentage of the total genetic variation was nearly equal within (53.5%;) and among sampling plots (46.5%; Table 2a) and within (54.1%) and among (45.9%) sites (Table 2b). BayeScan analysis did not detect any outlier loci.

3.3 | P_{st}-F_{st} comparison

The values of effect size for P_{st}-F_{st} comparison are summarised in Table 3. The distributions of trait values (Figure S2) and mismatches (Figure S5) were generally unimodal, suggesting that, in the case of P_{st} > F_{st} by a reasonable extent (Cohen's d > 0.8), directional selection could be the main force leading to population differentiation. Some exceptions were evident for Nmesophyll in PA for *P. australis*, and for LA in CH, IS, and MN or LMA in IS for *N. lutea*, indicating that in a minority of situations (specific sites and traits) the action of divergent selection cannot be excluded.

Source of variation	df	Sum of squares	Variance components	Percentage of variation	p-Values
a)					
Among sampling plots	49	9,200.153	18.92043	33.07	<0.0001
Within sampling plots	345	13,210.464	38.2912	66.93	<0.0001
Total	394	22,410.618	57.21164	100	
b)					
Among sites	4	3,955.551	12.32683	20.67	<0.0001
Within sites	390	18,455.067	47.32069	79.33	<0.0001
Total	394	22,410.618	59.64751	100	

TABLE 1 (a, b) The partition of genetic variance (AMOVA) for *P. australis* was represented at two different hierarchical levels: (a) within and among sampling plot, (b) within and among sites.

Note: Tables show degrees of freedom (df), sum of squared deviations, variance component estimates, percentages of total variance contributed by each component, and probability of obtaining a more extreme component estimate by chance alone (p). p-Values were estimated with 999 permutations.

TABLE 2 (a, b) The partition of genetic variance (AMOVA) for *N. lutea* was organised at two different hierarchical levels: (a) within and among sampling plot, (b) within and among sites.

Source of variation	df	Sum of squares	Variance components	Percentage of variation	p-Values
a)					
Among sampling plots	27	3,845.676	16.97461	46.55	<0.0001
Within sampling plots	175	3,411.496	19.49427	53.45	<0.0001
Total	202	7,257.172	36.46888	100	
b)					
Among sites	3	2,747.578	19.20076	45.87	<0.0001
Within sites	199	4,509.594	22.66128	54.14	<0.0001
Total	202	7,257.172	41.86204	100	

Note: Tables show degrees of freedom (df), sum of squared deviations, variance component estimates, percentages of total variance contributed by each component, and probability of obtaining a more extreme component estimate by chance alone (p). p-Values were estimated with 999 permutations.

In *P. australis* populations, directional selection is very evident as the main driver of phenotypic differentiation (Cohen's $d > 2$, i.e., P_{st} is higher than F_{st} by at least two times the pooled standard deviation) in MA, MN, and PA sites across many traits, i.e., four out of eight traits in the former two sites and two out of eight in the latter (plus *Nmesophyll* possibly driven by divergent selection). The patterns are generally leaning towards neutral divergence for IS populations (except for LDMC and LA, driven by directional selection with Cohen's $d > 1.9$), and a mixture of directional (*Nmesophyll*, LA, Chl-a) and stabilising (LMA, SLA, Chl-a/Chl-b) selection in CH populations. In addition, there was a significant, yet weak relationship between AGD values and the $P_{st}-F_{st}$ for LMA, suggesting a possible bias of $P_{st}-F_{st}$ for this trait.

As for *N. lutea*, stabilising selection (marked by large, negative Cohen's d of $P_{st}-F_{st}$ difference) appears to be the dominant driver of phenotypic differentiation in PA populations (especially marked for pigments-related traits, Cohen's $d < -1.4$). The populations in CH showed a mixed pattern, i.e., the relative dominance of neutral selection for several traits (five out of eight), with the exceptions of LA and Chl-a/Chl-b (apparently driven by divergent and directional selection, respectively), and LDMC (SS-driven). Similarly to what happens for *P. australis*, the dominance of neutral selection is observed for *N. lutea* populations in IS (five out of eight traits), while LA and LMA variation appears to be driven by divergent selection, and LDMC by directional selection. Directional selection seems to be the main force driving most of the FTs' differentiation in MN populations (four out of eight traits), in particular for SLA, scoring average P_{st} values higher than F_{st} by nine times the pooled standard deviation, while LA appears to be driven by divergent selection, from the bimodal distribution shown in Figure S2a.

4 | DISCUSSION

In all five investigated sites, the genetic structure of *P. australis* agrees with other Italian and European conspecific populations,

showing a higher genetic variation within rather than among plots and sites (Coppi et al., 2018; Lambertini et al., 2008). *N. lutea*, by contrast, exhibited a more complex genetic structure than expected at site level, possibly due to the combined effect of genetic isolation and its mixed mating system. Both species showed wide variability in leaf functional traits within and across sites, confirming their high plasticity (Dalla Vecchia & Bolpagni, 2022; Eller & Brix, 2012; Guo et al., 2016; Kordyum & Klimenko, 2013). As for evolutionary drivers, $P_{st}-F_{st}$ comparisons showed an overall tendency to directional selection for *P. australis* and a more complex pattern for *N. lutea*.

4.1 | Genetic structure and traits variation

The mean genetic diversity indices at both sampling plot and site levels showed no obvious signs of gene erosion for *P. australis*, apart from populations in Chiusi, characterised by lower genetic diversity than other sites. AGD and PPL values were in line with those observed for other conspecific populations in Italy and Europe (Coppi et al., 2018; Lambertini et al., 2008). Consistently, most of the genetic variation resided within rather than among sampling plots and sites, confirming previous studies on phylogeographic relationships in common reed stands on local to narrow range scales (Coppi et al., 2018; Lambertini et al., 2008; Qiu et al., 2016). Indeed, the high local genetic diversity observed for *P. australis* agrees with previous evidence, and it seems directly connected with the ecological and physiological characteristics of the species (Gao et al., 2012; Richards et al., 2012). As an example, invasiveness of common reed genotypes in North America has been attributed to the capacity of this species to combine sexual reproduction and vegetative propagation (Albert et al., 2015; McCormick et al., 2010).

As for *N. lutea*, the two previous works on this topic showed a tendency towards greater genetic diversity within than among populations (Fér & Hroudova, 2008; Vyšniauskienė et al., 2020). Our results, covering relatively distant sites (up to 370km), show

TABLE 3 Results of P_{st} - F_{st} comparison in terms of effect size (Cohen's d) for each trait within sites (CH = Chiusi; IS = Iseo; MA = Massaciuccoli; MN = Mantova; PA = Pusiano-Annone).

Trait	<i>P. australis</i>					<i>N. lutea</i>				
	CH	IS	MA	MN	PA	CH	IS	MN	PA	
Chl _{ab} µg/cm ²	0.74	-0.68	3.02	2.37	1.48	2.15	0.73	1.35	-1.77	
LMA g/m ²	-1.7	0.69	1.98	2.29	2.43	0.67	1.92	-0.29	-1.02	
LDMC g/g	-0.24	2.41	0.24	2.29	3.38	-0.89	1.49	-1.09	-0.38	
Nmesophyll	1.72	0.54	3.43	3.74	7.93	0.42	-0.35	0.93	0.85	
Leaf.area.tot mm ²	1.84	1.9	2.5	0.96	1.34	2.61	5.21	3.05	0	
Leaf.SLA. mm ² /mg	-1.26	-0.78	1.87	1.08	0.19	-0.54	0.25	9.05	-0.48	
Chl.a µg/g	1.84	0.1	1.14	0.81	1.63	-0.75	-0.35	2.06	-1.44	
Chl.a/Chl.b µg/g	-2.14	0.43	-0.43	0.44	-1.8	0.35	-0.69	-1.16	-3.84	

Note: The higher and positive Cohen's d scores are ($P_{st} > F_{st}$), the more evidently phenotypic differentiation of a specific trait is probably driven by directional or divergent selection (DS, highlighted in blue). Conversely, the higher and negative Cohen's d scores are ($P_{st} < F_{st}$), the more evidently phenotypic differentiation of a specific trait is probably driven by stabilising selection (SS, highlighted in red). In the middle, Cohen's d scores waggling around zero indicate that neutral divergence (NS, not highlighted) cannot be excluded as a possible cause of phenotypic diversity ($P_{st} = F_{st}$).

a partition of genetic variation nearly equal within and among both sampling plots and sites, thus revealing the existence of complex genetic structure characterising the targeted *N. lutea* populations. The ability of *N. lutea* to shift its reproduction strategy from out- or in-breeding (Ervik et al., 1995; Lippok & Renner, 1997; Padgett, 2007) to the vegetative and vice versa strongly affects the dynamics of populations and, consequently, their genetic structure. Merging the findings of previous works (e.g., Fér & Hroudova, 2008; Padgett, 2007) with our genetic outcomes—in particular the complete lack of clonal profiles—allows us to outline an interpretative framework for the establishment and expansion of *N. lutea* populations in lentic ecosystems. During a first *establishment step*, new germplasm of mainly generative origin arrives from nearby populations, entailing high genetic diversity within the new population and low genetic distance from donor populations. During a second *expansion wave*, vegetative reproduction leads to competition among genotypes, favouring individuals well adapted to local conditions that will tend to outcompete new migrants, and results in lower genetic diversity within the population and higher genetic distance from donor populations. Genetic drift may continue as long as the filtering of genotypes occurs, or new, competitive migrants succeed in establishing. However, it should not be excluded that genetic differentiation of the new population from the donor populations may also be increased due to sexual reproduction of the filtered genotypes.

In this context, the mixed reproductive strategy could play a role in how both *P. australis* and *N. lutea* respond to environmental variation. However, it is crucial to note that phenotypic variation can also promote adaptive evolutionary responses even if induced by the environment and not strictly controlled by genetics (Waddington, 1952, 1953; West-Eberhard, 2003). Regardless of the pre-eminent evolutionary process, evaluated by the P_{st} - F_{st} comparisons, high rates of leaf traits variation were observed across sites, suggesting a high phenotypic plasticity both in structural and biochemical traits. These results reinforce the outcomes by Dalla Vecchia and Bolpagni (2022), who found high rates of intraspecific variability for *N. lutea* at the micro-scale (Lake Chiusi), as well as strong links between traits variation and environmental drivers, especially for leaf size (LA and dry weight).

4.2 | P_{st} - F_{st} comparison

At present, local adaptation capabilities for *P. australis* and *N. lutea* have been studied from either a phenotypic (Henriot et al., 2019; Ren et al., 2020; Vretare et al., 2001) or a genetic point of view (Coppi et al., 2018; Fér & Hroudova, 2008; Lambertini et al., 2020; Naugžemys et al., 2021; Vyšniauskienė et al., 2020). To our knowledge, only one study has jointly investigated the complex relationships between population-based functional trait variation, genetic diversity, and environmental heterogeneity, i.e., the work of Wani et al. (2020) focusing on *P. australis*. For *P. australis*, the outcomes of our P_{st} - F_{st} comparison indicated that for a majority of sites (MA, MN,

and PA) most of the observed FTs variation was affected by directional selection, thus led by genetic adaptation.

In target *P. australis* populations, directional selection tends to move the traits mean towards the optimum for that environment, increasing the adaptability of individuals, thus confirming the observations of Wani et al. (2020) for the invasive haplotypes, and indicating the importance of the investigation P_{st} - F_{st} comparisons at a fine local scale (i.e., among sites within geographic regions). In this context, the proportion of variation in the phenotype due to neutral genetic differentiation approaches zero and the remaining phenotypic variation is from either environmental or adaptive genetic variation. This result, coupled with the genetic outcomes, confirms that the colonisation strategy of *P. australis* implies a prevalence of patches of individuals with high genetic diversity, well adapted to local conditions, across most of the studied sites. It is hypothesised that the isolation of patches of individuals and populations showing high local adaptation could be the effect of the prevalence of vegetative over generative reproduction (Alvarez et al., 2005; Koppitz & Köhl, 2000; Lebedeva et al., 2020). Exceptions to this pattern are the CH and IS populations, where traits variability is mainly shaped respectively by DS and SS, and by DS and NS. Our results show that CH and IS sites host lower genetic diversity and higher genetic differentiation for neutral markers than other sites, suggesting that patches of common reed are undergoing genetic drift at the micro-local scale, which in turn promotes SS on selected leaf traits. Despite this, in these sites the FTs did not show substantial differences compared to the others, except for the lowest values for Chl-a and the highest average value regarding the Chl-a and -b ratio in CH, the hypertrophic site.

By contrast, in *N. lutea* the P_{st} - F_{st} comparison showed differently mixed patterns, ranging from SS dominance for populations in PA to different balances of stabilising, neutral, and directional or divergent selection in the other sites. As the rate of plant adaptation to the environment depends on how close the plastic phenotype is to the environmental optimum (Price et al., 2003), when plasticity matches ideal conditions, the population should undergo SS without subsequent genetic differentiation; this is the case for *N. lutea* in PA across all the leaf pigments and LMA, contrasted by Nmesophyll (DS-affected). Instead, when traits tend to be far from the environmental optimum, the population should be subjected to directional selection. However, where divergent phenotypic patterns are present across populations at the same site, divergent selection may be the main driver, as in the case of LA in CH, IS and MN. These evolutionary trajectories seem to only partially reflect the trophic gradient among sites, with PA showing intermediate quality status (meso-eutrophic). Conversely, IS and the MN-CH pair, where the leading process is represented by DS, show oligo-meso- and hypertrophic conditions, respectively.

The use of P_{st} - F_{st} comparison allowed us to infer the relative role of genetic drift and natural selection on the diversification of their leaf traits across different lake systems. Our results showed an overall tendency to directional selection for *P. australis*, with the exclusion of the Chiusi site where stabilising selection drives the variability of three out of eight traits. By contrast, *N. lutea* showed

a more complex pattern of phenotypic differentiation drivers, with a mixture of stabilising, neutral and directional (divergent) selection in most of the investigated sites. These outcomes suggest the existence of species-specific behaviours in the diversification of leaf traits for both macrophytes, with marked differences between the two species in the PA site (Table 3). Conversely, populations of both species in IS showed similar differentiation patterns (dominated by neutral selection), indicating a sensitive effect of site conditions in affecting their functional plasticity. In this respect, it will be necessary to direct future analyses towards a better understanding of the implications of ecosystem quality status in driving local adaptation processes and functional responses of macrophytes, in order to define the extent and limits of their plastic adaptation across a wider range of conditions.

The sampling and characterisation of *P. australis* and *N. lutea* populations inhabiting the same sites allowed us, for the first time, to perform a joint analysis of the genetic structure of both species. This is a fundamental point for understanding the relative roles of genetic drift and natural selection on the diversification of phenotypic traits within the habitat of two keystone macrophyte species. As the relative contribution of selection, genetics, and plasticity to environmental adaptation appears to be strictly determined by the ecological context, further studies are needed to corroborate these initial findings and to deepen our understanding of the relative role of natural selection in the diversification of two target species and how this relates to eco-geographical variables.

AUTHOR CONTRIBUTIONS

Conceptualisation: A.C., P.V. Developing methods: M.B.C., A.C., R.B., P.V. Data analysis: M.B.C., A.C., A.D.V., P.V. Preparation of figures and tables: M.B.C., E.P. Conducting the research, data interpretation, writing: M.B.C., A.D.V., R.B., R.N., E.P., L.L., A.C., P.V. Main writing: M.B.C.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

ORCID

Alice Dalla Vecchia  <https://orcid.org/0000-0003-4974-0395>

Andrea Coppi  <https://orcid.org/0000-0003-4760-8403>

Paolo Villa  <https://orcid.org/0000-0002-6952-836X>

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SUPPORTING INFORMATION

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