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A multidisciplinary approach in the study of the genetic diversity of freshwater macrophyte communities

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SUMMARY (in Italian)

Questo lavoro di dottorato è finanziato da un progetto PRIN europeo chiamato "macroDIVERSITY", che mira a fondere la diversità spettrale e filogenetica per valutare i tratti e le funzioni delle macrofite negli ecosistemi di acqua dolce. Le macrofite sono elementi chiave degli ecosistemi di acqua dolce e svolgono un ruolo cruciale nelle reti trofiche, nei processi biologici e nei servizi ecosistemici connessi. La conservazione della diversità funzionale delle macrofite dovrebbe essere una priorità nella gestione della qualità degli ecosistemi di acqua dolce poiché si riflette sulla resilienza dell'ecosistema, sull'adattamento ai cambiamenti climatici, sulla sensibilità delle specie invasive e sugli impatti antropogenici. Le tecniche di telerilevamento, la genetica e gli approcci filogenetici hanno fornito strumenti innovativi per mappare i tratti e le funzioni delle piante, superando così i limiti del monitoraggio della diversità funzionale. Tuttavia, non è stata ancora stabilita una base solida e comune che colleghi le caratteristiche funzionali delle piante alle caratteristiche spettrali e genetiche. In questo contesto, il progetto "macroDIVERSITY" mira a unire la diversità spettrale, filogenetica e funzionale delle macrofite per preservare il loro potenziale evolutivo e comprendere meglio il funzionamento dell'ecosistemi acquatici. Il progetto è svolto da tre gruppi di ricerca: l'Istituto per il Rilevamento Elettromagnetico dell'Ambiente di Milano per l'analisi spettrale, l'Università di Parma per l'analisi dei tratti funzionali e l'Università di Firenze per l'analisi della diversità genetica. In quanto dottoranda del gruppo di ricerca di Firenze, il mio lavoro di dottorato si è concentrato sia sulle analisi molecolari strettamente correlate al progetto macroDIVERSITY che su altri casi studio che hanno approfondito la conoscenza della diversità (in particolar modo genetica) delle comunità macrofitiche nell'Italia centro-settentrionale.

SUMMARY (in English)

This thesis work is funded by a European PRIN project called 'macroDIVERSITY', which aims to merge spectral and phylogenetic diversity to evaluate the traits and functions of macrophytes in freshwater ecosystems. Macrophytes are key elements of freshwater ecosystems and play a crucial role in trophic webs, biological processes and connected ecosystem services. Conservation of macrophytes' functional diversity should be a priority in the quality management of freshwater ecosystems since it reflects on ecosystem resilience, climate change adaptation, invasive species sensitivity and anthropogenic impacts. Remote sensing techniques, genetics, and phylogenetics approaches have provided innovative tools for mapping plant traits and functions, thus overcoming functional diversity monitoring limitations. However, a sound and common basis linking plant functional characteristics to spectral and phylogenetic features have not yet been established. In such a framework, the project "macroDIVERSITY" aims to merge the spectral, phylogenetic, and functional diversity of macrophytes to preserve their evolutionary potential and better understand aquatic ecosystem functioning. In light of these objectives, my PhD work has focused on molecular analyses related to the project macroDIVERSITY and other case studies focused on deepening the knowledge of the macrophyte communities' diversity in central-northern Italy. Specifically, the investigations focused on relating the genetic and phylogenetic diversity results with the spectro-functional diversity data to decipher the processes shaping the evolution of macrophyte communities, with particular reference to two focal species, *Phragmites australis* and *Nuphar lutea*, dominating the wetlands under investigation.

1. INTRODUCTION: “macroDIVERSITY project”

This PhD work is funded by a European PRIN Project called "macroDIVERSITY", which aims to merge spectral and phylogenetic diversity to assess macrophytes’ traits and functions in freshwater ecosystems.

It is common knowledge that biodiversity promotes the ecosystems functioning (EF) due to functional diversity among organisms (Hooper et al. 2005). Plant functional diversity (FD) - i.e. the degree of variation of morphological, physiological, and phenological traits measured in a given community - can be a useful tool for describing and explaining spatial and temporal variability in primary production, and, therefore, can be used to conservation actions. FD of macrophytes reflects on ecosystem resilience, climate change adaptation, invasive species sensitivity and anthropogenic impacts (Cadotte et al., 2011; Flynn et al., 2011). Therefore, conserving aquatic plant diversity should be prioritized in managing the quality of freshwater ecosystems. In this context, macrophyte status and dynamics must be monitored regularly for reliable information. Many technological advances have been dedicated to studying plant functional diversity in the last decade, and remote sensing spectroscopy and techniques based on genetics and phylogenetics proved to have high potential in integrating measures of plant diversity and determining conservation priorities (e.g., Kokaly et al. 2009; Asner et al. 2011; Flynn et al., 2011; Asner, 2013; Faith, 2016). Since light is mainly absorbed by leaf pigments (Ustin et al., 1989; Curran, 1989), the morphology and size of the leaves influence spectral reflectance (Ollinger, 2011). Therefore, the spectral differences between plants capture the functional differences in biochemical and morphological traits, and the spectral diversity (SD, here intended as the metric of the variability of the spectral reflectance of plants at different wavelengths; Jacquemoud and Baret, 1990; Ustin and Gamon, 2010) could provide an integrated measure of the variability of phenotypes within plant communities (Schweiger et al., 2018). However, although remote sensing spectroscopy has demonstrated outstanding potential for monitoring ecological processes (e.g., Kokaly et al., 2009; Asner et al., 2011; Schaepman et al., 2015), how SD relates to plants FD is still not plenty established (Schweiger et al., 2017). Parallel, genetic approaches have widened the definition of biodiversity and improved conservation priorities. Phylogenetic diversity (PD) of plants integrates many functional traits and ecological differences and correlates with key ecosystem functions (Srivastava et al., 2012), including productivity (Cadotte et al., 2009) and community stability (Cadotte et al., 2012). The significance of PD derives from the hypothesis that closely related species have more similar functions than distantly related species; therefore, a community with high PD is more likely to provide complementary functions (Thompson et al., 2015;

Ritchie et al., 2021). However, evidence of PD's role as a proxy of FD is still not decisive (Winter et al., 2013).

In this perspective, the macroDIVERSITY project aims to develop a methodological approach that relates SD, PD, and FD of macrophytes, for a better understanding of aquatic ecosystems functioning and the conservation of the evolutionary potential of macrophyte communities. The project is carried out by three research groups: the Institute for Electromagnetic Sensing of the Environment in Milan for the spectral analysis, the University of Parma for the analysis of functional traits and the University of Florence for the genetic diversity analysis. In this context, my PhD work has focused on molecular analyses closely related to the project macroDIVERSITY and other case studies focused on deepening the knowledge of the macrophyte communities' diversity in central-northern Italy.

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2. STUDY BACKGROUND

2.1. Freshwater ecosystems: role, importance, and conservation status

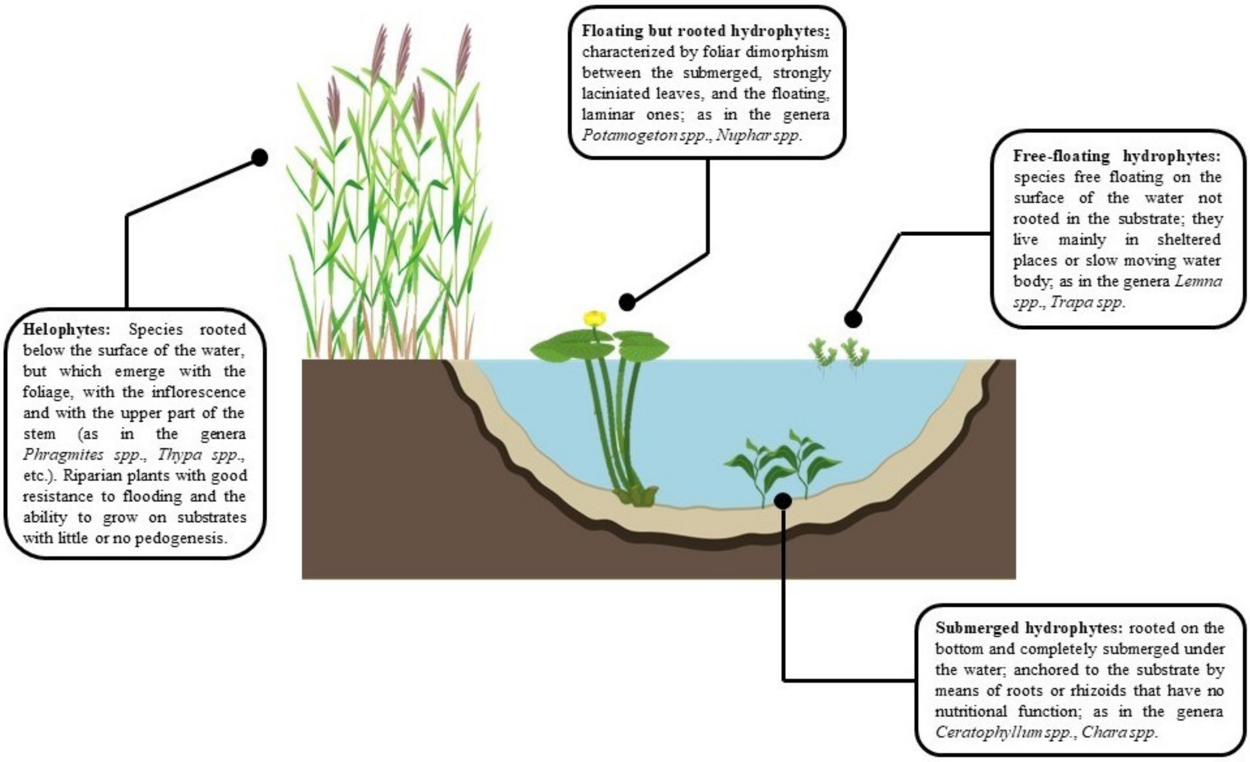
Wetlands are defined in Article 1.1 of the Ramsar's Convention as “areas of marsh, fen, peatland or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salt, including areas of marine water the depth of which at low tide does not exceed six meters”. In this context, freshwater ecosystems represent less than 1% of the Earth's surface and host a great variety of life forms, amounting to about 6% of the species described (Dudgeon at al., 2006; Hawksworth & Kalin-Arroyo, 1995). There are many typologies of environments related to freshwater, each representing a stand-alone system with its own physical and chemical characteristics, differing in local environmental conditions (e.g., habitat geometry, presence or absence of flow, time of water residence), as well as hydrological connectivity (Hutchinson, 1975; Wetzel, 2001). These different geomorphological and hydric conditions play a crucial role in determining plant community structure (France and Duffy, 2006; Starzomski and Srivastava, 2007) and, consequently, in the provision of several ecosystem services such as flood control, shoreline stabilization, water purification and carbon dioxide sequestration (Haines-Young and Pothschin, 2010; Lamarque et al., 2011). However, while some ecosystem services can be renewed (e.g., water supply), others (e.g., biodiversity and genetic resources) may be lost forever (Dodds et al., 2013). In this respect, increased urbanization, land use changes, pollution, alien plant invasion and other anthropogenic activities have been recognized as the most dramatic drivers impacting biodiversity loss and freshwater ecosystems degradation (Dudgeon et al., 2006; Vorosmarty et al. 2010, Seebens et al., 2017). The need to recover the functionality of these ecosystems has been sanctioned by many local and international guidelines, such as the UN resolution 58/217, which established "The International Decade for Action, Water for Life 2005-2015" or the European Water Framework Directive WFD 2000/60 for the protection and sustainable management of water resources. WFD tracks species composition and abundance of various Biological Quality Elements (BQEs) for accurately assessing ecological states and biodiversity. Understanding the relationships between functional structure, processes and services in the ecosystem remain the central node of debate, and to effectively protect and restore freshwater systems, it is crucial to use of a combined approach, for example by molecular, spectral, and ecological tools. The traits by which organisms transform resources, modify habitat structure and chemistry, or compete with other organisms may be excellent candidates to predict and preserve ecosystem properties. In freshwater habitats, macrophyte represent a critical structural and functional element and are recognized as model systems for many ecological and conservation studies (Feuchtmayr et al., 2009; Jeppesen et al., 2012; Svitok et al., 2018).

2.2. Macrophytes: classification, ecological role and conservation status

Macrophytes are defined on an ecological-functional basis and include the macroscopically visible plants in aquatic and marshy environments. This grouping comprises herbaceous angiosperms, pteridophytes, bryophytes and filamentous algae (Chambers et al. 2007). Based on the ecology of the species, concerning the hygrophilia of organisms, it is possible to distinguish macrophytes into four different groups: helophytes, submerged hydrophytes, floating but rooted hydrophytes and free-floating hydrophytes (Figure 1; Den Hartog & Segal, 1964).

Macrophytes are a valuable part of aquatic ecosystems. Besides providing habitats for many organisms, macrophytes can influence and significantly change the conditions of aquatic ecosystems (Carpenter and Lodge, 1986; Peters and Lodge, 2009; Choi et al., 2014). They play a significant role in water body's hydrological regime, nutrient cycle, and sediment dynamics by inhibiting resuspension (Barko et al., 1991; La Toya et al., 2013) and maintaining clear water status (Jeppesen et al., 2012). Both submerged and emergent species can affect water quality by binding and removing nutrients, organic contaminants, and even heavy metals (Dhote & Dixit, 2009). In addition, some macrophytes have narrow ecological niches that make them suitable as indicators of the ecological quality of freshwater environments (Penning et al., 2008; Søndergaard et al., 2010). Overall, maintaining healthy macrophyte communities is essential for freshwater ecosystems functioning. However, the survival of many aquatic plant communities in wetland and freshwater ecosystems is threatened by a wide range of direct or indirect factors, mainly linked anthropic activities. As a result of climate change, macrophytes are most impacted by changes in physicochemical water conditions, such as increased temperatures (Short et al., 2016), intense precipitation, increased nutrient loads and suspended matter. These factors, synergically with biotic interactions, may drastically influence the composition and dynamic of macrophytes communities (Scheffer & Nes, 2007; Van den Besselaar et al., 2013). The spread and establishment of invasive alien species in particular is considered the first main threat to this element of biodiversity of freshwater ecosystems (Havel et al., 2015; Sorte et al., 2013).

Therefore, the study of ecology, genetic differentiation, and the functional traits is crucial to represent and predict the adaptation mechanisms of macrophytes and to further maintain and manage aquatic ecosystems in the face of global changes and biodiversity loss.



Floating but rooted hydrophytes: characterized by foliar dimorphism between the submerged, strongly laciniated leaves, and the floating, laminar ones; as in the genera *Potamogeton spp., Nuphar spp.*

Free-floating hydrophytes: species free floating on the surface of the water not rooted in the substrate; they live mainly in sheltered places or slow moving water body; as in the genera *Lemna spp., Trapa spp.*

Helophytes: Species rooted below the surface of the water, but which emerge with the foliage, with the inflorescence and with the upper part of the stem (as in the genera *Phragmites spp., Thypa spp.,* etc.). Riparian plants with good resistance to flooding and the ability to grow on substrates with little or no pedogenesis.

Submerged hydrophytes: rooted on the bottom and completely submerged under the water; anchored to the substrate by means of roots or rhizoids that have no nutritional function; as in the genera *Ceratophyllum spp., Chara spp.*

Figure 1 *Cross–section of a typical zonation of aquatic macrophytes. Created with BioRender.com (2022).*

2.3. The study of genetic diversity

Genetic diversity is one of the most important components of biodiversity and plays a crucial role in the persistence and adaptation of species to environmental change over time (Spielman et al., 2004; Hoffmann and Sgro, 2011). The biological idea of "Adaptation" refers to a selection process of heritable traits that increase the fitness of an individual relative to other individuals in a population. Starting from this concept, studying the genetics of adaptation can entail identifying specific genetic locus responsible for the expression of an adaptive trait and variants which potentially depict phenotypic differences in a population. Moreover, it is assumed that populations with higher genetic diversity are more resilient to natural and anthropogenic disturbances (Barabás and D'Andrea, 2016) since they contain higher levels of variation on which natural selection can act (Lacy, 1987; Booy et al., 2000); the contrary, populations with low genetic diversity, are often considered at conservation risk (e.g., Spielman et al., 2004). Exploring the species' genetic structure and its link with functional diversity can let thus us know the processes affecting biological diversity and permit its protection. Currently, the "gold standard" for causally linking genetic and phenotypic variation is a direct measurement of fitness in the natural environment, ideally over multiple seasons (e.g., Anderson et al., 2014). However, with the fast progress in genetic techniques, particularly whit the advent of third- and fourth-generation sequencing approaches, it has been possible to develop more and more accurate studies in detecting the role of natural selection in shaping a trait. The most popular approach used in the past has been the restriction site-associated DNA sequencing (RAD-seq), a method for sequencing a small random part of each genome deeply in many individuals simultaneously to discover variants and genotypes with high confidence (Andrews et al., 2016; McKinney et al., 2017). Later, whole genome sequencing (WGS) allowed the detection of large stretches of the genome among the markers that remained unsampled by RAD-seq, thus revealing highly localized selection and divergence signatures (Tiffin & Ross-Ibarra, 2014; Lowry et al., 2017). Since WGS is still cost-prohibitive, sequencing pools of individuals (Pool-seq; Schlötterer et al., 2014) or low-coverage whole genome sequencing (lcWGS; Pasaniuc et al., 2012; Gilly et al., 2018) were considered as valid alternatives. However, when the number of individuals pooled and the sequencing depth are insufficient, individual-level analyses and identification of cryptic substructures among sampled individuals are impossible whit the Pool-seq method (Zhu et al., 2012; Fuentes-Pardo & Ruzzante, 2017). The lcWGS instead allows population-scale screening of the entire genome, retaining individual information. This approach sacrifices the depth of coverage in exchange for a much greater breadth of coverage. Another approach, which it does not necessarily use Next Generation Sequencing (NGS), compares phenotypic divergence among populations to neutral genetic divergence; the so-called QST/FST approach (Brommer 2011; Leinonen et al. 2013). However, phylogenetic tests for adaptation are also helpful for many evolutionary questions (Ackerly, 2004), particularly if extended from the population to the community level.

2.3.1 Inter-specific genetic diversity: the phylogenetic diversity approach

Phylogenetic diversity “PD” is a biodiversity measure based on phylogeny. It is well-accepted that phylogeny mirrors the cumulative phenotypic and genetic differences between evolutionary lineages (Harvey & Pagel, 1991). These differences could clarify or even predict ecological and biological processes. Therefore, phylogenetic information has increasingly been included in community studies, often providing critical details on the organization and assembly of ecological communities. The increasing availability of phylogenies for many taxonomic groups has given rise to several phylogenetic metrics (i.e., Faith, 1992; Webb et al., 2002; Hardy & Senterre 2007; Helmus et al. .2007). Faith's phylogenetic diversity index (1992) is the most widely used metric describing the evolutive diversity in a specie group. It is the most straightforward measure of the cumulative evolutionary age in a community, and it was commonly used in conservation studies and ecological research (Faith, 1992; Forest et al., 2007; Rodrigues and Gaston, 2002). However, the PD metric is based on the sum of the branch lengths connecting all the taxa in a phylogeny without considering their abundance. The Net Relatedness Index (NRI) and the Nearest Taxon Index (NTI) were thus proposed to quantify the degree of community-weighted phylogenetic relatedness among species within each consortium. NRI represents the standardized effect size of the mean phylogenetic distance (MPD), which estimates the average phylogenetic relatedness between all possible pairs of taxa in an assemblage (Webb, 2000). NTI is a standardized measure of the branch-tip phylogenetic clustering of the species on the plot and is usually indicated as Mean Nearest Taxon Distance, MNTD. Unlike NRI, the NTI metric is independent of the arrangement of the higher-level groups in the phylogenetic tree (Webb et al., 2002).

The relevance of phylogenetic diversity metrics in plant ecological studies depends on the hypothesis that closely related species share more functions than distantly related species. Consequently, a community characterized by high phylogenetic diversity could harbor species with complementary functions (Ritchie et al., 2021, Thompson et al., 2015); this, in turn, could lead to the maintenance of more biological properties and increase the number of ecosystems services. On the other hand, the smaller the phylogenetic diversity among species, the greater the functional redundancy (Naeem 1998, Petchey et al. 2007). Communities with more redundant species would be buffered from losses of any given species following disturbance events, resulting in stability in community structure or ecosystem function (Fonseca and Ganade, 2001). From this perspective, there is also a growing interest in using evolutive diversity metrics for conservation prioritization. However, phylogenetic diversity can be used as a proxy for functional diversity only if the species traits considered are phylogenetically conserved (Winter et al. 2013). Even though several functional traits are conserved enough to show an intense phylogenetic signal (e.g., Donoghue, 2008), closely related species may differ significantly in some functional traits due to rapid evolution or ecological convergence (Losos, 2008). In this context, the phylogenetic signal, intended as a measure of how informative the data is a relative underlying evolutionary process (Lemey, 2009), depends on community phylogenetic structure (e.g., Graham et al., 2012), environment (Burns & Strauss, 2012), and spatial scale (Kembel & Cahil, 2011).

Regarding aquatic macrophytes, the knowledge of functional and phylogenetic dimensions is limited by a low quality of data, and only since this century have research programs been focused on how macrophyte diversity patterns are affected by macroecological gradients (e.g., Les et al., 2003; Chambers et al., 2007; Alahuhta et al., 2017, 2018; Murphy et al., 2019; Zhang et al., 2019). Unfortunately, a robust phylogeny that represents all macrophyte lineages and includes detailed information on their functional ecology still has to be developed. The first reconstructions of evolutionary relationships are known for Potamogetonaceae (Lindqvist et al., 2006), Hydrocharitaceae (Chen et al., 2012) and Alismataceae (Ross et al., 2016). Later, García-Girón et al. (2020) constructed the first phylogeny, including several freshwater plants and explored critical functional related traits. In that perspective, further study combining phylogenetic and functional diversity of macrophytes should be required for integrating current knowledge and testing the hypotheses related to patterns in aquatic plants diversity, especially in the light of changing environmental conditions in an increasingly human dominated world (Devictor et al. 2010, Gianuca et al. 2018).

2.3.2 Intra-specific genetic diversity: the study of genetic diversity at population level

As a fundamental component of biodiversity, intraspecific genetic diversity intrinsically informs on populations' demographic and evolutionary history, biological connectivity level, and ability to adapt to environmental changes (Carroll et al., 2014; Rey et al., 2016; Mittel et al., 2015). It is based on the relationship between heterozygosity and population fitness (Reed and Frankham, 2003). Loss of genetic diversity (for example, due to smaller population sizes or decreased population connectivity) is related to inbreeding which in turn reduces reproductive fitness (Lande, 1988; Fahrig, 2003). However, this correlation may be weak or non-existent since genetic diversity is generally analyzed using the neutral genetic diversity (i.e., the genome portions that have not been affected by adaptive processes) through molecular markers capable of detecting the diversity (mutations) of homologous DNA regions in different individuals belonging to the same species (Pritchard et al., 2000; Putman and Carbone 2014; Blanchet et al., 2017). These markers are relatively easy to detect and provide an unbiased estimate of random processes like genetic drift (Luikart, 2003; Storz and Nachman, 2003). They are not necessarily referable to the activity of specific genes, as they can be located in both introns and exons regions. The measurement of genetic diversity through the investigation of molecular markers involves the identification of variations in the nucleotide sequence of the markers themselves or analyzing of their polymorphisms (Qi et al., 2014). The greater the number of polymorphisms associated with a marker, the better its discriminating power and, therefore, the information it will provide (Serrote, 2020). What is achieved is a molecular imprint (fingerprinting) of the individuals considered. There are a large panel of molecular markers usable for DNA-fingerprinting analysing, some more dated, such as Amplified Fragment Length Polymorphism (AFLP; Vos et al.,1995) and Simple Sequence Repeats (SSR; Morgante & Olivieri, 1993), than those NGS-derived allowing the rapid discovery of thousands of polymorphisms for several individuals in a single sequencing run, and the genotyping of large populations (Zhang et al. 2011).

As for aquatic plants, there have been several studies on intraspecific genetic diversity and trait variability as strategies for spreading and adapting to different ecosystems (Riis et al., 2010; Weyl and Coetzee, 2016). There is a paradigm whereby the broad tolerance of macrophytes to a range of environment conditions is often coupled with low levels of intraspecific genetic diversity due to clonal reproduction as preferred strategy (Santamaría, 2002). In fact, intraspecific variation may result from both heritable differences and phenotypic plasticity (Scheiner., 1993). Thus, the study of the intraspecific genetic diversity of macrophytes in relation to their trait variability is crucial to understand the contribution of phenotypic plasticity and heritable differences.

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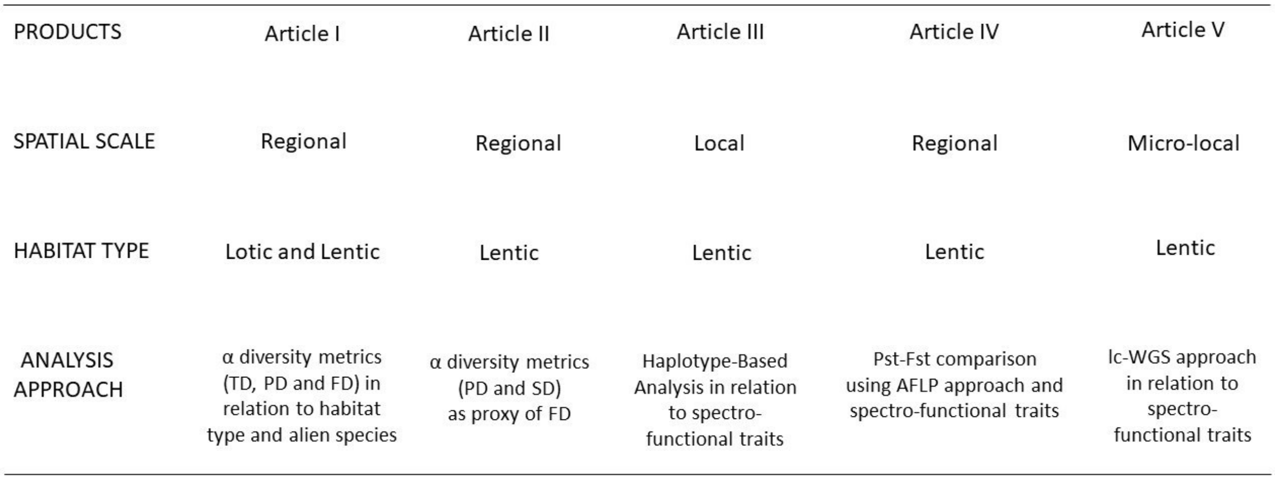
3. MAIN STUDY AIMS

The general aims of this thesis were i) to investigate the genetic diversity of freshwater macrophytes at both local and regional scales, from the population to community level; ii) to relate the genetic diversity results with the spectro-functional diversity data; iii) to decipher the adaptive processes shaping the evolution of two focal species dominating the wetlands under investigation. These objectives were faced within five scientific articles. Article I and II investigated the phylogenetic analysis of the macrophytes communities in selected sites of north-central Italy to outline their evolutionary relationships. Specifically, Article I (manuscript in elaboration phase) assessed the phylogenetic diversity metrics of the macroDIVERSITY project plant communities to evaluate the relationship with spectral and functional diversity. Article II (published in the journal "Knowl. Manag. Aquat. Ecosyst.") analyzed the incidence of alien species on the taxonomic, phylogenetic, and functional diversity of lentic and lotic communities dominated by *Phragmites australis*. Furthermore, articles from III to V have focused on the intraspecific genetic diversity of the helophyte *Phragmites australis* and the emergent hydrophyte *Nuphar lutea*. In particular, Article III (published in the journal "Hydrobiologia") has been investigating the relationship between the spectro-functional traits and the haplotype composition of *P. australis* at regional scale, discussing the possible connections with both the meteo-climatic conditions and the ecological status of stands. Article IV (manuscript under review on Freshwater Biology) has explored the Pst-Fst comparison to infer the relative role of genetic drift and natural selection on the diversification of both species' phenotypic traits across different wetlands. Finally, Article V (manuscript in elaboration phase) further deepened the analysis of *P. australis*' genetic diversity at the micro-local scale linking the spectro-functional traits with results obtained from the lcWGS approach.

4. STUDY SITES AND SPATIAL SCALES

The sampling sites (hereafter sites) cover the main centres of diversity of freshwater ecosystems of central-northern Italy (Bolpagni et al., 2018). Each sampling site includes a set of wetlands communities constituting sampling units (hereafter plots). Habitat type and spatial scales vary in different articles based on the used approach and the specific aims of the research (Figure 2). More specifically, the study extents range from a single site, at a very micro-local scale (Article V), to sites located in the same (Article III) or different (Articles I-II and IV) geographical regions. In Articles I, IV and V, data were obtained from one or more sites specifically selected for the macroDIVERSITY project. Each site covered the following environmental gradients: i) ecological conditions, i.e. trophic status derived from existing monitoring programs (Water Framework Directive) and satellite-based water quality proxies (turbidity and Chl-a concentration); ii) geographical features, i.e. surface area covered, the land cover of the watershed and habitat thematic maps (Habitats Directive); and iii) plant community structure, characterized from literature data and macrophyte growth form classification from satellite data (Villa et al., 2015). Article II integrated new data into a dataset extrapolated from published studies on lotic and lentic communities dominated by *P. australis*. To maximize the comparability between studies and plots, Article II considered only reed-dominated communities (cover ≥50%; Braun Blanquet values of 4 and 5), plot size from 2 to 100 m2, and sampling dates of relevés from 1989 to 2020. In addition, to further ensure comparability among data in terms of ecological and climatic conditions, an altitudinal threshold was fixed (500 m a.s.l.), excluding all the relevés recorded above this limit. In Article III, data were obtained from sites that differed in their pedo-morphological characteristics; from lowland and upland wetlands such as Colfiorito, Fucecchio, Massaciuccoli and Porta to shallow, turbid lakes such as Chiusi and Trasimeno, or volcanic basins (Vico Lake), characterized by deep and clear water. The surface areas of the sites were also variable, ranging from the smallest one, Colfiorito, covering 0.8 km^2, to the largest site Trasimeno, covering 121.5 km^2.

In general, strict quality control for selecting each data set was carried out: i) each site had to include both widespread macrophyte communities/populations and heterogeneous plots; ii) all sites had to be primarily natural systems, and iii) all macrophyte communities/populations within each data set had to have been empirically surveyed using similar methods to maintain data comparability. Including different types of sites (lotic and lentic environments) and different ecological conditions were considered essential in increasing the range of environmental features. Further descriptions of study areas and sampling designs are provided in the corresponding articles.



PRODUCTS	Article I	Article II	Article III	Article IV	Article V
SPATIAL SCALE	Regional	Regional	Local	Regional	Micro-local
HABITAT TYPE	Lotic and Lentic	Lentic	Lentic	Lentic	Lentic
ANALYSIS APPROACH	α diversity metrics (TD, PD and FD) in relation to habitat type and alien species	α diversity metrics (PD and SD) as proxy of FD	Haplotype-Based Analysis in relation to spectro-functional traits	Pst-Fst comparison using AFLP approach and spectro-functional traits	lc-WGS approach in relation to spectro-functional traits

Figure 2 *Overview, from the bottom up, of the analysis approach, habitat type and spatial scale considered for the production of each thesis article. PD = phylogenetic diversity, FD = functional diversity, SD = spectral diversity; AFLP = Amplified fragment length polymorphism; lc-WGS = Low-coverage whole genome sequencing.*

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5. RESULTS

5.1. Inter-specific genetic diversity

5.1.1. Article I: Merging spectral and phylogenetic diversity to assess macrophyte traits and functions along multiple gradients.

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Manuscript in elaboration phase.
Journal to which it will be submitted once completed: "Remote Sensing of Environment"

Contribution table:

	Maria Beatrice Castellani	Lorenzo Lastrucci	Andrea Coppi
Experimental Design	50		50
Field Work / Experiment Maintenance	50	50	
Sample Analysis	80		20
Manuscript Drafting	90		10

Author's contribution (percentage) relative to the different tasks carried out in this work. Only authors from the University of Florence are mentioned since this chapter has focused only on the phylogenetic diversity within the macroDIVERSITY project.

Introduction

In this era of global environmental changes, with relative loss of ecosystem functions and services, assessing biodiversity in a concise, scalable, and cost-effective way is becoming increasingly crucial. The mechanisms underlying the ecosystem benefits of biodiversity are a result of functional differences among organisms (Tilman et al., 1998; Hector et al., 2010) that facilitate complementary resource use (Tilman et al., 1997; Diaz and Cabido, 2001; Williams et al., 2017). Therefore, the conservation of high levels of functional diversity (FD) is a priority in the management of ecosystems (Zuppinger et al., 2014; Kraf et al., 2015; Cadotte, 2017). As a result of their FD, macrophytes contribute to freshwater ecosystems resilience, climate adaptation, invasive species sensitivity, and anthropogenic impacts (Cadotte et al., 2011; Flynn et al., 2011). In the last decade, spectroscopy and genetics-based techniques proved to be highly useful for monitoring macrophyte status and dynamics, integrating measures of plant functional diversity (e.g., Kokaly et al. 2009; Asner et al. 2011; Flynn et al., 2011; Asner, 2013; Faith, 2016; Villa et al., 2021; Garcia-Giron et al. 2020). Spectral diversity (SD) is a metric of the variability of the spectral reflectance of plants at different wavelengths. As spectral reflectance is primarily influenced by leaf pigments (Ustin et al., 1989; Curran, 1989), leaf morphology and size are determinant factors influencing it (Ollinger, 2011). Therefore, spectral differences among plants capture functional differences in morphological and biochemical traits (Jacquemoud and Baret, 1990; Ustin and Gamon, 2010). It follows that SD can be used as a proxy of FD, providing an integrated measure of phenotypes variability. Phylogenetic diversity (PD) of plants, similarly to SD, incorporates multiple functional traits and reflects key functions of ecosystems (Srivastava et al., 2012). It is generally assumed that functional differences increase with evolutionary divergence time (Felsenstein, 1985); however, phylogenetic distance does not necessarily correlate with traits dissimilarity (Losos, 2008). Therefore, the relationship between PD and ecosystem function can vary depending on the traits considered and their evolutionary conservatism level (Gravel et al., 2012). Combining SD and PD may then provide a better understanding of ecosystem function and evolutionary potential. The main objective of macroDIVERSITY is to develop a methodological approach that links spectral (SD), phylogenetic (PD) and functional (FD) diversity of macrophytes advancing the state of the art in terms of current knowledge and analysis techniques, regarding community ecology of aquatic ecosystems. To this, three targets are tested T1) the assessment of macrophyte traits (morphological, biochemical, physiological) from remote (and proximal) sensing data with high spatial and spectral resolutions (target of both Milan and Parma research unit); T2) the implementation of metrics based on phylogenetic data to measure macrophyte biodiversity (target of Florence research unit); T3) machine learning-based integration of traditional and new SD and PD metrics for mapping macrophyte FD at a synoptic scale (target of all the research units). As a member of the research unit of the University of Florence, part of my PhD work focused on the second target (T2) of the project, particularly assessing the phylogenetic diversity of the sampled macrophyte communities.

The results presented in this chapter will not be published individually but represent a milestone to complete the macroDIVERSITY project.

Materials and methods

Study area and sampling design

The research was conducted in six wetland ecosystems in central-northern Italy (Figure 1, Tab 1), which host widespread macrophyte communities. Sites are phytogeographical comparable areas based on different environmental gradients such as trophic status and plant community's structure. Trophic levels among sites ranged from oligo mesotrophic conditions (IS and BR) to mesotrophic (PA) or eutrophic (CH, MA and MN). In each site, macrophyte stands belonging to three main growth forms were considered: helophytes (e.g., *P. australis* dominated stands), emergent and floating hydrophytes (e.g., nymphaeids dominated stands), and submerged hydrophytes (e.g., potamids or Characeae dominated stands). A variable number of 4m^2 sampling plots were selected for each site, for a total of 166 plots. The floristic composition was surveyed from each plot, scoring all plants for cover percentage. Portions of leaf tissue belonging to taxa not available on Genbank were collected in subsequent resampling and dried in silica gel for molecular analysis.

Figure 1 *Geographic location of the study area with distribution of the sampling sites.*

SITE	CODE	LOCALITY	COORDINATES			
			N	E	N	E
Bracciano and Martignano Lakes	BR	north-western Lazio	42°07'16''	12°13'55''	42°06'46''	12°18'54''
Chiusi Lake	CH	south-eastern Tuscany	43°03'22''	11°57'55''		
Massaciuccoli Lake	MA	north-western Tuscany	43°50'00''	10°19'30''		
Mantua Lakes system	MN	south-eastern Lombardy	45°09'36''	10°47'48''		
Iseo Lake	IS	north-eastern Lombardy	45°43'00''	10°05'00''		
Pusiano and Annone Lakes	PA	north-western Lombardy	45°48'09''	09°16'34''	45°48'43''	09°20'58''

Table 1 *Table shows the study sites, their code abbreviation, geographical locality, and coordinates.*

Dataset

The species abundance matrix (Supplementary material_Table1), obtained from the vegetation analysis, was used to quantify the species richness (SR) and two phylogenetic metrics weighted on species abundance, the Net Relatedness Index (NRI) and the Nearest Taxon Index (NTI).

Selection of molecular markers, DNA isolation, sequence alignment and phylogenetic analysis

Phylogenetic diversity of the macrophyte communities in the plots was inferred from a super-tree obtained from the analysis of three markers of the nuclear DNA (Internal Transcribed Spacers of the nuclear ribosomal DNA, the ITS1-5.8S and ITS2 regions) and two markers of the chloroplast DNA (rbcL and matK). The first reason for using these markers as an indicator of evolutionary relationships is that they are the most widely used for phylogenetic inference in macrophytes. This way, it has been possible to retrieve the sequence data from open-source databases (http://www.ncbi.nlm.nih.gov/) for most of the species represented in the communities. Second, the genetic markers mentioned above have a great discriminatory power at low taxonomic levels and were widely used as DNA barcodes for higher plants (Kerss, 2017).

The source of the molecular data for 99 species found in our sampling sites is reported in Supplementary material Table 2. Most sequences have been obtained from GenBank. No account has been taken of the geographical origin of the accessions used since the evolutionary relationships between taxa has been investigated. Those taxa lacking sequences of one or more markers were processed for the isolation of genomic DNA following a modified 2 × CTAB protocol (Doyle and Doyle, 1990). The list of the used primers during the DNA amplification is summarized in Table 2. Polymerase chain reactions were performed in a total volume of 25 µl containing 2.5 µl of 10 × reaction buffer (Dynazyme II, Finnzyme, Espoo, Finland), 1.5 mM MgCl2, 10 pmol of each primer, 200 µM dNTPs, 1 U of TaqDNA polymerase (Dynazyme II) and 10 ng of template DNA. The MJ PTC-100 thermocycler was used to perform the reactions (Peltier ThermalCycler, MJ Research, Waltham, Massachusetts, U.S.A.). Subsequently, 5 µl of each amplification mixture was analyzed by agarose gel electrophoresis in TAE buffer (1.5% w / v) containing 1 µg / ml ethidium bromide. After purification (Roche, Mannheim purification kit, Germany), the PCR reactions were quantified with a spectrophotometric method (Biophotometer, Eppendorf). Amplification was unsuccessful for 9 species (as indicated in Supplementary material Table 2). In this case, accessions of congeneric species or confamilial genera were selected as "proxy species" from GenBank. ABI310 sequencer was used to sequence DNA directly from the purified PCR products (PE-Applied Biosystems, Norwalk, Connecticut, U.S.A.). Original sequences were checked for homology with Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and edited with BioEdit v.7.0 (Hall, 1999).

Three datasets were initially separately prepared, one for each marker ITS1-5.8s-ITS2, rbcL and matK, respectively. Multiple alignments were performed using two different strategies with MAFFT vs 7 (Katoh and Standley, 2013). The Q-INS-i method was used for ITS, whereas the GINS-I was used for both rbcL and matK. Gaps were coded separately for each alignment using FastGap 1.2 (Borchsenius, 2009) and appended at the end of the alignment according to Simmons & Ochoterena (2000). Phylogenetic reconstruction was finally performed on a concatenated alignment using the Maximum likelihood (ML). The analysis of ML was carried out using the RAxML tool (Stamatakis, 2014) via the CIPRES cluster (Miller et al., 2011). Bootstrap values were calculated after 999 iterations under the GTRþCAT approximation of rate heterogeneity (Stamatakis, 2006).

Target	Primer	Direnction	Sequence	Reference
ITS	ITS 36F	FW	GGCGCTGTGAGAAGTTCATTGAACC	Hall et al. (2010)
	ITS IR	RV	TTCTGCAATTCACACTACGTATCGC	Hall et al. (2010)
	ITS IF	FW	CAACTCTCGGCAAACGGATATCTTG	Hall et al. (2010)
	ITS R	RV	GGTTGGTCCCGACCGATCTGAGGTC	Hall et al. (2010)
	ITS5	FW	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990)
	ITS-u2	RV	GCGTTCAAAGAYTCGATGRTTC	Cheng et al. (2016)
	ITS-u3	FW	CAWCGATGAAGAACGYAGC	Cheng et al. (2016)
	ITS-u4	RV	RGTTTCTTTTCCTCCGCTTA	Cheng et al. (2016)
rbcL	rbcLa_f	FW	ATGTCACCACAAACAGAGACTAAAGC	CBOL Plant Working Group (2009)
	rbcL724_rev	RV	GTAAAATCAAGTCCACCRCG	CBOL Plant Working Group (2009)
matK	fEDR	FW	ATTCATTCRATRTTTTTATTTHTGGARGAYAGATT	Kuo et al. (2011)
	rAGK	RV	CGTRTTGTACTYYTRTGTTTRCVAGC	Kuo et al. (2011)
	matK472F	FW	CCC RTY CAT CTG GAA ATC TTG GTT C	Yu et al. (2011)
	matK1248R	RV	GCT RTR ATA ATG AGA AAG ATT TCT GC	Yu et al. (2011)

Table 2 *Table shows the primers used in this study.*

Indices of phylogenetic diversity and structure

The Net Relatedness Index (NRI) and the Nearest Taxon Index (NTI) were used to provide information regarding the phylogenetic divergence within the communities (Webb, 2000; Webb et al., 2002; Tucker et al. 2016). Positive values of NRI and NTI reveal phylogenetic clustering, i.e., the species within the community are more evolutionarily related than expected by the null hypothesis. Negative values reveal phylogenetic over-dispersion, i.e., the species within the community are less closely related than expected by chance. In general terms, NTI and NRI values higher/lower than 1.96/−1.96 are usually considered indicators of significant patterns. Both phylogenetic indices were obtained with the package Picante (Kembel et al. 2010) in R vers.3.4.3 (R Core Team 2017).

Results

Dataset

The vegetation survey showed 99 plant species, 89 Angiosperms, two Pteridophytes and eight Algae. The most represented families were Cyperaceae, Asteraceae, Potamogetonaceae, Characeae, Hydrocharitaceae and Lamiaceae, which count more than five taxa, whereas 25 families were underrepresented, with only one taxon registered. At site level, 52 plots were monospecific, dominated by *Nuphar lutea* or *Phragmites australis*. MA and MN lakes showed higher species richness SR (47 and 37 species, respectively) than the other four sites (BR = 26, CH = 26, PA = 21, IS =19).

Phylogenetic diversity and structure

The aligned matrix included a total of 2650 positions (383 constant and 2221 variable sites) for the combined markers (ITS+rbcL+matK). The coded gaps were attached from positions 1856 to 2650. The backbone of the resulting tree (Figure 2 and Supplementary material_Table 3 for the Newick format) was largely congruent with APG-IV topology (APG IV, 2016), that is considered the best available knowledge on the phylogenetic relationships of angiosperm lineages. M onocots and eudicots were both well supported as monophyletic, with Acorales and Alismatales being the successive sister groups to monocots and Ceratophyllales was classified as sister to eudicots. Nymphaeales were sisters of all seed-bearing plants, while Ferns diverged from the main Nymphaeales-Angiosperm group and resulting sister of Charales which constitute a well-supported terminal clade (Figure 2).

As for the phylogenetic metrics, NRI values ranged from 2.24 (BR13) to -3.80 (BR25), while NTI values ranged from 2.83 (BR09) to -3.13 (PA03) (Supplementary material_Table 4). It is important to note that almost a third of the plots showed no phylogenetic structuring because of having only one species, *P. australis* or *N. lutea*, that dominated the area. In particular, CH and PA sites showed a higher number of monospecific stands (16 and 14, respectively). Phylogenetic structure of communities showed that both NRI (Figure 3a) and NTI (Figure 3b) values were mostly positive but non-significant, indicating randomness with a tendency to a clustered pattern. However, there are several communities (mainly plots of MN, PA and BR sites) that instead showed significant negative values, indicating phylogenetic overdispersion both at the deepest and highest level of the phylogenetic tree. Moreover, seven plots, again belonging to the BR and PA sites, showed a significative phylogenetic clustering of the community. These communities are mainly composed of submerged macrophytes such as Characeae and Potamogeton mixed or of emerging floating macrophytes such as *Nuphar*, *Nelumbo* and *Trapa* and *Lemna*.

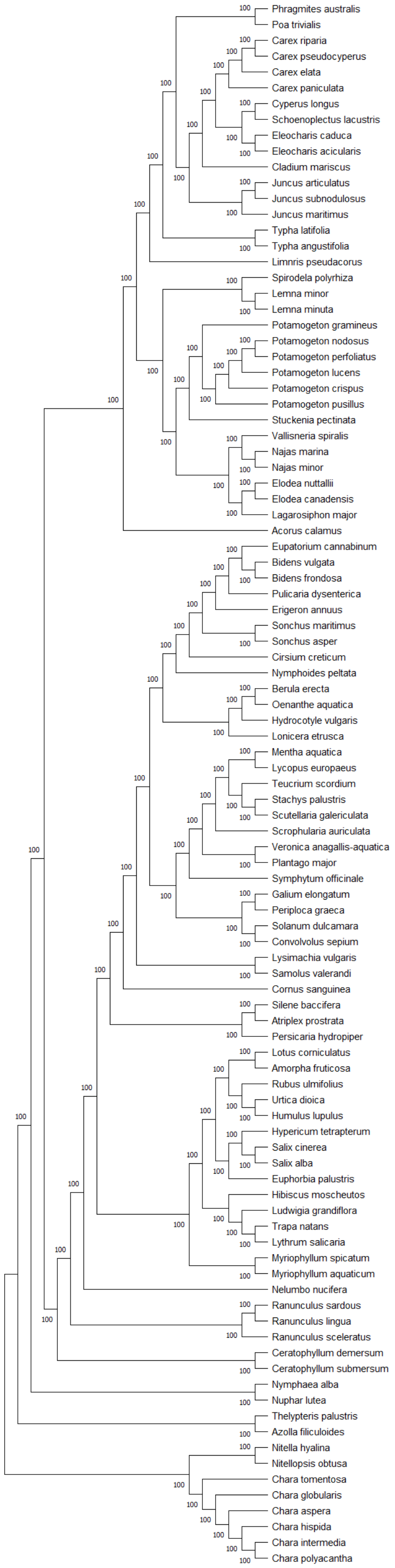

Figure 2 *Maximum likelihood tree based on combined ITS, rbcL and matK alignment. Bootstrap values are indicated near branches.*

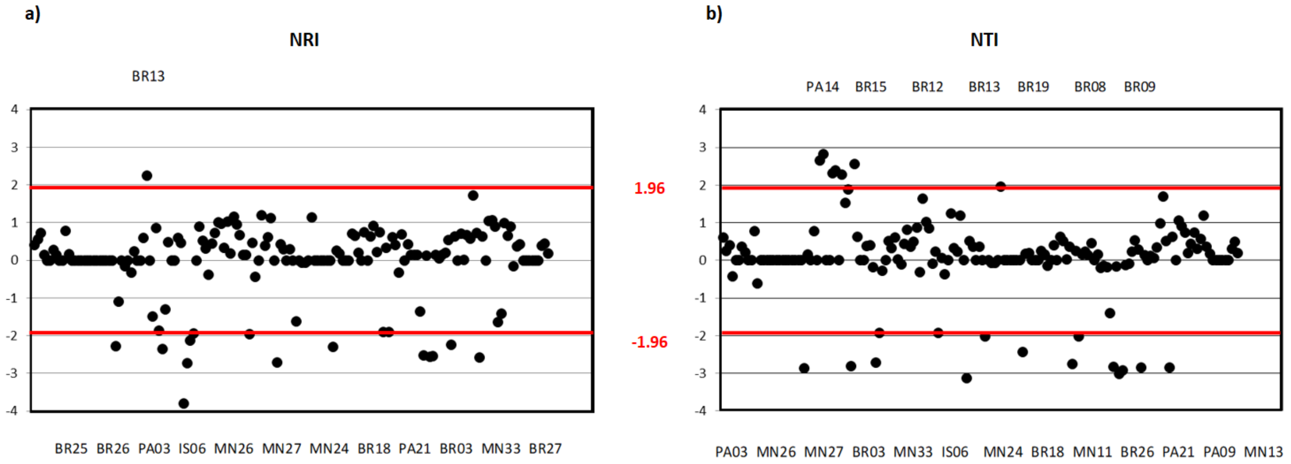

Figure 3a,b *The Net Relatedness Index (NRI) (a) and the Nearest Taxon Index (NTI) (b) of macrophytes community at each sampled plot. Positive NRI and NTI values indicate phylogenetic clustering while negative values indicate phylogenetic overdispersion. The figure reports the plots ID for those communities that showed statistically significant NRI and NTI values.*

Discussion

This work provided information on the distribution of phylogenetic diversity metrics of 166 indagated communities and solved the "molecular" task expected for the T2 objective of the macroDIVERSITY project. The phylogenetic structure (measured with NRI and NTI indices) showed a general, not significant tendency to a clustered pattern. Exceptions are some communities of the MN, PA and BR sites that showed a significant phylogenetic overdispersion or a significant phylogenetic clustering. Looking at plant communities' composition, it was possible to note that the submerged macrophyte component brings a high value to the calculation of the diversity metrics. Therefore, it is possible also to assume a relevant functional diversity impact. These results constitute a first step for mapping the investigated communities' macrophyte diversity and will be correlated to the spectral and proximal functional data as soon as available.

Supplementary material:

https://drive.google.com/drive/folders/1Ny3z3ddLGtkONi-Ud-t6ab4dq7irWI8C?usp=sharing

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5.1.2. Article II: The incidence of alien species on the taxonomic, phylogenetic, and functional diversity of lentic and lotic communities dominated by Phragmites australis (Cav.) Steud.

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Experimental Design		30				70
Field Work / Experiment Maintenance	80	15				5
Sample Analysis	80					20
Statistical Analysis	10		90			
Manuscript Drafting	70		10	5		15
Review and Edit	70	5	5	5	5	10

Author's contribution (percentage) relative to the different tasks carried out in this work.

Introduction

Alien plant invasion is one of the most important drivers of biodiversity loss and ecosystem degradation worldwide (Seebens et al., 2017; Bolpagni, 2021). Among the several deleterious impacts on native communities, invasive plants may produce dramatic changes in structure and function of invaded ecosystem (Blackburn et al., 2014; Lazzaro et al., 2020a). Among others, two main hypotheses attempt to predict the incidence of invasion based on the ecological differences between alien and native species within communities (Enders et al., 2020). Firstly, environmental filtering (Kembel and Hubbell, 2006) would ensure that alien species, with similar ecological optimum to native species, might have a greater possibility of establishing and spreading. This would mean that a part of the functional and phylogenetic space, originally occupied by native species in communities, being filled by the alien ones (Delle Fratte et al., 2019). Secondly, the "Darwin's naturalization hypothesis" (Rejmánek, 1996) claims that invasion would increase functional and phylogenetic diversity, when compared to non-invaded communities (Funk et al., 2008). In fact, alien species might exploit unfilled ecological niches if they are functionally and/or phylogenetically different from the native ones (Thuiller et al., 2010). Wetlands seem to be especially vulnerable to invasions: while they cover only a small portion of the earth's land mass, 24% of the world's most invasive plants are wetland species (Bolpagni, 2021). Several disturbances, such as creating canopy gaps, hydroperiod alteration and propagule dispersal routes, facilitates wetland invasion (Galatowitsch et al., 1999; Rejmánek, 2000; Miller and Zedler, 2003). Lentic and lotic habitats represent stand-alone systems, with its own physical and chemical characteristics, differing in local environmental conditions (e.g., habitat geometry, presence or absence of flow, time of water residence) as well as hydrological connectivity (Hutchinson, 1975; Wetzel, 2001). On the one hand, lentic habitats are closed depositional environments, often showing standing and shallow water, generally interconnected to the main canals of the drainage basin thanks to small water bodies, which in turn, limits the water level oscillations and influx-outflux of matter and organisms (Cristofoletti, 1981). Conversely, lotic habitats are open systems where the continuous water flow carries sediments and nutrients derived from underlying catchments (Cristofoletti, 1981) and serves as a vector of propagules dispersion. In this case, flowing water tends to be a limiting and controlling factor, stronger than in lentic environments. These different geomorphological and hydric conditions heavily affect the community structure (France and Duffy, 2006; Starzomski and Srivastava, 2007), the dispersion of aquatic taxa, and their coexistence in multiple sites (Bilton et al., 2001; Bolpagni and Dalla Vecchia, 2021). *Phragmites australis*-dominated stands are the predominant component of submerged and emergent shores of lakes, swamps, pools, ponds, riverbanks and hydrological networks (Landucci et al., 2013), most often with water level ranging from slightly below the soil surface to one meter above ground level (Brix, 1988; Ostendorp, 1993; Ailstock and Cente 2000). Indeed, reed beds play a key structural and functional role in freshwater ecosystems. They offer a suitable environment for the protection of land-water transition ecosystems and their components (Orsomando and Catorci, 1991). In addition, these areas provide multiple services for humans such as flood control and improving water quality (Carpenter and Lodge, 1986; Engelhardt and Ritchie, 2011; Smith et al., 2009). For these reasons, plant communities dominated by *P. australis* are fundamental for the biodiversity conservation, in particular, in the Mediterranean areas where the anthropic activities, such as water drainage, changes in land use, pollution and soil erosion upstream, lead to lower values of functional diversity, favoring the invasion of alien species (Gigante et al., 2011). Several studies described reed-dominated communities from floristic and phytosociological point of view or showed the effects of multiple factors, biotic and abiotic, on the integrity of these sensitive communities (i.e., Coppi et al., 2018; Lastrucci et al., 2016; Angelini et al., 2012; Gigante et al., 2011). On the other hand, to the best of our knowledge, no comparative analyses have been carried out in terms of plant diversity between reeds of lentic and lotic habitats and, as far as we know, studies integrating taxonomical, functional, and phylogenetic information are totally absent. As well, it lacks specific literature on the role of alien species on the structure and functions of reed-dominated communities. To date, studies only investigated the effect of *P. australis* as an alien species (i.e., Ailstock et al., 2001; Uddin and Robinson, 2017b). Recently, the increased availability of phylogenetic data, coupled with the huge development of informatics tools, has promoted the rapid expansion of studies on community ecology (Cadotte et al., 2010; Mouquet et al., 2012). The main idea behind this approach is to interpret taxa phylogenetic positions as result of evolutionary processes, such as extinction (Purvis et al., 2000), speciation (Winter et al., 2009) and biological invasion (Srivastava et al., 2012). It has already become widely accepted that merging the phylogenetic and functional information allows to better quantify the impact of disturbances and to understand the processes contributing on plant community dynamics (Flynn et al., 2011; Perronne et al., 2014; Dehling et al., 2014; Hao et al., 2018; Chun and Lee, 2019; Lazzaro et al., 2020b). In both lentic and lotic environments, *P. australis* dominated communities host a variable number of subordinate or alien species, whose presence can vary differently among sites. In this contest, we can hypothesize that the community assembly rules operate under a series of filters showing spatially nested effects (de Bello et al., 2013), which determine (i) stochastically and time-depending, the pool of colonizing species' of a location; (ii) which species should be selected locally through abiotic filters (i.e., water flooding or canopy gaps); (iii) the set of coexisting species modulated by the positive and negative interactions between organisms (biotic filters) within communities. This study aims to investigate, for the first time, (i) the taxonomical, functional, and evolutive diversity harbored in communities dominated by *P. australis* discriminating between lentic and lotic habitats; (ii) the contribution of alien species on lentic and lotic wetland vegetations dominated by *P. australis*, and (iii) their incidence on taxonomical, phylogenetic and functional diversity of these environments. The main hypothesis of this work was that lentic habitats would have a more incidence of competitive exclusion instead of lotic environments that, in turn, potentially showed a higher incidence of abiotic filters. This differentiated behavior may regulate the establishment and affirmation of alien species, with a higher expected number of alien taxa in lotic sites due to their greater vulnerability to physical and chemical disturbance (Rosset et al., 2017).

Materials and methods

Dataset

We assembled a dataset (Supplementary Material, Tab. S1) based on published studies of wetland plant communities from both central and northern Italy (Tab. S2), representative of the main lentic and lotic environments in the study area (S3a,b). In the present work, the “lentic sites” include the environments not subject to direct influence of current action, such as lakes, ponds, or pools. Whereas all sites influenced by recurring flooding events have been included in the “lotic” systems. As to consider only reed-dominated communities, only the relevés with *P. australis* cover ≥50% or with Braun-Blanquet values of 4 and 5 were extracted from the selected literature. In addition, new floristic and vegetational data from four localities (Lake Idro, Oglio riverscape, Busatello swamp, and the Reggio Emilia lowland) were collected to expand the geographical representativeness of our dataset. To maximize the comparability between studies and plots, both the size (from 2 to 100 m^2) and the sampling date (from 1989 to 2020) of all relevés were considered in the analysis. In addition, to further ensure comparability among data in terms of ecological and climatic conditions, an altitudinal threshold was fixed (500 m a.s.l.), excluding all the relevés recorded above this limit.

Quantifying biodiversity

The species abundance matrix (Tab. S1) was used to quantify three components of biodiversity: taxonomic, phylogenetic and functional diversity. For each component we calculated one metric based on species presence and one diversity metric weighted on species abundance. Taxonomic diversity (TD) was estimated by two traditional metrics: species richness (SR), as the number of species in each plot, and Shannon’s index (H), which accounts for species richness and their relative abundance (Lamb et al., 2009). All analyses were performed in R 3.0 software using the "vegan" package vers. 2.5-7 (Oksanen et al., 2020). Phylogenetic diversity (PD) was quantified using the Faith's phylogenetic diversity (PD_F) metric (Faith, 1992), a measure of the cumulative evolutionary age used in conservation research (Forest et al., 2007, Morlon et al., 2011, Rodrigues and Gaston, 2002), and the Mean Pairwise Distance (phy.MPD) metric, the average evolutionary distance between all pairwise species in a plot (Webb, 2000). In contrast to PD_F, this metric does not compare values to a null model and it does not consider the number of taxa. PD_F and phy.MPD calculations were performed with R software (RCoreTeam, 2017), usingthe "picante" package vers. 1.8.2 (Kembel et al., 2010) and the "PhyloMeasures" package vers. 2.1 (Tsirogiannis and Sandel, 2016) respectively. To compute these metrics, a phylogenetic tree was reconstructed using the online bioinformatics tool Phylomatic (Webb and Donoghue, 2005), matching the list of all our family/genus/ species according to APG III (Angiosperm Phylogeny Group, 2009). and the tip labels of phylogenetic megatree from Zanne et al. (2014). To compute functional diversity metrics, we selected four traits related to the ecosystem function of interest, i.e., leaf economic spectrum and morphology traits: Leaf mass (LM), Leaf Area (LA), Specific Leaf Area (SLA) and Leaf Dry Matter Content (LDMC) (see Perez-Harguindeguy et al., 2016). Functional traits for each species were obtained from international trait databases, LEDA (Kleyer et al., 2008) and TRY (Kattge et al., 2020). In case multiple values were available for a single species, the average value of the trait was used. In addition, traits values of eight species (*Cirsium creticum, Cyperus strigosus, Eclipta prostrata, Euphorbia palustris, Hibiscus moscheutos, Oenanthe silaifolia, Symphyotrichum squamatum and Typha minima*), which were missing in the databases, were measured in this study (Tab. S4). Functional diversity (FD) of each plot was estimated using two multi-trait metrics: the functional richness (FRic), given by the volume occupied by the community in the multidimensional space (Villéger et al., 2008), and the Rao's quadratic entropy (RaoQ), which reflects the abundance of each species, as well as the pairwise functional difference between species. Both these metrics were chosen for their adequate power to detect assembly rules (Mouchet et al., 2010) and were performed with R software using the package "FD" vers. 1.0-12 (Laliberté et al., 2014).

Data analysis

The differences in taxonomic, evolutive, and functional diversity harbored in both lentic and lotic habitats dominated by *P. australis* were investigated by means of linear mixed models (LMMs). The models included a random structure with two random effect factors, one accounting for the different source of the data, and one accounting for the relevés surface. In LMMs, response variables were transformed adopting the one-parameter Box–Cox transformation (Box and Cox, 1964) to achieve normality of residuals. The LMMs were carried out in R software using “lmerTest” package version 3.1-3. We evaluated the differences in alien species richness (A.SR) and in their relative abundance (A.RelAb) among the two types of habitat. Considering the high number of zeros (i.e., relevés not including alien species) we fitted two separated zero-inflated mixed models (ZIMMs), accounting for a double component in the models: a binary response in a logistic regression (i.e., the zero inflated model) and a non-zero response regression (i.e., the conditional model). The non-zero response regression was described by a Poisson family in case of A.SR, while a Gamma family has been adopted in case of A.RelAb. Indeed, A.SR was defined as the number of alien species in each plot (count data), while A.RelAb was calculated as relative cover of alien species at each plot (continuous percentage data). The models included a random structure with two random effect factors, one accounting for the different source of the data, and one accounting for the relevés surface. The ZIMMs were carried out in R software, using the package “glmmTMB” vers. 1.0.2.1 (Magnusson et al., 2017). To evaluate the effect of habitat, and respectively of A.SR or A.RelAb, on all three components of biodiversity (TD, PD and FD) we fitted two series of LMMs, with a Gaussian distribution, in a full factorial ANOVA design (thus including the interaction terms). In this case all the indices were calculated excluding the alien species from the community matrix. As above the models included a random structure with two random effect factors, one accounting for the different source of the data, and one accounting for the relevés surface. The LMMs were carried out in R software using “lmerTest” package version 3.1-3. All plots were drawn using “effect” package version 4.2-0 (Fox, 1987).

Results

Dataset

The final dataset included 231 vegetational plots, of which 176 representative of lentic habitats and 55 of lotic habitats. A total of 208 vascular macrophyte species was recorded, 104 in lentic and 34 in lotic habitats. Among the 208 macrophytes, 25 were identified as alien species, of which nine were exclusive for lentic habitats and eight for the lotic ones (Tab. 1). The range of A.SR was from 0 to 3 in the lentic stands and from 0 to 7 in the lotic ones. Regarding the A.RelAb, it varied from 0 to 15.5 in the lentic stands and from 0 to 37.5 in the lotic ones (Tab. S5).

Species	Lentic habitat		Lotic habitat	
	F	RelAb	F	RelAb
Acorus calamus	3	0.5		
Bidens connata	1	0.83		
Eclipta prostrata	2	1.17		
Hibiscus palustris	8	3.83		
Humulus scandens	12	19.53		
Lemna minuta	10	8.55		
Parthenocissus quinquefolia	3	2.17		
Periploca graeca	1	0.17		
Sorghum halepense	5	2.37		
Amaranthus tuberculatus			2	1.17
Cyperus glomeratus			2	0.33
Eragrostis pectinacea			1	0.17
Helianthus tuberosus			1	0.17
Lonicera japonica			1	5
Panicum dichotomiflorum			2	0.33
Populus xcanadensis			1	0.17
Sicyos angulatus			2	2
Amorpha fruticosa	17	3.29	1	3
Bidens frondosa	29	1.13	5	3.9
Galega officinalis	5	3.42	1	0.5
Paspalum distichum	7	4	2	0.5
Solidago gigantea	3	2.17	1	0.5
Symphyotrichum lanceolatum	1	0.05	2	18.78
Symphyotrichum squamatum	1	0.5	1	0.5
Xanthium italicum	4	0.39	7	1.21

Table 1 *List of alien species exclusive for each habitat and shared among habitats. For each species, frequency (F) and relative abundance (A.RelAb) were also shown.*

Quantifying biodiversity

Lentic and lotic habitats did not show significant differences in the diversity metrics (Tab. S6) The mean values of each diversity metric for both habitats are shown in Table 2. The SR metric varied from 1 to 21 (BU22) and from 1 to 14 (PANT11) species per plot, in lentic and lotic habitats, respectively. Moreover, lentic communities showed a higher number of monospecific stands (ms) than the lotic ones (21 and one respectively). Regarding H index, values ranged from 0 (ms) to 1.82 (BU64) in lentic communities, and from 0 (ms) to 1.98 (PANT6) in lotic ones (Tab. S5). The evolutive reconstruction of lentic and lotic macrophytes' communities was shown in supplementary materials (newick tree S7). Regarding PD_F metric, values of lentic communities ranged from 640.15 (14EVi05) to 2776.68 (BU06), while values of lotic ones ranged from 640.15 (ARNO15) to 2007.38 (PANT11). Looking at the phyMPDmetric, values ranged from 0 (ms) to 515.43 (SERR3) in lentic communities, and from 0 (ms) to 417.09 (ARNO11) in lotic ones (Tab. S5). The FRic metric ranged from 0 (ms) to 20.30 (MASSAC14) in lentic communities, and from 0 (ms) to 8.21 (PANT 5) in lotic ones. Looking at the RaoQ metric, values of lentic communities ranged from 0 (ms) to 11.52 (PORTA01), while values of lotic ones ranged from 0 (ms) to 3.44 (ARNO1) (Tab. S5).

Differences in A.SR and in A.RelAb as a function of habitat type

No significant effect of the habitat type was found as to both the alien species richness (A.SR) and the relative abundance of alien species (A.RelAb) (Tab. S8).

Effect of habitat, alien species and their interaction on TD, PD and FD

A.SR: Model results showed that only in lentic habitats, the taxonomic, phylogenetic and functional diversity increased at increasing species richness. Regarding lotic habitats, taxonomic and functional diversity metrics decreased at increasing species richness, while phylogenetic diversity remained almost unchanged (slightly higher). Specifically, the H and RaoQ metrics were influenced by the Habitat:A.SR interaction ($P = 0.044$, Fig. 1B; $P = 0.017$, Fig. 1F; Tab. S9), while the phyMPD metric was influenced by A.SR ($P = 0.039$, Fig. 1D; Tab. S9). In addition, despite having similar trends compared to the other biodiversity components, the SR, PD_F and FRic metrics were only marginally influenced by A.SR + Habitat: A.SR, Habitat:A.SR and Habitat, respectively ($P < 0.1$; Fig. 1A, C, E; Tab. S9).

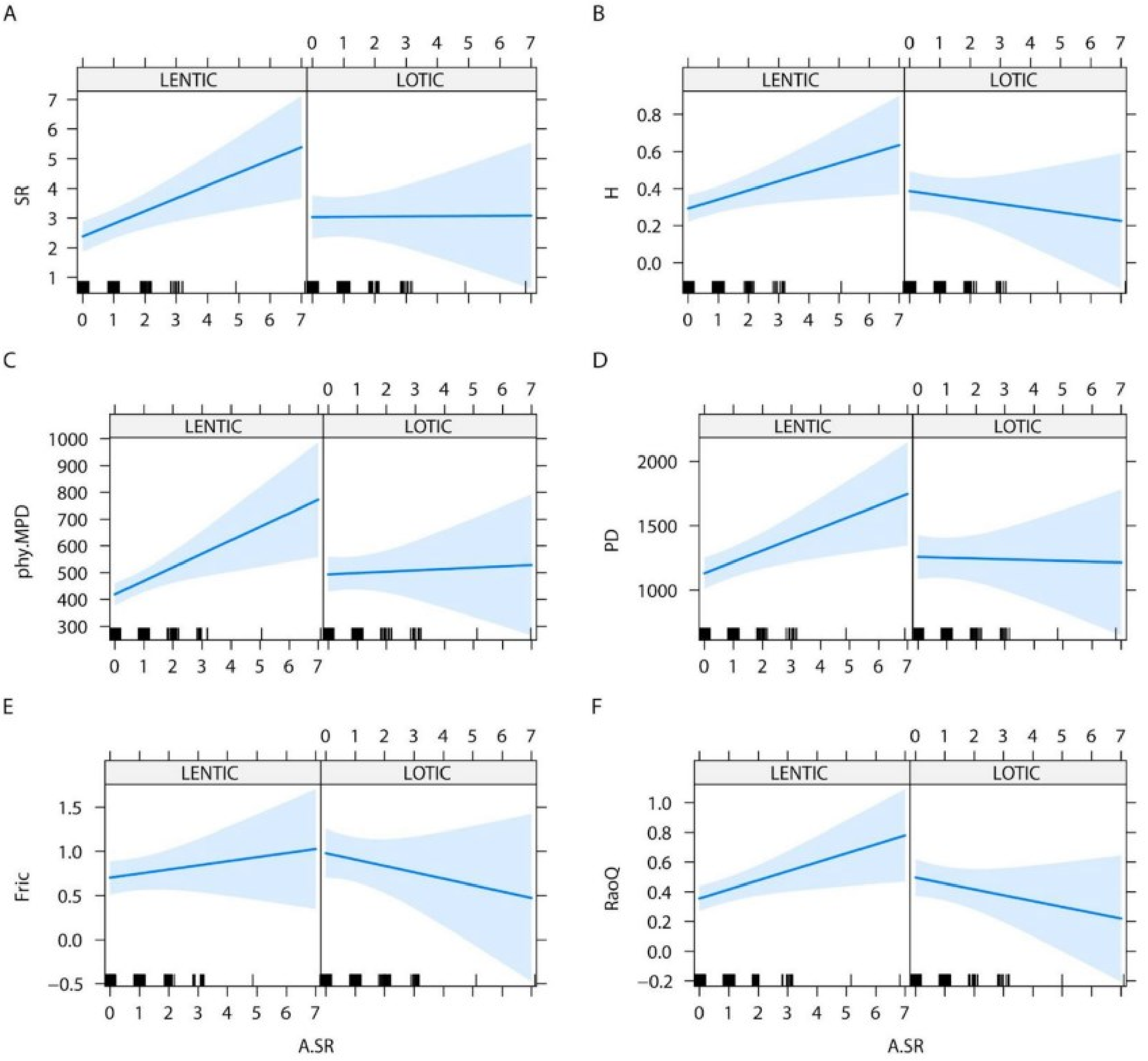

Figure 1 *Interaction plot from the linear mixed models showing the effect of the alien species richness (A.SR) on the different diversity metrics in both habitats. Species Richness (SR), Shannon's diversity index (H), Mean Pairwise Distance (phy.MPD), Faith's phylogenetic diversity (PD), functional richness (FRic), functional Rao's quadratic entropy (RaoQ).*

A.RelAb: The effect of the A.RelAb on the three components of biodiversity (TD, PD and FD) was not statistically different between both habitats (Tab. S10). However, there was a positive correlation between the A. RelAb and three metrics: H, phy.MPD and RaoQ ($P = 0.014$, Fig. 2B; $P = 0.032$, Fig. 2D; $P = 0.016$, Fig. 2F; Tab. S10). Even in this case, the SR metric was only marginally influenced by A.RelAb ($P < 0.1$, Fig. 2A; Tab. S10).

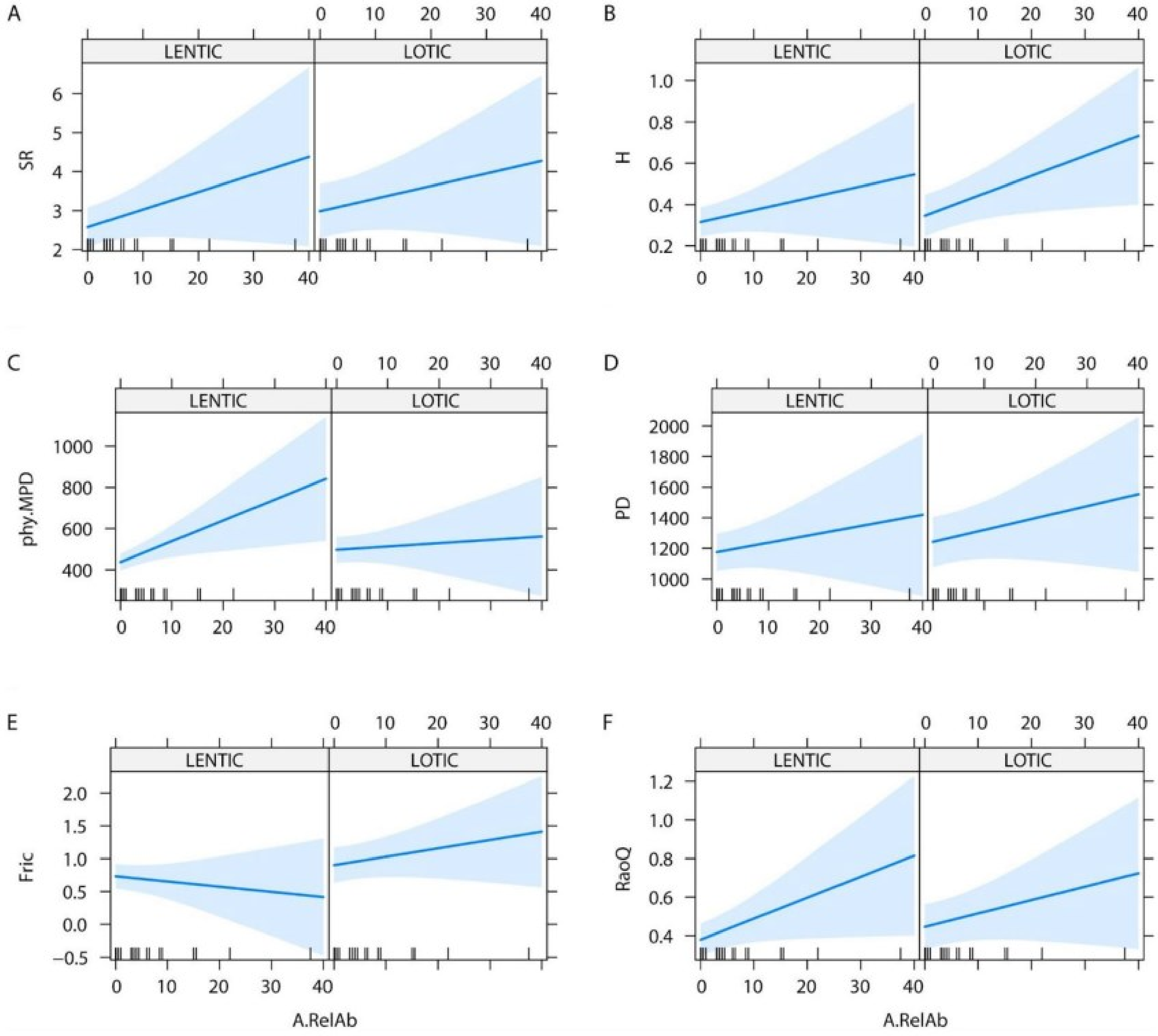

Figure 2 *Interaction plot from the linear mixed models showing the significant effect of the relative abundance of alien species (A.RelAb) on the different diversity metrics in both habitats. Species Richness (SR), Shannon's diversity index (H), Mean Pairwise Distance (phy.MPD), Faith's phylogenetic diversity (PD), functional richness (FRic), functional Rao's quadratic entropy (RaoQ).*

Discussion

In this study, we applied a multidimensional approach including taxonomic, functional and phylogenetic data to enhance our basic understanding of plant diversity between *P. australis* dominated communities. To the best of our knowledge, the outcome of this study carried out for the first time a comparison of the above-mentioned diversity facets of habitats dominated by *P. australis* across lentic and lotic conditions. Moreover, we analyzed, for the first time, the incidence of alien species on the "multiple diversity" of reed-dominated plant communities for lentic and lotic habitats. Although it was hypothesized that the ecological differences between lentic or lotic habitats can lead to plant adaptive trade-offs (Cristofoletti, 1981; Wetzel, 2001; Starzomski and Srivastava, 2007), thus shaping the assembly rules of plant communities, the outcomes of our work showed a substantial lack of differences in taxonomic, functional and phylogenetic diversity between lentic and lotic communities. Therefore, it is conceivable that the *P. australis* dominance affected the overall plant diversity in the same way in both target habitats. Uddin and Robinson (2017a) noted that the taxonomic diversity of reed-dominated plant communities differed significantly according to the density of *P. australis*. Since we considered a reduced range of coverage rates (the highest Braun-Blanquet values possible, notably 4 and 5), this may explain why no differences were observed in the taxonomic indices calculated. Miler et al. (2014) analyzed the biochemical and morphological differences between river and lake macrophytes. They showed that the observed differences could be explained by wind disturbance in lentic sites and by the water regime disturbance in lotic habitats. However, *P. australis* is an "ecosystem engineer species" par excellence, and its dense stands play an essential role as a wind and wave breaker (Takeda and Kurihara 1988; Vymazal 2011; Karstens et al., 2016). Therefore, the same high level of reeds coverage may have buffered different abiotic factors and led to similar levels of functional diversity across habitats. Moreover, the dominance of *P. australis* in dense stands may have promoted biotic interactions such as competitive exclusion, promoting random phylogenetic assemblage of communities in both habitats, and no phylogenetic differences across lentic and lotic stands. Similar outcomes were shown by Veldkornet et al. (2019) for riparian plant communities of streams and estuaries dominated by common reed. Most of the stands in estuaries showed no phylogenetic structure suggesting the coexistence of plant species structured mainly by biotic interactions (Veldkornet et al., 2019, and references therein: Mayfield and Levine, 2010; Barber et al., 2017). However, beyond the direct effects of *P. australis*, riparian macrophytes show a wide ecological adaptability, being able to establish and develop both in lotic and lentic ecosystems (such as *P. australis* itself). In the present study, we focused on lowland areas (below 500 m a.s.l.) where rivers tend to acquire physical features very similar to those of lentic habitats (e.g., due to slowed flows or damming), which could justify the compositional convergence between sites in terms of "companion species" of *P. australis*. Furthermore, the global alteration of inland waters, both in terms of water quantity and quality, has huge effects on aquatic species that are among the most threatened biodiversity facets (Dudgeon, 2019; van Rees et al., 2021). In heavily exploited landscapes, this translates into wet hyper-simplified plant communities characterized by an ever increasing structural similarity (Bolpagni and Piotti, 2016; Bolpagni et al., 2016). Similarly, we found that the lentic and lotic habitats host a comparable alien species richness and relative abundance. This is in line with the findings by Boggero et al. (2014) which did not notice differences between lentic and lotic habitats comparing different invaded biological communities. It should further be noted here that in our case indeed the definition of lotic ecosystems did include very different river ecosystems from each other, with a high variability of water regimes. Actually, the intensity of water flow is known as a pivotal factor that drives alien cover and richness in river ecosystems (see also below) (Heidbüchel and Hussner, 2020; Bolpagni, 2021), and it is conceivable that including it in our analysis could have led to different results. However, as previously discussed, we investigated a wide set of sites subjected to a huge range of human impacts that can completely change the hydrological functioning of aquatic ecosystems, greatly reducing our abilityto assess their local effects. Further efforts will be needed in this direction to improve our understanding of human-mediated effects to *P. australis*-communities invasibility.

Incidence of alien plant species

Regarding the incidence of alien species on the different components of biodiversity, different results were observed based on whether the alien species richness or their relative abundance were considered. Already Brummer et al. (2016), in similar environments (i.e., gravel floodplain ecosystems), recognized that alien cover and richness were shaped differently by different variables (such as water flow, substrate texture). This supports the idea that alien richness and relative abundance may also relate to different impacts on the colonized communities (Montanari et al., 2020; Bolpagni and Dalla Vecchia, 2021). As to the role of the relative abundance it should be noted that while it is generally agreed that there is a strong positive relationship among alien species abundance and subsequent impacts, it has been shown that even at low abundance, alien plants may impact native plant richness at both local and landscape scales (Bernard-Verdier and Hulme, 2019). The impact of alien species richness (A.SR) was different between lentic and lotic habitats, increasing A.SR in lentic habitats resulted in increased taxonomic, phylogenetic and functional diversity. Supporting the "Darwin's naturalization hypothesis" (DNH) (Rejmánek, 1996), alien species exploit unfilled ecological niches (Thuiller et al., 2010) and make the phylogenetic and functional space more saturated, providing a new subset of traits and evolutionary origins in invaded communities (Funk et al., 2008). On the other hand, the higher richness of alien species in lotic habitats promoted a decrease in taxonomic and functional diversity. Based on these observations, alien species established in lotic stands occupy a portion of the functional space within the native communities' range, thus leading to an increased similarity between species in invaded communities. In addition, the phy.MPD metric showed that the communities most invaded had similar or slightly higher phylogenetic distance than non-invaded ones, suggesting close phylogenetic relatedness of invaders to native species (Lososová et al., 2015). These results are consistent with the hydrologic disturbance to which lotic habitats are subjected. This abiotic factor must then be considered as the main driver in filtering alien species with ecological requirements and advantageous traits similar to those of native species (Campos, 2010). According to the hypothesis of environmental filtering (Kembel and Hubbell, 2006), alien species with high similarity to resident species tend to have similar ecological adaptations as native species and have a better chance to establish and spread. In contrast, the increase in phylogenetic diversity in lentic habitat is consistent with less intense hydrological disturbance and greater habitat stability than lotic ones. In these environments, the closed depositional conditions may promote coexistence of species from distant lineages via niche differences (Kitagawa et al., 2015; Lososová et al., 2015). In contrast to patterns of A.SR, the impact of relative abundance of alien species (A.RelAb) was not different between lentic and lotic habitats. The increase in A.RelAb resulted in increased taxonomic, phylogenetic and functional diversity in both habitats. On the one hand, results suggest that DNH is confirmed for lentic communities where the more abundant alien plant species entering the communities promote the taxonomic, functional and phylogenetic dispersion. On the other hand, choosing relative abundance vs richness of aliens in lotic stands can have a different impact in evaluating the effect of aliens on the different components of diversity. Previous studies supported that the influences of invasive alien species on plant taxonomic diversity may vary with degree of invasion (Dong et al., 2015). Two studies, based only on one alien species, showed that a low degree (less than 50%) of relative abundance of the alien species was associated with a significant increase in the taxonomic diversity of plant communities (Wang et al., 2018, 2021). As taxonomic diversity, also functional diversity may vary with degree of invasion. McGrannachan and McGeoch (2019), examining the effect of multispecies' invasion to the community level, showed that functional diversity of the understorey community increased until the relative abundance of alien species remained low (~20%). Within our framework, *P. australis* was the most abundant species (above 64.5% of mean coverage), and none of the alien species had a relative abundance greater than 35%. As in forest ecosystems, the low degree of relative abundance of alien species may not have negatively affected the diversity metrics, confirming the importance of this aspect in evaluating the diversity of reed communities.

Conclusions

Based on a large dataset (231 plots), referred to a key conservation area for wetland plant diversity at the global scale (Northern and Central Italy; Bolpagni et al., 2018), the present paper offered new insights on different levels of diversity (taxonomic, functional, and evolutionary) in communities dominated by *P. australis*. Main outcomes revealed a substantial comparability between lentic and lotic *P. australis* communities in terms of diversity facets. The dominance of common reed affected the diversity of plant communities of both target habitats more than the absolute presence of alien species. Further investigations are still required to widen the dataset to rebalance the number of relevés concerning the two macro-typologies of this study (lotic vs lentic), which are here strongly biased towards lentic habitats, and to include other geographical areas. This will allow to fully clarify the shaping role of *P. australis* on wetland plant diversity, offering essential information to design affective ecological recovery plans, as well as guaranteeing adequate levels of protection to one of the biodiversity components (i.e., the freshwater one) at greatest risk of global extinction.

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Supplementary material:
https://drive.google.com/drive/folders/1Ny3z3ddLGtkONi-Ud-t6ab4dq7irWI8C?usp=sharing

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5.2. Intra-specific genetic diversity

5.2.1. Article III: Assessing the haplotype and spectro-functional traits interactions to explore the intraspecific diversity of common reed in Central Italy

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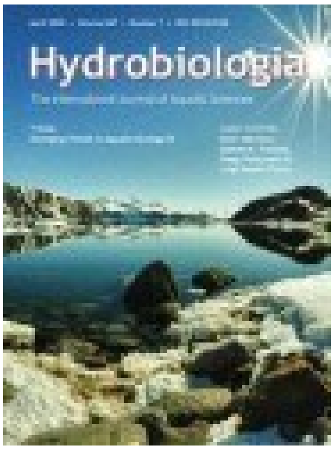

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Contribution table:

	Maria Beatrice Castellani	Andrea Coppi	Rossano Bolpagni	Daniela Gigante	Lorenzo Lastrucci	Lara Reale	Paolo Villa
Experimental Design	20	40					40
Field Work / Experiment Maintenance	30	35			35		
Sample Analysis	50	10					40
Statistical Analysis							100
Manuscript Drafting	50	10	5	5		5	25

Author's contribution (percentage) relative to the different tasks carried out in this work.

Introduction

Because of their sessile condition, plant species are largely prone to respond to changes in environmental conditions by varying multiple traits (Valladares et al. 2007). Even if the basic assumption underlying comparative ecology studies is that functions are more variable among than within species (McGill et al., 2006), recent studies focusing on intraspecific trait variability (ITV) have shown that this does not hold for all the traits of a species in equal ways (Albert el al., 2010).

Different environmental filters may drive the selection of different genotypes and phenotypes in the context of population assembly rules, with abiotic and biotic factors tending to act idiosyncratically (Violle et al., 2012; Garnier et al., 2016). Given the strong environmental filtering acting on aquatic and wetland habitats, species replacement is more difficult than in terrestrial ones and aquatic plants are generally showing a high degree of intraspecific variability to cope with their habitat changes (i.e., see Jung et al., 2010 regarding the variability of SLA along a flooding gradient). In highly dynamic wetland ecosystems, not only abiotic factors (e.g., water level, nutrients availability) can be extremely variable even at local scales, but also biotic factors (e.g., herbivore pressure, competition with phytoplankton, sediment microbiome composition) can show fine scale heterogeneity in spatial and temporal patterns, all together acting on performance and selection of aquatic vegetation communities (Yang et al., 2019; Stagg et al., 2020).

Within this frame, the geographic distribution of a species plays a role too, as it is widely accomplished that species broadly distributed tend to be more variable due to local adaptation and/or acclimation across a broad range of environmental conditions (Eller et al., 2017; Pither, 2003; Siefert et al., 2015). This intraspecific variation enables widespread species to have broad ecological amplitudes in response to biotic (e.g., herbivores, mutualists, pathogens) and abiotic (e.g., climatic, edaphic) environmental factors (Albert et al., 2010; Baythavong & Stanton, 2010).

In this respect, *Phragmites australis* (Cav.) Trin. ex Steud. is one of the most distributed of all flowering plants, showing a broad ecological amplitude and intraspecific variability (Meyerson et al., 2016, Coppi et al 2018). This species grows in very diverse wetland habitat types, such as riverbanks, lakeshores, brackish and salty marshes, in oligotrophic to eutrophic waters and soils (Landucci et al., 2013). In these habitats, *P. australis* has important ecological, economic, and social roles (Kiviat, 2013; Ostendorp, 1993), and forms large and monospecific stands (Granéli, 1989) that, in some cases, displace other wetland plants (Foggi et al., 2011; Próchnicki, 2005). Thanks to the analysis of two noncoding chloroplast regions, Saltonstall (2002) described a total of 27 haplotypes from worldwide specimens of *P. australis* and depicted a cryptic invasion process by a non-native genotype of common reed in North America. Subsequently Lambertini et al. (2012), based on analysis of chloroplast variation with parsimony and genetic distance methods, extended the knowledge on haplotype occurrence in natural populations of European *P. australis*. Indeed, four novel haplotypes were detected in Romania and Northern Europe. Remarkably, Coppi et al. (2018) showed the occurrence of five different common reed haplotypes in central Italy (M, K, CO, VI, and CHTR), mostly forming patches in the same wetland. Recently, Naugzemys et al., (2021) identified six new haplotypes (BM, BN, BO, BP, BQ and BR) in Lithuania river sites, as well as to confirming the presence of two European haplotypes (M and L).

Partitioning of intraspecific variability of regional populations (e.g., few genotypes and highly plastic *vs* many genotypes with low plasticity) is essential for assessing adaptation potential over time scales, also in face environmental change (Benito Garzón et al., 2011; King et al., 2018). Within this framework, there is a need for tools for assessing intraspecific plant diversity across scales, which are both reliable and efficient (Valladares et al., 2006). For its synoptic capabilities and the availability of abundant operational satellite constellation put on orbit in the last decade, the exploitation of remote sensing data for plant functional studies is nowadays a strong option (Jetz et al., 2016) - in particular towards the monitoring of plant canopy morpho-structural and biochemical characteristics and their spatial and temporal dynamics (see for example the reviews of Homolová et al., 2013; Verrelst et al., 2015; Gamon et al., 2019; Dalla Vecchia et al., 2020).

Remote sensing platforms acquiring data in the visible to shortwave infrared spectral range (400-2500 nm), the so-called passive optical systems, are used to measure vegetation spectral reflectance, a physical quantity (directly or indirectly) connected to light use and photosynthesis, and can be therefore used for characterizing plant functional types (e.g., Ustin & Gamon et al., 2010; Villa et al., 2015; Schweiger et al., 2017), as well as for modelling functional traits at leaf to canopy scales (e.g., Asner & Martin, 2008; Asner et al., 2015; Serbin et al., 2019). Due to their straightforwardness, spectral indices (SIs) derived from optical remote sensing data as algebraic combinations of reflectance in different spectral bands, can be used as proxies of specific functional traits: e.g., canopy density, biomass, leaf area index (e.g., Le Maire et al., 2008; Ustin et al., 2009; Villa et al., 2017; Feilhauer et al., 2018; Van Cleemput et al., 2019). As this, SIs could be easily employed within a framework for assessing intraspecific diversity in monospecific or quasi-monospecific communities, where satellite data pixels (usually metric to decametric resolution) are almost completely comprising one single species canopy. In similar plant community setup, such is typically the case with *P. australis* stands, decametric pixels of multispectral satellite scenes provide an unambiguous link to spectral response of the individual species in its natural environment at canopy scale, and satellite derived plant functional proxies can be used for assessing intraspecific diversity across sites and seasons.

The main aim of this study was to explore the links between haplotypes and spectral proxies of canopy functional traits (i.e. spectro-functional traits) in natural stands of *P. australis* from Central Italy, taking into account meteo-climatic and environmental conditions (site characteristics and ecological status of stands). The evaluation of spectro-functional variability over genotyped common reed patches should indeed represent a straightforward and efficient approach for monitoring the haplotype-phenotype relations across geographical and temporal scales, and eventually contextualize them in terms of environmental drivers and ecological significance.

Methods

Study area and plant materials collection

The study areas are seven wetlands in central Italy (Figure 1, Table 1), including two marshlands (Fucecchio and Colfiorito) and five lakes (Chiusi, Massaciuccoli, Porta, Trasimeno, and Vico). The seven sites differ in their pedo-morphological characteristics, passing from lowland and upland wetlands such as Colfiorito, Fucecchio, Massaciuccoli, and Porta to shallow, turbid lakes such as Chiusi and Trasimeno or a deep, clear, and volcanic basin as Vico. Surface areas are furthermore variable, with the smallest site (Colfiorito) covering 0.8 km^2 to the largest one (Trasimeno) covering 121.5 km^2. Leaf tissue was collected from each of the sampled stands, to implement the set of haplotypes described in Coppi et al. (2018). Repeated sampling during the growing seasons of 2020 was performed within selected plots. The final dataset was used to confirm the occurrence of haplotypes across the studied areas, expand the knowledge on the haplotypes' occurrence in other study areas (e.g. Porta and Massaciuccoli lakes), and identify spatial patches over which to extract spectro-functional traits from satellite data. The complete list of the specimens examined, their provenance and the GenBank information retrieved are given in Supplementary material S1.

P. australis stands were further classified following the model proposed by Lastrucci et al. (2016c) regarding canopy background conditions at the peak of summer in terms of soil moisture and submersion. The ecological status of sampled plots was summarized into three categories: flooded stands (F), when the rhizomes were found covered by water all the year; floating island stands (Fl), when plants were found forming mats with rhizomes floating above the water; and dry stands (D), when the surfaced of soil substrate showed different degrees of wetness and dried for at least one month in the hottest period of the year. Category Fl includes only some plots from Chiusi Lake, while categories F and D include plots from all surveyed sites.

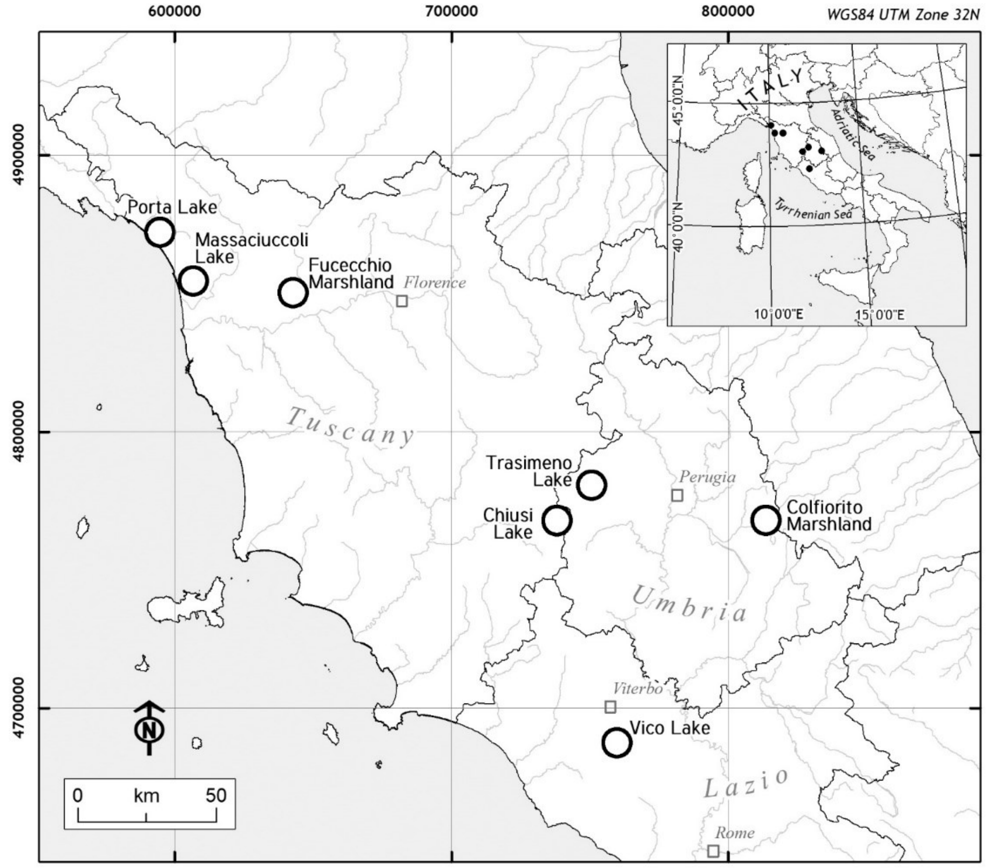

Figure 1 *Geographic location of the study area with distribution of the sampling sites (empty dots).*

Study site	Locality	Latitude	Longitude	Surface area (km^2)	Elevation (m a.s.l)	Type of site
Chiusi lake	South-eastern Tuscany	43° 03’ 22.11’’ N	11° 57’ 55.79’’ E	3.87	245	Shallow lake
Colfiorito marshland	Eastern Umbria	43° 01’ 23’’ N	12° 52’ 36’’ E	0.8	750	Palustrine wetland with small open-water areas
Fucecchio marshland	North-western Tuscany	43° 48’ 30.38’’ N	10° 48’ 20.14’’ E	1.02	15	Palustrine wetland with small open-water areas and canal nets
Massaciuccoli lake	North-western Tuscany	43° 50’ 34’’ N	10° 19’ 21’’ E	6.9	0	Shallow lake
Porta lake	North-western Tuscany	43° 59’ 27’’ N	10° 10’ 23’’ E	1,558	0	Palustrine wetland with small open-water areas
Trasimeno lake	North-western Umbria	43° 08’ 05.5’’ N	12° 06’ 04.6’’ E	128	255	Shallow lake
Vico lake	North-western Lazio	42° 18’ 58.40’’ N	12° 10’ 5.89’’ E	12.93	510	Deep lake

Table 1 *Overview of the geographical coordinates of the 7 study sites with locality, surface area, elevation and type of site information.*

DNA extraction and amplification

Genomic DNA was extracted from silica-gel-dried leaf samples using a modified 2× cetyltrimethylammonium bromide protocol GenElute Plant Genomic DNA Miniprep Kit (Sigma).

Amplification of the trnT-trnL and rbcL-psaI intergenic spacer regions of the cpDNA was performed following the protocol described by Taberlet et al. (1991) and Saltonstall (2001, 2002). Automated DNA sequencing was performed directly from the purified PCR products, using BigDye Terminator version 2 chemistry, and a sequencer (ABI310; PE-Applied Biosystems, Norwalk, CT, USA). To reduce artefacts, all the samples were amplified and sequenced twice, using both forward and reverse primers.

Sequence alignment and data analysis

A total of 178 original sequences of *P. australis* (89 for each of trnT-trnL and rbcL-psaI markers) were checked using BioEdit ver. 7.0 (Hall et al., 1999) through comparisons with accessions published in Coppi et al. (2018) and retrieved from the National Center for Biotechnology Information (NCBI). A single dataset that comprised the concatenated sequences of the intergenic spacer regions (trnT-trnL+rbcL-psaI) underwent fitting and analysis. The haplotypes identification and tagging follow the model described by Saltonstall (2001, 2002), implemented more recently by Hauber et al. (2011), Lambertini et al. (2012), Saltonstall (2016) and Coppi et al. (2018). Variation in the number of mononucleotides in the trnT-TrnL and rbcL-psaI sequences, was attributed to the variation at intra-haplotypic level and, as reported in Lambertini et al. (2012) and by Saltonstall (2016), has not been used to define new haplotypes.

Meteo-climatic data

ERA5-Land monthly averaged data from model reanalysis (Muñoz Sabater, 2019), managed by ECMWF and Copernicus, with 0.1° resolution and covering the period from January 1981 top December 2020: air temperature at 2 m above the surface, accumulated total evaporation from Earth's surface, accumulated total incoming solar (shortwave) radiation reaching the Earth's surface.

Satellite data

Satellite data used are surface reflectance products (L2A) derived from Sentinel-2 constellation, jointly managed by the EU Copernicus programme and European Space Agency (ESA). The two satellites composing Sentinel-2 constellation, i.e. Sentinel-2A (operational since July 2015) and Sentinel-2B (operational since July 2017), carry on board the same multispectral camera: the MultiSpectral Instrument (MSI), a push-broom imaging sensor with 13 spectral bands covering visible to shortwave infrared range (440-2250 nm) at medium spatial resolution (10 to 60 m pixel side on the ground), with a revisit time (in cloud free conditions) of 10 days with one satellite and of 5 days with both satellites (Drusch et al., 2012).

Cloud-free Sentinel-2 scenes acquired over each study area in July to early August, i.e. representing reed stands at their seasonal peak of density and greenness, were gathered from the existing archives for the years 2015-2020. After a preliminary assessment of differences in overall eco-environmental conditions of reed stands falling within the sampled sties at the time of satellite data acquisitions, compared to the season when *in situ* data collection took place (in 2014, 2017 or 2020, depending on the sampled reed stand), some years were deemed as not suitable for the analysis, i.e. year 2018 for Colfiorito, years 2017, 2019 and 2020 for Fucecchio, and year 2017 for Massaciuccoli, and were therefore excluded.

The satellite dataset used for the analysis of intraspecific variability of reed populations in terms of their spectral functional proxies was finally composed of the Sentinel-2 scenes listed in the following:

- Chiusi: 11/07/2015; 18/07/2016; 15/07/2017; 15/07/2018; 20/07/2019; 29/07/2020;
- Colfiorito: 11/07/2015; 04/08/2016; 15/07/2017; 20/07/2019: 24/07/2020;
- Fucecchio: 04/07/2015; 18/07/2016; 18/07/2018;
- Trasimeno: 11/07/2015; 18/07/2016; 15/07/2017; 15/07/2018; 20/07/2019; 29/07/2020;
- Vico: 04/07/2015; 18/07/2016: 10/07/2017; 10/07/2018; 05/07/2019; 29/07/2020;
- Massaciuccoli: 04/07/2015; 18/07/2016; 18/07/2018; 18/07/2019; 27/07/2020;
- Porta: 18/07/2019.

Spectral proxies of functional traits

Spectral indices known for their sensitivity to vegetation conditions at canopy scale were derived from Sentinel-2 scenes at 10m resolution as surrogates of specific reed functional traits, or spectro-functional traits: i) the Water Adjusted Vegetation Index (WAVI), a proxy of aquatic vegetation canopy density and fractional cover (Villa et al., 2014); ii) the Green Leaf Index (GLI), a proxy of canopy greenness and fraction of absorbed PAR (Hunt et al., 2011); and iii) the Normalized Difference Spectral Index for LMA ($NDSI_{LMA}$), a proxy of leaf mass per area in macrophytes, aggregated at canopy scale (Villa et al., 2021).

All values of the 3 spectral proxies falling within a polygon surrounding the plots of common reed communities sampled *in situ* (covering 3-13 pixels, equal to 300-1300 m^2 area) were extracted and used for calculating the mean and coefficient of variation (CV) of WAVI, GLI and $NDSI_{LMA}$.

Following this, matchups between Sentinel-2 derived spectro-functional traits for different years with *in situ* samplings were retained only when difference dates between satellite scene acquisition and in situ sample collection did not exceed 2 years. Moreover, some matchups were discarded in case of marked differences in eco-environmental conditions between the sampling date and satellite acquisition, i.e. due to ambiguities in plot recorded coordinates (one plot in Colfiorito and one in Trasimeno) or to strong inter-seasonal differences in water level (observed for Fucecchio and Trasimeno sites). Finally, polygons scoring strong internal variability in functional proxies for one year, i.e. CV higher than 0.5 in at least one of the spectral indices, were excluded from the final list of matchups (Table 2) as they were considered not consistently representing reed canopy features, but more a mixture of spectral targets.

Site	Type of site	CHTR	CO	K	M	nM	VI	TOT
Chiusi	Shallow lake	8 (8 F)	7 (5 F, 2 Fl)	8 (8 Fl)	20 (13 F, 2 Fl, 5 D)	11 (3 F, 6 Fl, 2 D)		54
Colfiorito	Wetland		10 (4 F, 6 D)		2 (2 F)			12
Fucecchio	Wetland				12 (4 F, 8 D)			12
Massaciuccoli	Shallow lake, wetland	5 (5 F)			12 (12 F)			17
Porta	Wetland				5 (4 F, 1 D)			5
Trasimeno	Shallow lake	10 (10 D)			5 (5 D)			15
Vico	Deep lake				16 (9 F, 7 D)		6 (4 F, 2 D)	22
	TOT	23	17	8	72	11	6	137

Table 2 *Spectral proxies – in situ samplings matchup table (F = flooded stand, Fl = floating island stand, D = dry stand), divided by reed haplotype.*

Data Analyses

The temporal dynamics of spectro-functional traits across 2015-2020 years range were assessed by fitting an ordinary least squares linear regression line to spectral proxies time series and deriving for each spectral index the base value, as the intercept of regression line for year 2015 - WAVIbase(2015-20), GLIbase(2015-20) NDSILMA_base(2015-20) - and the overall change throughout the 5 years, as the difference between the intercept of regression line for year 2020 and the base value - ΔWAVI(2015-20), ΔGLI(2015-20) ΔNDSILMA(2015-20).

The impact of meteo-climatic factors on *P. australis* in Central Italy was investigated by averaging the spectro-functional traits of all reed stands within each study area for a single year, separately for aquatic (flooded; N=25) and terrestrial (floating and dry; N=27) stands, and computing the reaction norms of WAVI, GLI and NDSILMA to Spring-Summer synoptic features (covering the 6 months spanning from March to August) derived from ERA5-Land model reanalysis data: average temperature (mean Temp. (Mar-Aug)), cumulated solar radiation (cum. Rad. (Mar-Aug)), and cumulated total evaporation (cum. Evap. (Mar-Aug)). The correlation between spectro-functional traits derived from Sentinel-2 data and meteo-climatic features was computed as Spearman's ρ (with 95% confidence intervals and p-value).

Due to non-normality of samples, the variability of spectro-functional reed traits (and derived features) across ecological statuses (N=3: F, Fl, D), sites (N=7: Ch, Co, Fu, Po, Tr, Vi, Ma) or haplotypes (N=6: CHTR, CO, K, M, nM, VI) was tested with non-parametric Kruskal-Wallis One Way ANOVA, and post-hoc pairwise comparisons were successively performed via Dunn's test, with p-value adjustment accounting for multiple comparisons computed with Benjamini-Hochberg method (FDR). The same statistical approach was used for assessing the differences among spectral-functional traits reaction to different meteo-climatic factors (temperature, radiation, evaporation quartiles).

The functional richness of each haplotype was calculated as the percentage of total trait space covered by each haplotype for each spectro-functional trait (Mason et al., 2005), separately for aquatic and terrestrial reed stands.

The partitioning of intraspecific variability in *P. australis* stands into components due to genotype (haplotype) or phenotypic plasticity was computed by fitting a linear mixed model (LMM) for each individual spectral proxy, including selected environmental factors as fixed effects (t_MarAug, rad_MarAug, ecol_stat, site) and haplotype as random effect.

All the analyses and graphing were performed using packages implemented in R v.4.0.3: i.e. ggplot2 3.1.0, ggstatsplot 0.7.2, nlme 3.1-152 (R Core Team, 2020).

Results

Haplotypes found

The combined alignment of trnT-trnL+rbcL-psaI regions used for the haplotype identification was 1627 bp length. Four out of five haplotypes previously detected for the study areas – namely M, CHTR, CO and K (Coppi et al., 2018) - were confirmed in samples collected during 2020, while haplotype VI was not identified again in 2020 data. Table 2 provides an overview of haplotypes distribution among sampled stands and sites. The haplotype M was the most distributed and described for all sites. The CHTR haplotype was instead confirmed for Chiusi and Trasimeno lakes and described for the first time for Massaciuccoli wetland. The haplotype CO was confirmed for Colfiorito wetland and Chiusi. The haplotype K was retrieved only for Chiusi, corroborating this haplotype as one of the less widespread and represented in the dataset. Lake Chiusi also hosts the new haplotype nM, which makes it the site featuring the higher level of haplotypic diversity (five haplotypes: CHTR, CO, K, M, nM) among those studied. The new haplotype nM differed from the haplotype M by the 16 bp insertion "AAAGTATTCTATAAAA" from the position 1494 of the intergenic spacer region rbcL-psaI.

Impact of meteo-climatic factors on spectro-functional traits of reed stands

Compared to the average values of the 1981-2010 period, in the last five years (2015-2020) the spring-summer meteo-climatic parameters (March-August) showed general increases of: average temperature (by 1.0-1.2 °C), cumulated incident solar radiation (by 66-105 MJ m^{-2}), and cumulated total evaporation (by 1.8-6.6 mm of water equivalent).

While no consistent correlation was found between Spring-Summer meteo-climatic features and *P. australis* spectro-functional traits for terrestrial stands (growing on non-flooded terrain with different degrees of moisture), a negative response to average temperature was found for aquatic reed stands in terms of WAVI (ρ=-0.53, p=0.006) and GLI (ρ=-0.55, p=0.005), as well as positive response, yet slightly weaker, of $NDSI_{LMA}$ to cumulated solar radiation (ρ=0.39, p=0.055), as shown in Figure 2.

Specifically, spectral surrogates of canopy density (WAVI) and green leaf area (GLI) for aquatic reed stands tend to decrease with increasing temperature: in hotter seasons - i.e. when spring-summer temperature falls in the 1st quartile of the 2015-2020 values (18.3-19.8 °C) - WAVI and GLI are lower on average by 19.0% (p=0.099) and 16.6% (p=0.078) respectively, than in the colder seasons - i.e. when spring-summer temperature falling in the 4th quartile of the 2015-2020 values (14.6-17.1°C).

The spectral proxy of leaf dry biomass at canopy scale ($NDSI_{LMA}$), responds positively to light availability: in the years that fall in the 1st quartile of the 2015-2020 spring-summer cumulated values (3900-4192 MJ m^{-2}), $NDSI_{LMA}$ is on average 7.9% higher (p=0.028) than when solar radiation falls in the 4th quartile of 2015-2020 spring-summer cumulated values (3572-3732 MJ m^{-2}).

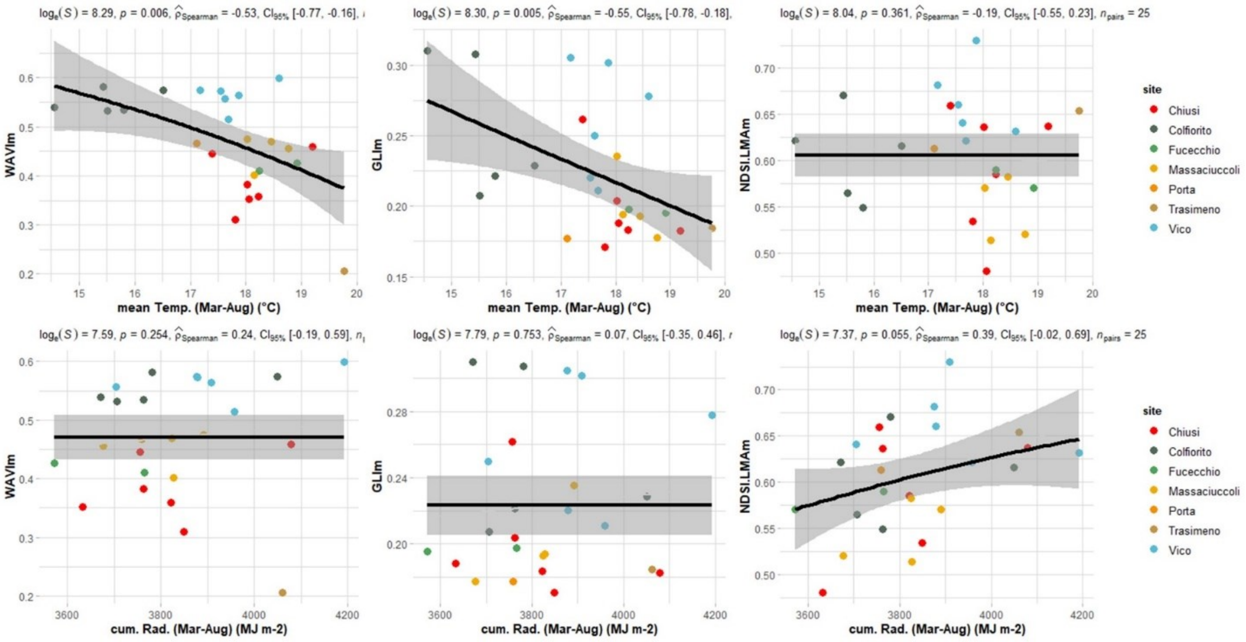

Figure 2 *Reaction norms of spectro–functional traits (WAVI, GLI, NDSILMA) derived from Sentinel–2 data to meteo–climatic features in Spring–Summer period (average temperature, cumulated solar radiation) for aquatic stands of P. australis in Central Italy, with superimposed generalized additive model (GAM) fitting (95% confidence intervals).*

Intraspecific variability partitioning (haplotype vs. phenotype plasticity)

The analysis of variance partitioning in spectro-functional traits of *P. australis* stands, computed according to LMM using environmental factors as fixed effects (meteo-climate data, ecological status and site) and haplotype as random effect - summarized in Table 3 - highlighted that in general, variability due to haplotype ranges between 15% and 17% of total intraspecific variability across all reed stands. In aquatic stands, variability explained by haplotypes is higher than the average, ranging from 21% for GLI to 24% for WAVI. In terrestrial reed plots, comprising both dry and floating stands, instead, the variability explained by haplotypes strongly depends on the individual trait considered, with virtually no effect of haplotype over $NDSI_{LMA}$ and a similar score to that of aquatic stands for GLI (22%).

	Spectro-functional trait	explained by env. factors	due to haplotype
All reed stands	WAVI (canopy density, biomass)	85.00%	15.00%
	GLI (green leaf area)	83.40%	16.60%
	$NDSI_{LMA}$ (LMA, productivity)	84.50%	15.50%
Aquatic stands (Flooded)	WAVI (canopy density, biomass)	76.20%	23.80%
	GLI (green leaf area)	79.10%	20.90%
	$NDSI_{LMA}$ (LMA, productivity)	77.60%	22.40%
Terrestrial stands (Floating + Dry)	WAVI (canopy density, biomass)	88.30%	11.70%
	GLI (green leaf area)	78.00%	22.00%
	$NDSI_{LMA}$ (LMA, productivity)	99.90%	0.10%

Table 3 *Partitioning of spectro-functional traits variance of reed stands.*

Differences in spectro-functional traits of reed haplotypes

Figure 3 shows the distributions (as violin and box plots) and groups characterized by significant pairwise differences ($p<0.05$) of spectral functional proxies among *P. australis* haplotypes in our study areas, highlighting different patterns for aquatic and terrestrial stands.

Among haplotypes, CHTR and M are the most common and most variable in terms of spectro-functional traits, showing a pronounced functional plasticity, while less common haplotypes, such as CO, K, nM, and VI, tend to be both specific of a single site (CO was found in 2 sites, but within 70 km of distance) and less variable, suggesting specialization and/or site acclimation.

When all reed stands are considered, comprising stands growing in flooded to dry substrates, both CO and VI (median GLI=0.31) show higher GLI ($p<0.02$) compared to M and CHTR (median GLI=0.22), and VI shows $NDSI_{LMA}$ (median $NDSI_{LMA}=0.72$) higher ($p<0.004$) than CHTR and M (median $NDSI_{LMA}=0.58-0.59$).

When separated by ecological status, differences among haplotypes are generally less evident in terrestrial than in aquatic reed stands. Aquatic CHTR stands score lower than homologous CO and VI stands, both in canopy density proxy (median WAVI relative difference>29%, $p<0.04$) and in LMA proxy (median $NDSI_{LMA}$ relative difference>12%, $p<0.03$). Aquatic VI stands are also characterized by systematically higher scores of spectro functional traits compared to stands of the most common M haplotype (relative difference in median scores >27%, $p<0.04$).

Comparing aquatic and terrestrial stands of the most common haplotypes (CHTR and M) shows that both score lower values of each spectro-functional canopy trait when growing in flooded conditions, more marked for WAVI and GLI (median relative difference>25%), than for $NDSI_{LMA}$ (median relative difference<16%).

While the GLI difference between aquatic and terrestrial stands tends to be similar for CHTR and M (aquatic stands score 29% lower median GLI), the decrement in WAVI for aquatic stands of CHTR is more marked than that of M (-43% vs -26% compared to terrestrial stands of the same haplotype, respectively).

Supplementary figure 1, as an example of the potential connected to the use of remote sensing data for deriving spatialized information about spectro-functional traits, portraits the patterns of relative variability in *P. australis* communities at within-site scale in Lake Chiusi (10 m spatial resolution), thus extending the information about local reed diversity beyond actually sampled points (marked with their respective haplotype on the map).

The breadth of the functional niche covered by different haplotypes is displayed by functional richness scores for the three spectro-functional traits considered, in Table 4.

The largest niche is occupied by the most common haplotype M, with functional richness >75%, followed by CHTR and CO, which cover >45% of total range across all traits. Narrower niches are occupied by less spread haplotypes – i.e. VI found only in Lake Vico, K found only in Lake Chiusi – which cover <41% of trait range across haplotypes. Different patterns are specific each spectro-functional trait, particularly when reed stands are separated by their ecological status (aquatic vs. terrestrial stands).

				Haplotype			
Spectro-functional trait	**Ecological status**	**CHTR**	**CO**	**K**	**M**	**nM**	**VI**
WAVI (canopy density, biomass)	Aquatic (Flooded)	45.50%	50.10%		92.90%	22.90%	11.40%
	Terrestrial	70.70%	70.20%	40.90%	85.20%	45.70%	34.10%
	(Floating)		27.90%	57.10%	9.00%	63.80%	
	(Dry)	78.00%	58.40%		94.00%	30.30%	37.70%
GLI (green leaf area)	Aquatic (Flooded)	78.50%	60.60%		81.00%	1.30%	12.70%
	Terrestrial	47.50%	87.80%	38.90%	75.40%	32.60%	23.10%
	(Floating)		0.90%	67.40%	19.30%	56.60%	
	(Dry)	47.50%	87.80%		75.40%	12.10%	23.10%
$NDSI_{LMA}$ (LMA, productivity)	Aquatic (Flooded)	61.70%	62.40%		100.00%	1.50%	14.70%
	Terrestrial	57.60%	65.90%	28.20%	85.20%	37.80%	16.30%
	(Floating)		40.50%	65.40%	10.50%	48.80%	
	(Dry)	57.60%	53.40%		85.20%	37.80%	16.30%

Table 4 *Functional richness (as percentage of total range) covered by each haplotype for each spectro-functional trait considered, separately for aquatic (flooded) and terrestrial (floating and dry) reed stands.*

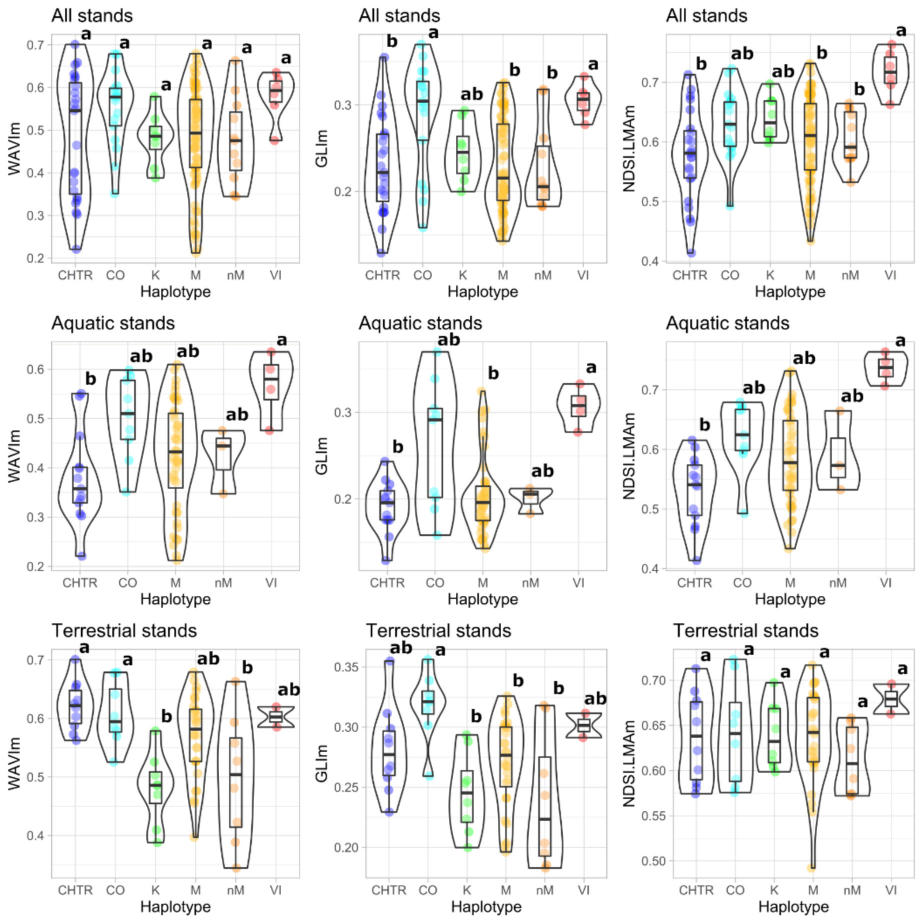

Figure 3 *Violin plots (with encompassed box plots) showing range and distribution of spectro–functional traits (WAVI, GLI, NDSILMA) across P. australis haplotypes in Central Italy: all stands pooled together (upper row); aquatic stands only (middle row); terrestrial stands only (lower row). Letters above the plots highlight groups characterized by significant differences (p<0.05) in pairwise haplotype comparisons, performed via Dunn's post–hoc test (Benjamini–Hochberg adjustment).*

Dynamics of spectro-functional traits of reed stands over the 2015-2020 period

Inter-seasonal dynamics of spectro-functional traits, summarized in Table 5, showed that floating reed stands (Fl) experienced a significant ($p<0.05$) decrement in WAVI (-0.032±0.003 y^{-1} for floating stands *vs.* -0.010±0.004 y^{-1} for flooded stands and -0.005±0.004 y^{-1} for dry stands) and $NDSI_{LMA}$ (-0.019±0.003 y^{-1} for floating stands vs. -0.007±0.003 y^{-1} for dry stands). The trend is evident even when starting conditions in terms of trait scores – i.e. base value - are similar: $WAVI_{base}$(2015-20) scores 0.583±0.019 for floating stands, and 0.609±0.011 for dry stands; while $NDSI_{LMA\_base}$(2015-20) scores 0.688±0.009 for floating stands, and 0.649±0.010 for dry stands. This seems to be framed into the general environmental change occurring in Lake Chiusi – the only site where floating reed stands were found - during the last years (2015-2020). At the whole site scale, Chiusi clearly showed the most intense signs of reed canopy density loss, as surrogated by inter-annual WAVI change (Figure 4). Complementary to this, multitemporal maps of GLI and MDSI$_{LMA}$ derived from Sentinel-2 clearly show the relatively fast dynamics of riparian plant communities in Lake Chiusi from 2015 to 2020 (Supplementary figures 2 and 3).

ΔWAVI(2015-20) amounts to -0.024±0.003 y^{-1} in Chiusi (significantly different, $p<0.001$, from Fucecchio and Vico) compared to -0.010±0.005 y^{-1} in Colfiorito ($p=0.06$), and -0.008±0.005 y^{-1} in Trasimeno ($p=0.09$); Massaciuccoli is the only site where of reed density loss through time is not much different from Chiusi (-0.015±0.012 y^{-1}, $p=0.36$). Reed stands conditions did not experience a statistically significant decrement of reed spectro-functional traits in 2015-2020, only in Fucecchio and Vico.

Less spread haplotypes (K, VI) are mainly separated from more common haplotypes (M, CHTR) by baseline NDSI$_{LMA}$ scores, which is higher in K and VI stands ($p<0.05$).

Spectro-functional trait	**Dynamics indicator**	**Ecological status (F, Fl, D)**	**Site (Ch, Co, Fu, Tr, Vi, Ma)***	**Haplotype (CHTR, CO, K, M, nM, VI)**
WAVI (canopy density, biomass)	ΔWAVI(2015-20)	Fl < (F, D)	Fu > (Ch, Tr, Ma) Vi > Ch	K < (M, Co, VI) nM < (M, Co, VI)
	WAVI$_{base}$(2015-20)	D > F	No differences	No differences
GLI (green leaf area)	ΔGLI(2015-20)	No differences	Fu > (Ch, Co, Tr, Vi)	No differences
	GLI$_{base}$(2015-20)	D > F	Ma < (Co, Vi, Tr)	No differences
NDSI$_{LMA}$ (LMA, productivity)	ΔNDSI$_{LMA}$(2015-20)	Fl < D	No differences	No differences
	NDSI$_{LMA\_base}$(2015-20)	Fl > (D, F)	No differences	K > (CHTR, M) VI > (CHTR, M)

* Porta (Po) excluded from this analysis because satellite data cover only 1 year (2019)

Table 5 *Summary of significant pairwise differences ($p<0.05$) of P. australis spectro-functional traits dynamics indicators, i.e., base values and change throughout 5 years (2015-2020), categorized on the basis of stand ecological status, site and haplotype.*

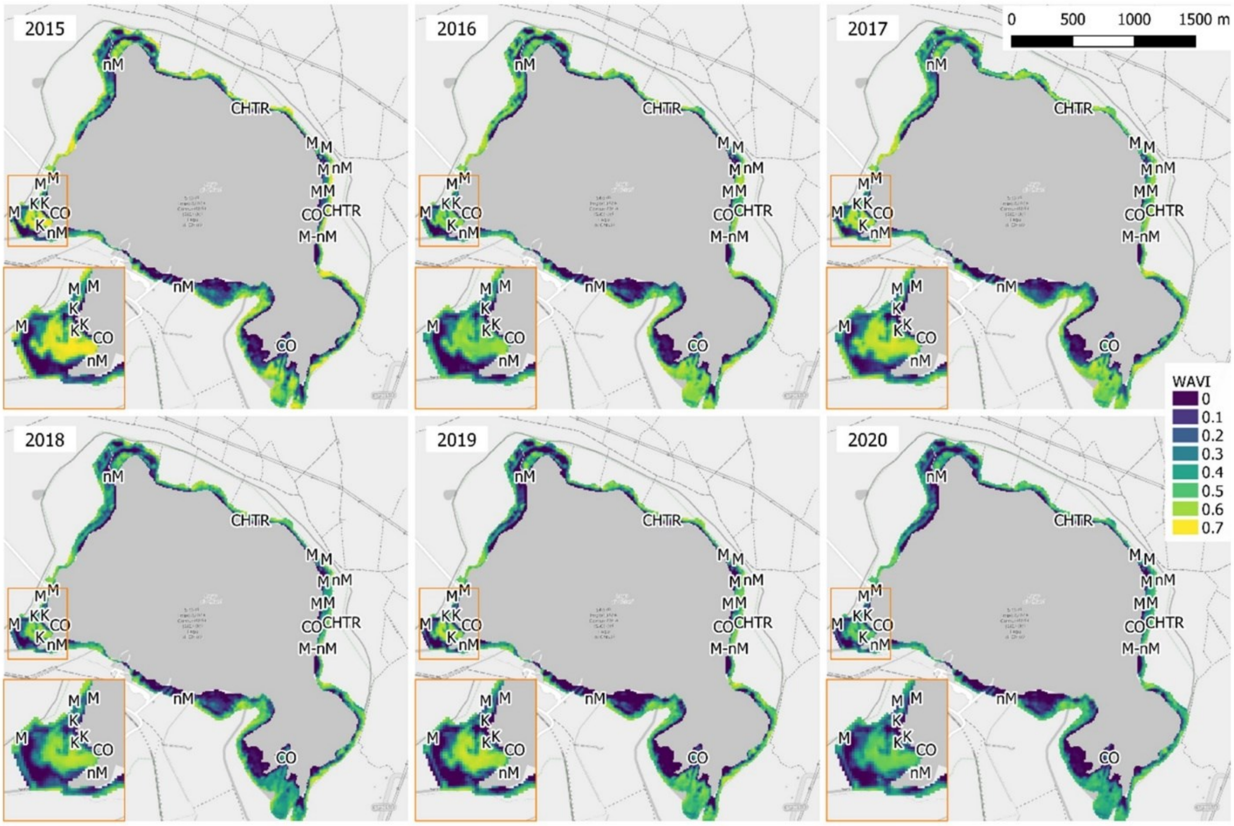

Figure 3 *Multitemporal maps showing the temporal dynamics of WAVI (as a spectral proxy of canopy density) over P. australis communities of Lake Chiusi derived from Sentinel–2 scenes acquired at peak of growth (mid–July) from 2015 to 2020, with superimposed positions of genotyped reed stands (marked with haplotype code).*

Discussion

Competition among plants can occur along different spatial and temporal axes: along the growth cycle, it unfolds both in horizontal and vertical directions, affecting functional traits and the adaptive trade-offs of interacting individuals (Bittebiere et al. 2017 and references therein). Following this framework, interaction among haplotypes in *P. australis* communities would involve variations of traits linked with the competition for light capture and use for optimising the photosynthetic efficiency (vertical growth, canopy density) and/or traits involved in gain of supremacy in space occupation (horizontal spreading of rhizomes). From a temporal dynamics point of view, competitive exclusion in common reed stands may be delayed by intraclonal aggregation in the short term (Silvertown et al., 1992), but clonal fragmentation may occur over medium to long time scales. The life span of *P. australis*, rhizomes seem to decay in a period of around 3–7 years (Silvertown et al., 1992), leaving space to different genetic integrated units which might compete vigorously against each other because of their converged functional traits constitution. Five out of the seven sites we investigated in the present study, Fucecchio and Porta being the exceptions, showed the simultaneous occurrence of more than one haplotype, with signs of dynamic interaction among genotypes that produces a fine-scale mosaic of functional variability of local *P. australis* stands. Moreover, in the same areas several reed stands have experienced marked change in their extension - mostly shrinking - in the last 25 years (Lastrucci et al., 2017; Gigante et al., 2017). Thus, we hypothesized that narrowly adapted clones have selected optimal microhabitat during the last years thanks to their functional trait plasticity. Indeed, previous research on the same species (Hu et al., 2015) has evidenced significant amount of variation in leaf economic traits across and intra-sites. In a time-oriented point of view, during the last growing seasons (2015-2020), some key meteo-climatic factors showed slight by significant increment compared to the average of 1981-2010 period across the study areas, in terms of mean temperature (by 1.0-1.2 °C), cumulated incident solar radiation (by 66-105 MJ m^{-2}) and cumulated total evaporation (by 1.8-6.6 mm of water equivalent). These variations were mirrored by changes in spectro-functional traits of *P. australis*, which showed a significant reduction in GLI in the hotter years. GLI can be considered as a spectral proxy for Leaf Area Index (LAI), which represents a key-variable parametrising productivity and growth at canopy scale, also linked to evapotranspiration capacity (Burba & Verma, 2001). Green LAI tend to respond to variation in temperature very differently from species to species (Iio et al., 2014), but negative association for *P. australis* has been observed by Anda et al. (2017) that have measured lower LAI scores for stands growing in dryland conditions, prone to periodic water stress. Contradictory results in morphological signatures were nevertheless provided for common reed stands growing in different water depth condition (Engloner et al., 2009). Indeed, our dataset showed values of GLI significantly higher for dry than for flooded stands, indicating a different response of two ecological statuses to temperature variation. Moreover, among the dry reed stands we evidenced higher values for CO haplotype compared to the most widespread M. Both haplotypes (CO and M) co-occur in two climatically well-differentiated sites (Colfiorito and Chiusi), showing differences in interaction outcomes: while the M haplotype dominates all the sites characterised by higher mean temperature, including Chiusi, the CO haplotype seems able to positively out-compete the M in Colfiorito, which is characterised by lower mean temperatures because of its altitude (750 m a.s.l.).

Similarly, to what was previously found across many other plant species (Poorter et al., 2010), the studied reed stands also respond to light availability showing an higher spectral proxy for LMA ($NDSI_{LMA}$) with increasing cumulated radiation, indicating a general rise in thickening of leaf blade or a denser tissue thereof, or both (Wright et al., 2004). Nevertheless, $NDSI_{LMA}$ dynamics among common reeds stands exhibited different patters depending on reed ecological status, site location and haplotype. In particular, floating island stands (Fl) evidenced a significant reduction of $\mathrm{NDSI}_{\mathrm{LMA}}$ and WAVI values, suggesting a decrease in biomass production and culms density of the stands during the last years (2015-2020). The finding suggested a general time-dependent deterioration in the state of the reeds in this site in recent years, compared to the work of Gigante et al. (2014), which described the floating reeds on Lake Chiusi as healthy and not susceptible to decay from data collected in summer 2011. Based on our framework, the time-dependant-reduction of the floating island reed stands could be indirectly related to haplotype composition: indeed, Chiusi is the only site that host floating stands, located in the northern and western part of the lake. Four out of six haplotypes were identified for this ecological category (CO, M, nM and K), and two of these haplotypes (nM, CO) showed a very reduced distribution. It is conceivable that under the pressure of environmental change in Lake Chiusi, those haplotypes have experienced intense competition and have narrowly adapted to the microhabitat constituted by floating condition. On the other hand, they may have become more sensitive to changes in local impact factors, such as wave movement. One possible interpretation of this finding is that the extensive reduction of common reed area in Lake Chiusi during the last 3 decades (Gigante et al., 2014; Lastrucci et al., 2019) could have reduced the protection of the open water from the wind, increasing the wave movement events and consequently their incidence on remnant reed stands (pers. obs.).

The dualism we observed between more common haplotypes and less spread ones across our study areas suggests the coexistence of two main evolutionary strategies deployed by this species: maximize phenotypic plasticity, which may favour colonizing new habitats (high functional richness, even with generally lower canopy traits implying suboptimal productivity, such as for stands with haplotypes CHTR and M), or maximizing productivity through specialization to peculiar site conditions (low functional richness, but higher scores of spectral proxies for traits connected to canopy density/biomass, and eventually productivity, such as for stands with haplotypes CO and VI).

Overall, our results showed that haplotypes explained a tangible portion of total intraspecific variability in spectral proxies of canopy traits across sampled reed communities, of variable magnitude (0-24%), depending on ecological status and/or spectro-functional trait considered. The higher portion of variability due to haplotype in aquatic stands could be linked to more intense selective pressure exerted on reeds by rhizome flooding, that cannot be tackled only by phenotypic plasticity (Engloner et al., 2010; Gigante et al., 2011, 2014; Lastrucci et al., 2016a, 2017)

The most common haplotypes (CHTR and M) showed significantly lower values of each proxy under flooded than in dry conditions, of major entity for WAVI and GLI, and minor one for $\mathrm{NDSI}_{\mathrm{LMA}}$. Moreover, while the difference between flooded and dry stands for GLI was similar for CHTR and

M, flooded CHTR stands scored a markedly larger decrement in WAVI compared to terrestrial ones. Such a difference suggests that canopy biomass of aquatic CHTR stands tends to be lower than that of other haplotypes, and this could be putatively connected to the higher impact of dieback on flooded CHTR stands. This outcome is in line with previous works that showed macro-morphological evidence, such as clumping habitus or lower culms diameter, commonly described for flooded reeds as generally associated with the dieback syndrome of *P. australis* (Lastrucci et al., 2016c; Coppi et al., 2018). Related findings may help shedding some light on drivers of common reed dieback in European aquatic stands, a largely investigated yet to date still poorly understood phenomenon, in terms of cause-effect processes (Ostendorp, 1989; Van der Putten, 1997; Gigante et al., 2011; Gigante et al, 2013; Gigante et al., 2014; Coppi et al., 2018).

Conclusion

Our results dealing with reaction norms of spectro-functional traits to macro-scale meteo-climatic factors bear implications with respect to ongoing scenarios of climate change and in particular summer warming, as trends of increasing temperature appear to be related to decreased canopy density (WAVI) and green leaf area (GLI) in flooded reed stands. This outcome appears particularly interesting and indicates the advisability of further investigations in this direction to understand better the processes underpinning common reed decline in Europe, and in particular the Mediterranean region.

Taking advantage of medium-high resolution satellite data collected by Sentinel-2 constellation, freely available since summer 2015 and now granting an observation frequency of up to 5 days over the majority of globe, we have investigated the canopy reflectance diversity in the dominant macrophyte *P. australis*, its relation to the haplotype diversity, the meteo-climatic parameters, and the different ecological condition of reed stands. Multispectral satellite data covering different years and systems have allowed exploring intraspecific variations in spectro-functional traits of *P. australis* within and among sites at spatial scales virtually impossible to assess from in situ survey only. Through this, we enlarged the knowledge on the distribution in Central Italy of previously known *Phragmites australis* haplotypes, as well as to identify a new haplotype (nM). Furthermore, we provided new evidence on haplotype-haplotype and haplotype-by-environment interactions for this species. In particular, the interaction among haplotypes and its relation to the spectro-functional variability of *P. australis* stands.

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Supplementary material:

https://drive.google.com/drive/folders/1Ny3z3ddLGtkONi-Ud-t6ab4dq7irWI8C?usp=sharing

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5.2.2. Article IV: How genetic drift and natural selection affect the evolution of P. australis and N. lutea populations from northern-central Italy.

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Experimental Design	20	20	20				40
Field Work / Experiment Maintenance	20	20	20	20	20		
Sample Analysis	70		10	15		5	
Manuscript Drafting	70	10	10				10

Author's contribution (percentage) relative to the different tasks carried out in this work.

Introduction

In the last decades, several studies on evolutionary processes inferred the relative role of genetic drift and natural selection for species diversification (Orsini et al. 2013, Andrews et al. 2016). However, the extent to which both evolutionary forces affect populations depends largely on species' ecological features. It is well known that intraspecific diversity plays a vital role in ecological and evolutionary dynamics (Bolnick et al., 2003), improving species and communities' resilience to disturbance (Moran et al. 2015) and leading species' response both to biotic and abiotic factors (Hughes et al. 2008; Bolnick et al. 2011; Eller et al., 2017). Therefore, a better understanding of patterns and processes of genetic and phenotypic intraspecific diversity becomes 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 versus neutral processes, several studies (as far as we know, none on aquatic plants) have compared phenotypic divergence (Pst) to differentiation of neutral alleles (Fst) (Pst–Fst 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 (Pst = Fst). On the other hand, if Pst > or < Fst, the differentiation of phenotypic features is more likely shaped by natural stabilizing – or directional – selection (Leinonen et al., 2008; Merilä and Crnokrak, 2001; Whitlock, 2008; Chapuis et al., 2008; Martin et al., 2008; Brommer, 2011; Leinonen et al., 2013).

In this regard, further efforts are needed to explore the interlinks between genetic differentiation and functional traits (FTs) variation, especially that referable to the leaf economic spectrum, a key tool for exploring species ecology (Pierce et al., 2012; Dalle Fratte et al., 2019a; Dalla Vecchia et al., 2020). FTs as leaf total area (LA, including petioles), specific leaf area (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 a-biotic 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 and 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 characterizing plant structure and physiology across sites and ecosystems can offer deeper insights into cross-feedback underlying the functioning of ecosystems and inform effective management strategies (Villa et al., 2017, Castellani et al., under review).

Here, we studied two different freshwater macrophytes, the floating hydrophyte *Nuphar lutea* (L.) Sm. (or yellow water lily) and the helophyte *Phragmites australis* (Cav.) Trin. ex Steud. (or common reed), as models to infer the relative role of genetic drift and natural selection on the leaf traits diversification within aquatic habitats. Both the target species are often dominant and widespread macrophytes and as such play essential roles as ecosystem engineers due to their ability to shape the habitats colonized through their physiology and physical structure. Yellow water lily is a floating-leaved macrophyte distributed across lower latitudes of Europe, northwest Africa (locally known for Algeria), and eastward 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 seemed to be regulated by water depth and sediment features in hyper-eutrophic environments (Dalla Vecchia and Bolpagni, 2022). Common reed is a sub-cosmopolitan helophyte, dominating the riparian vegetation of most of freshwaters and brackish wetlands globally. This species may also colonize disturbed wetlands, artificial ditches, mined areas, and garbage land, thus proving a broad ecological amplitude. These characteristics make *P. australis* a keystone species in freshwater ecosystems and a support several habitats and non-habitat ecosystem services (Kiviat, 2013). As *N. lutea*, it plays an essential role as a wind and wave breaker (Takeda and Kurihara 1988; Vymazal 2011; Karstens et al., 2016) and its functional traits seem to be determined by the water trophic content (Eid. et al., 2021).

The main aim of this work was to assess and compare the genetic variation and the 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 functional leaf traits directly measured and inferred from foliar reflectance - for both species sampled from different populations across several lakes in northern and central Italy. We then investigated the correspondence between the degree of population differentiation in neutral genetic markers and the quantitative FTs, expressed by Fst and Pst indices, to depict the forces (among natural stabilizing- or directional selection) shaping the differentiation of FTs. Comparing Fst and Pst, we assume that Fst, determined by neutral markers, reflects divergence caused only by genetic drift (Reynolds et al., 1983). Thus, Fst provides a null expectation and allows estimation of what would happen to populations if there were no selection (Merilä and Crnokrak, 2001). On the other hand, Pst also incorporates the effects of selective dynamics on the phenotype. Consequently, the Pst-Fst comparison involves three possibilities in deciphering the evolution of *P. australis* and *N. lutea* populations within different sites: I) Pst > Fst indicates that the degree of differentiation in traits overcome that attributable to the genetic drift alone, suggesting that natural directional selection (DS) is leading to population differentiation; II) Pst = Fst, indicates that neutral divergence cannot be excluded as a possible cause of phenotypic diversity (DS-SS); III) Pst < Fst indicates that phenotypic divergence is less than expected based on genetic drift alone, and trait differentiation is most likely caused by stabilizing selection (SS) (Leinonen et al., 2008; Merilä & Crnokrak, 2001).

Methods

Study area and Sampling Design

The study area included five wetland ecosystems (sites hereafter) of 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); Lakes Pusiano and Annone ("PA", 45° 48′ 40″ N, 9° 18′ 45″ E). They are phytogeographically comparable sites covering environmental gradients, in terms of ecological conditions (i.e., trophic status), geographical features (i.e. size and watershed land cover), and plant community structure (i.e. macrophyte community types, or growth forms). Specifically, the five sites differ by water body covered area, with the smallest site (Chiusi) covering ~3.8 km^2, and the largest one (Iseo) covering 65.3 km^2. In terms of trophic conditions, the sites range from oligo-mesotrophic (Iseo) and mesotrophic (Pusiano), up to eutrophic (Annone) and even hypertrophic (Chiusi, Massaciuccoli and Mantova).

Depending on area and relative coverage of different plant community types, a variable number of 10 m x 10 m sampling plots were selected in each site. Out of the total 78 sampled plots, 50 were dominated by *Phragmites australis* and 28 by *Nuphar lutea* (Supplementary material_Table 1). Based on the measures and analyses to be carried out (functional, spectral or genetic) a variable number of leaves (3 to 8) was taken from each plot. 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 were collected as matchups.

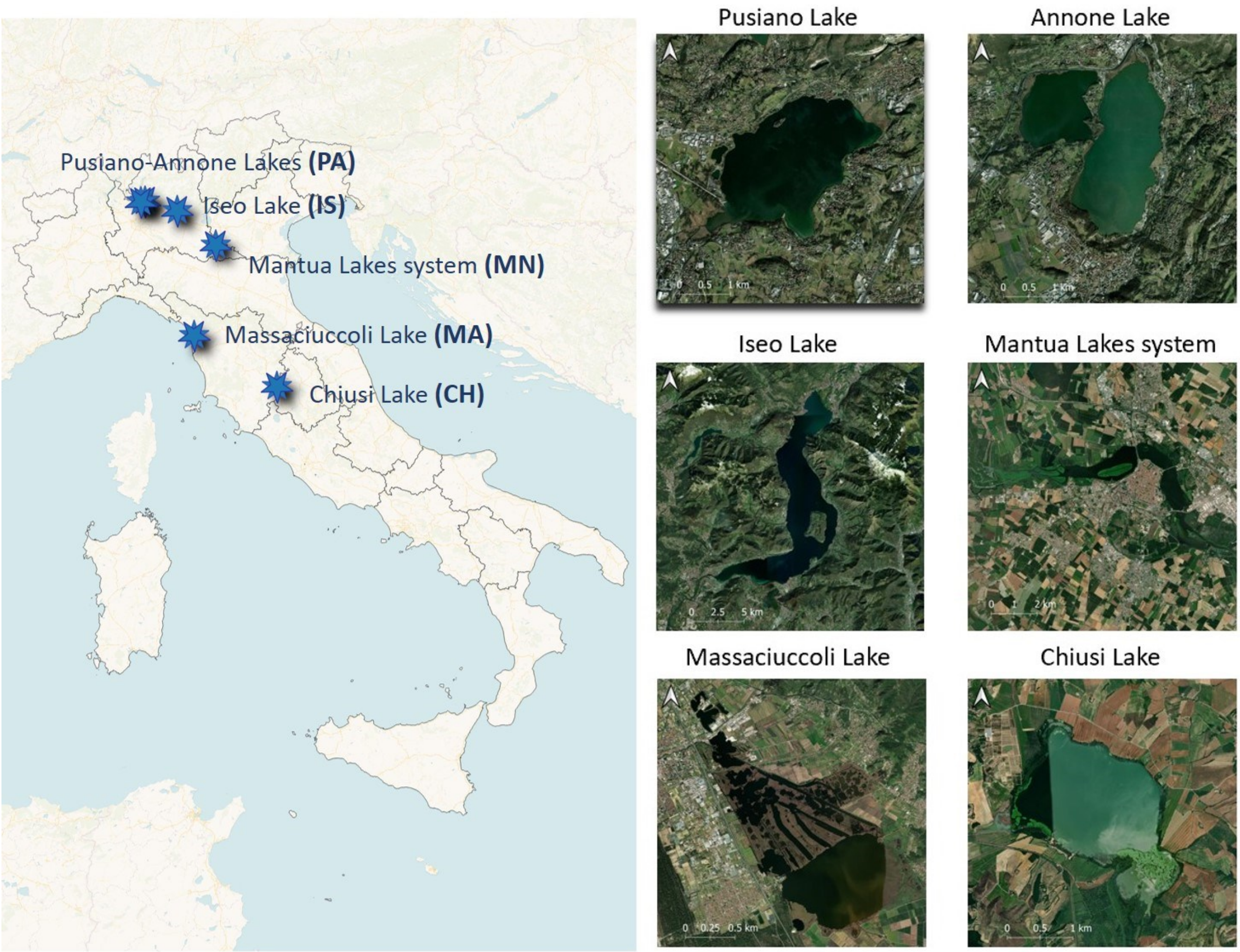


Figure 1 *Geographic location of the study area with distribution of the sampling sites. Satellite images of each site (at different scale), taken from QGis' Google Satellite Basemap, are shown on the right.*

Spectral data

Reflectance in the visible to shortwave infrared spectrum range (350 – 2500 nm) was measured from fresh leaves within seconds (maximum 1 minute) after cutting them from the plants. Measurements were done using a portable high resolution spectroradiometer (SR-3500, Spectral Evolution, Lawrence, USA), with a spectral resolution of 3 nm for wavelengths under 1000 nm, and < 8 nm up to 2500 nm. In order to minimize 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, USA; reflectance factor > 95%).

Functional traits

Eight intact well-developed leaves of *N. lutea* and *P. australis* were sampled to determine both structural (LA, mm^2; SLA, mm^2/mg) and bio-chemical (Chl-a, µg/g; Chl-a/Chl-b) traits. After kindly cleaning water-saturated leaves by debris and epiphytes, they were scanned for measuring the LA. Leaves were subsequently dried at 50°C until constant weight to quantify the dry weight (mg). LA was determined analyzing scanned images with the software ImageJ (Rasband, 1997-2018), whereas SLA was calculated as the ratio between the LA and the dry weight. High values of SLA indicate an acquisitive behavior, implying lower investments in structural tissues for photosynthetic organs and a higher photosynthetic capacity *per* mass unit (Dalle Fratte et al., 2019b). Chlorophyll content was determined after 24-h 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 2500 nm, based on leaf biochemical constituents (chlorophylls, carotenoids, anthocyanins, water, and dry matter) and a structural parameter, termed N (Féret et al., 2017). PROSPECT-D was inverted through iterative optimization, minimizing the RMSE between measured and simulated reflectance with optimal spectral domain and configuration for each individual trait (Spatford et al., 2021), using the R package "prospect" (Féret and de Boissieu, 2022). 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 models were designed and calibrated on terrestrial plant species, in order 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 = 238-394, varying with trait). Such recalibration reduced the relative error of modelled traits by 1 to 10 % depending on the trait, with nRMSE ranging from 6% (LDMC) to 12% (Chl_ab).

DNA extraction and AFLP protocol

A total of 400 and 206 samples were analyzed 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 2x cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1990). The quality and quantity control of the extracted DNA were performed by a spectrometric survey that used a Bio-Photometer (Eppendorf). AFLP analysis was performed in accordance with standard procedure, but with minor modifications of previous studies using molecular tools (see Coppi et al., 2014 and references therein). Two combinations of primers were selected for both species analysis: hex_EcoRI-CTA/MseI-ATG and fam_EcoRI-TAC/MseI-ATG for P. australis and hex_EcoRI-ACG/MseI-TTA and fam_EcoRI-CTA/MseI-CTC for N. lutea. AFLP profiles obtained by capillary electrophoresis were analyzed using GeneMarker v1.5 (SoftGenetics LLC, State College, PA, United States).

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). Genomic template stability was evaluated by comparing the differences in the percentage of polymorphic loci of each sampling plot's AFLP profiles within each site. The percentage composition of polymorphic bands was calculated as [(nfrag/ntotal)*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: I) within and among sampling plot, II) 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 a Slatkin's linearized pairwise Fst values (Slatkin 1995).

Following Yang et al. (2016), outliers were detected using BayeScan v2.01. Outliers are loci that fall over a threshold value set on the logarithm of posterior odds values (LogPO), determined as in Foll (2012). The number of pilot runs was kept at 20, with a length of 10 000 iterations each one (Coppi et al., 2018).

Pst-Fst comparison

Phenotypic variance (Pst), estimated from functional leaf traits, were compared to Fst values using "Pstat" package (Silva and Silva 2018) on R environment. Fst values for both species were extrapolated from the molecular variance analysis (see supplementary material_Table 4 and 5). For each site, Fst values at population level were averaged and bootstrapped 95 % confidence intervals were calculated using R environment. Pst values were determined with the bootstrap method (1,000

x) under a confidence level of 0.95% using “Pstat” package (Silva and Silva 2018) (Supplementary material_Table 3).

Pst is a proxy of Qst index (Spitze, 1993) and it is used when additive variance cannot be readily easily quantified (i.e., in field studies, Brommer, 2011). It was calculated from between population (σ^2_B) and within population (σ^2_w) components of variance for each trait following Brommer’s (2011) expression:

$$\text{Pst} = \frac{c/h^2\sigma_B^2}{c/h^2\sigma_B^2+2\sigma_W^2} \tag{1}$$

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). Since values of c/h^2 are unknown in natural populations (see Brommer 2011), 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, $c< h^2$) supposes 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 Pst exceeds Fst, the more likely it is that the trait is being shaped by selection (Brommer 2011). Because of this problem on the accurate estimation of the parameters c and h^2 for a set of traits, we assessed the strength of Pst–Fst comparisons exploring $c/h^2 = 0.5$.

Cohen’s d was used to calculate the effect size for Pst-Fst differences:

$$\text{Cohen's } d = \frac{\mu(Pst)-\mu(Fst)}{\sigma(Pst,Fst)} \tag{2}$$

where:

- $\mu(Pst)$ = Pst_Value from Pstat
- $\mu(Fst)$ = average Fst score computed
- $\sigma(Pst,Fst)$ = pooled standard deviation derived as $\sigma(Pst)+\sigma(Fst)$, with:
    - $\sigma(Pst)$ estimated using lower bound of 95% CI output of Pstat, as $\frac{2(Pst_{Value}-95\%_{lowCI\_Pst})}{3.92}$
    - $\sigma(Fst)$ estimated using 95% CI of Fst average, as $\sqrt{N}\frac{(95\%_{upCI\_Fst}-95\%_{lowCI\_Fst})}{3.92}$

As a first interpretation, we categorized the continuous range of Cohen’s *d* scores into three situations, following the scale proposed by Sawilowsky (2009), denoting dominant mechanisms underlying intraspecific diversity in terms of Pst-Fst, such as: SS situation, when $d \leq 0$; DS situation, when $d \geq 1.2$; and an intermediate situation driven by a mix of DS and SS (DS-SS situation), when $0<d<1.2$.

Results

Functional traits

The FTs values of each leaf for both species are shown in Supplementary material Table 2 and plotted in Figure 2 (*P. australis*) and 3 (*N. lutea*). In addition, the intraspecific leaf reflectance variability and the comparison between leaf traits measured and estimated from PROSPECT-D inversion are shown in Supplementary material Figure 1 and 2 respectively.

Average values of *P. australis* traits: As for structural traits, LA ranged from 6525.39 mm2 (IS) to 10536.02 (MN), while SLA from 12.12 mm2/mg (MN) to 13.90 (MA). As for bio-chemical traits, Chl-a ranged from 2021.38 µg/g (CH) to 3069.17 (MN), while Chl-a/Chl-b from 3.99 (CH) to 4.70 (PA). As for traits derived from PROSPECT-D model inversion, Chl_ab ranged from 51.83 µg cm-2 (CH) to 56.23 (PA) and LMA from 72.92 g m-2 (MA) to 81.54 (IS). In addition, LDMC ranged from 0.40 g g-1 (MA) to 0.45 (IS) and Nmesophyll from 1.41 (MA) to 1.57 (PA).

Average values of *N. lutea* traits: As for structural traits, LA ranged from 56748.92 mm2 (IS) to 80656.98 (PA), while SLA from 6.13 mm2/mg (PA) to 9.10 (CH). As for bio-chemical traits, Chl-a ranged from 693.29 µg/g (PA) to 934.92 (IS), while Chl-a/Chl-b from 3.41(CH) to 4.44 (IS).
As for traits derived from PROSPECT-D model inversion, Chl_ab ranged from 33.13 µg cm-2 (CH) to 40.77 (PA) and LMA from 76.75 g m-2 (CH) to 108.20 (PA). In addition, LDMC ranged from 0.16 g g-1 (CH) to 0.19 (PA) and Nmesophyll from 1.57 (CH) to 2.15 (PA).

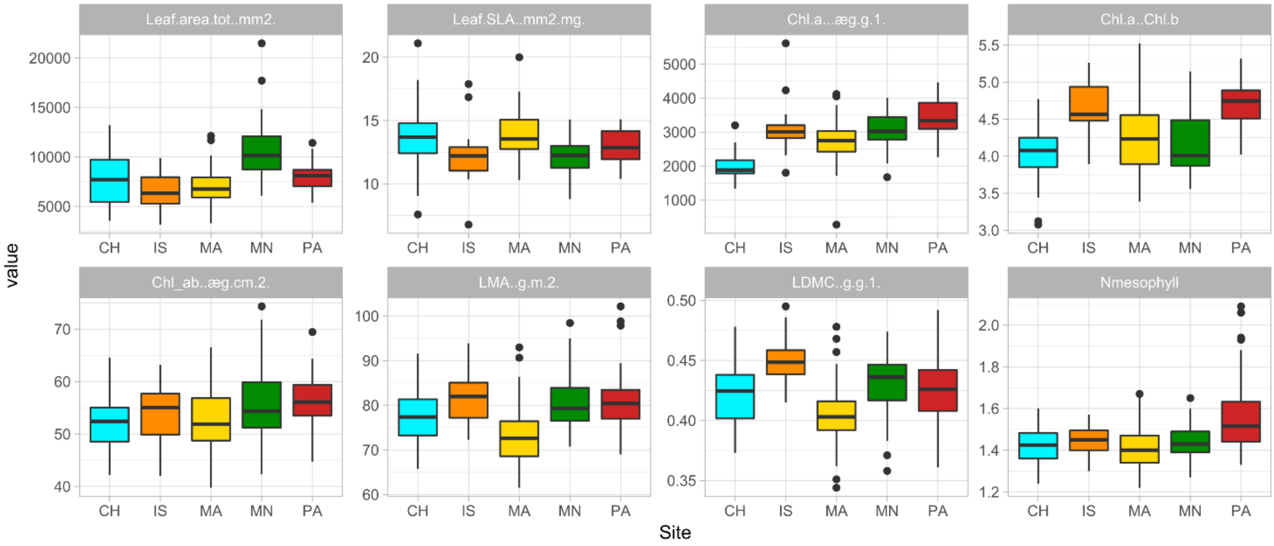


Figure 2 *Boxplot showing the variability of P. australis functional traits. As for structural and bio-chemical traits: LA, SLA, Chl-a, Chl-a/Chl-b. As for traits derived from PROSPECT-D model inversion: Chl_ab, LMA, LDMC and Nmesophyll. Results are grouped per site: CH = Chiusi, IS = Iseo, MA = Massaciuccoli, MN =Mantova, PA= Pusiano-Annone.*

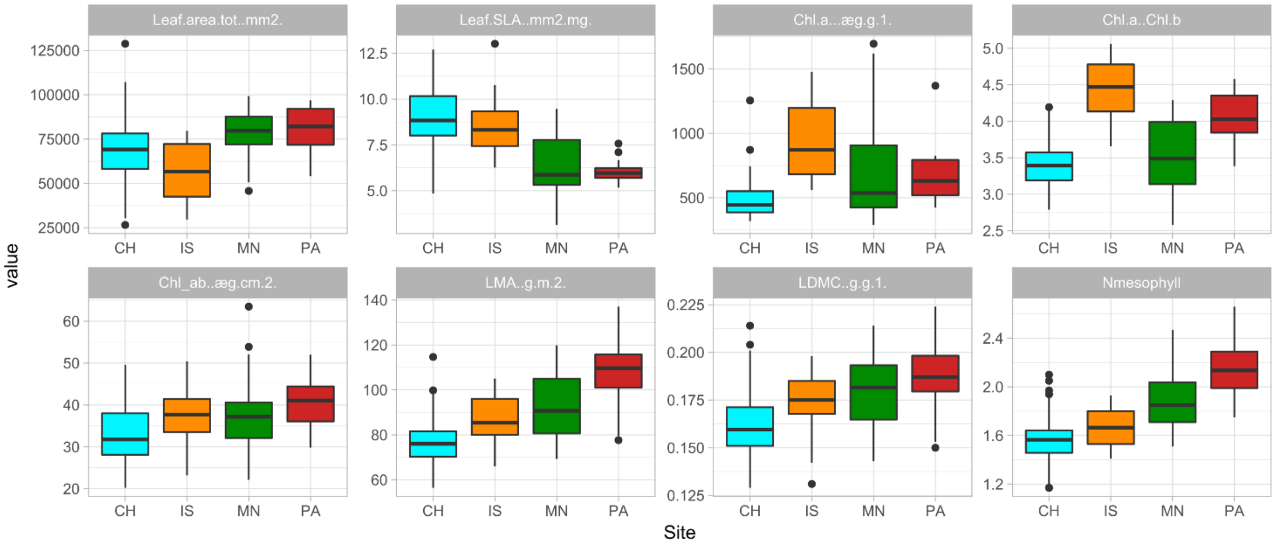


Figure 3 *Boxplot showing the variability of N. lutea functional traits. As for structural and bio-chemical traits: LA, SLA, Chl-a, Chl-a/Chl-b. As for traits derived from PROSPECT-D model inversion: Chl_ab, LMA, LDMC and Nmesophyll. Results are grouped per site: CH = Chiusi, IS = Iseo, MN =Mantova, PA= Pusiano-Annone.*

Amplified Fragment Length Polymorphisms Analysis

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 87.68% (MN14) down to a minimum of 29.61% (CH08). Within each site, the average percentage of polymorphic loci varied from 97.36% for Mantova to 76.83% for Chiusi. The AGD levels varied from 0.086 (CH02) to 0.353 (MA08) within sampling plot and from 0.176 to 0.321 (Chiusi and Mantova respectively) at site level. Genetic diversity values both at sampling plot and site level were shown in supplementary material (Tab 4a,b). Regarding AMOVA, the greatest percentage of the total genetic variation occurred within sampling plots level (66.9%), rather than among sampling plots (33.1%) (Table 1a). The same can be observed at the site level 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 greater than 0.78 (at a threshold of log10 PO > 0.5).

a)

Source of variation	**d.f.**	**Sum of squares**	**Variance Components**	**Percentage of variation**	***P- values***
Among sampling plots	49	9200.153	18.92043	33.07	<0.0001
Within sampling plots	345	13210.464	38.2912	66.93	<0.0001
Total	394	22410.618	57.21164	100	

b)

Source of variation	**d.f.**	**Sum of squares**	**Variance Components**	**Percentage of variation**	***P- values***
Among sites	4	3955.551	12.32683	20.67	<0.0001
Within sites	390	18455.067	47.32069	79.33	<0.0001
Total	394	22410.618	59.64751	100	

Table 1a,b *Partition of genetic variance. AMOVA was performed at two different hierarchical levels: a) within and among sampling plot, b) within and among sites. The table shows: degrees of freedom (d.f.), 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.*

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 percentage of polymorphic loci (PPL) ranged from a maximum of 92.67% (IS06) down to a minimum of 35.60% (CH17). Within each site, the average percentage of polymorphic loci varied from 86.39% for Iseo to 77.49% for Pusiano - Annone. The AGD levels varied from 0.098 (CH05) to 0.287 (MN33) within sampling plot and from 0.212 to 0.267 (Chiusi and Iseo, respectively) at site level. Genetic diversity values both at intra- and inter- site level were shown in supplementary material (Tab 5a,b).

Regarding AMOVA, the percentage of the total genetic variation was nearly equal within (53.45%;) and among sampling plots (46.55%) (Table 2a) and within (54.13%) and among (45.87%) sites (Table 2b). BayeScan analysis did not detect any outlier loci.

a)

Source of variation	d.f.	Sum of squares	Variance Components	Percentage of variation	***P- values***
Among sampling plots	27	3845.676	16.97461	46.55	<0.0001
Within sampling plots	175	3411.496	19.49427	53.45	<0.0001
Total	202	7257.172	36.46888	100	

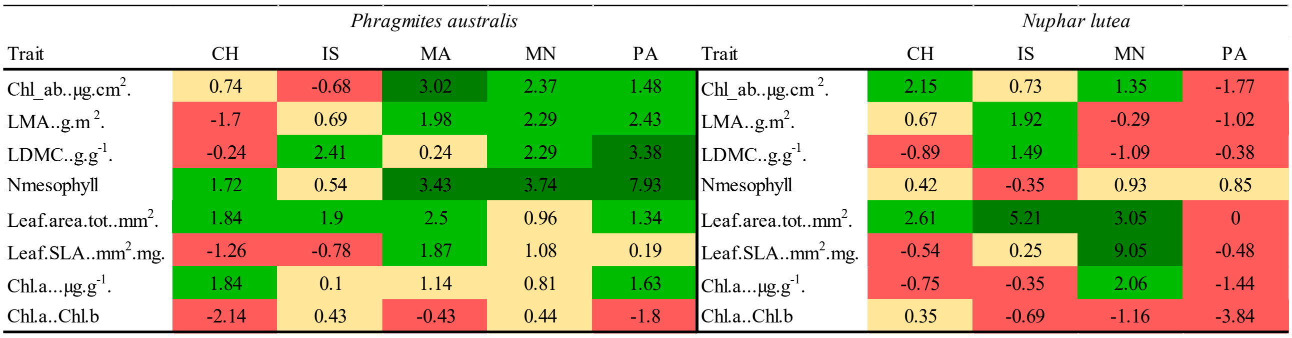
b)

Source of variation	d.f.	Sum of squares	Variance Components	Percentage of variation	***P- values***
Among sites	3	2747.578	19.20076	45.87	<0.0001
Within sites	199	4509.594	22.66128	54.14	<0.0001
Total	202	7257.172	41.86204	100	

Table 2a,b *Partition of genetic variance. AMOVA was performed at two different hierarchical levels: a) within and among sampling plot, b) within and among sites. The table shows: degrees of freedom (d.f.), 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.*

Pst-Fst comparison

According to equation 2, the table of effect size (Cohen's *d*) for Pst-Fst differences in the conservative case $c/h^2 = 0.5$ was the following:



	Phragmites australis						*Nuphar lutea*			
Trait	CH	IS	MA	MN	PA	Trait	CH	IS	MN	PA
Chl_ab..μg.cm^2.	0.74	-0.68	3.02	2.37	1.48	Chl_ab..μg.cm^2.	2.15	0.73	1.35	-1.77
LMA..g.m^2.	-1.7	0.69	1.98	2.29	2.43	LMA..g.m^2.	0.67	1.92	-0.29	-1.02
LDMC..g.g^{-1}.	-0.24	2.41	0.24	2.29	3.38	LDMC..g.g^{-1}.	-0.89	1.49	-1.09	-0.38
Nmesophyll	1.72	0.54	3.43	3.74	7.93	Nmesophyll	0.42	-0.35	0.93	0.85
Leaf.area.tot..mm^2.	1.84	1.9	2.5	0.96	1.34	Leaf.area.tot..mm^2.	2.61	5.21	3.05	0
Leaf.SLA..mm^2.mg.	-1.26	-0.78	1.87	1.08	0.19	Leaf.SLA..mm^2.mg.	-0.54	0.25	9.05	-0.48
Chl.a...μg.g^{-1}.	1.84	0.1	1.14	0.81	1.63	Chl.a...μg.g^{-1}.	-0.75	-0.35	2.06	-1.44
Chl.a..Chl.b	-2.14	0.43	-0.43	0.44	-1.8	Chl.a..Chl.b	0.35	-0.69	-1.16	-3.84

Which brings to the following categorized table (Table 3):

	Phragmites australis					*Nuphar lutea*				
Trait	CH	IS	MA	MN	PA	Trait	CH	IS	MN	PA
Chl_ab..µg.cm^{2}.	DS-SS	SS	DS	DS	DS	Chl_ab..µg.cm^{2}.	DS	DS-SS	DS	SS
LMA..g.m^{2}.	SS	DS-SS	DS	DS	DS	LMA..g.m^{2}.	DS-SS	DS	SS	SS
LDMC..g.g^{-1}.	SS	DS	DS-SS	DS	DS	LDMC..g.g^{-1}.	SS	DS	SS	SS
Nmesophyll	DS	DS-SS	DS	DS	DS	Nmesophyll	DS-SS	SS	DS-SS	DS-SS
Leaf.area.tot..mm^{2}.	DS	DS	DS	DS-SS	DS	Leaf.area.tot..mm^{2}.	DS	DS	DS	SS
Leaf.SLA..mm^{2}.mg.	SS	SS	DS	DS-SS	DS-SS	Leaf.SLA..mm^{2}.mg.	SS	DS-SS	DS	SS
Chl.a...µg.g^{-1}.	DS	DS-SS	DS-SS	DS-SS	DS	Chl.a...µg.g^{-1}.	SS	SS	DS	SS
Chl.a..Chl.b	SS	DS-SS	SS	DS-SS	SS	Chl.a..Chl.b	DS-SS	SS	SS	SS

Table 3 *Table showing results of Pst/Fst comparison for each trait within sites (CH= Chiusi; IS= Iseo; MA= Massaciuccoli; MN= Mantova; PA= Pusiano-Annone). DS indicates that the trait has a greater genetic divergence than neutral expectations, which could indicate directional selection, i.e. Pst>Fst. SS indicates that phenotypic divergence is less than expected under neutral conditions, likely due to stabilizing selection, i.e. Pst<Fst; DS-SS that neutral divergence cannot be excluded as a possible cause of phenotypic diversity, i.e. Pst=Fst.*

As for *P. australis*, results showed that most traits tended to show genetic divergence in line or greater than neutral expectations, indicating directional selection, except for Chiusi site (CH) where trait variability was shaped both by directional and stabilizing selection, depending on trait. Further exceptions were three traits: Leaf SLA and Chl-ab for Iseo, and the Chl-a/Chl-b ratio for Massaciuccoli and Pusiano-Annone sites (Table 3).

As for *N. lutea*, results were more heterogeneous: both directional and stabilizing selection appeared to have affected the traits variability within each site differently (similarly to what happens in Chiusi for *P. australis*), except for Pusiano-Annone where traits variability was mostly due to stabilizing selection (Table 3).

Comparing the patterns at trait levels across sites, we observe that LA and Chl-a/Chl-b are consistently marked by the same situation in both species, i.e. directional and stabilizing selection respectively, why a strong contrast between the two species is shown for LDMC, generally driven by directional selection in *P. australis* and by stabilizing selection in *N. lutea*. The other traits show complex, often site-dependent variability in Pst-Fst categorized situations, with an overall tendency pending towards directional selection for *P. australis* and the other way (balanced or stabilizing selection) for *N. lutea* populations.

Discussion

In the present work we studied two keystone macrophytes species, *N. lutea* and *P. australis*, representing two dominant growth forms - floating-leaved and riparian helophytes, respectively – for the first time using the Pst-Fst comparison to infer the relative role of genetic drift and natural selection on the diversification of their leaf FTs across different wetlands. With some rare exceptions, our results showed an overall tendency to directional selection for *P. australis* and to balanced or stabilizing selection for *N. lutea* populations.

Genetic structure and FTs variation

The mean genetic diversity indices at the sampling plots and site levels showed no obvious signs of gene erosion for *P. australis*. AGD and PPL values were thus in line with those observed for other Italian and European populations (Coppi et al., 2018, Lambertini et al., 2008). The AMOVA analysis has shown that most of the genetic variation resided within rather than among sampling plots and sites, confirming previous studies of the phylogeographic relationships in common reed stands at local or narrow range scale (Coppi at al., 2018 Lambertini et al., 2008; Qiu et al., 2016). The high "local" genetic diversity of *P. australis* was largely demonstrated and related to several ecological and physiological aspects of the species (Gao et al., 2012; Richards et al., 2012). In particular, the high competitivity of common reed, becoming invasive in North America, has been described because of the capacity of the plants to combine sexual reproduction and vegetative propagation (Albert et al., 2015; McCormick et al., 2010). This is mirrored by the FTs that resulted quite variable among sites with rather wide standard deviations (both for measured and derived leaf features; Figure 2).

As for *N. lutea*, the only two previous works on the species' genetic diversity showed a greater genetic variability within rather than among populations (Vyšniauskiene et al., 2020; Fér, & Hroudova, 2008). In our study, differently, the partition of genetic variation was nearly equal within and among both sampling plots and sites, revealing a more complex genetic structure than expected at site level. Comparing our AFLP results (Supplementary material_Table 5) to those associated with the breeding systems for other plants (Nybom, 2004) a possible explanation could be that the genetic structure of studied *N. lutea* populations may be influenced by the prevalence of a mixed mating system. In confirmation, although *N. lutea* is defined a long-living clonal species, both the in- and out-breeding is well demonstrated, and bees, bumblebees, and hoverflies are considered the most important pollinators of the species (Ervik et al. 1995; Lippok and Renner 1997; Padgett, 2007). As found for *P. australis*, the FT values of *N. lutea* exhibited wide variations across sites, highlighting a high phenotypic plasticity both in structural and in biochemical traits. This is in line with the results by Della Vecchia & Bolpagni (2022) which found high rates of intraspecific variability for *N. lutea* at the micro-scale (Lake Chiusi), as well as observed strong links between traits variation and environment drivers. These authors demonstrated that the LA and the fresh and dry leaf weight increased with water depth, and open new perspectives on the relevance of petiole traits for nymphaeids.

In this context, the mixed reproductive strategy could play a role in facing environmental variation for both *P australis* and *N. lutea*. 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 (West-Eberhard 2003; Waddington, 1952, 1953). At present, local adaptation capabilities for these two species have been studied from either a phenotypic (Ren et al., 2020; Vretare et al., 2001; Henriot et al., 2019;) or a genetic point of view (Vyšniauskiene et al., 2020; Fér & Hroudova, 2008; Naugžemys et al., 2021; Coppi et al., 2018; Lambertini et al., 2020); but 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) for *P. australis*.

Pst-Fst comparison

The Pst-Fst comparison indicated that, for *P. australis*, most of the observed FT variation was affected by directional selection (DS), thus led by genetic local adaptation. On the other hand, for *N. lutea*, the comparison showed an overall tendency to balanced or stabilizing selection.

Regarding *P. australis*, directional selection tends to move the traits mean towards the optimum for the environment, increasing adaptability of individuals. 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. The present result confirms the theory concerning the colonization strategy of *P. australis*, where vegetative reproduction prevails over the generative one (Lebedeva et al., 2020; Alvarez et al., 2005), and populations become more and more dominated by a small number of clones well adapted to the prevailing site condition (Koppitz and Kuehl, 2000). An exception is the Chiusi's populations where traits variability was shaped both by directional and stabilizing selection. It is well known that the Chiusi site hosts different common reed haplotypes (Coppi et al., 2018). Moreover, our results show that this site hosts 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 and promote the more decisive influence of SS on several leaf traits.

Because freshwater environments are typically heterogeneous in space and time (Palmer et al., 1997) and water-level fluctuations are a key disturbance factor of hydrophytes (Maltchik et al., 2005, Lenssen et al., 2004), is likely that the rate of adaptation to environment differ depending on how closely the plastic phenotype is to the optimum for the environment (Price et al. 2003). Following Price and colleagues (2003), when plasticity matches the optimum, the population should undergo stabilizing selection, without subsequent genetic differentiation. Instead, when plasticity is in the direction favored by selection but below the optimum, the population should be subjected to directional selection as the case of the other sites MA, MN and PA. In this context, comparing *P. australis* and *N. lutea* populations from the same sites may provide more in deep knowledge on how different reproductive strategies and traits differentiation are related and acts in shaping the functional diversity and adaptation of both species. In this framework, the also more complex genetic structure of *N. lutea* due to a mixed mating system, combined with the degree to which phenotypic plasticity closely matches with the optimum for the environment, could explain the heterogeneous results displayed in the Pst-Fst comparison where both directional and stabilizing selection act on traits variability within each site differently. As for Pusiano-Annone lakes, contrarily to *P. australis*, the proportion of variation in the phenotype was largely due to genetic divergence caused only by genetic drift and the stabilizing selection tended to favor individuals with intermediate traits.

In summary, this study amplified the knowledge of the genetic structure of *N. lutea* and *P. australis* populations in the lakes of central and northern Italy and it was the first step to understanding the relative role of genetic drift and natural selection on the diversification of their phenotypic traits within habitats. Since the relative contribution of selection, genetics, and plasticity to environmental adaptation, seem to be strictly determined by the ecological context, further study is needed to analyze the relative role of natural selection on the diversification of the two species (especially in Chiusi and Pusiano-Annone lakes) and try to find its relationship with eco-geographical variables.

Supplementary material:
https://drive.google.com/drive/folders/1Ny3z3ddLGtkONi-Ud-t6ab4dq7irWI8C?usp=sharing

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5.2.3. Article V: Genome-Wide Association analysis of leaf spectro-functional traits in Phragmites australis.

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Contribution table:

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Experimental Design	10		20	20					50
Field Work / Experiment Maintenance	20		20	20	20	20			
Sample Analysis	70	10							20
Bioinformatic analysis	35	35					25	5	
Manuscript Drafting	90								10

Author's contribution (percentage) relative to the different tasks carried out in this work at the current state of research.

Introduction

Studies conducted on different types of natural environments, which measure both genetic diversity and functional diversity together, suggested that these two measures give differing, but complementary information. In fact, they showed how genetic diversity associated with functional traits allows to both quantify the effect of disturbances and to better understand the factors that drive community dynamics (Jones et al., 2019; Hao et al., 2018; Flynn et al., 2011). However, the methods used to assess functional diversity have often neglected the variation of traits within species, rather comparing mean trait values per species. Since competition for resources, niche width expansion and natural selection occur at the individual level, rather than on species means, these processes could promote considerable progress in the field of community (e.g., Pachepsky et al. 2001; Bown et al., 2007; DeAngelis, 2018).

Both genetics and environmental factors contribute to the observed variation of traits within a species (Hufford & Mazer, 2003; Nicotra et al., 2010; Sultan, 2000; de Bello et al., 2011; Violle et al., 2012) and Waitt and Levin (1998) demonstrated positive correlations between phenotypic variation of morphology-related functional traits and genetic variation in several species from different plant families. Genetic diversity, per se, is considered essential for population fitness and evolutionary processes and affects the adaptive ability of a species to environmental changes and competitors (Nicotra and Davidson, 2010; Karbstein et al., 2019). Nevertheless, if trait variation is plastic, it does not necessarily correlate with genetic variation (see also Chevin and Hoffmann, 2017). Whole genome sequencing (WGS) has recently allowed to understand how genomes evolve among closely related organisms (De Pristo et al., 2011; Pasaniuc et al., 2012). At a more affordable price, low-coverage whole genome sequencing (lcWGS) has emerged as a successful approach for population genomic studies also in nonmodel species, improving the ability to multiplex samples in a single sequencing run (Lu et al., 2020). Although, lcWGS allows to sequence random genomic regions at a reduced sequencing coverage, it can be a powerful resource for Genome-wide association studies (GWAS), in trait-associated variant discovery (eg., Chat et al., 2021; Perumal et al., 2021; Zhang et al., 2022). GWAS is an effective method for identifying trait-associated variants by comparing the allele frequencies between individuals who are ancestrally similar but phenotypically different (Korte and Farlow, 2013). GWAS usually report blocks of correlated SNPs with statistically significant associations with the trait of interest. Although the literature on forest ecosystems is just being (e.g., Ćalić et al., 2017; Browne et al., 2019; Bai et al., 2020), studies of aquatic plant intraspecific trait variation in freshwater ecosystems are sparse. Based on the results obtained in the studies of chapters

5.2.1 and 5.2.2 (Articles III and IV), we contribute to fill this gap by investigating how genetic diversity is related to intraspecific functional diversity in *Phragmites australis* populations in Chiusi Lake. This helophyte species (known as common reed), characteristic of wet sites, is an important resident of submerged and emergent shores (Landucci et al., 2013) and may shows high genotypic variation as it reproduces both sexually and asexually. Different environmental filters, such as eutrophication, mechanical damage, presence of heavy metals and permanent flooding exposure may play a crucial role in the population dynamics of common reed, and over time contribute to shape its genetic and phenotypic diversity (Van der Putten, 1997; Ostendorp, 1999; Gigante et al., 2014; Lastrucci et al., 2016; 2017; Kettenring et al., 2011). Based on our previous results, Chiusi Lake populations were quite interesting since they have shown the highest genetic diversity and a significant number of haplotypes (see also Coppi et al., 2018; Castellani et al., 2022). Using a lcWGS analysis coupled with the GWAS approach would deepen the knowledge of the intraspecific diversity of common reed at the micro-local scale and attempt to identify, for the first time, trait-associated variants for this ecosystem engineers of wetland habitats.

This work is in preparation phase. Results are still being processed.

Methods

Study area and plant materials collection

Lake Chiusi (43°03’22.11’’N, 11°57’55.79’’E) is a shallow lake in southern Tuscany (Siena Province), at 252 m a.s.l. (Fig. 1). Due to its tributaries, the subcatchment covers 105 km^2 despite having a surface area of only 3.9 km^2. Lake depth varies with the seasons, averaging approximately 5.7 m (Arrigoni and Ricceri, 1982). It is part of a protect natural reserve named A.N.P.I.L. “Lago di Chiusi”. It is affected by rapid silting and sedimentation and is characterize by a severe condition of eutrophication (Balsamo, 1990).

Eight homogeneous reed-dominated plots (each 4 m × 1 m in size) were randomly selected across the site from an oversample of plots obtained from the processing of very-high resolution satellite data (<5 m pixel).

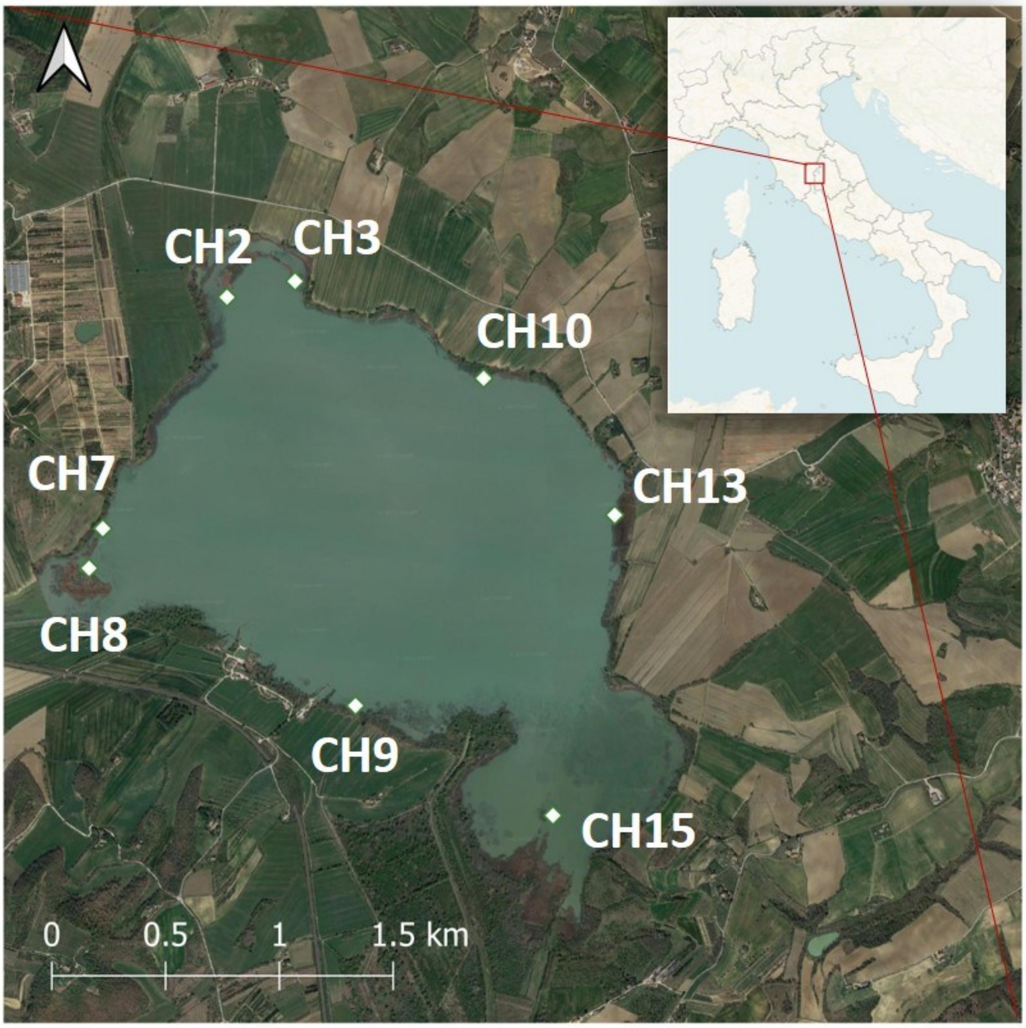

Figure 1 *Geographic location of the study area with distribution of the sampling plots.*

Functional traits

Functional traits were estimated using both proximal measures on the leaves and inversion of the PROSPECT model starting from leaf reflectance. To ensure comparability of the results, the same traits were used as in Article IV (chapter 2.5.5).

Regarding proximal measures, eight intact well-developed leaves of *P. australis* were sampled from each plot to determine both structural (Leaf Area, mm^2; Specific Leaf Area, mm^2/mg) and bio-chemical (Chlorophyll a, µg/g; ratio of Chlorophyll a and Chlorophyll b) traits. As for spectral measures, four leaf traits were derived from the inversion of PROSPECT-D model starting from leaf reflectance in the visible to shortwave infrared spectrum range (350 – 2500 nm): Total chlorophylls (µg/cm^2), dry matter content on area basis, or leaf mass per area (g/m^2); dry matter content on weight basis, or leaf dry matter content (g/g); and the mesophyll structure parameter, as a proxy for mesophyll complexity.

For more details see chapter 2.5.5.

Genetic data

The DNA was obtained from the same leaf samples used to analyze functional traits. Genomic DNA was extracted from 64 leaves (stored on silica gel) using Norgen's Plant/Fungi DNA Isolation Kit (Norgen Biotek Corporation, Canada), according to the provided protocol. The quality and the quantity of extracted DNA were evaluated using DS-11 spectrophotometer (De Novix, Wilmington, DE, USA) and Qubit 4.0 (Thermofisher, Waltham, Massachusetts, USA), respectively. The library preparation and the whole genome sequencing were carried out by the Novogene Co (Novogene, Oxford, UK) using the high-throughput Illumina sequencing platform (NovaSeq 6000).

The variant calling will be performed using the Ovarflow workflow in the Docker container (Bathke and Lühken, 2021). Particularly, this workflow uses FastQC for quality control (Andrewes, 2010), BWA for the alignment of the reads to the Reference Genome (Li and Durbin, 2009), GATK for the variant calling (McKenna et al., 2010) and SnpEff for the annotation of the variant calls (Cingolani et al., 2012). The snakemake file of the workflow will be modified in accordance with the raw data output produced by the Novogene company (Novogene, Oxford, UK). As for the reference genome, we will use the *P. australis* whole genome reported by Oh et al. (2021), downloading at https://genomevolution.org/coge/GenomeInfo.pl?gid=59768..

The GWAS analysis will be carried out using the "vcf2gwas" pipeline in the Docker container (Vogt et. al, 2022). This pipeline is based on the usage of bcftools (Danecek et al., 2021), PLINK (Purcell et al., 2007) and GEMMA (Zhou ad Stephens, 2012).

Preliminary results

Summary of Sequencing Data Information, including detail statistics for the quality of sequencing data, are shown in Table 1 (supplementary material).

Supplementary material:

https://drive.google.com/drive/folders/1Ny3z3ddLGtkONi-Ud-t6ab4dq7irWI8C?usp=sharing

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6. DISCUSSION AND CONCLUSIONS

This thesis work investigated the genetic diversity of freshwater macrophytes at both local and regional scales, from the population to community level. Specifically, it aimed to relate the genetic and phylogenetic diversity results with the spectro-functional diversity data to decipher the processes shaping the evolution of macrophyte communities with particular reference to two focal species dominating the sites under investigation.

As for the analysis at inter-specific level, the PhD products were organized in two main arguments (up until now, Article I and II), which contribute to depicting the phylogenetic diversity of macrophytes communities in selected wetlands of northern-central Italy. Article I assessed the phylogenetic diversity of the sampled macrophyte communities in the macroDIVERSITY project areas. This first work provided information regarding the phylogenetic divergence of the species within the communities. In particular, the combined analysis of one marker of the nuclear DNA (ITS region) and two of the cpDNA (rbcL and matK) allowed the evolutive history reconstruction of the sampled macrophyte communities and the implementation of PD's metrics NTI and NRI. The phylogenetic structure showed a general, not significant tendency to a clustered pattern, whit the exception of some communities that instead showed a significant phylogenetic overdispersion both at the profound and highest level of the phylogeny. The latter communities mainly comprised submerged hydrophytes such as Characeae and *Potamogeton* highlighting the high value assumed by the submerged macrophyte component to the diversity metrics, and, therefore, the hypothesized high impact also on the functional diversity of the site. The correlation between functional and evolutionary diversity is thus fundamental to describing the ecology and dynamics of the indagated macrophyte communities and therefore consists in the last step of macroDIVERSITY activity, which will be finalized with a recapitulative article of the PRIN project.

Article II analyzed the incidence of alien species on the taxonomic, functional, and phylogenetic diversity of communities of macrophytes dominated by *P. australis* in two different types of habitats, lentic and lotic. Although it was hypothesized that ecological differences between habitats (lentic vs. lotic) lead to plant adaptive trade-offs (Cristofoletti, 1981; Wetzel, 2001; Starzomski and Srivastava, 2007), results showed that *P. australis* dominance affected overall plant diversity in the same way in both target habitats. This was explained by the fact that the considered stands density (only the highest Braun-Blanquet values, notably 4 and 5) and its role as a wind and wave breaker may have buffered different abiotic factors and led to similar levels of functional diversity across habitats (Uddin and Robinson; 2017a; Miler et al., 2014; Karstens et al., 2016). Moreover, as already suggested by

Veldkornet et al. (2019), *P. australis* dominance may have promoted competitive exclusion and the random phylogenetic assemblage of the communities, leading to similar level of genetic diversity in both habitats. Similarly, the two habitats hosted a similar alien species richness (A.SR) and relative abundance (A.RelAb), confirming previous finding by Boggero et al. (2014) on different invaded biological communities. Regarding the incidence of alien plant species, different results were observed based on whether the A.SR or A.RelAb. This agrees with what is found both by Brummer et al. (2016) about factors that differently shape alien cover and richness, and by Montanari et al. (2020) about the different impacts of the two variables on the colonized communities. In lentic habitats, A.SR increased taxonomic, phylogenetic, and functional diversity. While, in lotic habitats, it promoted a decrease in taxonomic and functional diversity. As for lentic habitats, the alien species saturated the phylogenetic and functional space of invaded communities, exploiting unfilled ecological niches, thus confirming the "Darwin's naturalization hypothesis" (Thuiller et al., 2010; Rejmánek, 1996). In fact, because of less intense hydrological disturbances and greater habitat stability, species from distant lineages may coexist via niche differences (Kitagawa et al., 2015; Lososová et al., 2015). The greater hydrologic disturbance of lotic habitats was the main driver infiltering alien species with ecological requirements and advantageous traits like those of native species, thus leading to an increased similarity between species in invaded communities (Campos, 2010; Kembel and Hubbell, 2006). In contrast to patterns of A.SR, the impact of A.RelAb was not different between lentic and lotic habitats. As the A.RelAb increases, the taxonomic, phylogenetic and functional diversity raised in both habitats. On the one hand, results confirmed the Darwin's naturalization hypothesis for lentic communities. On the other hand, choosing A.RelAb vs A.SR in lotic stands had a different impact on evaluating the incidence of alien species on the diversity components. It has been demonstrated that a low degree of invasion (A.RelAb less than 50%) can be associated with a significant increase in plant communities diversity (Dong et al., 2015; Wang et al., 2018, 2021; McGrannachan and McGeoch, 2019). Since *P. australis* was the most abundant species (above 64.5% of mean coverage) in all communities and none of the alien species had a relative abundance greater than 35%, the impact of A.RelAb in lotic stands has been congruent with literature. In conclusion, this work provided new insights on the taxonomic, functional, and phylogenetic diversity of plant communities dominated by *P. australis* of Northern and Central Italy, a key conservation area for wetland plant diversity at the global scale (Bolpagni et al., 2018). In particular, it revealed a significant comparability between lentic and lotic communities in terms of diversity facets. The dominance of *P. australis* affected the diversity of communities of both target habitats more than the absolute presence of alien species.

As for the analysis at the intra-specific level, the PhD project faced the study of the genetic structure of the helophyte *Phragmites australis* and the emergent hydrophyte *Nuphar lutea* and its correlation with the spectro-functional dimension. Three Articles were thus organized, one published (III), one under review (IV), and the last one in the elaboration phase (V). Article III assessed the haplotype and spectro-functional traits interactions to explore the intraspecific diversity of common reed in Central Italy. Taking advantage of satellite data, the canopy reflectance diversity of *P. australis* was investigated in relation to the haplotype diversity, the meteo-climatic parameters, and the different ecological condition of reed stands (terrestrial vs aquatic). Six haplotypes were identified, including a new one ("nM"). Differences in haplotype compositions in different ecological statuses (aquatic vs terrestrial stands) explained a significant portion of intraspecific variability in spectral proxies of canopy traits. In aquatic stands, the specro-functional diversity was higher than the average, probably because it was associated with stronger selective pressure caused by rhizome flooding, which cannot be overcome only through phenotypic plasticity (Engloner et al., 2010; Gigante et al., 2011, 2014; Lastrucci et al., 2016a, 2017). The haplotypes CHTR and M have been confirmed the most spread across sites and showed a higher variation at the spectro-functional traits. Instead, the remaining haplotypes (CO, VI, K and nM) tended to be more localised and also less variable. The variation in the values of the spectral traits between broadly distributed haplotypes versus more localised ones suggested the coexistence of different evolutionary strategies in *P. australis*. As for the more spread haplotypes M and CHTR, the maximisation of phenotypic plasticity (high functional richness, even with generally lower values for canopy traits) was hypothesised, which may promote the colonisation of new habitats. On the contrary, the more localised haplotypes maximised productivity through specialisation to peculiar site conditions, thus low functional richness but higher scores of spectral-functional traits. Moreover, different patterns of each spectro-functional trait were observed for reed stands in different ecological statuses (aquatic vs terrestrial). In particular, differences among haplotypes were generally less evident in terrestrial than in aquatic reed stands. From a temporal point of view, during the last growing seasons (2015-2020), meteo-climatic factors showed mild or significant increments compared to the average of the 1981-2010 seasons. This was related to the variation of spectro-functional traits of aquatic stands but not over terrestrial ones. As previously hypothesized by Anda et al. (2017) and Engloner et al. (2009), we confirm the role of ecological statuses in response capacity to the meteo-climatic variation of *P. australis*. This work enlarged the knowledge of the distribution in Central Italy of *Phragmites australis* haplotypes, the dynamics in reed canopy traits (related to the ecological status and haplotype) and the implications of ongoing climate change. This involves the opportunity to advance further investigations to better understand the processes underpinning common reed decline in the Mediterranean region.

Article IV investigated, for the first time, the Pst-Fst comparison (Brommer, 2011; Leinonen et al., 2013) to infer the relative role of genetic drift and natural selection on the diversification of phenotypic traits of *N. lutea* and *P. australis* in five sites of Northern and Central Italy. We first quantified and described the genetic structure and phenotypic diversity for both species. As for *P. australis*, the large portion of genetic variation observed resided within rather than among sampling plots and sites, thus confirming several other studies conducted for the species at different spatial scales (Coppi at al., 2018 Lambertini et al., 2008; Qiu et al., 2016). The high genetic diversity of *P. australis* was demonstrated at local scale and it seemed related to its ecological and physiological characteristics (Gao et al., 2010), and to the ability to combine vegetative propagation and sexual reproduction (Albert et al., 2015). The high genetic diversity was mirrored by the functional traits that resulted quite variable among sites with rather wide standard deviations. As for *N. lutea*, genetic variation was nearly equal within and among both sampling plots and sites, revealing a more complex genetic structure than expected at the site level. Comparing these results to those associated with the breeding systems for other plants (Nybom, 2004) a possible explanation could be that the genetic structure of studied *N. lutea* populations may be influenced by the prevalence of a mixed mating system. In fact, despite it is considered as a clonal species, the in- and out-breeding are well demonstrated (Ervik et al. 1995; Lippok and Renner, 1997). According to Dalla Vecchia & Bolpagni (2022), *N. lutea* exhibited wide variations of traits values across sites. In general, the mixed reproductive strategy could play a role in facing environmental variation for both *P. australis* and *N. lutea*. Secondly, we assessed the Pst-Fst comparison, assuming that Fst (determined by neutral markers) reflects divergence caused only by genetic drift (Reynolds et al., 1983) while Pst also incorporate the effects of selective dynamics on the phenotype. With some rare exceptions, our results showed an overall tendency to directional selection for *P. australis* and to balanced or stabilizing selection for *N. lutea* populations. As for *P. australis*, the results confirmed its colonization strategy according to which vegetative reproduction rules over generative one (Lebedeva et al., 2020; Alvarez et al., 2005). An exception is Chiusi's population, where trait variability was shaped by both DS and SS, suggesting that patches of reed stand underwent genetic drift at the micro-local scale. As for *N. lutea*, results were more heterogeneous. It was probably due both to the more complex genetic structure of *N. lutea* (due to a mixed mating system) and to the degree to which the plastic phenotype closely matches with the optimal phenotype for the environment (Price et al. 2003). In summary, this work increased the knowledge of the genetic structure of *N. lutea* and *P. australis* populations in the lakes of central and northern Italy as well as the relative role of genetic drift and natural selection on the diversification of their phenotypic traits within habitats.

Based on the results obtained in Articles III and IV and those already published by Coppi et al. (2018), Article V has laid the foundation for going deeper into the high level of *P. australis'* intraspecific diversity at Lake Chiusi, employing GWAS for identifying trait-associated variants.

In conclusion, the five articles provided in this thesis allowed for a better understanding of macrophytes diversity in northern and central Italy. The main outcomes were accomplished through a multidisciplinary approach, considering taxonomic, functional, phylogenetic and spectral facets of diversity. This allowed i) to better describe the biodiversity occurring in the investigated communities, ii) to integrate spectral and phylogenetic metrics for mapping macrophyte functional diversity, iii) and to decipher the adaptive processes shaping the evolution of two focal species.

This thesis work represents a starting point for future research aimed at analyzing different facets of macrophytes diversity.

In conjunction with the closure of the macroDIVERSITY project, the next step will be to correlate the preliminary results of Article I and Article V with the missing spectro-functional diversity data.

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ABBREVIATIONS

A.RelAb	Relative abundance of alien species
A.SR	Alien species richness
AFLP	Amplified fragment length polymorphism
AGD	Average genetic diversity
AMOVA	Molecular variance analysis
Chl_ab	Total chlorophylls
Chl-a	Chlorophyll-a
Chl-a/Chl-b	Ratio between chlorophyll-a and -b
DNH	Darwin's naturalization hypothesis
DS	Directional selection
DS-SS	Neutral divergence cannot be excluded as a possible cause of phenotypic diversity
EF	Ecosystems functioning
FD	Functional diversity
FRic	Functional richness
Fst	Differentiation of neutral alleles
FTs	Functional traits
GLI	Green leaf index
GWAS	Genome-wide association studies
H	Shannon's index
ITV	Intraspecific trait variability
LA	Leaf area
LA	Leaf total area
lcWGS	Low-coverage whole genome sequencing
LDMC	Leaf dry matter content
LM	Leaf mass
LMA	Leaf mass per area
LMMs	Linear mixed models
ms	Monospecific stands
$\mathbf{NDSI_{LMA}}$	Normalized Difference Spectral Index for LMA
Nmesophyll	Proxy for mesophyll complexity
NRI	Net relatedness index
NTI	Nearest taxon index
PD	Phylogenetic diversity
$\mathbf{PD_F}$	Faith's phylogenetic diversity
phy.MPD	Mean pairwise distance
Pool-seq	Pooled sequencing
PPL	Percentage of polymorphic loci
Pst	Phenotypic divergence
RAD-seq	Restriction site-associated DNA sequencing
RaoQ	Rao's quadratic entropy
SD	Spectral diversity
SIs	Spectral indices
SLA	Specific leaf area

SR	Species richness
SS	Stabilizing selection
SSR	Simple sequence repeats
TD	Taxonomic diversity
WAVI	Water adjusted vegetation index
WGS	Whole genome sequencing
ZIMMs	Zero-inflated mixed models

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“Ovunque sarai, ovunque sarò,
in ogni gesto io ti cercherò.
Se non ci sarai, io lo capirò
e nel silenzio io ti ascolterò”

OVERALL ACTIVITIES

Publications presented in this thesis

Maria Beatrice Castellani, Lorenzo Lastrucci, Rossano Bolpagni, Alice Dalla Vecchia, Erika Piaser, Paolo Villa and Andrea Coppi [in preparation]. Merging spectral and phylogenetic diversity to assess macrophyte traits and functions along multiple gradients.

Maria Beatrice Castellani, Lorenzo Lastrucci, Lorenzo Lazzaro, Rossano Bolpagni, Alice Dalla Vecchia and Andrea Coppi (2022). The incidence of alien species on the taxonomic, phylogenetic, and functional diversity of lentic and lotic communities dominated by *Phragmites australis* (Cav.) Steud. *Knowledge & Management of Aquatic Ecosystems.*

Maria Beatrice Castellani, Andrea Coppi, Rossano Bolpagni, Daniela Gigante, Lorenzo Lastrucci, Lara Reale, Paolo Villa [minor revision]. Assessing the haplotype and spectro-functional traits interactions to explore the intraspecific diversity of common reed in Central Italy. *Hydrobiologia.*

Maria Beatrice Castellani, Rossano Bolpagni, Paolo Villa, Alice Dalla Vecchia, Lorenzo Lastrucci, Erika Piaser and Andrea Coppi [in preparation]. How genetic drift and natural selection affect the evolution of *P. australis* and *N. lutea* populations from northern-central Italy.

Maria Beatrice Castellani, Lorenzo Bini, Rossano Bolpagni, Paolo Villa' Lorenzo Lastrucci, Alice dalla Vecchia, Cheng Li, Aboubakr Moradi, Andrea Coppi [in preparation]. Genome-Wide Association analysis of leaf spectro-functional traits in *Phragmites australis*.

Other publications

Colzi, I., Gonnelli, C., Vergata, C., Golia, G., Coppi, A., **Castellani, M. B**., ... & Martinelli, F. (2022). Transgenerational effects of chromium stress at the phenotypic and molecular level in Arabidopsis thaliana. *Journal of Hazardous Materials*, 130092.

Coppi, A., Colzi, I., Lastrucci, L., **Castellani, M. B**., & Gonnelli, C. (2022). Improving plant-based genotoxicity bioassay through AFLP technique for trace metal-contaminated water: insights from Myriophyllum aquaticum (Vell.) Verdc. and Cd. *Environmental Science and Pollution Research*, 1-9.

Di Nuzzo, L., Masoni, A., Frizzi, F., Bianchi, E., **Castellani, M. B**., Balzani, P., ... & Benesperi, R. (2022). Red wood ants shape epiphytic lichen assemblages in montane silver fir forests. *iForest-Biogeosciences and Forestry*, *15*(1), 71.

Colzi, I., Renna, L., Bianchi, E., **Castellani, M. B.,** Coppi, A., Pignattelli, S., ... & Gonnelli, C. (2022). Impact of microplastics on growth, photosynthesis and essential elements in Cucurbita pepo L. *Journal of hazardous materials*, *423*, 127238.

Castellani, M. B., Bianchi, E., Coppi, A., Nascimbene, J., & Benesperi, R. (2021). Revision of the Parmelia saxatilis group in Italy based on morphological, chemical, and molecular data. *Phytotaxa*, *512*(1), 28-40.

Congresses and Seminars

Poster at International Conference: Analysis of genetic diversity and phenotypic plasticity in *Phragmites australis* and *Nuphar lutea*. A case study from Northern and Central Italy. (07/09/2022 – 10/09/2022) 117° Congress of Italian Botanical Society.

Oral presentation at seminar: Lo studio della diversità di comunità vegetali di ambienti umidi. (07/12/2021) "Sarò matricola". Facoltà di Scienze Matematiche, Fisiche e Naturali, Università di Firenze.

Oral presentation at seminar: La riflettanza: uno strumento per valutare la biodiversità vegetale. (08/05/2021) BioSaturdays 2021, Università di Firenze.

Oral presentation at International Conference: The incidence of alien species on taxonomic, functional and phylogenetic diversity of macrophyte communities dominated by *Phragmites australis* (cav.) Steud. in two different types of environments, lotic and lentic. (01/03/2021 – 05/03/2021) 10° Shallow Lakes Conference.

Oral presentation at International Conference: An evolutionary approach for the study of spatial diversity of freshwater macrophytes in Central and Northern Italy. (06/02/2020 – 07/02/2020) CYBO, Conference of Young Botanists. Genova, Italia.