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Research article

Multidimensional trait variability in a widespread, Paleoarctic macrophyte: functional, spectral and genetic drivers

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Understanding how environmental conditions and plant functional variation are mutually related is critical to improving our comprehension of plant adaptations. In this context, our knowledge of the interlinks between plant functional, spectral and genetic traits and environmental filters is still very limited, especially for wetland species. To gain new insights on this topic, a multidimensional dataset, centred on the widespread macrophyte species *Nuphar lutea*, was assembled by collecting data on functional traits (including spectral traits), genetic metrics and environmental determinants from 28 plots spanning north-central Italy. A strong environmental filter acts on all traits (morphological, biochemical, spectral and the genetic diversity metrics) resulting in significant local control over trait patterns, exemplified by the discrimination value of water electrical conductivity. This is further reinforced by the key contribution of sediment variables in explaining traits variation. Site-specific environmental conditions were reflected in different patterns of genetic diversity, suggesting a long-term effect of environmental filters on genotypes as well. High water conductivity – in our study sites indicative of long-term hydrogeological settings – is linked to more acquisitive behaviour in *N. lutea* and a progressive reduction in its genetic diversity, while high nutrients availability in sediments promotes higher leaf traits performance. This study better explores how high variability in leaf traits reinforces current genetic and mechanistic knowledge about competitive strategies in the key aquatic plant *N. lutea*, by testing the effectiveness of a novel integrative approach to assess multiple sources of plant functional variation.

Keywords: aquatic plants, environmental gradients, functional traits, genetic diversity, leaf reflectance, nymphaeids

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Introduction

Functional traits are widely used in plant studies and have shown great potential to describe plant adaptations (Díaz et al. 2016, Dalla Vecchia et al. 2020). As traits reflect plant functions and responses to environmental conditions, they are useful to answer a number of ecological questions and for understanding the drivers of plant performance (Violle et al. 2007). Among other things, leaf traits can describe tradeoffs in resource-use strategies and reveal variations in strategies adopted even within species (Wright et al. 2004). Intraspecific trait variability is key to ensure successful plant adaptation to environmental gradients and it becomes increasingly relevant at finer scales of observation and in species-poor communities (Violle et al. 2012, Siefert et al. 2015).

In addition to typically measured leaf traits, reflectance spectroscopy has been increasingly employed over the last few years as a high-throughput tool to quantify foliar spectral properties, which can be related to structural, biochemical (e.g. pigments) and physiological characteristics of plants (Klančnik et al. 2018, Jacquemoud and Ustin 2019, Villa et al. 2021), providing integrative descriptions of plant phenotypes (Kothari and Schweiger 2022).

Because phenotypic expression of traits is determined by the interplay between plasticity and genetics (Violle et al. 2012), the degree of genetic differentiation within and among populations may also explain some of the variation in observed traits and may provide insights into future adaptive potential as well as past selection (Agrawal et al. 2008, Parsons et al. 2011). In this regard, the degree of genetic differentiation in a population could also be, in part, a response to environmental pressures that select certain characteristics, offering a view on past driving forces (Pavoine et al. 2011).

Numerous studies have linked genetic metrics to variation in functional traits to describe trait plasticity or to understand what the evolutionary trajectories of plant adaptation are (García-Girón et al. 2020, Roubeau Dumont et al. 2020, Castellani et al. 2022, 2023a). Also, there are many examples that combine functional traits and spectral features of plants to assess the effect of environmental conditions and overcome limitations dealing with direct trait measurements across scales (Lausch et al. 2016, Kamoske et al. 2021, Wang et al. 2022). However, to our knowledge, there are no studies that simultaneously ground functional, spectral and genetic variability to understand the effect of environmental gradients on plant performance at species level. In fact, as spectral traits may integrate wide aspects of plant phenotypic variability (Kothari and Schweiger 2022), they can be valuable in advancing functional plant ecology and biodiversity studies across spatial scales (Abelleira Martínez et al. 2016, Cavender-Bares et al. 2022, Castellani et al. 2023a, b) – especially when joined with commonly measured functional traits. Besides, determining whether and to what extent environmental gradients influence genetic differentiation in populations is a key aspect of understanding patterns of trait variability (Lehmair et al. 2022).

Freshwater plants (referred to as macrophytes) are ideal candidates for investigating these aspects, as the aquatic environment naturally offers strong gradients of conditions (e.g. temperature, light, nutrient availability) at relatively narrow spatial scales (Chou et al. 2022). Among macrophytes, *Nuphar lutea* is a rooted floating-leaved macrophyte (hereafter referred as nymphaeids) that is widespread in lowland lentic water bodies in temperate to subtropical regions. Within its habitat, *N. lutea* can be considered a dominant species: it usually forms large monospecific stands, represents a top competitor plant, and naturally inhabits a wide range of conditions showing high plasticity (Pierce et al. 2012). This provides a good premise to offer innovative insights on within-species plant trait variability and its linkage to environmental conditions. With this study we investigated this topic by integrating different approaches, merging multidimensional measures of functional (including spectral features) and genetic variability of *N. lutea* to evaluate intraspecific diversity drivers along an environmental gradient.

Water level, light and nutrients availability (and consequently derived eutrophication processes) have been associated with plants performance, and nymphaeids in particular (Khanday et al. 2017, Henriot et al. 2019, Klok and van der Velde 2022). Water depth has an influence on plant economics (Richards et al. 2011, Dalla Vecchia and Bolpagni 2022), as well as determining a change in reproduction strategies in *Nymphoides peltata* (Khanday et al. 2017). Water depth, along with turbidity, also influences light availability, which in turn is a major determinant of submerged leaf development and seedling survival in nymphaeids (Smits et al. 1990). Moreover, increased light availability increases the photosynthetic activity and the carbon stock, together with changes in the root/shoot ratio, have been observed in the rooted floating-leaved species *Nymphaea advena* (Cronin and Lodge 2003). Increased nutrients have been shown to promote growth and reproduction traits in *N. lutea* (Klok and van der Velde 2017, Henriot et al. 2019), although no effect of eutrophication on photosynthesis traits was observed (Brykov et al. 2022). However, it also caused toxicity-induced responses in *N. lutea* and *N. advena* (Cronin and Lodge 2003). So far, there is only limited evidence showing that water quality (in this case expressed in terms of pH and salinity) affects the genetic diversity in macrophytes, particularly *Ranunculus* sp. (Wu et al. 2019).

The effect of all these drivers on plant traits, however, may vary depending on the spatial or temporal scale observed (Alahuhta et al. 2018). Previously, *N. lutea* leaf traits have been shown to vary with water depth and conductivity on a local scale (Dalla Vecchia and Bolpagni 2022). Here we aim at testing the effect of environmental filtering on leaf traits and genetic diversity of 28 plots of *N. lutea* in four lake systems in Italy. Among the trait variability drivers mentioned above, we hypothesize that nutrient availability represents the main driver of trait and genetic variability in *N. lutea* at the regional scale. We expect the effect of the depth gradient covered by the study sites at this scale to be less than the effect of trophic differences between sites, given the eutrophication

pressure exerted on some of the lakes studied. Moreover, we expect the pattern of trait diversity to be reflected in the genetic diversity of the populations analysed.

Material and methods

Study sites

This study was carried out in four lake systems located in central and northern Italy, grouped by proximity that reflected similar watershed conditions: lakes Pusiano and Annone, Lake Iseo and Torbiere del Sebino, Mantua lakes system and Lake Chiusi, sorted by location from north to south (Fig. 1). Main morphological and trophic features of studied lakes are presented in Table 1. Both Pusiano and Annone are sub-alpine lakes located south of Lake Como, between its two branches, and Lake Annone is further divided into an eastern and a western basin connected by an isthmus. Lake Iseo is a deep sub-alpine lake, regulated by a dam built in the first reach of its outflow, the Oglio River. Immediately south of Lake Iseo shores lies the Torbiere del Sebino wetland, a protected area hosting well developed macrophytes stands. Mantua lakes system is composed of three smaller lakes (Superior, Middle and Inferior) and two connected wetlands: the Valli del Mincio and Vallazza, respectively located upstream and downstream of the lakes. The lakes system is located along the city of Mantua and fed by the Mincio river, emissary of Lake Garda, and has been regulated since 1190 (Pinardi et al. 2015). The system hosts different submerged, floating, and emergent macrophyte communities (Pinardi et al. 2021). Lake Chiusi is the southernmost sampling site, located in Tuscany, central Italy, and it is part of a Natura 2000 protected area.

Sampling design

In the four investigated lake systems (hereafter: sites) we sampled 28 plots each of 4 m^2 area where *Nuphar lutea* was present: 12 in Chiusi, 7 in Mantua, 5 in Iseo–Torbiere and 4 in Pusiano–Annone (Fig. 1). Plots were chosen among *N. lutea* stands in the sites, starting from floating macrophyte maps derived from very-high-resolution satellite images (2 m pixel) and drawing plots randomly within 3 m of the waterfront of stands that were larger than 100 m^2 and logistically reachable with a light boat. Samples were further stratified by three levels of canopy density (modelled via spectral proxies), to ensure representativeness of within-site variability. In each plot, species presence and percentage cover were obtained, and water, sediment and plant samples were collected for environmental characterization, traits and genetic analyses (Table 2). Samplings were carried out in July 2020 in lake Chiusi and in July 2021 for all other sites, during the peak season of *N. lutea* development.

Environmental characterization

In each plot one measure of water depth (hereafter depth, m), water specific electrical conductivity (SPC, μS cm^{-1}), water nitrate concentration (NO$_3$, mg l^{-1}), sediment organic matter content (sed.OM, %) and sediment total phosphorus content (sed.TP, μg g^{-1} dry weight) was collected. SPC was determined in situ using a multiparameter probe (YSI 556 MPS), while site water was filtered with glass fibre filters (Whatman) of 0.7 μm pore size and kept refrigerated. Within two days from collection, samples were filtered a second time with 0.2 μm pore size nylon filters, and NO$_3$ concentration was determined by means of ion chromatography (883 Basic IC plus Metrohm). Ammonium ion and soluble reactive phosphorus
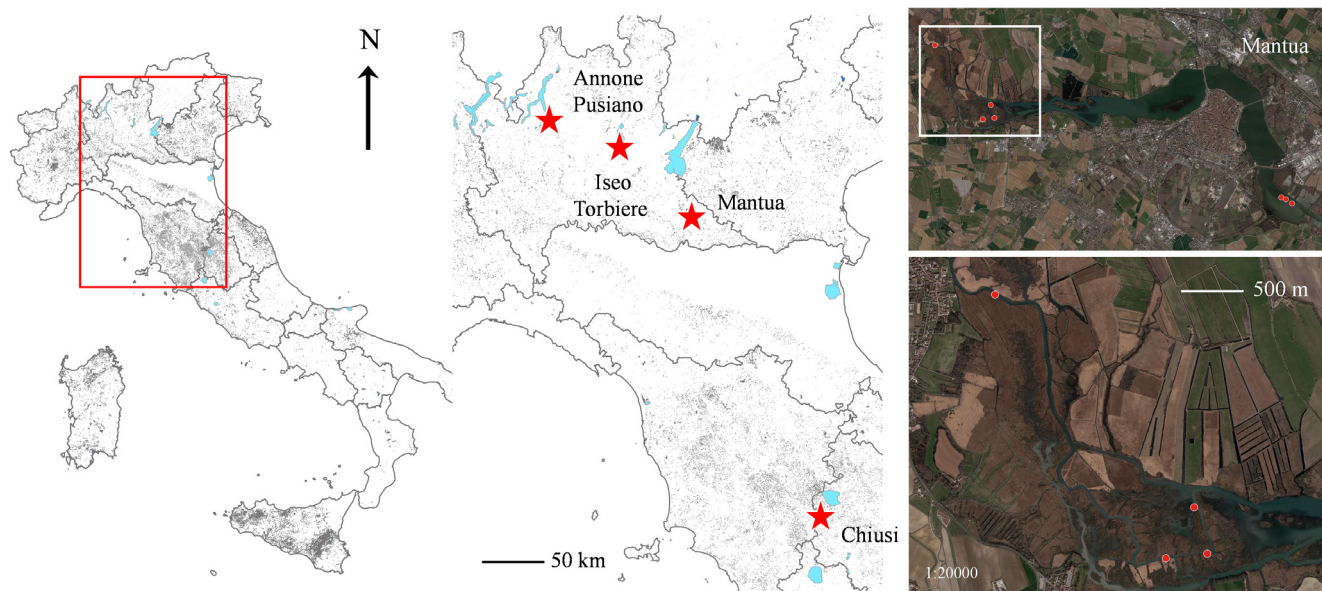

Figure 1. Map of the study sites located in north-central Italy, and a focus on the Mantua lakes sampling area, showing an example of the spatial arrangement of sampling plots.

Table 1. Main morphological and trophic characteristics of the investigated lakes.

Lake	Coordinates	Surface area (km^2)	Max depth (m)	Mean depth (m)	Trophic state	Reference
Pusiano	45°48′12″N, 09°16′17″E	5.26	24	14	phosphorus-limited	Legnani et al. 2005
Annone	45°49′05″N, 09°19′57″E	5.71	11	5	eutrophic	Rusconi et al. 2022
Iseo	45°40′13″N, 10°01′32″E	60.8	256	123	meso-eutrophic	Pilotti et al. 2018, Scibona et al. 2022
Torbiere del Sebino	45°38′51″N, 10°01′45″E	3.6	<3	2	eutrophic	Cappelli 2014
Mantova	45°09′42″N, 10°46′08″E	6.22	17.5	3.5	eEutrophic	Pinardi et al. 2021
Chiusi	43°03′25″N, 11°57′55″E	6.44	5.7	2.7	hyper-eutrophic	Lastrucci et al. 2014, Cavalieri et al. 2018

were also measured with ion chromatography and following Valderrama (1977), respectively, but they were not considered in this study because the concentrations were often below detection limits (0.072 mg l^{-1} for ammonium ion and 0.004 mg l^{-1} for soluble reactive phosphorus). A sample of 50 ml of sediment was collected from the upper 5 cm sediment layer in each plot, stored in falcon tubes and frozen as soon as possible after collection. In the laboratory, sediment samples were defrosted, homogenized and a subsample was dried at 60°C for 24 h. After grounding the dried sediment to fine powder, an aliquot of ca 0.3 g was weighed and incinerated at 450°C for 4 h. Sed.OM was determined gravimetrically from the weight loss of the ashes compared to the dry sample (Buchanan 1984). Sed.TP includes both organic and inorganic phosphorus pool in the sediments, and was analysed spectrophotometrically from the ashes, after extraction in HCl (Aspila et al. 1976).

Leaf traits

At each plot, eight branches were haphazardly selected from different plants, located at similar depth. One leaf from each branch was used for analysis, and to standardize for life stage, only young, fully-developed floating leaves without mechanical damages or herbivory signs were selected and gently washed from dirt and epiphytic algae. Reflectance spectra of fresh leaves were collected within seconds of leaves detachment with a portable spectroradiometer (SR-3500, Spectral Evolution; range covered: 350–2500 nm). Radiance reflected from the adaxial side of each leaf laid on dark background (black neoprene with absolute reflectance factor < 5%) was measured and converted to reflectance via readings taken over white standard panel (Spectralon, with absolute reflectance factor > 95%).

After spectra readings, leaves (including whole petioles) were stored in sealed plastic bags and kept cool until processing, which occurred within few hours. A small blade portion was removed from each of the eight leaves, weighed, dehydrated in sealed plastic bags with silica gel and stored for genetic analyses. Then, five leaves per plot were used to determine the following structural traits: leaf area (LA, mm^2), specific leaf area (SLA, mm^2 mg^{-1}), leaf dry matter content (LDMC, mg g^{-1}) and proportion of leaf dry weight allocated to petioles (pet.propDW, %). Fully hydrated leaves, including

Table 2. List of functional traits (including spectral traits and genetic metrics) and environmental variables analyzed in this study, with related abbreviations, units of measurements and functional or genetic meaning (for traits and genetic metrics).

Trait	Abbreviation	Unit of measurement	Meaning
Leaf area	LA	mm^2	leaf size
Specific leaf area	SLA	mm^2 mg^{-1}	acquisitive resource-use strategy
Leaf dry matter content	LDMC	mg g^{-1}	conservative resource-use strategy
Petiole dry weight proportion	pet.propDW	%	leaf investment in petioles
Leaf chlorophylls content	chlab	µg g^{-1}	photosynthetic capacity
Genetic diversity	AGD	%	average genetic diversity within plot
Percentage of outlier loci	OUTLIER	%	percentage of loci falling out of a certain threshold within plot
Average leaf reflectance between 430–450 nm	r440	ratio	leaf surface roughness and composition
Average leaf reflectance between 510–520 nm	r515	ratio	leaf carotenoids and anthocyanins content
Average leaf reflectance between 610–640 nm	r625	ratio	leaf chlorophylls content
Average leaf reflectance between 780–820 nm	r800	ratio	mesophyll structure complexity
Environmental variable	**Abbreviation**	**Unit of measurement**	
Water depth	Depth	m	
Water electrical conductivity	SPC	µS cm^{-1}	
Water nitrate content	NO$_3$	mg l^{-1}	
Sediment organic matter content	sed.OM	%	
Sediment total phosphorus content	sed.TP	µg g^{-1}	

petioles, were weighed and scanned with a portable scanner at 300 dpi (before collecting the sample for genetic analyses), and successively dried at 60°C for 48 h. LA was calculated using the software imageJ (Rasband 1997–2018), SLA is the ratio between LA and leaf dry weight, LDMC is the ratio between leaf dry and fresh weight (Pérez-Harguindeguy et al. 2013). All these traits were calculated on full leaves, including blades and petioles. We then calculated pet.propDW, as the ratio between petiole and whole leaf dry weight, expressed in percentage. Leaf chlorophylls content (chlab, µg cm^{-2}), expressed as sum of chlorophyll-a and chlorophyll-b on an area basis, were analysed spectrophotometrically on the remaining three fresh leaves after extraction in 80% acetone for 24 h of grinded fresh blade tissue (Wellburn 1994). Only blades were used for this analysis, omitting petioles. These traits were chosen as broadly representative of the tradeoffs underlying the leaf economic spectrum (Wright et al. 2004).

We derived four specific reflectance features connected to pigment content and leaf structure to be analysed. These foliar traits were: 1) r440, i.e. the mean reflectance within 430 and 450 nm, linked to leaf surface roughness and composition, e.g. presence of trichomes (Sims and Gamon 2002); 2) r515, i.e. the mean reflectance within 510 and 520 nm, linked to absorbance band for carotenoids and anthocyanins (Féret et al. 2017, Peters and Noble 2020); 3) r625, i.e. the mean reflectance within 610 and 640 nm, linked to chlorophyll-a and -b content and their balance (Villa et al. 2021); and iv) r800, i.e. the mean reflectance within 780 and 820 nm, linked to mesophyll structure complexity (Féret et al. 2017).

Genetic analyses

The DNA extraction and AFLP protocol were carried out on 206 dried leaf samples (from five to eight individuals per plot; Supporting Information 1). When it was not possible to use the very same leaf selected for functional traits, leaves from the same rosettes were collected as matches. DNA extraction was performed using the 2x cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1990), and the quality and quantity control of the extraction product was assessed by a spectrometric survey using a Bio-Photometer (Eppendorf). AFLP analysis followed the standard procedure (Vos et al. 1995), modified at the final amplification step (Coppi et al. 2014). Two combinations of primers were selected for the final PCR amplification: hex_EcoRI-ACG/MseI-TTA and fam_EcoRI-CTA/MseI-CTC. AFLP profiles obtained by capillary electrophoresis were analyzed using GeneMarker ver. 1.5 (SoftGenetics LLC, State College). The analyses of genetic variation at plot and site level were performed as average genetic diversity over loci (hereafter AGD) using Arlequin ver. 2.000 software (Schneider et al. 2000). AGD was computed as the probability that two randomly chosen homologous sites are different (Nei 1987). Moreover, the partition of genetic variation was evaluated by an analysis of variance framework using analysis of molecular variance (AMOVA). The AMOVA was performed using Arlequin

software ver. 3.5.2.2 (Excoffier et al. 2009) at three different hierarchical levels, 1) within plots, 2) among plots and 3) among all hypothetical groups of sites (i.e. all possible combinations of sites grouped together). Statistical support for the different hypothetical groups of sites, based on geographical distribution, was tested in terms of the variance components and the percentage of explained variation. The population structure was then explored using STRUCTURE ver. 2.3.3 (Pritchard et al. 2000). To determine the optimal K value (most likely number of genetic groups), assumed to be in the range between 1 and 5, the Evanno method (ΔK; Evanno et al. 2005) was used as implemented in STRUCTURE Harvester ver. 0.6.94 (Earl and vonHoldt 2012). The analysis was carried out adopting the admixture model and 100 000 burn-ins followed by 200 000 MCMC (Markov chain Monte Carlo) runs (structure manual). The percentage of outlier loci (OUTLIER) was detected following Yang et al. (2016) using BayeScan ver. 2.01. Following Foll (2012), outliers were determined as loci that fall over a threshold value set on the logarithm of posterior odds values (LogPO). The number of pilot runs was kept at 20, with a length of 10 000 iterations each one (Coppi et al. 2018).

Statistical analyses

To investigate the interlinks between ecological drivers and variation in functional traits (including reflectance features) and genetic metrics, GAM models were used, because traits responses to environmental variables were often not linear. GAM models were built in R environment (www.r-project.org). All traits were tested in separate models (11 models in total) including all five environmental variables (depth, SPC, NO$_3$, sed.OM and sedTP), using the function {gam} of the package 'mgcv' (Wood 2011). For traits with multiple measures per plot (i.e. all except genetic diversity metrics) the plot number was added to the model as a random factor. The function {gam.check} was used to check the model assumptions, and response variables where log-transformed when necessary. Model selection was performed with aid of the function {dredge} of the package 'MuMIn' (Barton 2020), and the model with the lowest AIC criterion was selected. In case the trend of the model was linear (few cases), the additive model was used nonetheless to keep consistency with all other traits' models. GAM models were graphically visualized with the implementation of the packages 'ggplot2' (Wickham 2016), 'gratia' (Simpson 2022) and 'gridExtra' (Auguie 2017), aiming at emphasising the contribution of each smoothing term to the shape of the relationship between traits and environmental variables (Zuur et al. 2009). To investigate if the variability of environmental conditions is correlated to a site effect, and thus if sites themselves are related to trait variation, the differences in traits and environmental variables were tested among sites. Towards this, a preliminary PCA (principal component analysis) was performed with environmental data, to visualize if sites were distinguished in the environmental space. The package 'ggbiplot' (Vu 2011) was used to visualize the first two axes of the PCA and the distribution of sites. Then,

one-way ANOVA tests were performed using site as a categorical explanatory variable and traits and environmental variables as continuous response variable, after visually checking if model assumptions were acceptable. The ANOVA was then followed by Tukey post hoc comparisons to identify differences among pairs of sites.

Results

Environmental gradients

The plots investigated showed a wide variability in terms of environmental conditions: depth ranged between 0.1 m in Mantua and Iseo–Torbiere and 1.9 m in Chiusi and Iseo–Torbiere; SPC ranged between 216 μS cm^{-1} in Pusiano–Annone and 608 μS cm^{-1} in Chiusi; NO_3 had values < 0.01 mg l^{-1} in 13 plots from Chiusi, Iseo–Torbiere and Pusiano–Annone, and peaked in Mantua with 9.49 mg l^{-1}; sed.OM ranged between 1.2% in Iseo–Torbiere to 38.1% in Chiusi, and sed.TP ranged between 277.5 μg g^{-1} in Chiusi and 1788.5 μg g^{-1} in Mantua. The first two axes of the PCA on environmental variables together explain 62.1% of the variation and showed that Chiusi is environmentally distinct from the other sites, mainly due to higher SPC and lower NO_3 (Supporting information). The other three sites cluster together; however, Mantua system occupies a bigger portion of the environmental space, having plots with higher water and sediment nutrients content (Supporting information). The ANOVA tests showed no difference among sites for depth and sed.OM, whereas significant differences were detected for SPC, NO_3 and sed.TP (Supporting information for complete sites comparisons). SPC was significantly higher in Chiusi, intermediate in Mantua and lower in Iseo-Torbiere and Pusiano-Annone. NO_3 was significantly higher in Mantua, where it also showed the highest variance, than in Chiusi and Pusiano–Annone, while Iseo–Torbiere was not statistically different from any other site, although this is mainly due to one single plot (IS24) with particularly high values of NO_3 (5.63 mg l^{-1}). Sed.TP was again statistically higher in Mantua.

Genetic diversity

The AFLP analysis was successfully performed on 203 samples and produced a total of 190 polymorphic loci. Three samples out of the initial 206 samples were discarded due to incomparable genetic profile (Supporting information). For the hex_EcoRI-ACG/MseI-TTA primer combination, 94 loci were detected, whereas the amount of 96 was shown for the fam_EcoRI-CTA/MseI-CTC pair of primers combination. The range of bp varied from 50 to 442 and 50 to 347 for the hex_EcoRI-ACG/MseI-TTA and fam_EcoRI-CTA/MseI-CTC, respectively. No clones were detected.

The mean value of AGD and the mean OUTLIER were 0.207 and 34, respectively. The site with lower levels of AGD and the frequency of OUTLIER was Chiusi (0.169 and 27

respectively), whereas the other sites showed comparable higher levels of AGD (Supporting information). A higher percentage of outliers was registered for Lake Iseo (45), indicating a possible higher impact of selective pressure on the gene pool of the five plots analysed. The AMOVA analysis (Table 3a) showed that genetic variation among plots is high ($F_{ST} = 0.465$). The higher portion of the total genetic differentiation (53.5%) was due to intra-plot differences rather than among-plots differences (46.5%). The relatively high level of differentiation among plots indicates a possible genetic separation among geographically separated sites. Among all hypothetical groupings of sites examined, the one formed by the Chiusi plots, separated from the Mantua, Iseo and Pusiano–Annone plots, accounted for the highest percentage of among groups percentage of variation (51.2%; p < 0.0001; Table 3b). In addition, as evidenced by ΔK scores (Supporting information), the structure analysis supports the detection of two genetic groups (K = 2) showing that Chiusi populations result to be distinct from all the others (Supporting information).

The variance of genetic metrics explained by GAM models is relatively low (< 48%), also due to low cardinality (n = 28). Yet, AGD and OUTLIER showed significantly negative correlations with SPC (p < 0.002) (Fig. 2a–b). A site effect was also detected for AGD, which was significantly lower in Chiusi than in Iseo–Torbiere and Mantua (p = 0.019 and p = 0.009 respectively); however, no difference among sites was highlighted for OUTLIER.

Traits drivers

The GAM models indicated that most traits (except r515) were significantly correlated with one or more of the environmental variables included in this study (Table 4, Supporting information). The most important environmental variables were SPC, significantly related to six out of nine traits, and nutrient-related sediment features, like sed.TP and sed.OM. The role of SPC is not the same for every trait and implies

Table 3. (a) Partition of genetic variance among plots. AMOVA was performed at two hierarchical levels testing the differentiation among and within plots. The table shows: degrees of freedom (df), Sum of squared deviations, Variance component estimates, percentages of total variance contributed by each component; (b) Partition of genetic variance among groups of plots performed on a hypothetical subdivision in two groups of sites. Data show the degrees of freedom (df), the Sum of squared deviations, the Variance component estimates, the Percentage of total variance contributed by the among-site level.

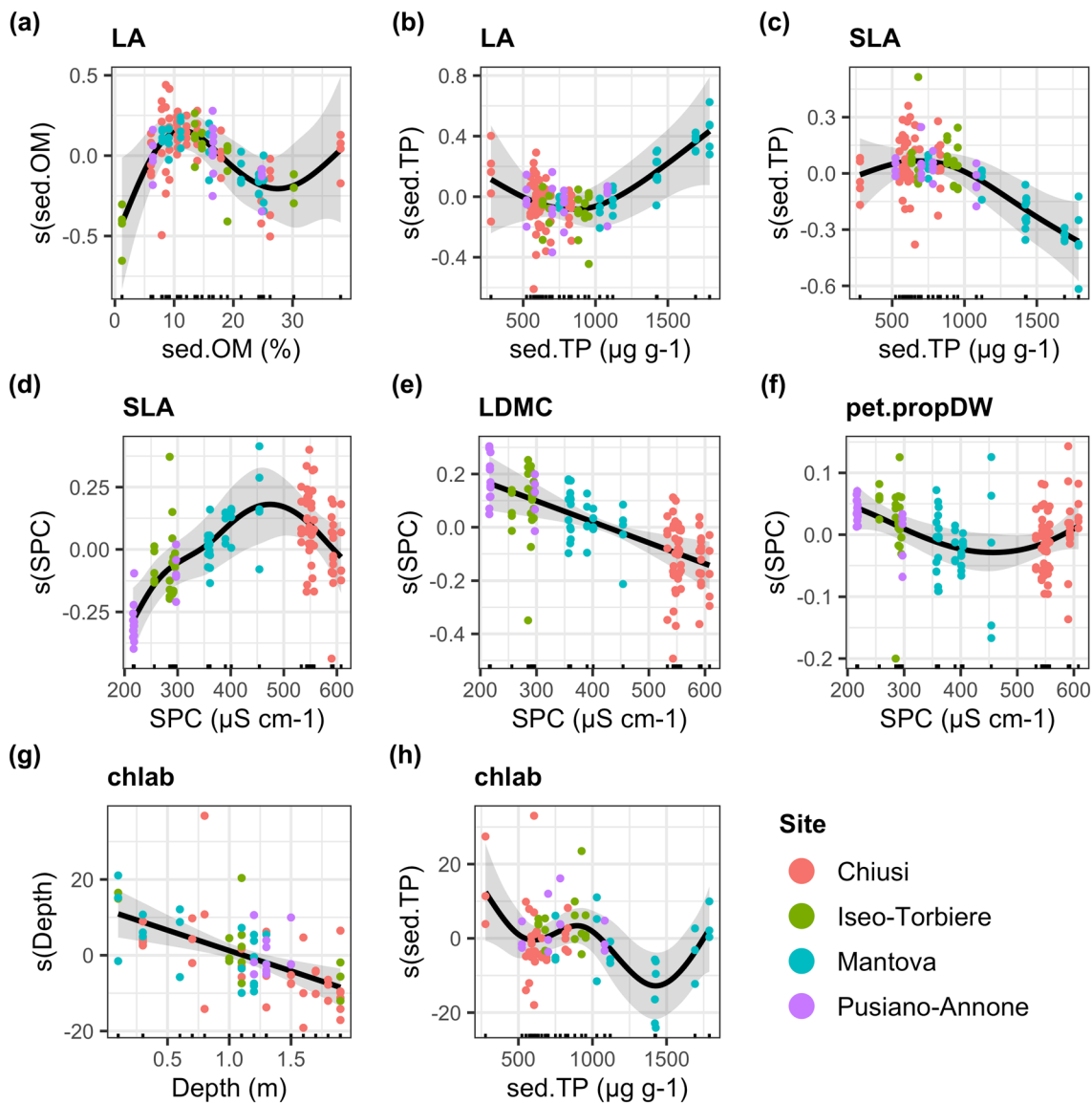| Source of variation	df	Sum of squares	Variance components	Percentage of variation
(a)				
Among plots	27	3845.676	16.97461 Va	46.55
Within plots	175	3411.496	19.49427 Vb	53.45
Total	202	7257.172	36.46888	
(b)				
Within plots	1	2554.961	24.80706 Va	51.18
Among plots	26	1290.715	4.16863 Vb	8.6
Among sites	175	3411.496	19.49427 Vc	40.22
Total	202	7257.172	48.46996	

Figure 2. Results of the GAMs showing the relationships between environmental variables and measured traits. The y-axes are centred and standardized, and indicate the contribution of each smoothing term to the variation of each trait as reported in the title; different point colours refer to residuals of different study sites. s(x) indicates the smoothing term. Gray shade shows 95% confidence interval, and only significant relationships are shown. LA = leaf area, SLA = specific leaf area, LDMC = leaf dry matter content, pet.propDW = proportion of leaf dry weight allocated to petioles, chlab = leaf chlorophylls content, Depth = water depth, SPC = specific conductivity, sed.OM = sediment organic matter content, sed.TP = sediment phosphorus content.

non-linear responses: it often determined a negative trend in traits values, especially at high SPC levels, but it is not always the case, with SLA as a notable exception to this pattern. SPC seems to reflect general site conditions, as significant differences were found among lakes. It had an opposite effect on SLA (linear positive, $p < 0.001$) and LDMC (linear negative, $p < 0.005$) (Fig. 3d–e), while it determined a unimodal response of spectral traits r440, linked to leaf surface roughness and composition ($p = 0.003$, Fig. 2c), r625, linked to leaf chlorophyll content ($p < 0.0001$, Fig. 2d), and r800, linked to mesophyll structure complexity ($p < 0.0001$, Fig. 2f). A significant relation with pet.propDW was

also observed ($p = 0.035$, Fig. 3g), however the model only explained $< 40\%$ of the variance, and the slope of the trend was not pronounced.

Conversely, sed.TP determined greater traits performance in LA ($p = 0.037$) and chlab ($p = 0.027$) at high concentrations, however it also determined negative traits response for SLA ($p < 0.005$, Fig. 3c) and r625 ($p = 0.001$, Fig. 3e).

Water depth and nutrients content (NO_3) appeared to have a marginal role in shaping *N. lutea* leaf traits variability at the regional scale (they were significantly related only to chlab, $p < 0.001$, and r625, $p < 0.005$, respectively), due to their high spatial and temporal dynamicity in wetland
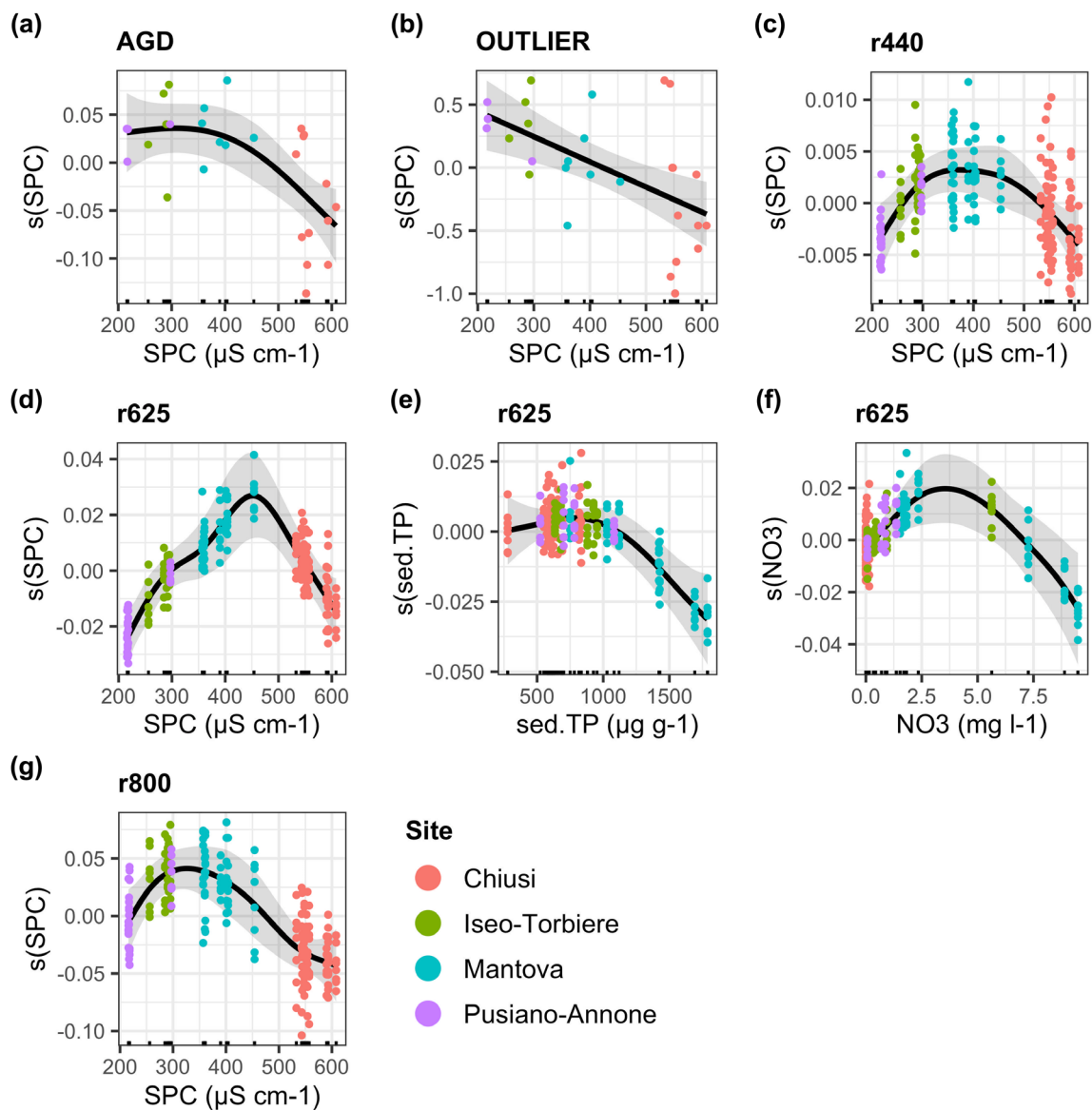
Figure 3. Results of the GAMs showing the relationships between environmental variables and genetic metrics or spectral traits. The y-axes are centred and standardized, and indicate the contribution of each smoothing term to the variation of each trait as reported in the title; different point colours refer to residuals of different study sites. s(x) indicates the smoothing term. Gray shade shows 95% confidence interval, and only significant relationships are shown. Plots titles (c–g) refer to the mean leaf reflectance at the given wavelength, AGD = average genetic diversity, OUTLIER = percentage of outlier loci, depth = water depth, SPC = specific conductivity, NO_3 = water nitrate concentration, sed.OM = sediment organic matter content.

environments. Depth had a negative effect on chlab, showing a linear trend (Fig. 3g), while the influence of NO_3 was unimodal and similar to that observed in all models for spectral traits (Fig. 2f). Detailed comparisons of traits among lakes can be found in Table 3 and in the Supporting information.

Discussion

Environmental drivers of multiple functional variation

In the present study, non-linear trends in the relationships between *N. lutea* traits and the environment appear to be due

to site-specific effects and are therefore informative of the processes taking place within the systems. A strong site-specific component was evident for SPC, which generally exerted a negative influence on LDMC, leaf reflectance traits (r440, r625 and r800) and genetic diversity metrics, especially at Mantua and Chiusi, where the SPC range cover was wider (from 357 to 454 μS cm^{-1} at Mantua and from 533 to 608 μS cm^{-1} at Chiusi). The specificity of environmental conditions at Chiusi is also reflected on a genetic basis (Table 3b, Supporting information). In fact, both the AMOVA and the STRUCTURE Harvester analysis highlighted this site as separate from other sites in terms of genetic diversity. This corroborates our results of lower genetic diversity in plots with

Table 4. Summary of the results of GAM models and traits difference among sites. Significant variables in GAM models are marked with 'x'. In the comparisons among sites, '=' indicates no difference, '+' indicates first site has higher values than second site, the opposite for '-'. Depth = water depth (m), SPC = specific conductivity ($\mu S\ cm^{-1}$), NO_3 = water nitrate concentration (mg l^{-1}), sed.OM = sediment organic matter content (%), sed.TP = sediment phosphorus content ($\mu g\ g^{-1}$), Dev.Ex = percentage of deviance explained, R-sq (adj.) = adjusted R square, CH = Chiusi, IS = Iseo-Torbiere, MN = Mantua, PA = Pusiano-Annone. LA = leaf area, SLA = specific leaf area, LDMC = leaf dry matter content, pet.propDW = proportion of leaf dry weight allocated to petioles, chlab = leaf chlorophylls content, AGD = average genetic diversity, OUTLIER = percentage of outlier loci, r440 to r800 refer to the mean leaf reflectance at the given wavelength.

Trait	Depth	SPC	NO_3	sed.OM	sed.TP	Dev.Ex (%)	R-sq. (adj.)	IS-CH	MN-CH	PA-CH	MN-IS	PA-IS	PA-MN
LA				x	x	83.0	0.792	=	+	+	+	+	=
SLA		x			x	71.5	0.661	=	−	−	−	−	=
LDMC		x				74.2	0.687	=	+	+	+	=	=
pet.propDW		x				37.3	0.285	+	=	=	=	=	=
chlab	x				x	59.8	0.495	+	=	=	=	=	=
AGD		x				45.8	0.414	+	+	=	=	=	=
OUTLIER		x				31.8	0.292	=	=	=	=	=	=
r440		x				65.6	0.614	+	+	−	=	−	−
r515						//	//	+	+	=	=	−	−
r625		x	x		x	75.7	0.727	+	+	−	=	−	−
r800		x				72.6	0.692	+	+	=	−	−	−

higher SPC, suggesting that peculiar environmental conditions have selected distinct *N. lutea* genotypes over time. Indeed, the decrease in OUTLIER as water conductivity increases suggests that the higher incidence of neutral loci at high SPC is favoured by balanced selection rather than local genetic adaptation (Excoffier et al. 2009). Other studies have already confirmed the effect of environmental conditions on genetic variation of aquatic species *Ranunculus subrigidus* (Wu et al. 2019), *Phragmites australis* (Coppi et al. 2018, Castellani et al. 2023a, b), *Ranunculus baudotii* (Coppi et al. 2015) and *Ceratophyllum demersum* (Li et al. 2022), although the literature on this topic remains sparse.

SPC reflects the ions content in the water and could therefore be related to the trophic level of the system (Shimoda 1997, Yuwono et al. 2015). At the lake scale, Dalla Vecchia and Bolpagni (2022) found that SPC was highly correlated with water depth, reflecting the influence of sediment metabolism on dissolved ions in the overlying water column. By expanding the spatial scope of investigation, the links between SPC, depth and, more in general, water trophic conditions were weaker. These findings lead to two main considerations. First, sediments emerge as major environmental drivers of trait variability in the meso-eutrophic conditions investigated in this study. Indeed, in our systems, nutrient availability is much higher in the sediments than in the water column. This is possibly due to the high metabolism rates in these systems leading to very fast recycling of nutrients (Twinch and Ashton 1983, Nedwell et al. 1999), so they are not detected in the water during the peak of the growing season. Second, SPC seems to provide information on the basin scale, medium to long term hydrogeological setting (and biogeochemical) of each lake, so the relations between traits and SPC would show site-specific patterns. This is not surprising, considering that SPC is often more associated with dissolved carbon and other ions than with nutrients, depending on the hydrogeological context (Zhao et al. 2020). After all, SPC is one of the most used descriptors for water chemistry and is known to show a much wider variation among sites than within sites (Borowiak et al. 2020). Therefore, the effect of SPC on traits and genetic variability should be understood as an indirect effect, reflecting general and long-term hydrogeological conditions at sites.

Trait–environment relationships

Sediments are characterized by nutrient concentrations at least one order of magnitude higher than in the water column (Hopkins et al. 2018), as a result of human impacts (e.g. pollution) or natural eutrophication processes. Besides sed.TP, sed.OM is also a proxy for trophic level, as it reflects water column productivity and the decomposition capability of benthic organisms and is often associated with phosphorus availability (House and Denison 2002). Higher nutrient availability is often related to higher trait performance, notably so for LES traits like LA, SLA and pigments (Fan et al. 2013, Dalle Fratte et al. 2019, Zervas et al. 2019). Our results are generally in line with this trend for LA and chlab, which showed a positive response to sed.TP increase at high phosphorus concentrations, although chlab appears to be less sensitive for sed.TP < 1500 $\mu g\ g^{-1}$. The same trend was observed also in r625, the spectral trait inversely related to leaf chlorophyll content, which decreased at high levels of sed.TP and NO_3. Interestingly, plots showing a weak sed.TP–traits relation also showed almost undetectable (< 0.15 mg l^{-1}, at Chiusi) or relatively low (< 1 mg l^{-1} in most of the plots at the Iseo and Pusiano–Annone sites) water nitrate concentrations. The relative scarcity of NO_3 in the water of most sites confirms the importance of sediments as nutrients source for rooted species (Bornette and Puijalon 2011), though the LA and chlab behaviour with respect to the relative availability of potential limiting nutrients will require further investigation. Chlab also showed a negative a correlation with depth, which could be interpreted as the effect of less favourable conditions for *N. lutea* individuals growing in deeper waters (Richards et al. 2011), negatively affecting the investment in photosynthetic efficiency.

SLA and LDMC represent opposite sides of the tradeoff between 'fast and acquisitive' leaves (high SLA) and 'slow and conservative' leaves (high LDMC) (Wright et al. 2004), our findings suggest that, at the regional scale, SPC tends to promote a more acquisitive behaviour in *N. lutea*, given its positive influence on SLA and negative influence on LDMC. Again, this is in line with the pattern observed for r800, the spectral trait related to leaf structural complexity, which decreases at high SPC levels. This result is in contrast with the trend found at lake scale (Lake Chiusi) by Dalla Vecchia and Bolpagni (2022) of a negative relation between *N. lutea* SLA and SPC, which suggested a stressful effect of SPC. The reduced investment in leaf structure found at higher SPC, namely in Chiusi, may suggest that in this site this species is able to exploit the more extreme environmental conditions to implement a more acquisitive resource-use strategy, becoming the dominant hydrophyte species within this lake. These results are in line with Klok and van der Velde (2017), who observed, on the other hand, a more conservative strategy of *N. lutea* under limited nutrients availability.

Even if it constitutes a physical constraint for aquatic plant species, water depth does not emerge as a key driver of *N. lutea* traits variability in this study, according to our expectation. Previous studies reported contrasting effects of depth on structural traits, namely SLA (Richards et al. 2011, Fu et al. 2014), supporting the idea of the authors that depth alone should be considered a partial driver of functional variability, and the relation between depth and leaf structural investment can be mediated by other important parameters that vary along the depth gradient, like nutrients and light availability.

Integrative relevance of the outputs

With this work, we adopted an integrative approach merging different dimensions of plants variation (functional, spectral and genetic) to better understand variability in leaf resource-use strategies under different ecological conditions. Although these aspects are seldom considered together, their joint use allowed us to draw comprehensive conclusions about the effect of environment on a key component of primary production in aquatic ecosystems (i.e. nymphaeids). On the one hand, leaf reflectance features can provide abundant and relatively easy to collect foliar traits, alongside commonly measured functional traits (e.g. eight spectral traits replicates in each plot, vs three replicates for pigments concentration in this case). On the other hand, testing whether environmental conditions also have an effect on genetic diversity allowed us to better understand and confirm the observed patterns of variation in leaf traits.

Furthermore, information on spectral traits variability – especially in large, horizontally leaved species such as *N. lutea* – can be exploited to derive intra-site patterns of leaf traits surrogated by reflectance features through the generation of synoptic, continuous, and fine scale maps from very-high-resolution multispectral remote sensing images (see the Supporting information for example maps at 2 m spatial resolution over Lake Chiusi). Such spatial-wise information allows to increase the coverage and level of information detail over the target plant stands under investigation (Villa et al. 2021) and can be greatly useful in detecting patterns and trends in functional traits that might be difficult to capture following a point-sampling strategy.

Conclusions

Our findings provide evidence for strong covariation of leaf traits with changing environmental conditions, using *N. lutea* as ecologically crucial plant. The main environmental drivers of trait variability are water electrical conductivity and sediment phosphorus and organic matter content. Overall, the effect of conductivity was related to site-specific conditions, and the addition of genetic insights allowed us to detect a genetic specificity of the populations at the different study sites, which was ordered by local environmental conditions and could thus support the observed patterns in trait variability. In this regard, we strongly encourage the collection of plant functional traits together with detailed ecological descriptors, accounting for variability in both time and space. Orienting research in this direction could foster a real understanding of the role of traits in plant strategies, not only for aquatic plants, and promote upscaling from describing single species to defining community variability. Finally, we emphasize the advantages of multidimensional, integrated approaches linking different aspects of plant diversity – e.g. functional, spectral and genetic – as they allow a quantitative and exhaustive investigation of population responses to environmental drivers at multiple scales. On one hand, upscaling the information associated with spectral traits at the site scale, through high-resolution maps based on remotely sensed data, could enable the study of plant functional ecology over spatial scales and levels of detail unattainable using only punctual sampling data. Genetic diversity metrics, on the other hand, could help to explain hidden patterns in trait variation due to responses mediated across generations.

Author contributions

Paolo Villa and **Rossano Bolpagni** share senior authorship. **Alice Dalla Vecchia**: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Investigation (equal); Methodology (equal); Visualization (lead); Writing – original draft (lead); Writing – review and editing (equal). **Andrea**

Coppi: Formal analysis (supporting); Investigation (equal); Writing – original draft (supporting); Writing – review and editing (equal). **Maria Beatrice Castellani**: Data curation (equal); Investigation (equal); Writing – review and editing (supporting). **Lorenzo Lastrucci**: Investigation (equal); Methodology (equal); Writing – review and editing (supporting). **Erika Piaser**: Data curation (supporting); Investigation (equal); Writing – review and editing (supporting). **Paolo Villa**: Conceptualization (supporting); Data curation (equal); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project administration (lead); Visualization (supporting); Writing – original draft (equal); Writing – review and editing (equal). **Rossano Bolpagni**: Conceptualization (lead); Investigation (equal); Methodology (equal); Supervision (lead); Writing – review and editing (equal).

Data availability statement

Supporting information

The Supporting information associated with this article is available with the online version.

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