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MACROPHYTES FUNCTIONAL TRAITS

Intraspecific leaf trait variation of the heterophyllous amphibious plant *Luronium natans*: a trait-based study

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Abstract Heterophylly, a type of intraspecific trait variation (ITV), is commonly observed in aquatic and amphibious plants. In this study, we focused on *Luronium natans* (L.) Raf., as a model species to investigate ITV, specifically looking at variations within different leaf types. The objective was to test general hypotheses regarding ITV across various leaf traits. We collected 210 leaves from the homogeneous environments of softwater lakes in northwestern Poland to measure six leaf traits: leaf area (LA), leaf dry weight (LDW), specific leaf area (SLA), leaf dry mass content (LDMC), circularity, and leaf width/length ratio (shape traits). These leaves were categorized into three types based on the shoot: heterophyllous shoots with floating leaves (HF), heterophyllous shoots with submerged leaves (HS), and submerged rosettes with linear leaves (S). Significant differences were observed in LA and LDMC within these leaf types and in SLA between HF and S types. ITV within each shoot type exhibited a wide range of variation, particularly in LA, LDW, SLA, and LDMC, with shape traits displaying stronger variability than functional traits. Our results indicate lower ITV of functional leaf traits within the same heterophyllous shoots compared to fully submerged leaves in a rosette form. We also noted negative correlations between SLA-LDMC and LA-circularity trait pairs, suggesting a variation–covariation pattern in leaf traits with minimal influence from water environmental factors.

Keywords Macrophytes · Heterophyllous · Amphibious plants · Leaf traits

Introduction

Phenotypic variation has been recognized for centuries, with Darwin's work in 1859 being a cornerstone in this area (Darwin, 1859). Subsequent studies by Gleason (1926) and Clements (1936) further elucidated how trait variation within plant species contributes to the structure of plant communities. Numerous studies have highlighted the role of phenotypic variation in shaping the diversity of plant forms, species coexistence, community structure (Jung et al., 2010), and ecosystem processes (Lavorel & Garnier, 2002; Funk et al., 2017). In recent decades, functional and trait-based ecology have provided conceptual

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frameworks and methodologies to study intraspecific trait variation ITV (Albert et al., 2010, 2011). However, trait variation among different species exceeds ITV (Siefert et al., 2015; Westerband et al., 2021). A global meta-analysis found that ITV accounts for approximately 25% of total trait variation within communities (Siefert et al., 2015). ITV can impact competitive interactions, community composition (Violle et al., 2012), and ecosystem functions, such as productivity (Ma et al., 2022). Moreover, ITV manifests across different biologic organizations, from organs and individuals to ecosystems (de Bello et al., 2021). At the individual level, the relative extent of ITV tended to be greater for whole-plant traits (e.g., plant height) vs. organ-level traits (e.g., leaf area, leaf economics spectrum; Siefert et al., 2015).

There are two major complementary mechanisms driving ITV: (1) genetic variability and (2) phenotypic plasticity, which reflects each genotype's potential to produce different phenotypes in response to environmental conditions (Garnier et al., 2016). Many studies have demonstrated high phenotypic plasticity in macrophytes (Wells & Pigliucci, 2000; Santamaria 2002) and ITV (Fu et al. 2013), differentiation of growth forms with wide variability in leaf size, leaf economics spectrum (LES), and shape (Willby et al. 2000; Pierce et al., 2012; Zhang et al., 2017; Chmara et al., 2021). Consequently, macrophyte species often exhibit higher specific leaf area (SLA) and relative growth rates (Poorter et al., 2009; Fu et al., 2014; Liu et al., 2021). Heterophylly, a form of phenotypic plasticity in plants (Wells & Pigliucci, 2000), is particularly common in aquatic and amphibious plants (Li et al., 2019). Amphibious plants possess the unique ability to produce vastly different leaf types on the same shoot (Koga et al., 2020; van Veen & Sasidharan, 2021). The water surface acts as a distinct boundary between aerial and submerged conditions, necessitating different leaf morphology based on function (Wanke, 2011; Li et al., 2019). Therefore, heterophyllous plants serve as excellent models for studying ITV because they produce two or more leaf types on the same shoot, each adapting to different environments. For example, submerged leaves typically tend to be thinner, narrower, lack a cuticle, and may exhibit dissection, whereas aerial leaves are thicker, cutinized, feature stomata, and have been morphologic and anatomic similarities to terrestrial plants (Sculthorpe, 1967; Wells & Pigliucci, 2000). However, the extent of ITV in heterophyllous plants remains largely unexplored.

In this study, our focus was on *Luronium natans* (L.) Raf., a known heterophyllous species (Hyldgaard & Brix, 2011; Chmara et al., 2019). The present study includes data measured on six leaf traits from different shoots of *L. natans* in relatively homogeneous softwater lakes. We hypothesized that heterophyllous amphibious plants represent a unique case of ITV in leaves, with ITV varying across different shoot types. Our specific questions were as follows: (1) Do traits and the extent of ITV vary within different shoot types? (2) What are the trait–trait relationships within leaves in different shoot types? (3) How do environmental conditions influence leaf trait variation at a local scale?

Materials and methods

Study sites and field sampling

The study took place in the Pomeranian Lakeland (54° 07′ 42″ N, 17° 36′ 08″ E), situated on the outwash plain of the Brda River in northwestern Poland. This region represents a young glacial landscape, with the southern portion falling within the Tuchola Forest UNESCO-MAB Biosphere Reserve (53° 46′ 12″ N, 18° 01′ 05″ E; central point). Currently, the study area is characterized by a high concentration of lakes and peatlands, predominantly covered by *Pinus sylvestris* forests. The Chojnice weather station reports a mean annual air temperature of 7.4 °C and mean annual precipitation of 580 mm, based on data from 1952 to 2019 (Polish Institute of Meteorology and Water Management—National Research Institute).

The study focused on three relatively homogeneous softwater lakes where *L. natans* was observed (Fig. 1). These lakes represent a narrow range of softwater habitats, with water pH (5.68–6.19) and calcium concentrations (1.04–4.39 mg L^{-1}). Situated at elevations between 124 and 153 m a.s.l., these shallow lakes were sampled during vegetation seasons in June and August from 2020 to 2021 by SCUBA divers. To investigate trait variation in *L. natans*, individuals were collected from two depth zones in each lake: 0–1 and 1–2.5 m. Field measurements (from a boat) included (1) visibility (m; Secchi disk) and (2) photosynthetic active radiation (PAR), expressed as a

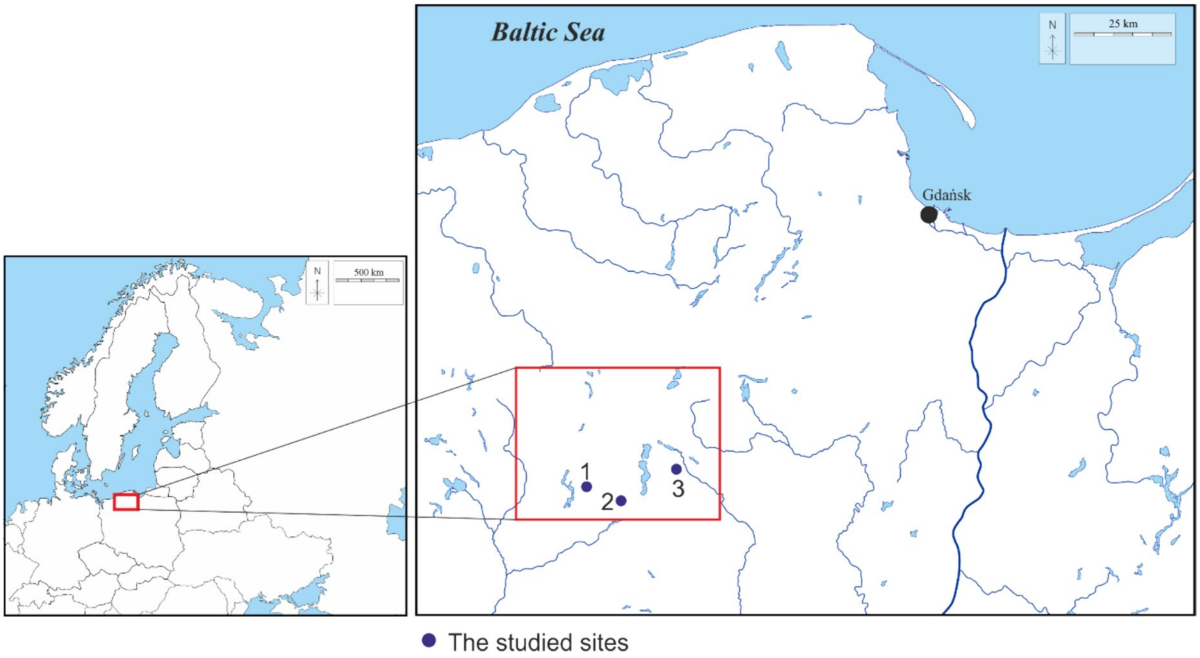

Fig. 1 Location of the studied sites (1–3; explanations can be found in Supplementary Materials 1)

percentage of light reaching the water surface, measured with a Licor LI-250 Light Meter. A total of 25 water samples were collected from different depth zones in the lakes, alongside additional water samples from near-sediment areas, each containing 500 ml, for physicochemical analyses. Physicochemical properties of the water samples were assessed as follows: (1) pH using a pH meter 320/SET1 with a SENTIX 97 T measuring electrode; (2) conductivity; (3) calcium concentration (Ca^{2+}, determined through complexometric titration with calconcarboxylic acid as an indicator); (4) water color assessed comparatively using the Platinum–Cobalt Reference Standards; (5) concentration of humic acids measured spectrophotometrically at 330 nm using a UV–VIS Aquamate spectrophotometer; and (6) total nitrogen (TN) and total phosphorus (TP) analyzed using Merck Spectroquant Cuvette Tests.

Species

L. natans (Alismataceae) is an endemic plant species in Europe, primarily found in the Atlantic and subAtlantic climatic zones in the western and northwestern parts of Europe (Lansdown, 2011). It is included in Annex II of the Europe Habitats Directive 92/43/EEC (European Commission, 1992), which highlights its conservation significance. *L. natans* is a perennial and evergreen plant that reproduces through both vegetative and generative means. It comprises unevenly aged ramets interconnected by stolons, with new shoots emerging from pseudostolons (Nielsen et al., 2006). The species thrives in aquatic and amphibious environments, exhibiting two distinct growth forms: a fully submerged isoetid form as a bottom-dwelling plant with a rosette and a nymphaeid form with long-petioled oval floating leaves (Fig. 2; Greulich et al., 2001). *L. natans* can be found in various habitat types, ranging from oligotrophic lakes to eutrophic lakes, canals (Willby & Eaton, 1993; Szańkowski & Kłosowski, 2001; Nielsen et al., 2006), and even dystrophic lakes (Lansdown, 2011).

Leaf traits measurements

Healthy leaves were specifically collected from different shoots of *L. natans*. The leaves were categorized into three types based on their position on the shoot: heterophyllous shoots with floating leaves (HF, $n=45$), heterophyllous shoots with submerged leaves

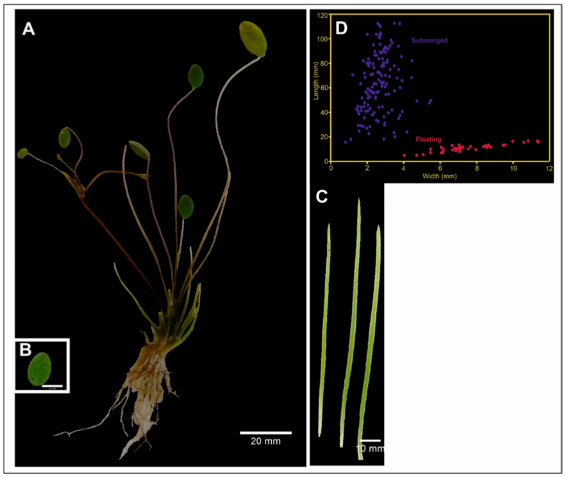

Fig. 2 Heterophylly of *L. natans* showing the morphology of **A** basal rosette, **B** floating leaf, and **C** submerged linear leaves. Additionally **D** presents length–width plots of submerged (violet dots) and floating leaves (pink dots)

Table 1 List of leaf traits, abbreviations, and units

Trait	Abbreviation	Units
Leaf area	LA	mm^2
Leaf dry weight	LDW	mg
Specific leaf area	SLA	$mm^2\ mg^{-1}$
Leaf dry mass content	LDMC	(%)
Circularity	Circ	
Leaf width/length ratio	w/l	Ratio

(HS, $n=31$), and submerged bottom-dwelling plants with rosette linear leaves (S, $n=134$), totaling 210 leaves (Supplementary Information S1). The individuals with floating leaves and submerged leaves with a rosette, belonging to the same shoot (HF and HS types), were collected from the 0 to 1.0 m depth zone, while submerged individuals with a rosette (S type) were collected from the 1.0 to 2.5 m depth zone.

Six leaf traits were measured, including leaf area (LA), leaf dry weight (LDW), specific leaf area (SLA), leaf dry mass content (LDMC), and shape traits: circularity (Circ) [calculated as 4π (area × perimeter^{-2})] and leaf width/length ratio (w/l; Table 1). The measurement methods followed the standardized protocols of Pérez-Harguindeguy et al. (2013). Each leaf was weighted and scanned while fresh using a balance and ImageJ software, respectively (http://imagej.nih.gov/ij), to determine LA and shape traits (Circ, w/l). Subsequently, the same leaves were dried at 80 °C until a constant weight was achieved and their final dry mass was measured. SLA was calculated as the LA (mm^2) per unit of leaf dry mass (mg), which is a component of the leaf economics spectrum representing the trade-off between rapid resource acquisition and conservation (Wright et al., 2004). LDMC is the ratio of dry weight to fresh weight (*g*).

Data analysis

We conducted basic statistical analyses to assess the differences and extent of ITV within shoot types for various traits, including LA, LDW, SLA, LDMC, Circ, and w/l. To quantify the extent of ITV, we calculated the coefficient of variation (CV) using the formula: CV = traits (SD)/traits (mean) × 100%, where SD represents the standard deviation. The nonparametric Kruskal–Wallis test, followed by Dunn's multiple comparisons post hoc test, was employed to compare leaf trait values among different leaf types. Spearman rank correlations were utilized to determine trait–trait relationships and trait–environmental variable correlations, and correlation heat maps were generated using OriginPro software (OriginLab Corporation, USA). Kernel density plots were used to visualize the overlap of traits within shoot types, focusing on LDMC, LA, and SLA distributions. To analyze leaf trait variation in the environmental space of sites (lakes), we conducted nonmetric multidimensional scaling (nMDS) separately for four leaf traits and water properties, as well as for shape leaf traits (circularity and w/l ratio) and water properties. Furthermore, an analysis of similarities (ANOSIM) test was performed using Bray–Curtis distance.

For comparing the functional traits of the investigated plant, we utilized R 4.2.1 software (R Core Team, 2022) and employed ggstatsplot to create violin plots with statistical descriptions (Patil, 2021). Additionally, density plots were also generated and visualized using the ggpubr package (Kassambara, 2023). Finally, for the nMDS analysis and ANOSIM test, we utilized the vegan package (Oksanen et al., 2019).

Results

Trait differences and extent of ITV

The ITV values for leaf traits were notably high, ranging from 47.3% for LDMC to 56.6% for LDW (Supplementary Information S2). For LA, LDW, SLA, and LDMC, the ranges were 10.0–333.7 mm^2, 0.2–7.0 mg, 7.3–176.8 $\mathrm{mm}^2\ \mathrm{mg}^{-1}$, and 0.8–46.6%, respectively (Supplementary Information S2). Kruskal–Wallis tests revealed significant differences in LA and LDMC within the leaf types (Fig. 3a, d; $\chi^2 = 30.3$, $P < 0.001$ and $\chi^2 = 29.9$, $P < 0.001$). The values of LA and LDMC indicate that floating leaves (HF) are smaller and possess higher LDMC compared to submerged leaves (S). Additionally, there were differences in SLAs between submerged leaves and other shoot types (Fig. 3c; $P < 0.001$). However, there were no differences in SLA between leaves on the same heterophyllous shoot (HF vs. HS). LDW within the leaf-type groups did not exhibit significant differences (Fig. 3b; $P = 0.08$).

The analysis of shape traits revealed high ITV and significant differences in circularity and the ratio of leaf width to length (Fig. 4a, b; $\chi^2 = 91.2$, $P < 0.001$ and $\chi^2 = 93.5$, $P < 0.001$). In general, submerged leaves from individuals with rosettes and heterophyllous shoots were narrower compared to floating leaves. We observed high intraspecific variation in shape traits within shoot types, with CV values of 137.5% for circularity and 144.5% for the leaf w/l ratio (Supplementary Information S2). Specifically, circularity values exhibited a wide range, representing the full spectrum of leaf shapes, ranging from extremely narrow leaves to perfectly circular (0.01–1.0; Supplementary Information S2). Overall, the extent of ITV for shape traits varied more strongly than for functional traits.

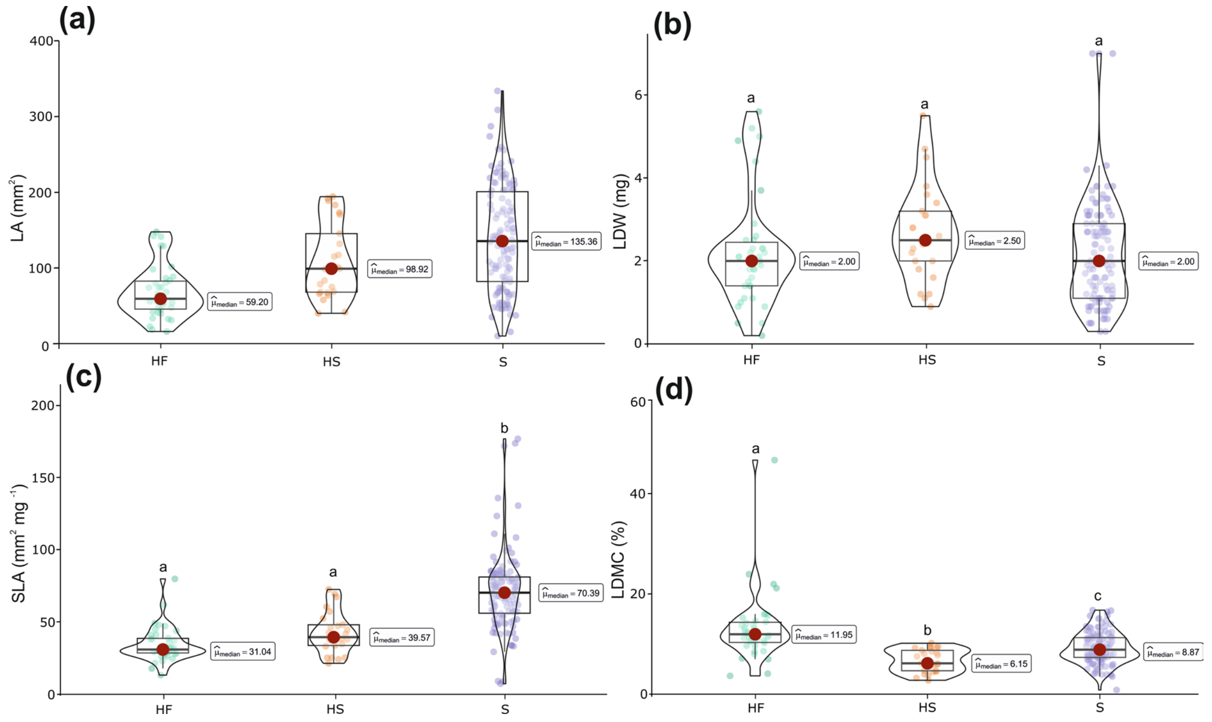

Fig. 3 Functional leaf traits related to shoot types: *HF* heterophyllous shoot with floating leaves ($n = 45$), *HS* heterophyllous shoot with submerged leaves ($n = 31$), *S* submerged rosette with linear leaves ($n = 134$). Differences between leaf traits: **a** *LA* leaf area, **b** *LDW* leaf dry weight, **c** *SLA* specific leaf area, and **d** *LDMC* leaf dry mass content. Different letters indicate significant differences (Dunn's post hoc test of multiple comparisons of independent $P < 0.01$). The violin plot shows the estimated density of sample distributions with boxes showing interquartile ranges and the red dot indicating the median

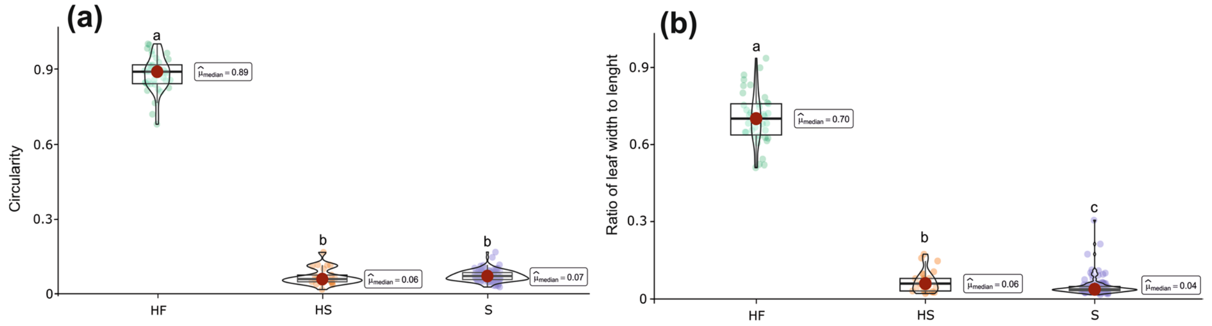

Fig. 4 Comparisons of shape traits: **a** circularity and **b** ratio of leaf to width length between shoot types. For abbreviations, see Fig. 3

Trait–trait relationship and trait overlap

When comparing traits within shoot types, we identified several trait–trait relationships. The SLA values in HF type and S type were negatively related to LDMC ($r = -0.63$, $r = -0.41$; Fig. S1a, c, Supplementary Materials 1). LDMC was negatively correlated with circularity in the HS shoot type and not correlated with SLA ($r = -0.44$, $r = -0.02$; Supplementary Information S2). However, SLA for all leaf types was not correlated with leaf shape traits (Circ, w/l). LA showed a negative correlation with circularity in HF type and S type (Fig. S1a, c, Supplementary Information S2). Trait–trait relationships conducted separately for each site confirmed correlation patterns between SLA and LDMC (negatively significantly correlated; Supplementary Information S2) and SLA not significantly correlated with shape traits (Circ and w/l).

We observed a relatively low trait overlap of LDMC and SLA (Fig. 5a, c) and a relatively high overlap of LA within shoot types (Fig. 5b).

Environmental effects on the leaf traits variation

The environmental conditions of the studied sites (lakes) are available in the supplementary materials (S2). These sites exhibited limited diversity in terms of environmental variables: water pH ranged narrowly from 5.68 to 6.19, TN ranged from 0.669 to 2.267 mg N dm^{-3} water color ranged from 5 to 70, humic acid concentration ranged from 0.50 to 10.25 mg dm^{-3}, and PAR ranged from 23.3% to 59.9%. In general, there was minimal differentiation in environmental variables among the studied lakes. The ANOSIM analysis indicated that environmental conditions in the lakes were not significantly different (ANOSIM statistic: $R = 0.12$, $P = 0.0001$). Additionally, environmental conditions in the study lakes explained little of the leaf trait variation within shoot types (ANOSIM statistic: $R = 0.14$, $P = 0.0001$).

The nMDS analysis was performed to assess how leaf traits varied within different shoot types, exhibiting distinct separation of the three shoot type groups in ordination space (Fig. 6a; ANOSIM statistic: $R = 0.37$, $P = 0.0001$). Another analysis, which was conducted for shape leaf traits showed a clear distinction between shoot types (Fig. 6b; ANOSIM statistic: $R = 0.74$, $P = 0.0001$).

In general, environmental variables had limited effects on leaf trait variation within shoot types. Some correlations could be partially explained by environmental effects. We observed a significant positive correlation for the LA–pH, LA–Calcium and negative correlation for the LA–TP, LA–TN, LA–Color, and LA–HA trait–environmental variable pairs (Supplementary Information S2). SLA was negatively related to pH, TP, TN, Color, HA, and PAR. However, LDMC and Circularity within leaf types were not correlated with environmental variables.

Discussion

Advancements in trait-based methods in recent decades have contributed significantly to our understanding of ITV (Jung et al., 2014; Ma et al., 2022). Our study, utilizing ITV approaches, revealed clear leaf differences and high ITV (Figs. 3, 4; Supplementary Materials 1) in *L. natans*, affected by contrasting

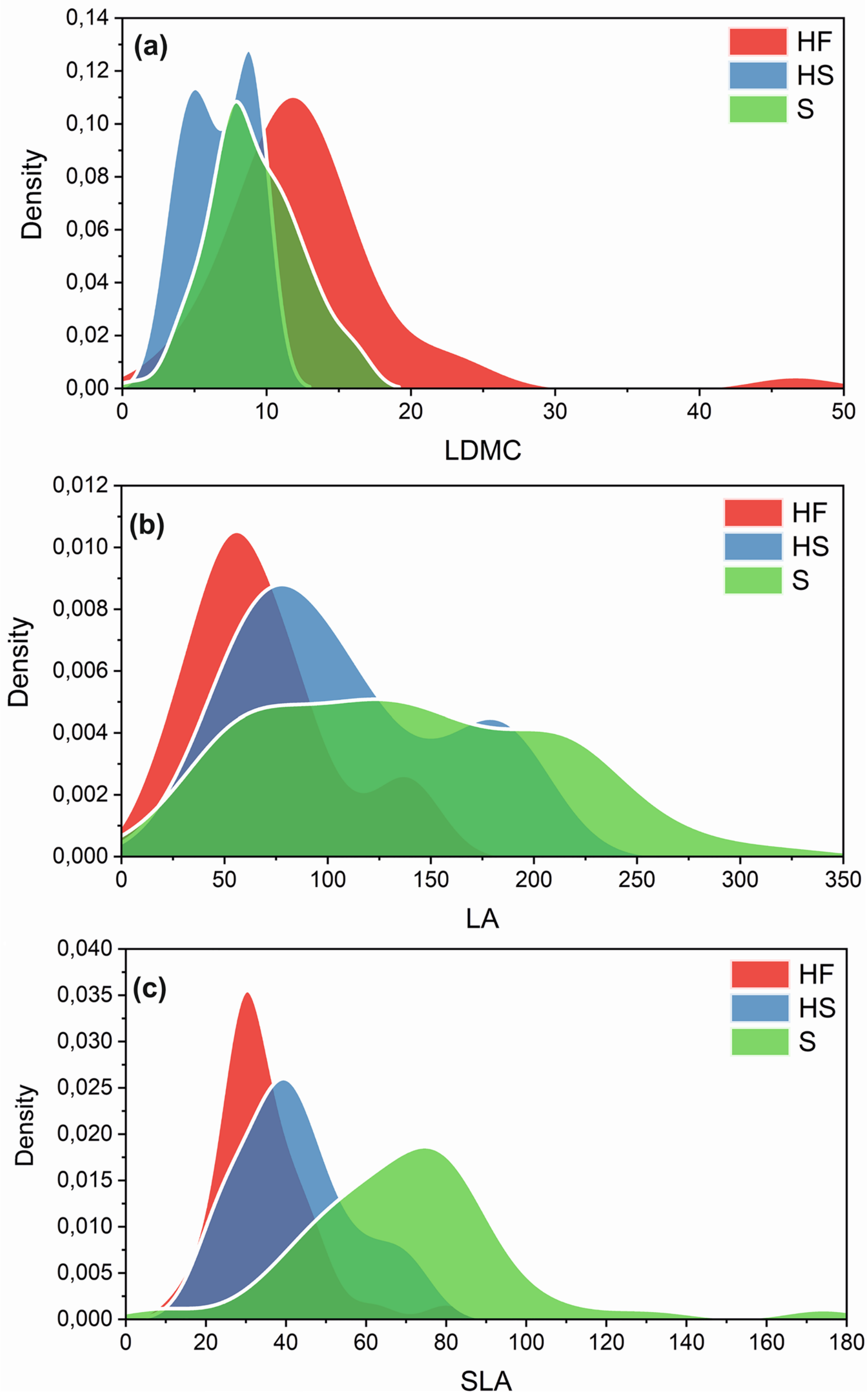

Fig. 5 Kernel density distribution of leaf functional traits in shoot types: **a** *LDMC*, *HF* heterophyllous shoot with floating leaves, **b** *LA*, *HS* heterophyllous shoot with submerged leaves, and **c** *SLA*, *S* submerged rosette with linear leaves

air–water environments. Overall, we found high ITV across all leaf traits, ranging from 47.3% to 144.4% (Supplementary Information S2). A recent study at the intraspecific level reported significantly lower ITV values (only 10–15%) for leaf functional traits compared to our results (Martin et al., 2017; Bloomfield et al., 2018). For example, traits belonging to the leaf economics spectrum exhibited ITV values similar

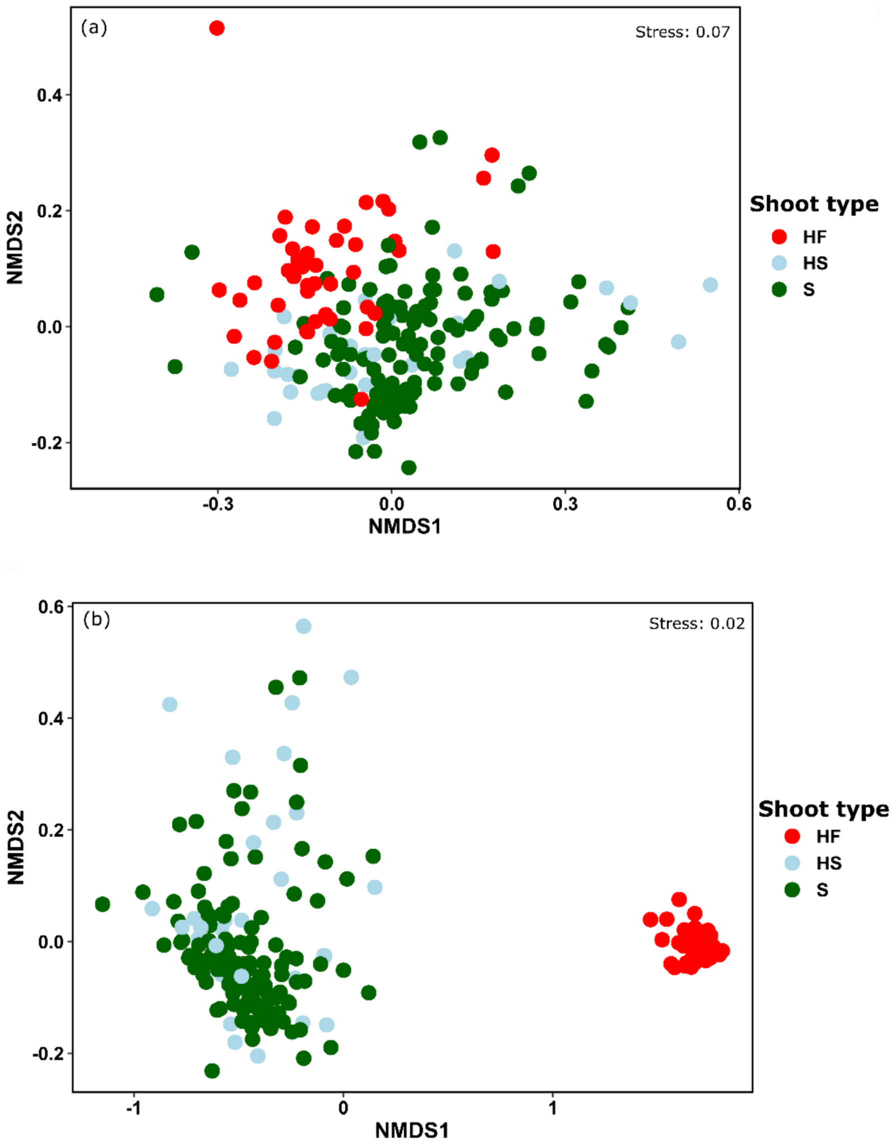

Fig. 6 Nonmetric multidimensional scaling (nMDS) of leaf trait within different shoot types in ordination space. The color circle represents shoot type: *HF* heterophyllous shoot with floating leaves, *HS* heterophyllous shoot with submerged leaves, *S* submerged rosette with linear leaves. The nMDS analysis was performed for **a** four leaf traits (LA, LDW, SLA, LDMC) and **b** shape leaf traits (circularity and w/l ratio)

to mean ITV values within community levels (Siefert et al., 2015). This confirms the well-known high phenotypic plasticity and high ITV of macrophytes and at the same time provides insight into the relatively unexplored extent of ITV at the individual level of macrophytes, especially concerning heterophyllous plants. Several studies have indicated that ITV can be equivalent to or similar to trait variation between species (BTV) for leaf mass per area (LMA; Fajardo and Siefert, 2018) and LDMC (Read et al., 2017). A recent study on grassland species revealed higher ITV than BTV (Tautenhahn et al., 2019). However, it has been shown that ITV measurements may be substantially underestimated (Yang et al., 2020). The authors argue that ITV values can be biased for low sample sizes, emphasizing the need for more extensive sampling. According to these studies, samples greater than 20 are recommended for almost all traits and species. Our research based on the leaf traits of *L. natans* complies with these requirements.

Even higher ITV were found in leaf shape traits, specifically circularity and the w/l ratio (Fig. 4; Table in Supplementary Information S2). Within the shoot types, we noted a high ITV for circularity and the w/l ratio, expressed as coefficients of variation. Different studies show that leaf shape, being a crucial phenotypic trait, can reflect the adaptation of macrophytes to environmental constraints (Pierce et al., 2012; Maberly & Gontero, 2018; Dalla Vecchia et al., 2020; Dalla Vecchia & Bolpagni, 2022), adaptive strategies related to lake eutrophication and the relationship between water depth and leaf shape (Liu et al., 2021), as well as adaptation to carbon acquisition in air–water environments (Chmara et al., 2021; Pan et al., 2023).

Our investigation revealed differences in SLAs between submerged leaves and other leaf types, but no differences in SLA were observed among leaves on the same heterophyllous shoot (Fig. 3b, c). The highest values of specific leaf area were found in a submerged rosette with linear leave groups. Leaves from these groups tend to be thinner and narrower, characteristics adapted to submerged conditions, categorizing them as true aquatic plants (sensu Veen & Sasidharan 2021). Consequently, high-SLA leaves facilitate better gas exchange and inorganic carbon acquisition (Mommer et al., 2005). In contrast, floating leaves are smaller and have lower SLA and higher LDMC (Fig. 3c, d). Aquatic plants have limited access to carbon and experience reduced light levels (Pedersen et al., 2013). The process of inorganic carbon assimilation varies depending on leaf types. Floating leaves may utilize atmospheric carbon dioxide (CO_2), while submerged leaves lack stomata, requiring CO_2 to diffuse through their leaf surface (Hyldgaard & Brix, 2011). This suggests that different leaf types within the same heterophyllous shoot serve to optimize carbon concentration mechanisms (CCMs) for photosynthesis and confirm the carbon acquisition plasticity of *L. natans* (Greulich et al., 2001; Hyldgaard & Brix, 2011).

Overall, we found that environmental variables had minimal impact on trait variation. Why were the leaf trait–environmental variable relationships observed in our study so weak? Firstly, the study was conducted in relatively homogeneous lakes with a narrow range of water environmental conditions (refer to Table in Supplementary Information S2). It is possible that factors at the boundary between water and land, particularly water-level fluctuations, play a significant role. According to Banaś et al. (2023), reduced water levels can lead to a significant reduction in the amphibious form of *L. natans*, which typically thrives in deep-water populations (submerged rosette with linear leaves). This form is particularly common in lakes in northern Poland.

On the other hand, clear trait–trait correlations were found (Supplementary Information S2). The negative and significant correlations observed for the SLA–LDMC within shoot types may indicate a trade-off relationship in resource allocation within these shoot types. These findings align with previous studies conducted at the intraspecific level in homogeneous environments (Gorné et al., 2021). The authors of those studies suggest that there is a high degree of variation–covariation in leaf traits that is not primarily driven by environmental variables.

Conclusion

Our results highlight high ITV observed in the heterophyllous amphibious plant *L. natans*. Specifically, ITV of functional leaf traits within the same heterophyllous shoot is lower compared to fully submerged leaves with a rosette. These results offer valuable insights into the extent of ITV in aquatic plants and suggest the inclusion of heterophyllous plants in trait-based studies. Furthermore, it is recommended to consider heterophyllous amphibious plants in global leaf economics spectrum research.

On the other hand, fully submerged leaves with a rosette exhibit significantly different traits from leaves on heterophyllous shoots, indicating their adaptation to submergence. These adaptations are likely driven by ITV within shoot types in *L. natans*, which plays a crucial role in facilitating adaptation to contrasting air–water environments. Moreover, our findings highlight a variation–covariation pattern in leaf traits and indicate minimal influence from water environmental factors.

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Author contributions RC contributed to the conception and design of the study. RC and EP co-organized the field study and collected the samples in the field. RC collected the plant samples, analyzed the data, and wrote the manuscript. Finally, RC also prepared all samples for functional trait analyses. Data analysis was conducted by RC and EP. All authors read and approved the final manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author (RC) upon request.

Declarations

Conflict of interest The authors declare no competing interests.

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