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Seed Diversity and Evolutionary Trends in Boraginaceae from Pakistan

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Abstract

Seed morphology and micromorphology are important for the taxonomy and evolutionary interpretation of Boraginaceae. In this study, 20 species from Pakistan were examined using scanning electron microscopy (SEM) and phylogenetic analyses to evaluate variation in seed traits. A total of 10 nutlets per species were analyzed, and both qualitative and quantitative characters were recorded. The results revealed substantial variation in seed morphology, particularly in shape, color, texture, and surface sculpturing. Quantitative traits (seed length, width, and length–width ratio) also showed significant interspecific variation. Principal component analysis (PCA) indicated that PC1 (61.5%) was mainly influenced by seed length and width, while PC2 (38.2%) was associated with seed elongation (length–width ratio), effectively separating species based on size and shape. Micromorphological characters exhibited high diversity, whereas some features, such as anticlinal and periclinal walls, showed limited diagnostic value. Ancestral state reconstruction and phylogenetic signal analyses suggested that most seed traits are evolutionarily labile and exhibit high homoplasy. Overall, seed characters are useful for species-level identification but less informative at higher taxonomic levels.

Introduction

The morphology of nutlets has contributed valuable data to the systematics and evolutionary classification of flowering plants (Johnston, 1949; Corner, 1976). Nutlet characters are also considered one of the most significant diagnostic attributes of the Boraginaceae (Al-Shehbaz 1991; Juan et al., 2000). Due to their structural stability and taxonomic relevance, nutlet features have long been employed in the delimitation of taxa within Boraginaceae. Based on nutlet macromorphology, the family was previously divided into 13 tribes (Akçin, 2007; Otero et al., 2014). However, advances in molecular phylogenetics have led to a revised and more robust classification system, which condenses tribal delimitations into four major tribes (Akçin, 2008; Kahraman et al., 2011): Boragineae, Lithospermeae, Echiochileae, and Cynoglosseae (Långström and Chase, 2002; Långström and Oxelman, 2003; Weigend et al., 2010; Nazaire and Hufford, 2012; Weigend et al., 2013; Cohen, 2014). This shift highlights the importance of integrating morphological and molecular evidence to better understand evolutionary relationships within the family (Akçin and Binzet, 2011).

Phylogenetic investigations of Boraginaceae genera have revealed important patterns in the evolution of morphological characters (Långström and Oxelman 2003; Akçin et al., 2010; Cohen 2014). In particular, seed micromorphology has emerged as a powerful tool for uncovering hidden variation that is often not apparent at the macromorphological level (Alam et al., 2023). These micromorphological traits, including surface sculpturing, cell arrangement, and wall structure, provide critical insights into evolutionary processes and phylogenetic relationships among species (Al-Shehbaz, 1991; Karcz et al., 2005). Despite considerable progress over the past 17 years, most studies have focused on phylogenetic relationships within genera or among closely related taxa, with relatively limited emphasis on quantitative assessments of trait evolution across broader phylogenetic frameworks (Cohen 2014; Arabameri et al.,

2014; Kayani et al., 2024). Consequently, a comprehensive understanding of how seed traits evolve and diversify across lineages remains incomplete (Attar et al., 2018; Khatamsaz, 2001).

Distinctive nutlet characters are associated with different tribes within Boraginaceae, reflecting their evolutionary divergence (Kubitzki, 2013; Attar et al., 2021). The tribe Echiochileae is characterized by a basal or submedial adhesion scar and a smooth to pyramidal gynobase (Långström & Chase 2002; Kurşat et al., 2023). Members of Lithospermeae typically possess ovoid, keeled, somewhat compressed, and strongly incurved nutlets with a prominent basal attachment scar and a flat gynobase (Weigend et al. 2010; Yu et al. 2012). In Boragineae, nutlets are superficially attached, often exhibiting a basal annulus around the scar and a flat gynobase (Hilger et al. 2004; Binzet and Akçin, 2009). In contrast, Cynoglosseae, identified through molecular phylogenetic analyses, represents a morphologically heterogeneous group that exhibits the highest diversity in nutlet morphology and ornamentation (Bobrov et al., 2005; Binzet and Akçin, 2011). This diversity includes deeply dentate margins, glochidia, papillae, and entirely smooth surfaces, along with variation in gynophore configuration ranging from nearly flat to pyramidal (Boyd, 2002; Buys and Hilger, 2003). Such diversity underscores the evolutionary plasticity of seed traits within the family (Långström and Chase, 2002; Chacón et al., 2016).

Seed ultrastructural features, particularly seed surface characteristics and epidermal cell morphology, are considered important diagnostic traits at both intrageneric and intraspecific levels (Kubitzki 2013; Cohen, 2014). These traits are often less influenced by environmental conditions and therefore provide more reliable taxonomic signals. Seed size has been shown to have a direct relationship with nutrient reserves, influencing early seedling establishment and growth (Corner, 1976; Veloso et al., 2017). Additionally, variation in seed color, shape, and size plays an important role in plant identification and classification (Ninkaew et al. 2017). Among these characteristics, surface sculpturing is regarded as the most reliable taxonomic feature, whereas seed color, size, and shape are often considered less dependable due to their high variability and environmental plasticity (Oran and Sciences 1996; Ebadi and Nikzat, 2021; Sadia et al. 2024).

Despite these advances, the evolutionary dynamics of seed morphological traits in Boraginaceae remain insufficiently explored within an explicit phylogenetic framework, particularly in regional floras such as Pakistan (Långström and Oxelman, 2003; Gillani et al., 2024a). Pakistan represents a biogeographically diverse region, encompassing a wide range of ecological zones that may influence morphological diversification (Hassan et al., 2005; Gillani et al., 2024b). However, integrative studies combining seed morphology with phylogenetic methods in Boraginaceae from this region are still scarce (Hilger, 2014). Moreover, quantitative analyses linking morphological variation with evolutionary patterns, including ancestral state reconstruction and phylogenetic signal assessment, are largely lacking (Hilger, 1985; Hilger et al., 2004).

The present study aims to investigate seed morphological diversity and character evolution in 20 species of the family Boraginaceae from Pakistan using an integrative phylogenetic framework (Majeed et al., 2022; Jabeen et al., 2023). It further examines both quantitative traits, including seed length, width, and

length-to-width ratio, alongside a range of qualitative characters to assess patterns of intra- and interspecific variation among the studied taxa. Ancestral state reconstruction is conducted under an equal-rates (ER) model to infer evolutionary transitions of key seed traits, while phylogenetic signal is evaluated using Pagel's λ and Blomberg's K to determine the extent of trait conservatism and evolutionary dependence. Moreover, by integrating detailed morphological data with phylogenetic analyses, the study provides comprehensive insights into evolutionary trends, trait stability, and diversification patterns of seed morphology in Boraginaceae. These findings contribute to improved taxonomic resolution and enhance our understanding of evolutionary processes within the family.

Methodology

Collection of nutlet samples

The plant species used in this study were collected from their natural habitats across different regions of Pakistan, depending on the availability of material. Pakistan is geographically located at approximately 30°00' N and 70°00' E and covers an area of about 796,096 km² (Rasul and Ahmad 2012; Shinwari et al. 2017; Manzoor et al. 2023; Kayani et al. 2024; Jabeen et al. 2023). Plant specimens representing selected Boraginaceae species were collected across various geographical regions of Pakistan, encompassing a wide range of habitats and altitudinal gradients. Information regarding species identification, collection localities, habitat characteristics, and voucher details is summarized in Table S1. Collected specimens were carefully preserved and processed for further analysis. The list of investigated species, including locality details and voucher numbers, is provided in Table 1. All plant specimens were identified by Dr. Rhat Ullah, Assistant Professor, Ghazi Umara Khan Degree College, Samarbagh, Dir Lower, Khyber Pakhtunkhwa, Pakistan. Voucher specimens of wild-collected plants were deposited at Ghazi Umara Khan Degree College Herbarium. Collection sites, habitats, collector names, years of collection, and voucher numbers are provided in Table S1. All collections complied with national and local biodiversity regulations, and permissions were obtained from landowners or appropriate authorities where necessary. A total of 20 species belonging to the family Boraginaceae were examined in this study. For each species, up to 10 nutlets were selected, depending on material availability, and analyzed using commonly used morphological descriptors (Manzoor et al., 2023). Both qualitative and quantitative seed characters were recorded and evaluated (Tables S2 and S3) to assess variation in seed morphology and micromorphology. All collections followed ethical guidelines for botanical research and legal requirements for access to wild flora.

Scanning electron microscopy (SEM)

The nutlets were cleaned by immersing them in alcohol for 3–4 minutes to remove surface impurities. Cleaned samples were mounted on standard aluminum stubs (two per taxon/accession) using double-sided adhesive tape. The prepared specimens were examined under a PHILIPS/FEI XL 20 scanning electron microscope operated at 15 kV. High-resolution images were captured to observe diagnostic

features, including seed size, shape, color, cell boundaries, epidermal cells, anticlinal and periclinal walls, and surface sculpturing. SEM analysis allowed detailed visualization of micromorphological characters, particularly epidermal features and surface ornamentation, which are considered important for species and genus delimitation (Boyd 2002). The terminology and parameters used for describing seed morphological and micromorphological features followed established standards (Özkan, Aktaş et al. 2009). All SEM observations were conducted at the University of Peshawar, Khyber Pakhtunkhwa, Pakistan (Manzoor et al., 2024).

Trait Selection and Standardization

Morphological and micromorphological traits were selected based on their taxonomic relevance as reported in the source study. To ensure consistency and suitability for comparative and phylogenetic analyses, all traits were standardized into three-state categorical variables. Seed shape was categorized into ovate, rounded, and irregular forms (Mirzaman et al., 2023). Seed color was grouped into light, medium, and dark categories based on pigmentation intensity. Seed texture was classified into smooth, intermediate, and strongly ornamented types. Apex morphology was categorized into rounded, acute, and specialized types, while base morphology was grouped into rounded, elongated, and acute-truncate forms. Seed coat cell outline was classified into random, organized, and modified categories (Mohsenzadeh et al., 2023). Periclinal wall structure was categorized into not visible, smooth, and prominent states. Surface sculpturing was grouped into smooth, granular, and complex categories, reflecting the increasing complexity of epidermal ornamentation. All categorical traits were encoded numerically (1–3) to facilitate statistical and phylogenetic analyses.

Phylogenetic Framework Construction

The phylogenetic framework for Boraginaceae was constructed based on a classification-derived Newick tree extracted from the comprehensive dated phylogeny of vascular plants presented by Feng et al. (2025), which provides a robust evolutionary context for angiosperm lineages. The tree representing the sampled taxa was imported into the R programming language environment using the ape R package. The extracted phylogeny included branch-length information; therefore, no additional transformation or branch-length estimation was required. Species names were standardized by replacing spaces with underscores to ensure consistency with tree tip labels. The trait dataset was then matched with the phylogenetic tree, and taxa not shared between the dataset and the phylogeny were excluded. The phylogenetic tree was pruned using the drop.tip () function, and the dataset was reordered to correspond with the final tip order. The resulting pruned and standardized phylogenetic framework was subsequently used for ancestral state reconstruction and phylogenetic signal analyses (Moon and Hong, 2006).

Ancestral State Reconstruction

Ancestral state reconstruction was performed using maximum likelihood under the equal-rates (ER) model implemented in the ace () function of the ape package. Each trait was analyzed independently. The probabilities of ancestral states at internal nodes were estimated and visualized using pie charts. Trait evolution was mapped onto the phylogeny using the phytools package, and all figures were exported as high-resolution PDF files. For ancestral character reconstruction, qualitative seed traits were transformed into discrete character states. Seed shape was coded as rounded (0), irregular (1), and ovate (2), whereas seed color was categorized into light colors (0), medium brown (1), and dark colors (2). Seed texture was classified as smooth (0), moderately ornamented (1), and strongly ornamented (2). Apex morphology was divided into acute (0), rounded (1), and specialized (2), while base morphology was coded as acute (0), round (1), and elongated (2). Hilum position was recorded as terminal (0), central (1), and slightly terminal (2), and the seed surface level was coded as raised (0) or depressed (1). Cell outline was classified into modified surface (raised/depressed) (0), random or unorganized (1), and organized or aligned (2). The central line was coded as prominent with glochids (0), present (1), or absent (2). Ventral keel was categorized into absent (0), middle (1), and present (2), whereas prickles/tubercles were scored as present (0) or absent (1). Periclinal wall characters were divided into smooth or slightly developed (0), prominent or raised (1), and not visible (2). Anticlinal wall characters were categorized as smooth straight (0), thick raised (1), undulate (2), or not visible. Wall ornamentation was scored as thin (0), thick (1), very thick (2), and very thin (3). Surface sculpturing was classified into protruding ornamentation (0), irregular pattern (1), and weakly ornamented conditions. Epidermal cells were coded as pentagonal to hexagonal (0), irregular (1), and not visible (2). These character states were used as the basis for ancestral state reconstruction and comparative evolutionary analyses in Boraginaceae. Qualitative morphological traits were transformed into discrete numerical states to generate a coded character matrix for phylogenetic analyses; the complete dataset is provided in Table S4.

Phylogenetic Signal Analysis

Phylogenetic signal was assessed to evaluate the extent to which trait variation reflects phylogenetic relationships. Pagel's λ and Blomberg's K were calculated after converting categorical traits into numeric form. These analyses were conducted using functions implemented in phytools and ape.

Principal component analysis

Quantitative seed traits (length, width, and length–width ratio) were analyzed using principal component analysis (PCA) based on standardized data (Nasrollahi et al., 2013).

Statistical Environment

All data processing, statistical analyses, and visualizations were performed in R (R Core Team). The primary packages used included ape, phytools, readr, and dplyr. Variation among species was further

illustrated using bar plots.

Results

Morphometric characters variation

Seed length exhibited substantial interspecific variation among the studied Boraginaceae species (Fig. 9A). The length of the seed ranged from a maximum of 6.90 ± 0.14 mm observed in *Alkanna tinctoria* to a minimum of 1.90 ± 0.12 mm in *Euploca strigosa*. The longest seeds were recorded in *Gastrocotyle hispida*, followed by *Heliotropium crispum* and *Anchusa arvensis*, all showing relatively large seed dimensions. In contrast, the smallest seeds were observed in *Onosma dichroantha*, *Nonea edgeworthii*, and *Nonea caspica*. Most species displayed intermediate seed lengths, including *Cynoglossum lanceolatum*, *Buglossoides arvensis*, *Alkanna tinctoria*, and *Onosma hispida*. Species of *Heliotropium* showed moderate variation, with *H. bacciferum* and *H. europaeum* having relatively larger seeds compared to *H. supinum* and *H. curassavicum*. Overall, the results indicate pronounced variability in seed size across taxa, reflecting potential ecological and taxonomic differentiation within the family (Nazaire and Hufford, 2012).

Seed width showed marked interspecific variation among the studied Boraginaceae species (Fig. 9B). The seed width of the investigated species ranged from 4.10 ± 0.19 mm recorded in *Alkanna tinctoria*, to a minimum of 0.84 ± 0.13 mm in *Euploca strigosa*. The greatest seed width was recorded in *Gastrocotyle hispida*, followed by *Anchusa arvensis* and *Asperugo procumbens*, indicating relatively broader seeds in these taxa. In contrast, the narrowest seeds were observed in *Onosma dichroantha*, *Nonea edgeworthii*, and *Nonea caspica*. Most of the remaining species exhibited intermediate seed width values, including *Cynoglossum lanceolatum*, *Arnebia decumbens*, *Heliotropium europaeum*, and *Heliotropium crispum*. Species within *Heliotropium* displayed moderate variation, with *H. europaeum* and *H. crispum* having relatively wider seeds compared to *H. supinum* and *H. curassavicum*. Overall, seed width demonstrates considerable variability across taxa, reflecting differences in seed size and morphology within the family (Ninkaew et al., 2017).

The length–width ratio exhibited considerable interspecific variation among the studied Boraginaceae species (Fig. 9C). The highest length-by-width ratio was found in *Heliotropium supinum*, 2.38, while the lowest was present in *Cynoglossum lanceolatum*, 1.08. The highest ratios were observed in *Onosma hispida*, *Heliotropium crispum*, and *Onosma dichroantha*, indicating relatively more elongated seed shapes in these taxa. In contrast, the lowest ratios were recorded in *Euploca strigosa* and *Nonea caspica*, reflecting comparatively broader and less elongated seeds. Most of the remaining species, including *Heliotropium bacciferum*, *Trichodesma indicum*, *Buglossoides arvensis*, and *Heliotropium supinum*, exhibited intermediate ratio values. Species such as *Alkanna tinctoria*, *Asperugo procumbens*, and *Gastrocotyle hispida* also showed moderate elongation, while taxa like *Anchusa arvensis* and *Heliotropium europaeum* clustered around similar intermediate values. Overall, the results highlight

significant variation in seed shape among species, ranging from broadly ovate to distinctly elongated forms (Noor et al., 2024).

Characters evolution

Seed shape

Ancestral state reconstruction of seed shape across subfamilies revealed distinct patterns among Heliotropioideae, Boraginoideae, and Cynoglossoideae. In Heliotropioideae, seed shape was predominantly irregular, with occasional transitions to other forms, indicating moderate variability within the clade. Boraginoideae exhibited higher morphological diversity, with ovate seeds being most frequent, while rounded and irregular forms appeared sporadically across different lineages. In contrast, Cynoglossoideae showed a mixture of ovate and rounded shapes, suggesting multiple independent transitions. Overall, seed shape demonstrates heterogeneous distribution with repeated evolutionary shifts among subfamilies (Fig. 1A).

Seed color

Seed color showed clear variation across subfamilies, with three main states: light colors, medium brown, and dark colors. In Heliotropioideae, seed color was predominantly medium brown, indicating a relatively conserved condition within the clade. Boraginoideae displayed broader variation, with all three-colors states present, although medium brown and dark colors were more frequent. In Cynoglossoideae, seed color was mainly light to dark, with fewer occurrences of medium brown, suggesting greater evolutionary flexibility. Overall, seed color exhibits subfamily-specific tendencies but with considerable homoplasy across lineages (Fig. 1B).

Seed texture

Ancestral state reconstruction of seed texture revealed three states: smooth, moderately ornamented, and strongly ornamented, with clear variation among subfamilies. In Heliotropioideae, seed texture was predominantly moderately ornamented, with occasional transitions to smooth and strongly ornamented states. Boraginoideae exhibited higher variability, with all three states present, although smooth and moderately ornamented textures were more frequent. In Cynoglossoideae, seed texture was mainly smooth, with limited occurrence of ornamented conditions. Overall, seed texture shows subfamily-level differentiation but with repeated independent transitions across the phylogeny, indicating homoplasy (Fig. 7A).

Seed apex

Ancestral state reconstruction of the seed apex identified three states: acute, rounded, and specialized. In Heliotropioideae, the apex was predominantly acute, with occasional occurrences of rounded forms. Boraginoideae exhibited greater variability, with both acute and rounded apices widely distributed, while specialized forms were restricted to a few taxa. In Cynoglossoideae, the apex was mainly rounded, with

limited transitions to acute conditions. Overall, seed apex shows subfamily-level variation with multiple independent transitions, indicating homoplasy within Boraginaceae (Fig. 3A).

Seed base

Ancestral state reconstruction of the seed base identified three states: acute, rounded, and elongated. In Heliotropioideae, the seed base was predominantly rounded, with a few transitions to elongated and acute conditions. Boraginoideae showed greater variation, with rounded bases being most common, while acute and elongated forms occurred in scattered taxa. In Cynoglossoideae, the base was mainly rounded, with occasional elongated states and rare acute conditions. Overall, the seed base exhibited subfamily-level variation but was dominated by the rounded condition, with repeated independent transitions to acute and elongated forms (Fig. 3B).

Hilum position

Ancestral state reconstruction of hilum position revealed three states: terminal, central, and slightly terminal. In Heliotropioideae, the hilum position was predominantly central, indicating a conserved condition within the clade, with occasional transitions to terminal states. Boraginoideae also showed a strong dominance of the central position, although terminal conditions appeared in several taxa, reflecting moderate variability. In contrast, Cynoglossoideae exhibited greater variation, with all three states present, including the rare slightly terminal condition. Overall, hilum position is largely conserved as central across subfamilies, with independent shifts to terminal and slightly terminal states, indicating limited but notable evolutionary transitions (Fig. 5A).

Hilum level

Ancestral state reconstruction of the hilum level revealed two states: raised and depressed. In Heliotropioideae, the hilum level was predominantly raised, indicating a conserved condition within the clade, with rare occurrences of depressed states. Boraginoideae showed moderate variation, although the raised condition remained dominant, while depressed states were limited to a few taxa. In Cynoglossoideae, the hilum level was consistently raised, with no or minimal variation observed. Overall, hilum level is largely conserved as raised across subfamilies, with limited independent transitions to the depressed condition, suggesting low evolutionary variability (Fig. 5B).

Cell outline

Ancestral state reconstruction of the cell outline identified three states: modified surface, unorganized, and organized. In Heliotropioideae, the cell outline was predominantly unorganized, indicating a conserved pattern within the clade. Boraginoideae exhibited greater variability, with all three states present, although unorganized outlines were more frequent, while modified and organized conditions appeared in specific lineages. In Cynoglossoideae, the cell outline was mainly modified surface, with occasional occurrences of organized and unorganized states. Overall, the cell outline shows subfamily-

level differentiation with multiple independent transitions, suggesting homoplasy in Boraginaceae (Fig. 6A).

Central line

Ancestral state reconstruction of the central line identified three states: prominent with glochids, present, and absent. In Heliotropioideae, the central line was predominantly absent, indicating a conserved condition within the clade. Boraginoideae showed greater variation, with present and absent states commonly observed, while the prominent condition with glochids was restricted to a few taxa. In Cynoglossoideae, the central line was mainly present, with occasional absence and rare occurrences of the glochidiate condition. Overall, the central line exhibits subfamily-level variation with multiple independent transitions, suggesting homoplasy across Boraginaceae (Fig. 6B).

Ventral keel

Ancestral state reconstruction of the ventral keel identified three states: present, absent, and middle. In Heliotropioideae, the ventral keel was predominantly present, indicating a relatively conserved condition within the clade. Boraginoideae exhibited greater variation, with all three states present, although the absent condition was more frequent, while the middle state occurred in a few taxa. In Cynoglossoideae, the ventral keel was mainly present, with occasional transitions to absent and middle states. Overall, the ventral keel shows subfamily-level variability with multiple independent transitions, suggesting homoplasy across Boraginaceae (Fig. 8A).

Prickles/tubercles

Ancestral state reconstruction of prickles/tubercles identified two states: present and absent. In Heliotropioideae, prickles/tubercles were predominantly absent, indicating a conserved condition within the clade, with only occasional occurrences of the present state. Boraginoideae showed moderate variation, with both present and absent conditions observed, although absence was more frequent. In Cynoglossoideae, the present condition was more common, particularly in several taxa, while absence occurred less frequently. Overall, prickles/tubercles exhibit subfamily-level variation with multiple independent gains and losses, indicating homoplasy within Boraginaceae (Fig. 8B).

Periclinal wall

Ancestral state reconstruction of the periclinal wall identified three states: slightly developed, raised, and not visible. In Heliotropioideae, the periclinal wall was predominantly not visible, indicating a conserved condition within the clade. Boraginoideae showed moderate variation, with the not visible state being most common, while slightly developed and raised conditions occurred in a few taxa. In Cynoglossoideae, the periclinal wall was mainly not visible, with rare occurrences of the other states.

Overall, the periclinal wall exhibits low variability across subfamilies, with the not visible condition dominating and limited independent transitions to developed or raised states (Fig. 2B).

Anticlinal wall

Ancestral state reconstruction of the anticlinal wall identified four states: smooth, straight, thick raised, undulate, and not visible. In Heliotropioideae, the anticlinal wall was predominantly undulate, with occasional transitions to thick, raised conditions. Boraginoideae exhibited moderate variation, although the undulate state was most common, while smooth, straight, and thick raised conditions were restricted to a few taxa. In Cynoglossoideae, the anticlinal wall was mainly undulate, with limited occurrences of other states. The not visible condition was rare and scattered across the phylogeny. Overall, the anticlinal wall shows dominance of the undulate state with few independent transitions, indicating relatively low variability across subfamilies (Fig. 2A).

Wall ornamentation

Ancestral state reconstruction of wall ornamentation identified four states: low ornamentation, thick, very thick, and very thin. In Heliotropioideae, wall ornamentation was predominantly low ornamentation, with occasional transitions to thick conditions. Boraginoideae exhibited greater variation, with all four states present, although thick ornamentation was more frequent, while very thick and very thin conditions occurred in fewer taxa. In Cynoglossoideae, wall ornamentation was mainly thick, with limited occurrences of other states. Overall, wall ornamentation shows moderate variability across subfamilies with multiple independent transitions, indicating homoplasy in Boraginaceae (Fig. 4A).

Surface sculpturing

Ancestral state reconstruction of surface sculpturing identified three states: protruding ornamentation, irregular pattern, and weakly ornamented. In Heliotropioideae, surface sculpturing was predominantly protruding ornamentation, with occasional transitions to irregular and weakly ornamented states. Boraginoideae showed greater variation, with all three states present, although protruding ornamentation was most common, while irregular and weakly ornamented conditions occurred in several taxa. In Cynoglossoideae, surface sculpturing was mainly protruding ornamentation, with limited occurrences of other states. Overall, surface sculpturing exhibits subfamily-level variation but is dominated by protruding ornamentation, with multiple independent transitions indicating homoplasy (Fig. 7B).

Epidermal cells

Ancestral state reconstruction of epidermal cells identified three conditions: pentagonal to hexagonal, irregular/not visible, and not visible. In Heliotropioideae, epidermal cells were predominantly not visible, indicating a conserved condition within the clade. Boraginoideae showed moderate variation, with both not visible and irregular conditions being common, while the pentagonal to hexagonal state was restricted to a few taxa. In Cynoglossoideae, epidermal cells were mainly not visible, with occasional

occurrences of the other states. Overall, epidermal cell characters exhibit low variability across subfamilies, dominated by the not visible condition, with limited independent transitions (Fig. 4A).

Principal Component Analysis (PCA)

PCA analysis. Principal component analysis (PCA) was performed to evaluate variation among Boraginaceae species based on seed length (mm), seed width (mm), and length–width ratio. The relationship among these three variables is illustrated in Fig. 9C. According to the PCA results, the first two principal components (PC1 and PC2) had eigenvalues greater than 1 and were therefore considered significant. PC1 explained 61.5% of the total variation and was mainly influenced by seed length and width, indicating that this axis represents overall seed size. Species such as *Gastrocotyle hispida*, *Heliotropium crispum*, and *Anchusa arvensis* were positioned on the positive side of PC1, whereas *Euploca strigosa*, *Nonea edgeworthii*, and *Onosma dichroantha* were located on the negative side, reflecting smaller seed dimensions. PC2 accounted for 38.2% of the total variation and was primarily associated with the length–width ratio, representing seed shape (elongation). Species including *Onosma hispida*, *Heliotropium crispum*, and *Onosma dichroantha* were distributed on the positive side of PC2, indicating more elongated seeds, while *Euploca strigosa*, *Heliotropium curassavicum*, and *Lappula spinocarpos* were positioned on the negative side, reflecting relatively broader seeds. Overall, the PCA clearly separated species based on variation in seed size and shape, demonstrating the importance of these quantitative traits in distinguishing Boraginaceae taxa.

Discussion

In the present study, scanning electron microscopy (SEM) was used to investigate both macro- and micromorphological features of nutlets in Boraginaceae. In recent years, plant systematics has increasingly relied on SEM as a powerful tool for analyzing micromorphological diversity due to its ability to provide highly accurate observations despite challenges in sample preparation. The micromorphology of Boraginaceae seeds at different taxonomic levels revealed substantial variation in distinctive attributes, emphasizing the importance of seed traits for taxonomy and evolutionary interpretation (Akçin 2007; Binzet and Akcin 2011; Arabameri et al. 2014; Nasrollahi et al. 2013; Akcin 2008; Attar et al. 2021; Ebadi and Nikzat 2021). The investigated species showed considerable variation in seed morphology, including seed shape, color, texture, hilum position and level, apex, base, cell outline, surface sculpturing, and wall structures (Oran, 1996). Such variation highlights the taxonomic significance of nutlet morphology, particularly micromorphological traits, which have been previously reported as useful in Boraginaceae genera such as *Onosma* (Akçin 2007; Binzet and Akcin 2011; Arabameri et al. 2014), *Anchusa* (Nasrollahi et al. 2013), *Cynoglossum* (Akcin 2008; Attar et al. 2021), and *Lappula* (Ebadi and Nikzat 2021). In agreement with earlier studies, seed color, shape, and surface sculpturing were found to be particularly important diagnostic characters (Akcin and Binzet 2011; Arabameri et al. 2014; Ebadi and Nikzat 2021), which is strongly supported by the present findings. This study represents the first detailed investigation of nutlet micromorphology of selected Boraginaceae

species from Pakistan. The results revealed that seed shape varied widely, with obovate and ovate forms being dominant, followed by kidney-shaped, oblong, ovoid, spherical, and pyramidal forms. Seed color ranged from grey-brown and greenish-brown to dark brown, black, yellow-brown, and mustard-brown, consistent with previous findings that reported significant variation in seed coloration across Boraginaceae taxa (Akcin and Binzet 2011; Arabameri et al. 2014; Ebadi and Nikzat 2021). Seed texture was predominantly smooth but also included scabrate, baculate, echinate, rough, and slightly smooth conditions, further supporting the high diversity of surface features observed in earlier studies (Nasrollahi et al. 2013; Attar et al. 2021).

Micromorphological traits such as cell outline, surface sculpturing, and wall ornamentation also showed considerable diversity (Otero et al., 2014). Cell outlines ranged from random and protuberant to organized rows, while surface sculpturing included gemmate, papillate, reticulate, granulate, verrucose, striate, fossulate, and irregular patterns (Ovchinnikova, 2006). Wall ornamentation varied from very thick to very thin, confirming previous observations that seed surface micromorphology is highly variable and taxonomically informative in Boraginaceae (Arabameri et al. 2014; Attar et al. 2021; Ebadi and Nikzat 2021). In contrast, anticlinal and periclinal walls, as well as epidermal cells, were often not visible, although in some species they were expressed as smooth, undulate, convex, or thick-raised structures (Ovchinnikova, 2009). When interpreted within a phylogenetic framework, the ancestral state reconstruction demonstrated that most seed characters exhibit distinct subfamily-level patterns combined with high variability (Özkan et al., 2009). Seed shape showed that Heliotropioideae is dominated by irregular forms, while Boraginoideae is characterized mainly by ovate seeds, and Cynoglossoideae exhibits a mixture of ovate and rounded forms, indicating repeated evolutionary transitions across lineages (Rasul and Ahmad, 2012). Similarly, seed color displayed medium brown dominance in Heliotropioideae, whereas Boraginoideae and Cynoglossoideae showed broader variation including light and dark colors, supporting earlier findings that seed color is highly variable and taxonomically informative (Akcin and Binzet 2011; Arabameri et al. 2014).

Seed texture also followed clear evolutionary trends, with moderately ornamented seeds dominating in Heliotropioideae, high variability in Boraginoideae, and predominantly smooth seeds in Cynoglossoideae, which is consistent with previous reports emphasizing variation in seed surface structures (Nasrollahi et al. 2013; Attar et al. 2021). Reproductive traits such as the seed apex and base further supported these patterns (Retief and Van Wyk, 1997). The apex was predominantly acute in Heliotropioideae, while Boraginoideae exhibited both acute and rounded forms, and Cynoglossoideae was mainly rounded. In contrast, the seed base was largely rounded across all subfamilies, indicating a relatively conserved character.

Hilum characteristics exhibited comparatively higher stability (Sadia et al., 2024). The hilum position was predominantly central in Heliotropioideae and Boraginoideae, while Cynoglossoideae showed additional variation, including slightly terminal states, suggesting moderate evolutionary flexibility. Similarly, hilum level was mainly raised across all subfamilies, indicating a conserved structural feature (Selvi and Bigazzi, 2003). Micromorphological characters revealed complex evolutionary patterns. Cell outline was

predominantly unorganized in Heliotropioideae, while Boraginoideae displayed all states, and Cynoglossoideae was mainly characterized by modified surfaces (Selvi et al., 2006). The central line was mostly absent in Heliotropioideae, whereas Boraginoideae and Cynoglossoideae showed mixed conditions, with the prominent glochidiate state restricted to a few taxa (Selvi et al., 2019). The ventral keel exhibited all three states (present, absent, and middle), particularly in Boraginoideae, indicating high variability (Shinwari et al., 2017). Similarly, prickles/tubercles were mostly absent in Heliotropioideae, while Boraginoideae showed moderate variation, and Cynoglossoideae exhibited a higher frequency of presence, reflecting repeated evolutionary changes. Structural traits such as periclinal and anticlinal walls showed relatively low variability, with the not visible and undulate states dominating, respectively (Song et al., 2005). Wall ornamentation and surface sculpturing exhibited moderate variation, with protruding ornamentation and thick walls being most common, consistent with previous studies (Arabameri et al. 2014; Attar et al. 2021; Ebadi and Nikzat 2021).

Epidermal cells were predominantly not visible across most taxa, with pentagonal to hexagonal and irregular forms observed only in a few species, suggesting limited taxonomic importance compared to other characters (Veloso et al., 2017). At the species level, our findings showed both agreement and differences with previous studies. The seed morphology of *Onosma*, *Anchusa*, *Cynoglossum*, and *Lappula* generally agrees with earlier reports, particularly in surface sculpturing and wall ornamentation (Akçin 2007; Nasrollahi et al. 2013; Akcin 2008; Ebadi and Nikzat 2021). However, discrepancies in seed shape, cell outline, and epidermal structures were observed, which may reflect regional variation in Pakistani taxa or intraspecific diversity (Weigend et al., 2010). Overall, the results indicate that most seed morphological and micromorphological traits in Boraginaceae exhibit high variability and heterogeneous distribution across subfamilies, with clear evidence of multiple independent evolutionary transitions (homoplasy) (Weigend et al., 2013). While some traits, such as hilum position and level, show relative conservatism, most characters appear to be influenced by ecological and adaptive factors rather than strict phylogenetic constraints (Yu et al., 2012). These findings confirm that seed morphology is highly valuable for species-level identification and provides important insights into the evolutionary and taxonomic diversity of Boraginaceae.

Taxonomic Key to the Studied Boraginaceae Species (Based on Seed Characters)

1

1. a. Seed strongly ornamented (echinate / protruding / rough) …… 2
2. b. Seed smooth or moderately ornamented …… 7

2

1. a. Central line with glochids or tubercles …… 3
2. b. Central line absent …… 4

3

1. a. Central line prominent with glochids; epidermal cells pentagonal–hexagonal …… *Arnebia decumbens*
2. b. Central line with tubercles; epidermal cells not visible …… *Buglossoides arvensis*

4

1. a. Seed ovate …… 5
2. b. Seed irregular or rounded …… 6

5

1. a. Apex specialized; epidermal cells pentagonal–hexagonal …… *Nonea caspica*
2. b. Apex rounded; epidermal cells not visible …… *Gastrocotyle hispida*

6

1. a. Seed irregular; light colored; very thick wall …… *Trichodesma indicum*
2. b. Seed rounded; medium brown; weakly ornamented …… *Onosma hispida*

7

1. a. Seed shape ovate …… 8
2. b. Seed shape irregular or rounded …… 13

8

1. a. Hilum slightly terminal …… *Asperugo procumbens*
2. b. Hilum central or terminal …… 9

9

1. a. Central line present …… *Onosma bracteosa*
2. b. Central line absent …… 10

10

1. a. Seed smooth …… 11
2. b. Seed moderately ornamented …… 12

11

1. a. Base elongated …… *Heliotropium curassavicum*
2. b. Base round …… *Lappula spinocarpos*

12

1. a. Hilum terminal …… *Heliotropium europaeum*
2. b. Hilum central; depressed …… *Nonea edgeworthii*

13

1. a. Seed rounded …… 14
2. b. Seed irregular …… 16

14

1. a. Central line present …… 15
2. b. Central line absent …… *Cynoglossum lanceolatum*

15

1. a. Surface irregular pattern …… *Onosma dichroantha*
2. b. Surface weakly ornamented …… *Euploca strigosa*

16

1. a. Seed strongly ornamented …… *Heliotropium crispum*
2. b. Seed moderately ornamented …… 17

1

1. a. Hilum depressed …… *Heliotropium supinum*
2. b. Hilum raised …… 18

18

1. a. Color medium brown …… *Heliotropium bacciferum*
2. b. Color dark …… 19

19

1. a. Ventral keel middle …… *Alkanna tinctoria*
2. b. Ventral keel absent …… *Anchusa arvensis*

Conclusion

The present study provides the first comprehensive investigation of the seed macro- and micromorphology of selected Boraginaceae species from Pakistan using scanning electron microscopy (SEM) and phylogenetic approaches. The results revealed substantial variation in seed characters,

including shape, color, texture, hilum position and level, cell outline, surface sculpturing, and wall structures, demonstrating their importance for species identification and taxonomic delimitation within the family. Ancestral state reconstruction showed that most seed traits exhibit heterogeneous distribution across subfamilies, with clear differences among Heliotropioideae, Boraginoideae, and Cynoglossoideae. Traits such as seed shape, color, and texture displayed high variability, whereas hilum position and level were relatively conserved. Phylogenetic signal analysis further indicated that most characters possess low phylogenetic signal, suggesting multiple independent evolutionary transitions (homoplasy) and highlighting the evolutionary plasticity of seed morphology in Boraginaceae. Overall, the integration of morphological and phylogenetic analyses demonstrates that seed characters are highly valuable for species-level identification but show limited consistency at higher taxonomic levels. This study enhances our understanding of seed diversity and evolutionary patterns in Boraginaceae and provides a foundation for future studies that integrate morphological, molecular, and ecological data to improve systematic and evolutionary interpretations.

Limitations of the Study

This study provides insights into the seed morphology and micromorphology of Pakistani Boraginaceae; however, the results should be interpreted within the scope of the sampled taxa. The analysis was limited to 20 species and partly based on available specimens for morphological and SEM examination. Future studies incorporating broader taxon sampling, fresh material, and integrated molecular datasets may provide a more comprehensive understanding of seed trait evolution in Boraginaceae.

Declarations

The authors confirm that all plant materials were legally collected, correctly identified, properly vouchered, and deposited in a publicly accessible herbarium. Complete collection and voucher information is provided in Table S1 to ensure transparency, reproducibility, and future verification of the studied material.

Conflict of Interest

The authors declared no conflict of interest.

Ethics approval and consent to participate

Dr. Rhat Ullah, Assistant Professor, Ghazi Umara Khan Degree College, Samarbagh, Dir Lower, Khyber Pakhtunkhwa, Pakistan, identified all plant specimens used in this study. Plant materials and seeds were collected from natural populations in different localities of Khyber Pakhtunkhwa, Pakistan. Detailed information on species names, collection localities, habitats, geographic coordinates, collector names, collection dates, voucher numbers, and herbarium accession details is provided in Table S1 (Plant species, localities, habitats, and voucher information). Voucher specimens of all studied taxa were prepared and deposited in the Ghazi Umara Khan Degree College Herbarium, Samarbagh, Dir Lower,

Khyber Pakhtunkhwa, Pakistan, where they are available for future reference and verification. Permission for field sampling and plant collection was obtained from relevant local authorities and landowners where required. No endangered, protected, or legally restricted species were collected during this study. All plant collections and research activities complied with applicable institutional, local, and national guidelines and regulations for the collection, handling, and study of wild plant materials in Pakistan. The study was conducted in accordance with accepted ethical standards for botanical and biodiversity research.

Consent for publication

Not applicable.

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Author Contribution

Shakir Ullah conceived and designed the study, conducted the experiments, performed data analysis, and wrote the original draft of the manuscript. Lubna Shakir assisted in data collection and contributed to manuscript writing and revision. Mushtaq Ahmad provided supervision, conceptual guidance, and critical revision of the manuscript. Ghani Subhan contributed to fieldwork, specimen collection, and data interpretation. Mohammad Sohail, Falak Naz, Azmat Noreen, Sajid Ali, and Hasnain Khan assisted in laboratory work, data processing, and compilation of results. Naveen Dilawar contributed to data analysis and manuscript editing.

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Data availability

The datasets generated and/or analyzed during the current study are not publicly available due to ongoing related research, but are available from the corresponding author upon reasonable request.

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Figures

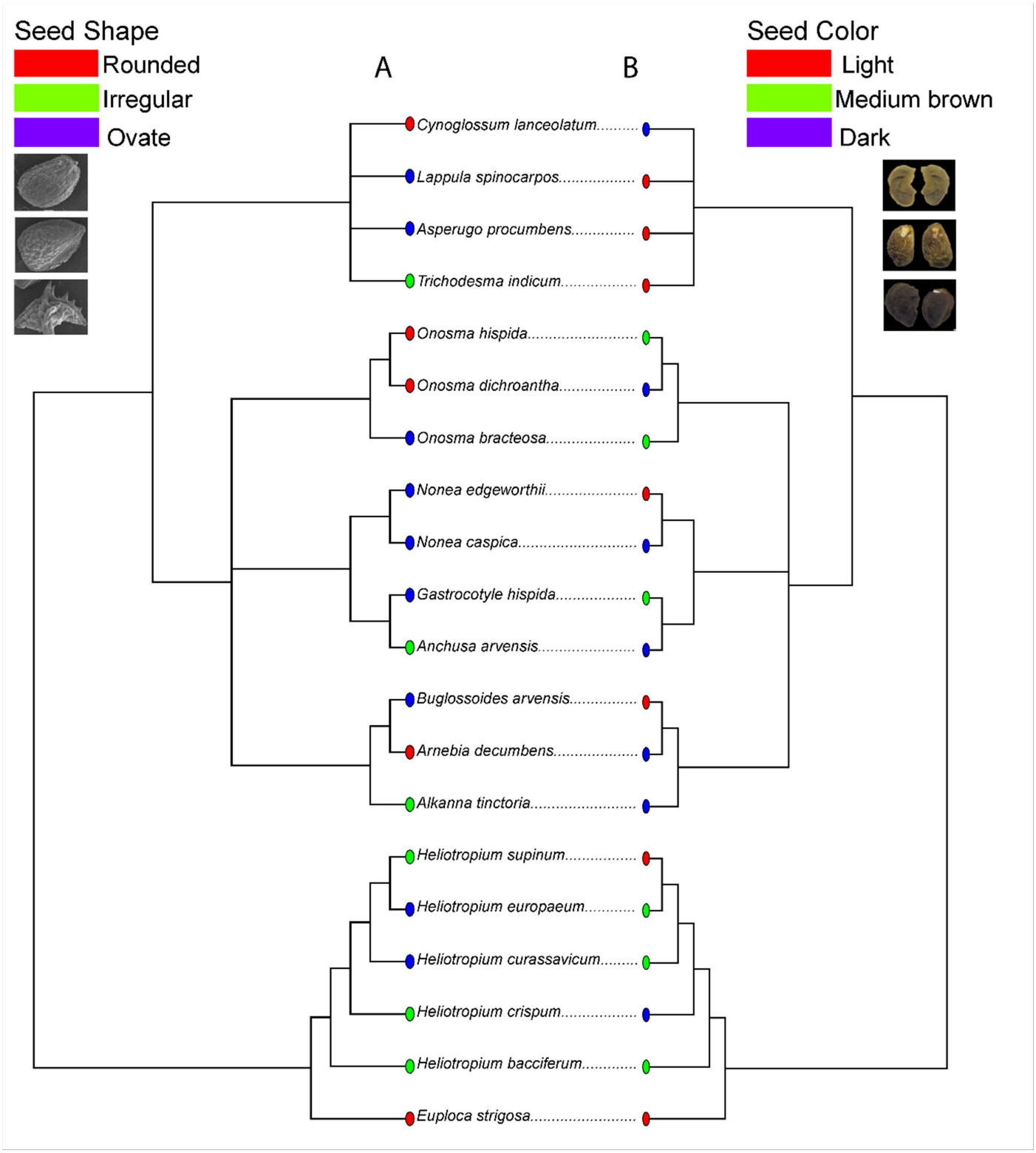


Figure 1

Ancestral state reconstruction of seed characters in Boraginaceae: (A) seed shape; (B) seed color. Tip colors indicate observed states, and node pie charts represent likelihoods under the ER model.

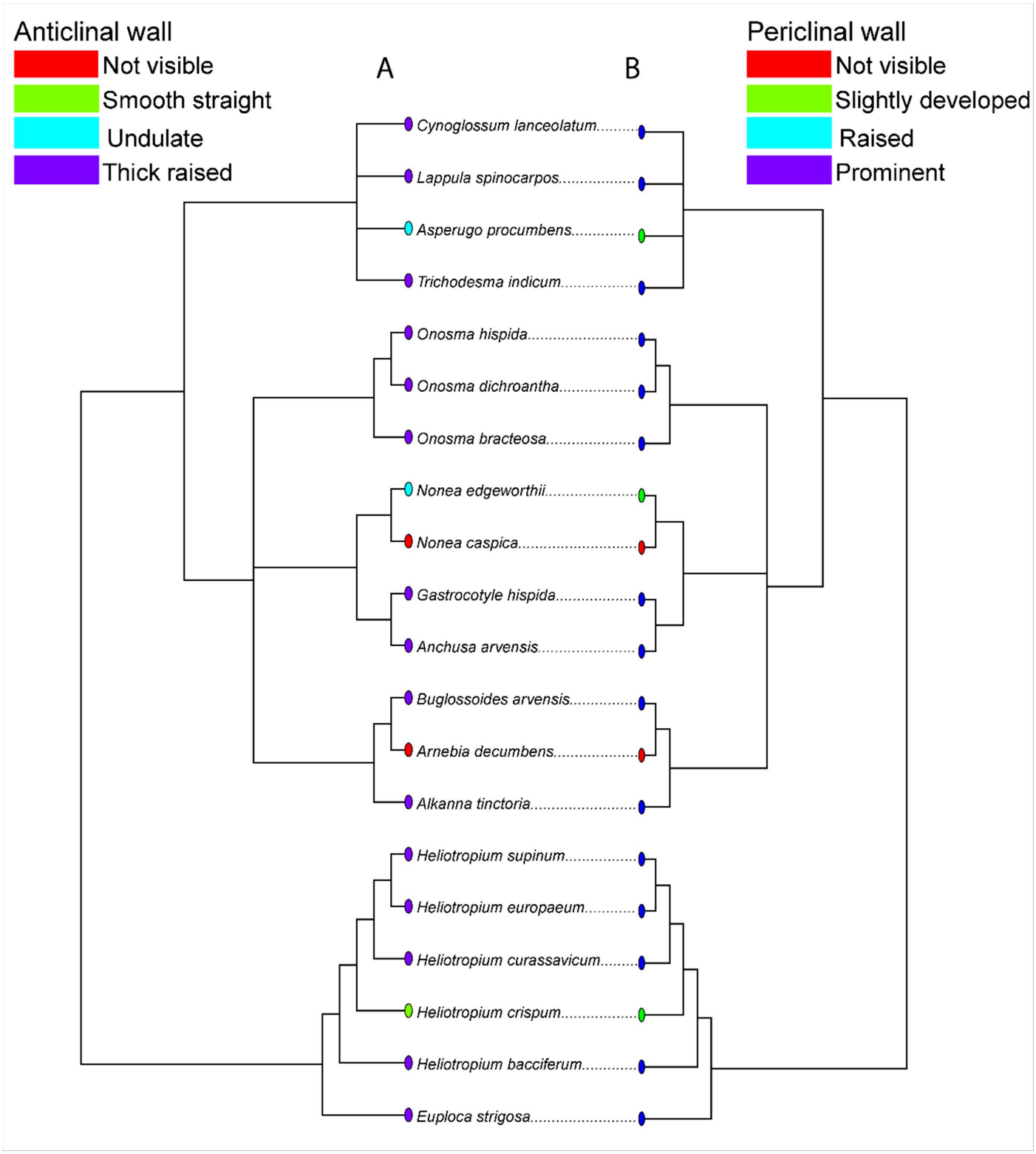


Figure 2

Ancestral reconstruction of seed coat epidermal characters: (A) anticlinal wall structure; (B) periclinal wall ornamentation. Tip states are shown by colors, and node pie charts indicate marginal likelihoods under the ER model.

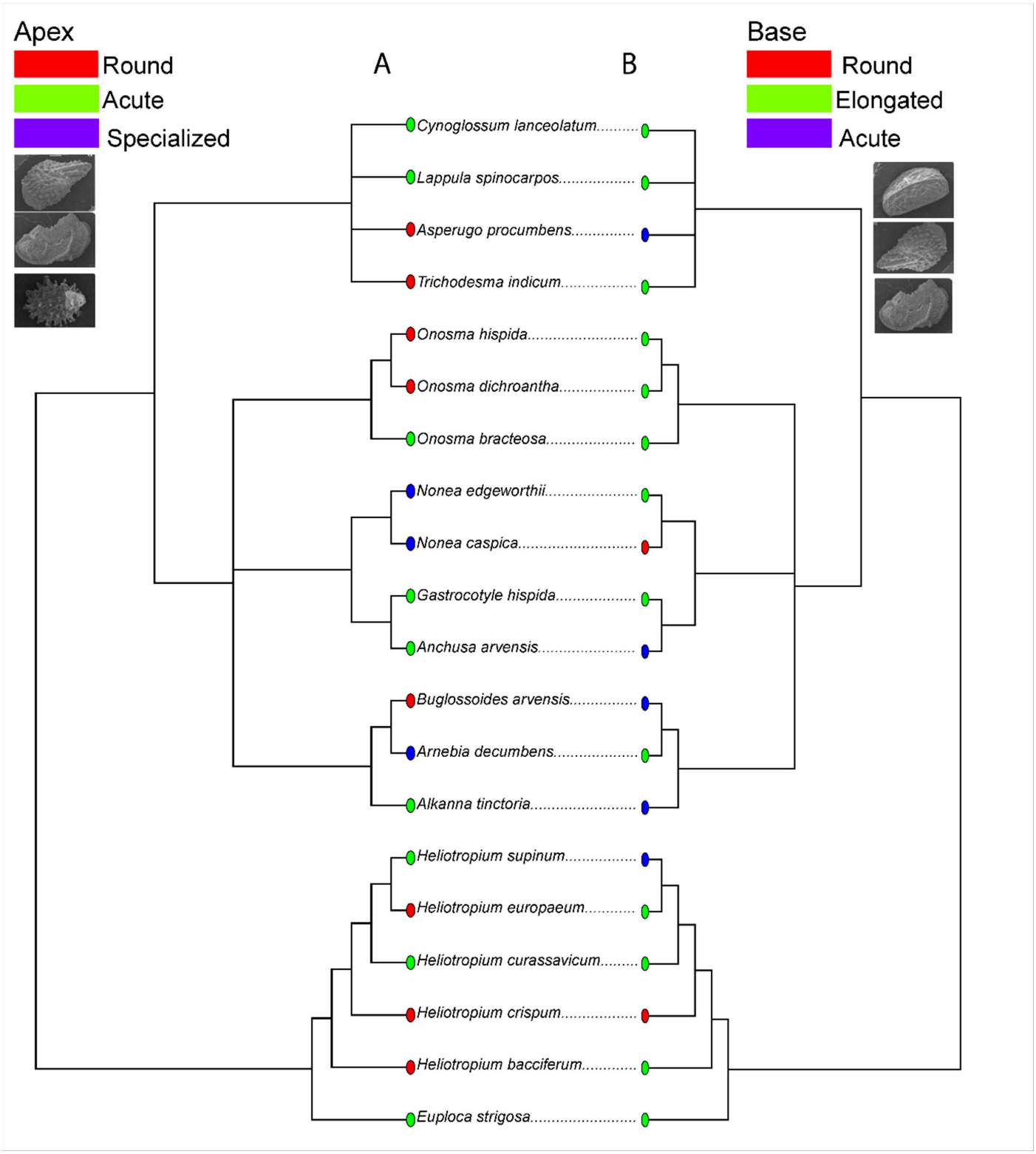


Figure 3

Ancestral state reconstruction of seed characters in Boraginaceae: (A) seed apex; (B) seed base. Tip colors indicate observed states, and node pie charts represent likelihoods under the ER model.

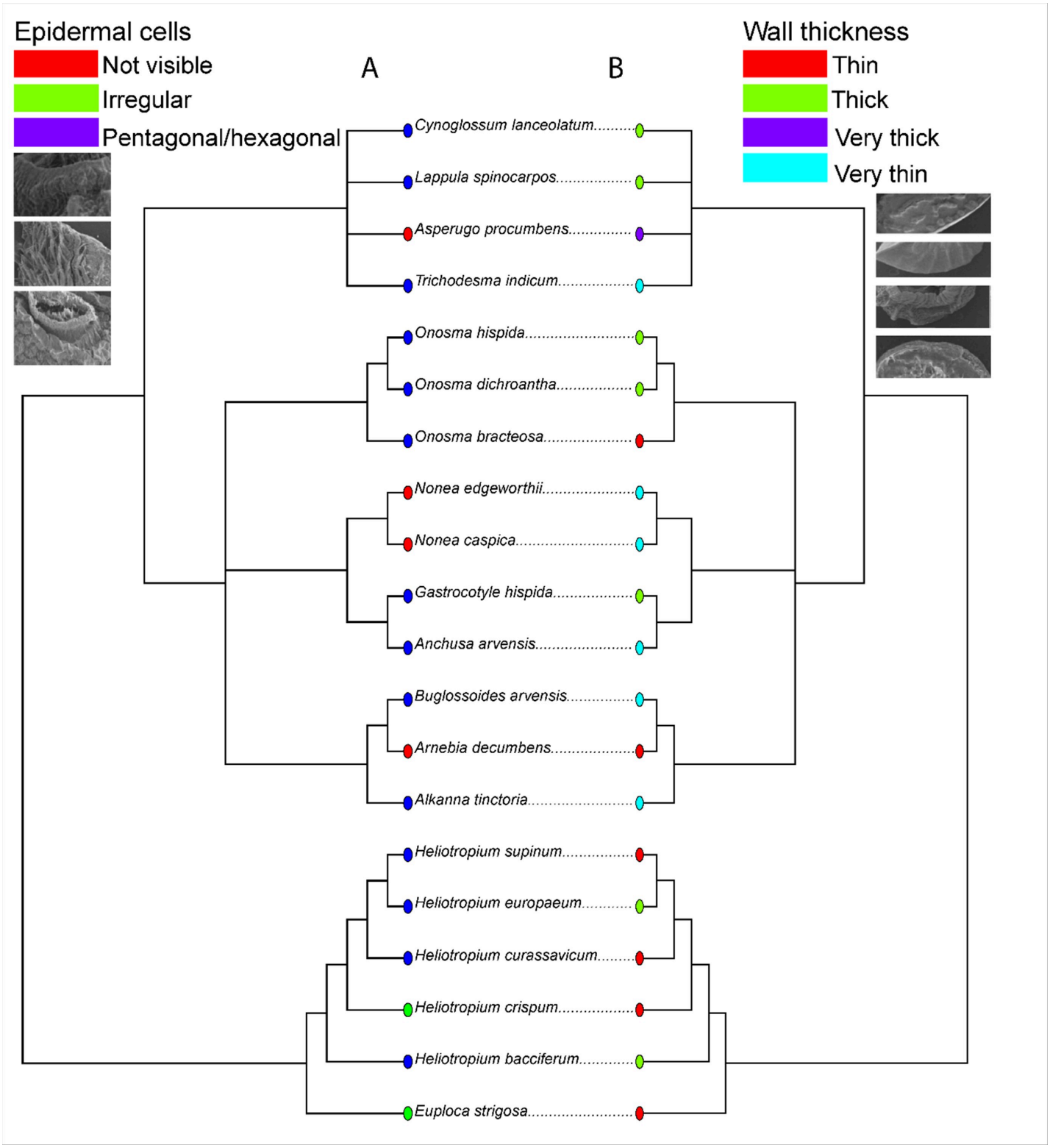


Figure 4

Ancestral state reconstruction of seed coat anatomy: (A) epidermal cell type; (B) wall thickness. Tip states are indicated by colors, and node pie charts represent marginal likelihoods under the ER model.

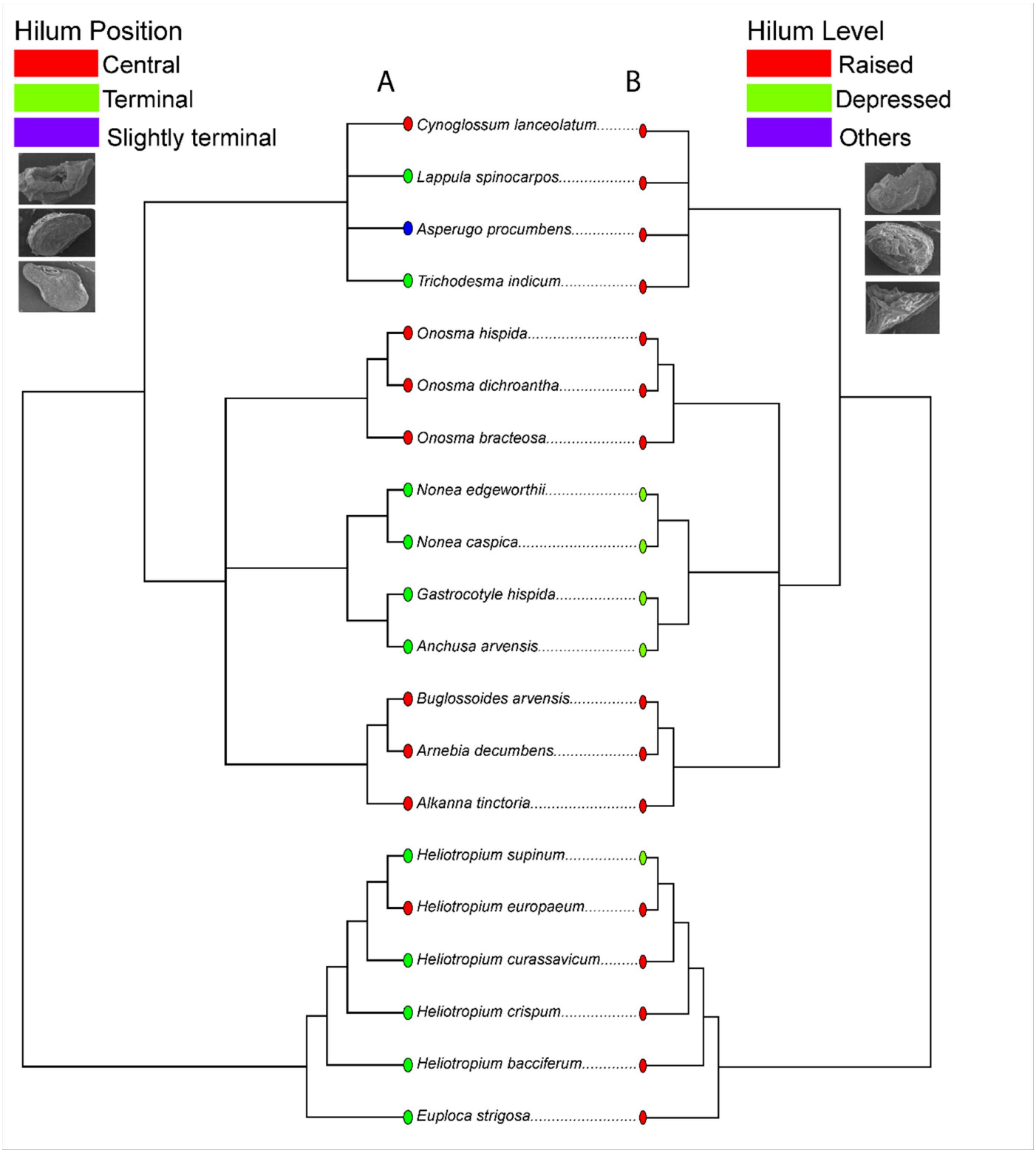


Figure 5

Ancestral reconstruction of seed hilum traits: (a) hilum position; (b) hilum level. Tip states are shown by colors, and node pie charts represent marginal likelihoods under the ER model.

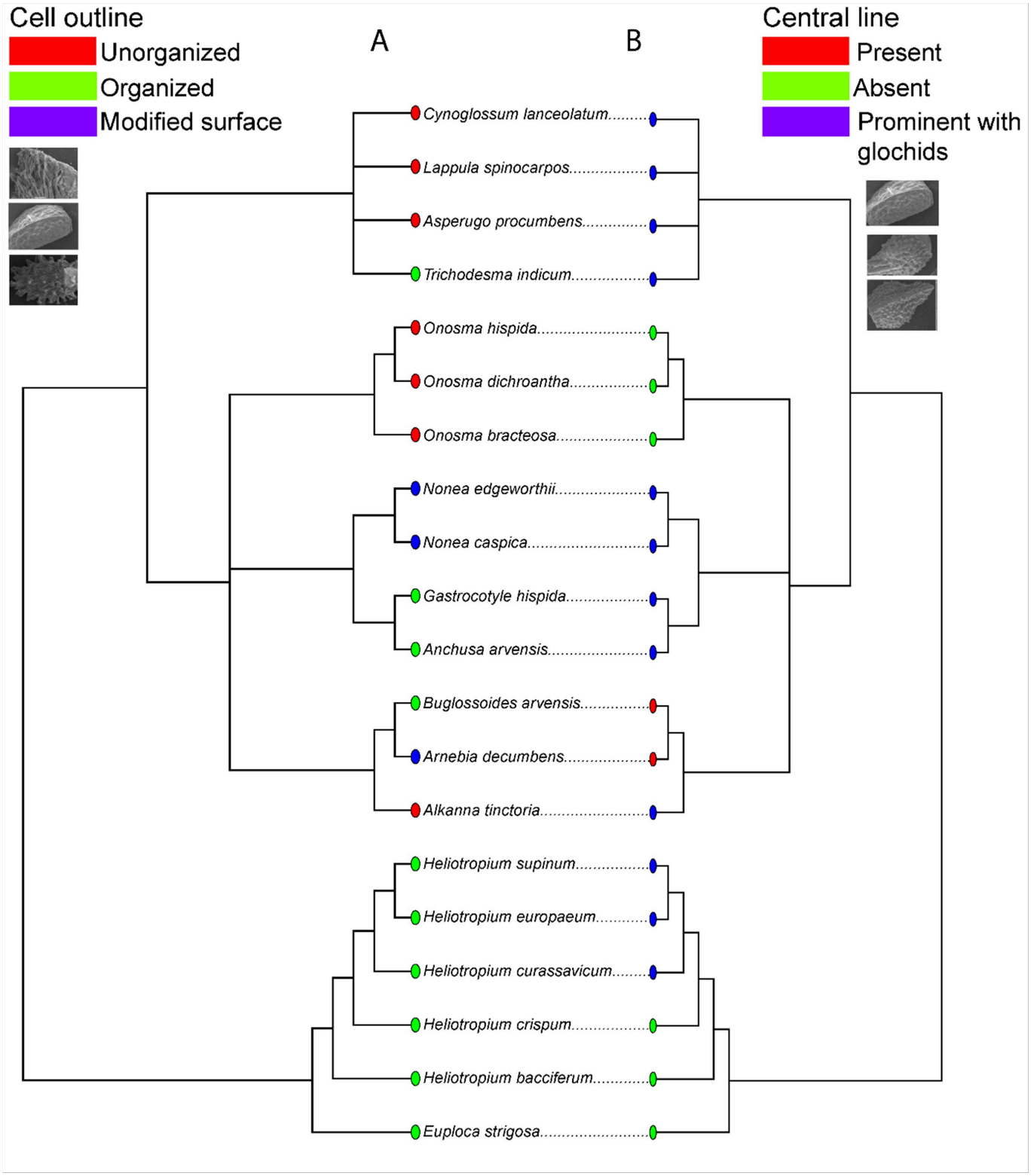


Figure 6

Ancestral reconstruction of seed epidermal micromorphology: (a) cell outline; (b) central line. Tip states are shown by colors, and node pie charts represent marginal likelihoods under the ER model.

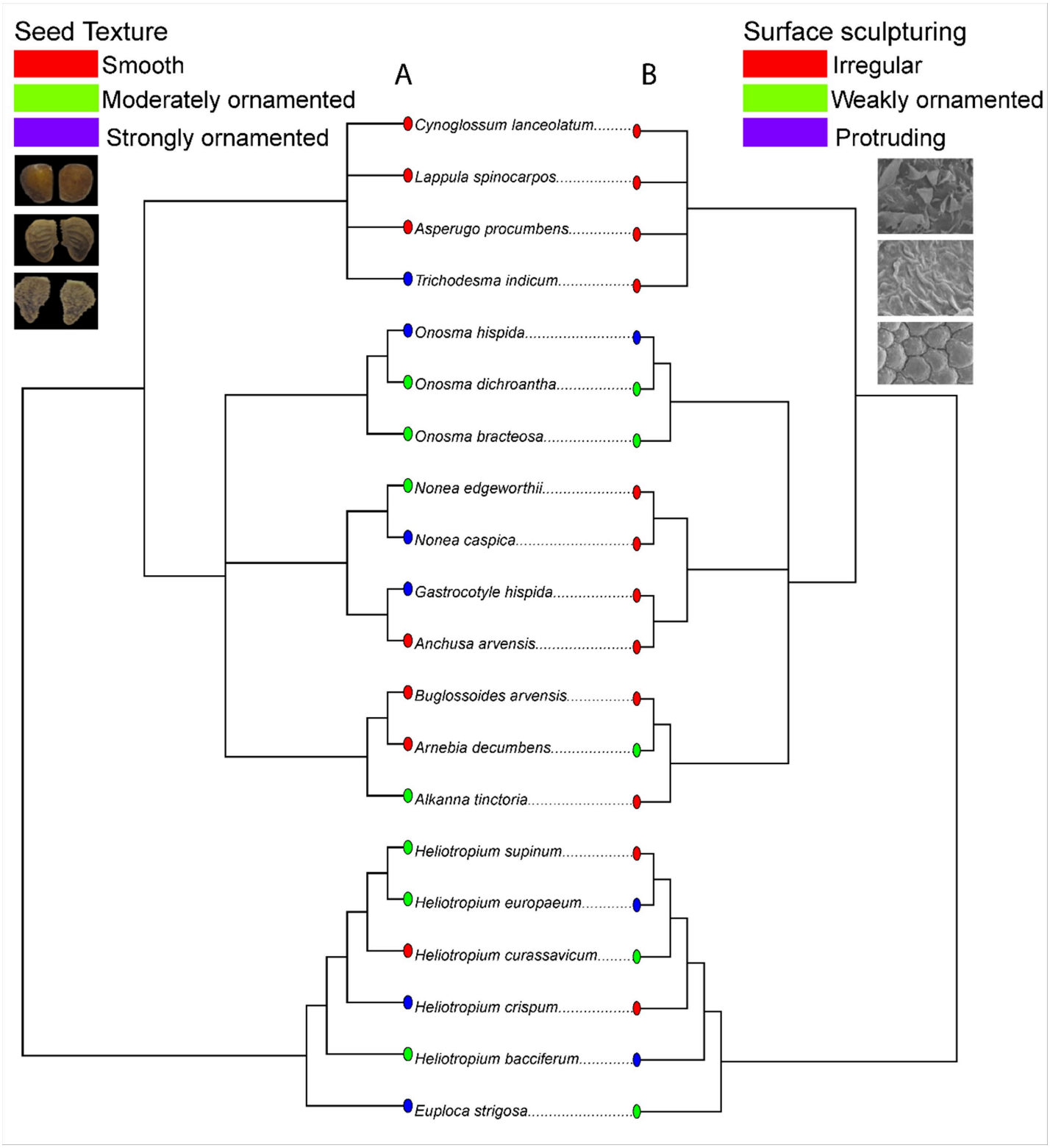


Figure 7

Ancestral reconstruction of seed surface traits: (a) seed texture; (b) seed surface sculpturing. Tip states are shown by colors, and node pie charts represent marginal likelihoods under the ER model.

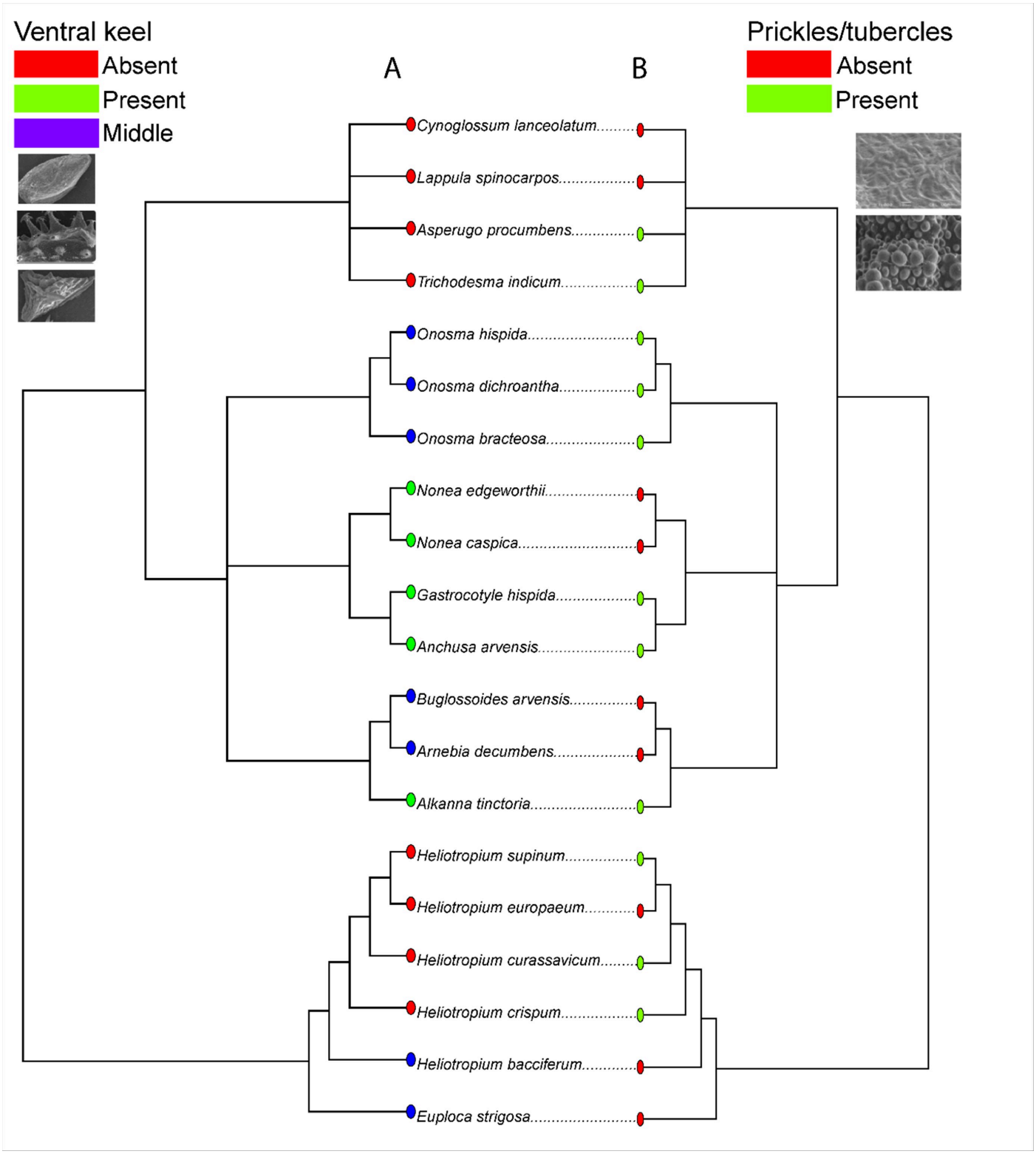


Figure 8

Ancestral reconstruction of seed micromorphological traits: (a) ventral keel; (b) prickles/tubercles. Tip states are shown by colors, and node pie charts represent marginal likelihoods under the ER model.

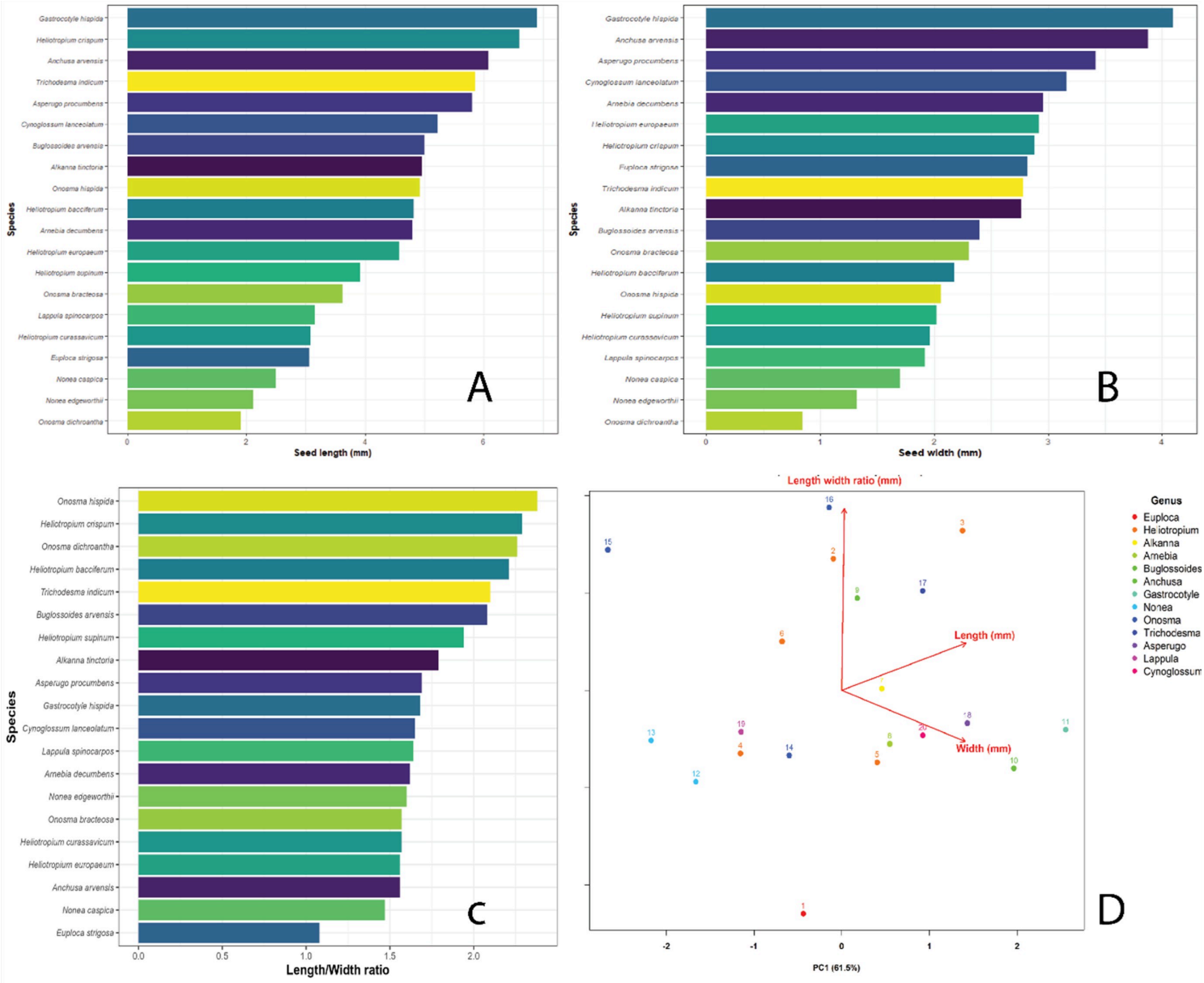


Figure 9

Seed morphometric variation in Boraginaceae: (a) seed length; (b) seed width; (c) seed length-to-width ratio; (d) principal component analysis (PCA) showing multivariate differentiation among species.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table20260520.docx