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Research Article

Integrative Taxonomy of *Diplazium* Sw. (Athyriaceae) in Peninsular Malaysia: Insights from Morphological and Spore Ornamentation Data

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ABSTRACT

The genus *Diplazium* Sw. (Athyriaceae) exhibits significant morphological diversity and complex taxonomy in Peninsular Malaysia. Building upon previously published anatomical and molecular phylogenetic data, this study integrates detailed morphological traits and novel spore ornamentation analyses to provide a comprehensive regional taxonomic assessment. Morphological data were collected from field and herbarium specimens, while spore surface ornamentation was examined using scanning electron microscopy on selected species representing key subclades identified through combined molecular datasets (plastid markers: *rbcL*, *atpB*, *atpA*, *trnL-F*; nuclear marker: ITS) as well as anatomical datasets from previous studies. Phylogenetic analyses based on these previously published molecular and anatomical data robustly support the monophyly of *Diplazium* species in Peninsular Malaysia. Morphological traits alone were insufficient to fully resolve species boundaries due to overlaps with related genera such as *Athyrium*, *Deparia*, and *Cornopteris*. Spore ornamentation revealed five distinct micro-morphological patterns that provide additional diagnostic characters, enhancing species delimitation and complementing classical morphological and molecular approaches. A dichotomous key to the *Diplazium* species of Peninsular Malaysia is also provided in supplementary material. This study presents an integrative taxonomic framework for *Diplazium* in Peninsular Malaysia by combining previously published molecular and anatomical phylogenies with new spore micromorphological data. The addition of spore ornamentation as supplementary evidence strengthens taxonomic resolution and supports future floristic, conservation, and evolutionary research in this biodiversity hotspot.

Key words: *Diplazium*, morphology, Peninsular Malaysia, spore ornamentation, taxonomy

INTRODUCTION

The genus *Diplazium* Sw., belonging to the family Athyriaceae, represents a taxonomically challenging and ecologically significant group of ferns with a global distribution, especially prominent in tropical and subtropical regions. Since its initial description, *Diplazium* has been the subject of extensive taxonomic scrutiny due to its remarkable morphological diversity and phenotypic plasticity, which have historically complicated its systematic placement. Early classifications predominantly relied on morphological characteristics such as the distinctive sori arrangement, presence of false indusia, venation patterns, and rhizome structure. However, these traits often exhibited considerable overlap with allied genera such as *Athyrium* and *Deparia*, leading to conflicting genus delimitations and a fragmented taxonomic framework (Christensen, 1938; Copeland, 1947). To provide a comprehensive overview of the historical taxonomic treatments and classifications of *Diplazium*, a synopsis of key previous classifications is summarized in Table 1. This table highlights the evolving perspectives on genus delimitation and species concepts, illustrating the complexity and dynamic nature of *Diplazium* taxonomy.

In the mid-20th century, taxonomic interpretations of *Diplazium* underwent significant revisions. Copeland (1947) controversially subsumed *Diplazium* under *Athyrium*, advocating for a broad genus concept that emphasized shared morphological features at the expense of finer diagnostic characters. This treatment was met with resistance by subsequent taxonomists, notably Holttum (1947) and Alston (1956), who reinstated *Diplazium* as a distinct genus based on consistent differences in sorus morphology, indusium structure, and rhizome anatomy. These morphological re-evaluations underscored the necessity for integrative approaches to resolve genus boundaries.

The incorporation of cytogenetic data represented a pivotal advancement in elucidating the taxonomy of *Diplazium*. In the study of Manton and Sledge (1954) cytological studies in Sri Lankan ferns revealed a chromosome base number of n=41 for *Diplazium*, distinct from the n=40 observed in *Athyrium*, providing compelling genetic evidence for their separation. Cytogenetic analyses have since illuminated the role of polyploidy and chromosomal rearrangements in the diversification of *Diplazium*, emphasizing the importance of karyological characters in systematic fern biology.

The advent of molecular phylogenetics in the late 20th and early 21st centuries revolutionized the understanding of *Diplazium*'s evolutionary relationships. Studies utilizing chloroplast gene regions, notably *rbcL* and *trnL-F*, have consistently

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confirmed the monophyly of *Diplazium* and clarified its phylogenetic placement within Athyriaceae (Sano *et al.,* 2000; Wang *et al.,* 2003). These molecular phylogenies resolved previous ambiguities, including the placement of closely related genera such as *Diplaziopsis* and *Allantodia*, which were shown to be either nested within or sister to *Diplazium*, prompting a re-evaluation of generic limits and infrageneric classifications. Wang *et al.* (2004) proposed a revised familial framework for Athyriaceae, designating *Diplazium* within the subfamily Diplazioideae, thus reflecting its distinct evolutionary lineage corroborated by both morphological and molecular evidence.

The contemporary classification endorsed by the Pteridophyte Phylogeny Group I (PPG I, 2016) has stabilized *Diplazium* as a valid genus encompassing approximately 400 species worldwide. This taxonomic consensus integrates traditional morphological taxonomy with cutting-edge molecular and cytogenetic data, highlighting the genus's evolutionary significance. Importantly, *Diplazium* species serve as valuable genetic resources due to their wide ecological amplitude (Li *et al.,* 2025), diverse reproductive strategies (Cicuzza, 2021), and potential for adaptation to environmental stresses (Sareen *et al.,* 2019), rendering them relevant for studies on plant evolution, biodiversity conservation, and crop improvement, particularly in the context of abiotic stress tolerance.

Table 1. Summary of historical and contemporary classification systems involving the genus *Diplazium*, showing shifts in taxonomic placement and rationale

Author(s) & Year	Higher Taxonomy Placement	Treatment of *Diplazium*	Basis of Classification	Notes
Christensen (1938)	Polypodiaceae, Subfam. Asplenioideae, Tribe Athyrieae	Recognized *Diplazium*, *Athyrium*, *Cystopteris*	Morphological (sorus, rhizome, vascular bundles)	Early broad classification under large Polypodiaceae
Ching (1940)	Aspleniaceae, Athyrieae	*Diplazium* recognized, linear sorus type	Morphological (sorus shape, indusium, rhizome scale)	Distinguished *Diplazium* by sorus type and sporangium features
Copeland (1947)	Aspidiaceae	Lumped many genera into *Athyrium*, including *Diplazium*	Morphology (broad genus concept)	Considered *Diplazium* insufficiently distinct
Holttum (1947)	Dennstaedtiaceae, Athyrioideae	Treated *Diplazium* under *Athyrium*	Morphology (rhizome, frond dissection)	Lacked clear morphological separation
Alston (1956)	Athyriaceae (newly described)	Recognized both *Athyrium* and *Diplazium*	Morphology	First to define Athyriaceae as a separate family
Ching (1964)	Athyriaceae	Separated *Diplazium* and *Athyrium*, total 9 genera	Morphology + Cytology	Used spore count and clear genus boundaries
Nayar (1970)	Dennstaedtiaceae, Subfam. Athyrioideae	*Diplazium* in *Athyrium* group	Cytology, gametophyte morphology	Phylogenetic approach to homosporous ferns
Tryon and Tryon (1982)	Dryopteridaceae, Tribe Physematieae	*Diplazium* recognized	Morphology, cytology	Included *Diplazium* with *Athyrium*, *Cystopteris*
Wang *et al.* (2003; 2004)	Athyriaceae	*Diplazium* monophyletic; suggested 5 subfamilies	Molecular (trnL-F, rbcL), morphology	Supported *Diplazium* as distinct clade (Diplazioideae)
Smith *et al.* (2006)	Woodsiaceae (broad circumscription)	*Diplazium* included with *Cystopteris*, *Athyrium*	Combined molecular & morphological data	Later split by Rothfels et al. (2012)
Rothfels *et al.* (2012)	Athyriaceae reinstated	*Diplazium* in own family (Athyriaceae)	Molecular phylogenetics	Split Woodsiaceae into 5 families
PPG I (2016)	Athyriaceae	*Diplazium*, *Athyrium*, and *Deparia* accepted as core genera	Monophyly using multilocus DNA data	Recognized ~650 spp. in Athyriaceae; accepted monophyly

Recent advances in molecular phylogenetics have reshaped our understanding of the genus *Diplazium*, revealing complex evolutionary relationships that are often incongruent with morphology-based classifications alone (Sano *et al.,* 2000; Wang *et al.,* 2003; Adjie *et al.,* 2008; Wei & Zhang, 2020). In Peninsular Malaysia, *Diplazium* represents a taxonomically challenging group due to its high morphological variability (Fig.1). While molecular studies confirm the monophyly of *Diplazium* in Peninsular Malaysia (Aliah *et al.,* 2024), morphological evidence alone has proven insufficient for clear species delimitation, with some species from related genera nested within *Diplazium* clades (Sano *et al.,* 2000; Wang *et al.,* 2004). Therefore, an integrative taxonomic approach, combining detailed morphological analysis, spore ornamentation via scanning electron microscopy, combined with previously established anatomical and molecular data, is essential to resolve species boundaries and clarify phylogenetic relationships within this genus. This study aims to apply such an integrative framework to *Diplazium* in Peninsular Malaysia, providing an updated taxonomy supported by multiple lines of evidence, and facilitating more accurate identification and conservation strategies for this diverse fern group.

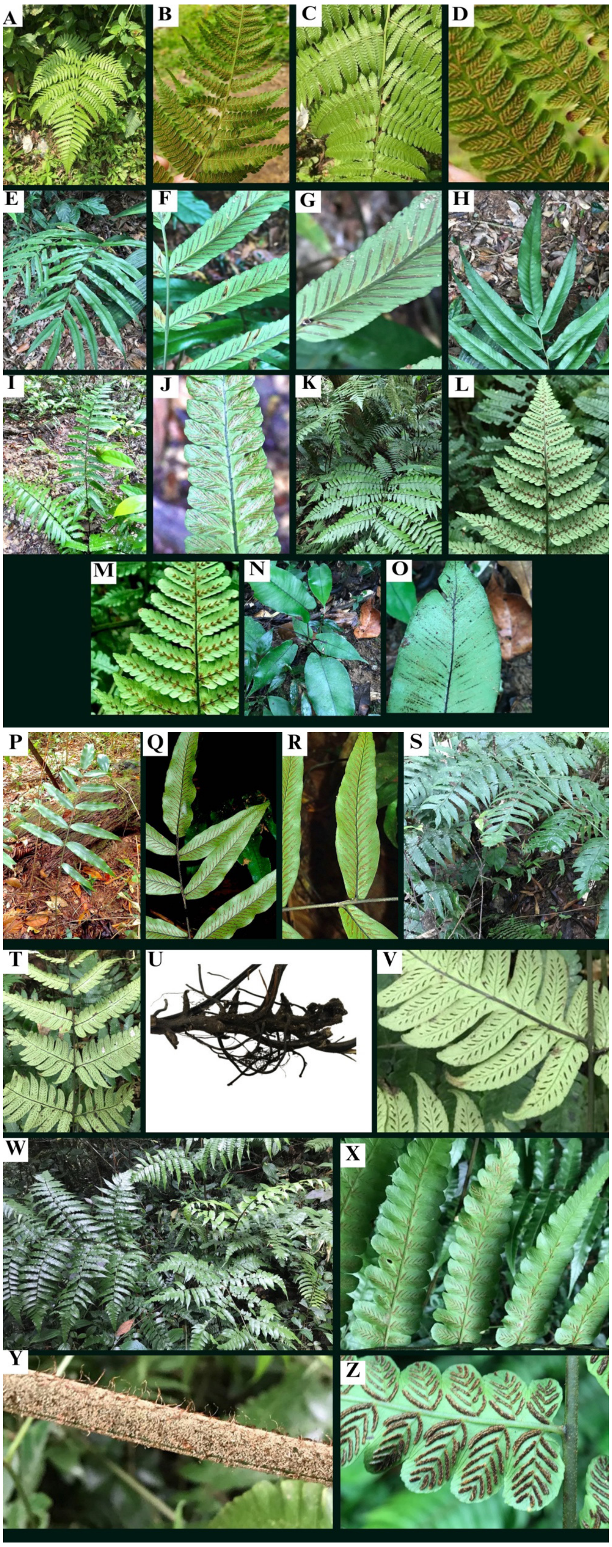

Fig. 1. Examples of morphological diversity in *Diplazium* taxa from in this study A–D: *D. polypodioides*. E–H: *D. pallidum* var. *montanum*. I&J: *D. malaccense*. K–M: *D. latisquamatum*. N&O: *D. cordifolium* var. *cordifolium*. P–R: *D. angustipinna*. S-T: *D. kunstleri.* U: Creeping rhizome of *D. procumbens*. V: *D. kunstleri*. W&X: *D. procumbens*. Y&Z: D. *simplicivenium.*

MATERIALS AND METHODS

Plant Materials

A total of 31 taxa from the Athyriaceae family were successfully sampled across Peninsular Malaysia, out of the 38 taxa known to occur in the region (Parris & Latiff, 1997, Maideen *et al.,* 2019). Detailed information on collectors, localities, and herbarium codes is presented in Supplementary Material (S1). Field collections were conducted across various habitats throughout Peninsular Malaysia. For species that were difficult to obtain in the field, herbarium specimens were examined and borrowed from the Herbarium of Universiti Kebangsaan Malaysia (UKMB), the Herbarium of the University of Malaya (KLU), the

Forest Research Institute Malaysia Herbarium (KEP), and the Singapore Botanic Gardens Herbarium (SING). Images of type specimens were accessed from the SING and Kew (K) online herbarium databases. All collected specimens were deposited at the UKMB Herbarium.

Morphological Observations

Morphological observations were carried out on rhizomes, stipes, scales, frond morphology, sori arrangement, and indusium type. These characters (Supplementary Material 2) were examined using a Meiji EMZ-5TRD stereo microscope. Observations were based on both fresh specimens collected during fieldwork and herbarium specimens (Supplementary Material 3). Particular attention was paid to characters relevant for generic and species delimitation within Athyriaceae.

Spore Ornamentation Analysis

Spore morphology was examined using field emission scanning electron microscopy (FESEM) to assess micro-morphological variation among selected *Diplazium* species: *D. kunstleri*, *D. latisquamatum*, *D. procumbens*, *D. malaccense*, *D. sorzogonense*, *D. tomentosum*, *D. esculentum*, and *D. crenatoserratum*. These species were chosen as representatives of distinct subclades identified in the phylogenetic analysis by Aliah et al. (2024). For FESEM analysis, mature spores were directly mounted without chemical treatment onto aluminum stubs using adhesive carbon tape. Samples were then coated with a thin layer of iridium via vacuum sputter coating to enhance conductivity. Observations were performed under high vacuum using a Zeiss Supra 55VP Field Emission Scanning Electron Microscope at the i-CRIM Centralised Lab, Centre for Natural and Physical Laboratory Management UKM (ALAF-UKM), Research Complex, Universiti Kebangsaan Malaysia (UKM). Spore images were captured and organized to illustrate increasing complexity of perispore ornamentation across species. Morphological terminology for perispore structures follows established conventions in pteridological literature, primarily from Tryon and Lugardon (1991), with modifications from Lellinger and Taylor (1997) and Liu *et al.,* (2000). This subset of representative *Diplazium* species provides an overview of diagnostic variation in spore surface features within the genus.

Anatomical Observations

For anatomical data, two anatomical characteristics (stele shape and type of venations) for all *Diplazium* species studied were obtained from previously published datasets from Aliah *et al.* (2024). Transverse sections of the stipe were prepared using standard microtomy techniques following the protocol described in Aliah *et al.,* (2024). Stele morphology was examined under a compound light microscope, and stele types were categorized based on cross-sectional shape. Leaf venation patterns were also observed and classified as either free or anastomosing. Full anatomical procedures and classification criteria are detailed in Maideen *et al.,* (2024) and Aliah *et al.,* (2024).

Molecular Phylogenetic Framework

This study integrates molecular phylogenetic findings from a previous analysis of the Athyriaceae in Peninsular Malaysia (Aliah *et al.,* 2024), which included multiple representatives of *Diplazium* along with related genera such as *Athyrium*, *Deparia*, and *Cornopteris*. The earlier study employed five DNA markers—four chloroplast regions (*rbcL*, *atpB*, *atpA*, and *trnL-F*) and the nuclear ribosomal internal transcribed spacer (ITS)—to reconstruct the phylogeny of Athyriaceae species in the region. Phylogenetic inferences were carried out using both Maximum Likelihood (ML) and Bayesian Inference (BI) methods, generating a robust and well-supported topology for the family. While no new molecular data were generated for the present study, the *Diplazium*-specific subtrees from Aliah *et al.,* (2024) were re-examined with a focus on topology, species relationships, and clade structure. These molecular insights were then interpreted in light of new morphological assessments and spore micromorphological observations, allowing for a more integrative evaluation of species boundaries within *Diplazium*. Voucher specimens for sequenced individuals were rechecked and cross-referenced with herbarium materials to confirm their taxonomic identity and to ensure consistency across morphological and molecular datasets. This integrative approach allowed for the identification of potential cryptic lineages, clarified species delimitations, and informed the construction of a revised taxonomic key for *Diplazium* species in Peninsular Malaysia.

RESULTS

Morphological data

To provide a comprehensive taxonomic assessment of *Diplazium* in Peninsular Malaysia, morphological data were collected not only from *Diplazium* species but also from closely related genera within the family Athyriaceae, including *Athyrium anisopterum*, *Cornopteris opaca*, and *Deparia japonica*. Including these taxa allows for comparative analysis that helps clarify diagnostic characters and resolve species boundaries within *Diplazium*, given the morphological similarities and overlapping traits observed among these genera.

a. Rhizome (Character 1)

Two types of rhizomes were observed among the studied Athyriaceae taxa: erect and creeping. Most taxa possessed erect rhizomes, with the exception of *Deparia japonica* and *Diplazium procumbens*, which exhibited creeping rhizomes.

b. Stipe (Characters 2–10)

The stipe length across species fell into two categories: less than 20 cm and more than 20 cm. Only *Athyrium anisopterum* and *Diplazium subserratum* had stipes shorter than 20 cm. Regarding scale shape (Fig. 2), most species had linear scales; however, *D. latisquamatum* and *D. kunstleri* featured ovate scales. Two scale attachment types were noted at the stipe base: basifixed and peltate. Basifixed attachment was observed in *D. sorzogonense*, *D. tomentosum*, and *D. polypodioides*, whereas peltate attachment occurred in *D. crenatoserratum*, *D. malaccense*, *D. esculentum*, *D. kunstleri*, and *D. latisquamatum*. Meanwhile, for scale coloration varied across taxa: yellowish-brown (e.g., *A. appendiculiferum*, *C. opaca*, *Dep. japonica*), reddish-brown (e.g., *D. esculentum*, *D. prescottianum*, *D. velutinum*), and dark brown to blackish (e.g., *D. crenatoserratum*, *D. malaccense*, *D. polypodioides*).

Fig. 2. Variation in scale morphology among the studied taxa. **a** *Athyrium anisopterum* **b** *Cornopteris opaca* **c** *Deparia japonica* **d** *Diplazium angustipinna* **e** *D. cordifolium* var. *cordifolium* **f** *D. cordifolium* var. *pariens* **g** *D. malaccense* **h** *D. pallidum* var. *pallidum* **i** *D. pallidum* var. *montanum* **j** *D. prescottianum* **k** *D. riparium* **l** *D. sorzogonense* **m** *D. subintegrum* **n** *D. tomentosum* **o** *D. velutinum* **p** *D. xiphophyllum* – linear scales with entire margins **q** *D. kunstleri* **r** *D. latisquamatum* – ovate, peltate scales with entire margins. Scale attachment: basifixed (e.g. l, n); peltate (q, r). Scale coloration: yellowish-brown (e.g. b, c), reddish-brown (e.g. e, j), dark brown to blackish (e.g. f–i). Presence of a dark vascular strand near the scale margin indicated in d, l.

The margin of stipe scales was particularly useful for distinguishing morphologically similar species such as *D. crenatoserratum* and *D. tomentosum*. Most species exhibited entire (smooth) margins, while others had dentate margins (Fig. 3), which were further classified into branched teeth, unbranched teeth, or blunt teeth. Additionally, the presence of a black marginal band on scales was observed in species like *D. angustipinna*, *D. dilatatum*, and *D. polypodioides*.

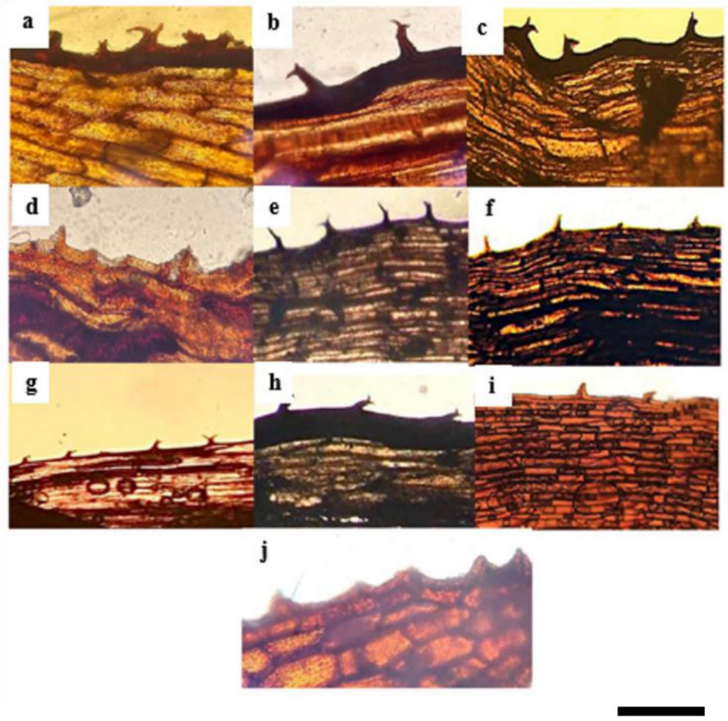

Fig. 3. Scales with toothed/serrate margins among the studied taxa. **a** *Diplazium bantamense* **b** *D. dilatatum* **c** *D. esculentum* **d** *D. crenatoserratum* **e** *D. polypodioides* **f** *D. procumbens* **g** *D. proliferum* **h** *D. simplicivenium* **i** *D. speciosum* var. *major* **j** *D. subserratum*. Margins show variation including branched, unbranched, and blunt teeth. A dark marginal band is visible in some species (e.g. b, e).

c. Lamina (Characters 11–18)

Lamina morphology ranged from simple to tripinnatifid forms. Simple laminas were only found in *D. cordifolium* var. *cordifolium* and *D. subserratum*. Pinnate laminas were seen in species such as *D. malaccense* and *D. simplicivenium*, while bipinnate forms occurred in *D. kunstleri* and *D. prescottianum*. *Deparia japonica* exhibited a bipinnatifid lamina, and tripinnatifid laminas were observed in *C. opaca*, *D. dilatatum*, *D. latisquamatum*, and *Dep. boryana*.

d. Basal Pinna Petiole (Character 19)

Most species had petiolated basal pinnae, while five species—*Dep. japonica*, *C. opaca*, *D. cordifolium* var. *cordifolium*, *D. cordifolium* var. *pariens*, and *D. subserratum*—had sessile basal pinnae. Petiole length varied and was grouped into three ranges: <2 mm (e.g., *D. sorzogonense*, *A. appendiculiferum*), 2–6 mm (e.g., *D. dilatatum*, *D. tomentosum*), and >6 mm (e.g., *D. proliferum*, *Dep. boryana*).

e. Pinna Margin Lobation (Character 20)

Species were also distinguished by whether the pinna margins were lobed or entire. Unlobed margins (entire) were found in taxa such as *D. cordifolium* var. *cordifolium*, *D. angustipinna*, *D. bantamense*, *D. cordifolium* var. *pariens* and *D. riparium*. Lobed pinnae were divided into two categories: those with sinuses reaching ½ the pinna width such as *D. dilatatum*, *D. kunstleri*, *D. malaccense*, and *D. velutinum*; and those with sinuses reaching ¾ of the pinna width, including *C. opaca*, *Dep. japonica*, *D. latisquamatum*, *D. polypodioides*, *D. procumbens*, *D. sorzogonense*, *D. tomentosum*, *D. speciosum* var. *major*, and *Dep. boryana*.

f. Sorus (Characters 21–22)

Sori arrangement varied significantly among taxa and could be categorized into four types based on their position: basal (e.g., *D. latisquamatum*), medial (e.g., *C. opaca*), extending from base to margin (e.g., *D. malaccense*), and terminal (e.g., *A. anisopterum*). Most species had sori that extended from the base to the margin of the pinnae. Sorus shape also proved taxonomically informative: rounded sori were noted in *A. anisopterum* and *C. opaca*, while linear sori were typical of all *Diplazium* species.

g. Indusium (Character 23)

Indusia were present in all species studied except *C. opaca*, which lacked this structure. The presence or absence of the indusium provides a reliable character for distinguishing *C. opaca* from other Athyriaceae taxa.

Morphological Phylogeny

The phylogenetic tree reconstructed using Maximum Parsimony based solely on morphological characters revealed two major clades (Fig. 4).

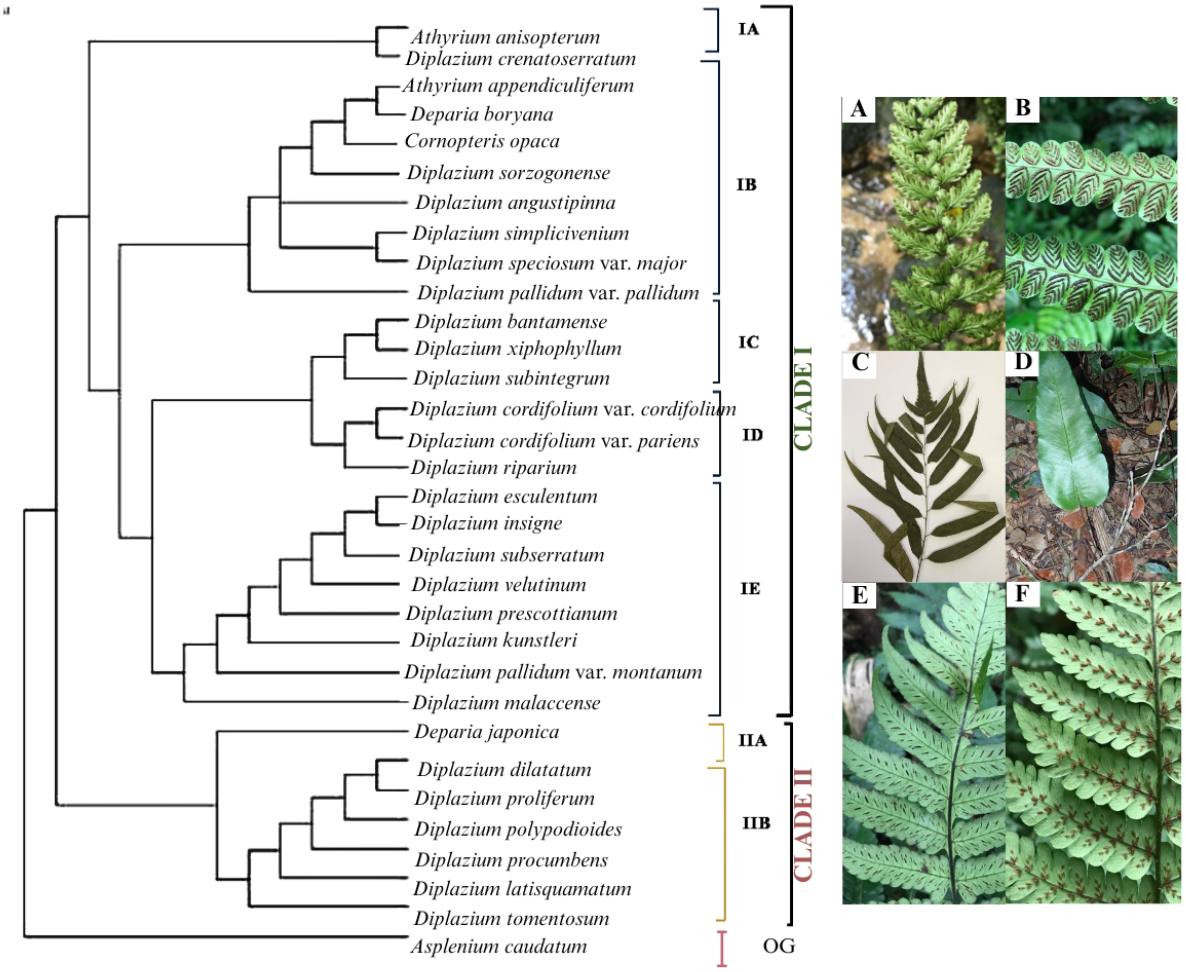

Fig. 4. Phylogenetic tree based on morphological characters of Athyriaceae species from Peninsular Malaysia. The tree illustrates the relationships among taxa inferred from a matrix of morphological traits. Subclade IA, A: *A. anisopterum*. Subclade IB, B: *D. simplicivenium*. Subclade IC, C: *D. pallidum* var. *pallidum*. Subclade ID, D: *D. cordifolium* var. *cordifolium*. Subclade IE, E: *D. kunstleri.* Subclade IIB, F: D. *latisquamatum*. Outgroup, OG: *Asplenium caudatum*.

Clade I comprised a heterogeneous assemblage of species including *Athyrium anisopterum*, *Diplazium crenatoserratum*, *Athyrium appendiculiferum*, *Deparia boryana*, *Cornopteris opaca*, and several *Diplazium* species such as *D. sorzogonense*, *D. angustipinna*, *D. simplicivenium*, *D. speciosum* var. *major*, *D. pallidum* var. *pallidum*, *D. bantamense*, *D. xiphophyllum*, *D. subintegrum*, *D. cordifolium* var. *cordifolium* and var. *pariens*, *D. riparium*, *D. esculentum*, *D. insigne*, *D. subserratum*, *D. velutinum*, *D. prescottianum*, *D. kunstleri*, *D. pallidum* var. *montanum*, and *D. malaccense*.

Within this clade, five subclades were recovered with varying degrees of support. The first subclade (IA) grouped *Athyrium anisopterum* and *D. crenatoserratum* together, suggesting close morphological affinities. The second subclade (IB) included *Athyrium appendiculiferum*, *Deparia boryana*, *Cornopteris opaca*, and several *Diplazium* species such as *D. sorzogonense*, *D. angustipinna*, *D. simplicivenium*, *D. speciosum* var. *major*, and *D. pallidum* var. *pallidum*. Another subclade (IC) united *D. bantamense*, *D. xiphophyllum*, and *D. subintegrum*. The fourth subclade (ID) consisted of *D. cordifolium* var. *cordifolium*, var. *pariens*, and *D. riparium*, while the fifth (IE) grouped *D. esculentum*, *D. insigne*, *D. subserratum*, *D. velutinum*, *D. prescottianum*, *D. kunstleri*, *D. pallidum* var. *montanum*, and *D. malaccense*. Clade II was resolved into two subclades as well, with subclade IIA consisting solely of *Deparia japonica*, and subclade IIB comprising *D. dilatatum*, *D. proliferum*, *D. polypodioides*, *D. procumbens*, *D. latisquamatum*, and *D. tomentosum*. Overall, the morphology-based tree reflects broad species groupings consistent with previous molecular and anatomical studies; however, internal relationships within several subclades were weakly supported or unresolved, indicating that morphological characters alone provide limited resolution for fully resolving species-level phylogenetic relationships in *Diplazium*.

Anatomical data *(Character 10 and Character 18)*

Anatomical observations of stipe vascular strands (stele) revealed three distinct morphological types among the studied species: V-shaped, U-shaped, and two parallel plates, consistent with previous findings (Aliah et al. 2024). The V-shaped stele type was predominant and found in species including *Athyrium anisopterum*, *Cornopteris opaca*, *Deparia japonica*, and multiple

Diplazium species such as *D. angustipinna* and *D. malaccense*. The U-shaped stele was observed in species like *D. bantamense*, *D. dilatatum*, and *D. kunstleri*, while *D. riparium* and *D. subserratum* exhibited a stele consisting of two parallel plates.

Leaf venation patterns were mostly free venation across the taxa studied, including *Athyrium anisopterum*, *Cornopteris opaca*, *Deparia japonica*, and most *Diplazium* species. Anastomosing venation was restricted to five *Diplazium* species (*D. angustipinna*, *D. cordifolium* var. *cordifolium*, *D. cordifolium* var. *pariens*, *D. esculentum*, and *D. proliferum*). These anatomical data corroborate previous studies and provide additional characters useful for delimiting species within Athyriaceae in Peninsular Malaysia (Aliah et al. 2024).

Although molecular analyses were not conducted in the present study, we incorporated and compared our morphological findings with the previously published molecular phylogeny of *Athyriaceae* species from Peninsular Malaysia (Aliah *et al.,* 2024) in Fig. 5. This prior work employed a comprehensive dataset combining plastid markers (*rbcL, atpB, atpA, trnL-F*) and nuclear ITS sequences. The phylogeny recovered two major clades within *Athyriaceae*, consistent with the previous topology in Aliah et al. (2024). From our result, *Diplazium* was strongly supported as monophyletic with 100% BS value, and the *Diplazium* clade further can be subdivided into four distinct subclades (IA–IE).

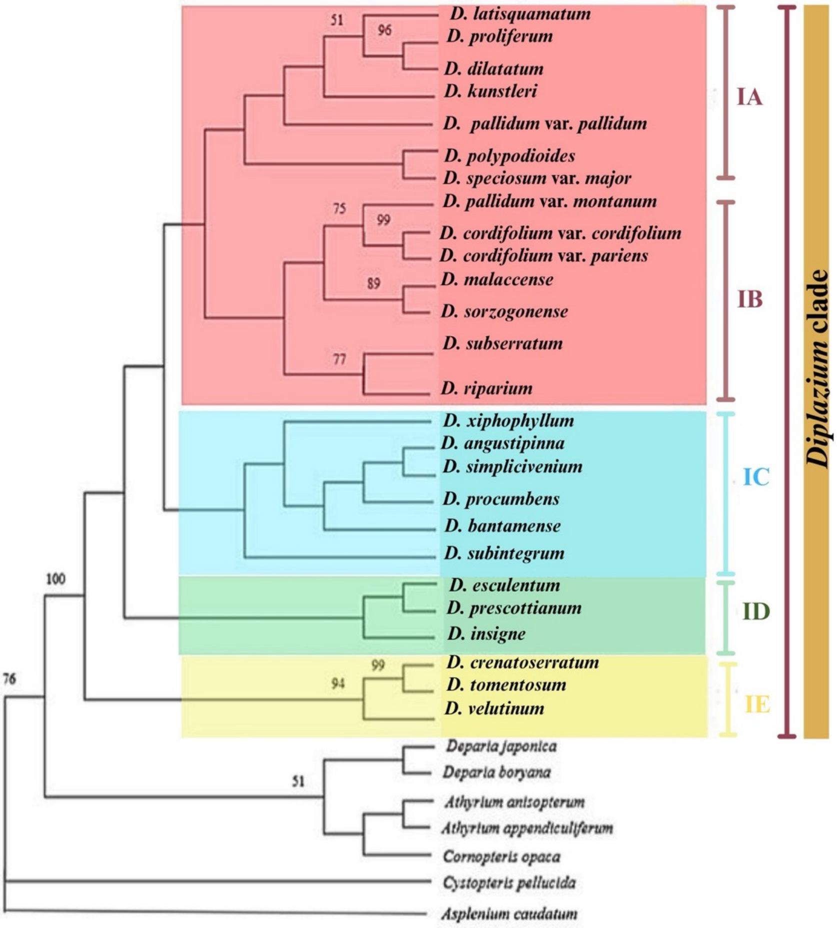

Fig. 5. Most parsimonious (MP) tree based on combined morphological, anatomical, and molecular characters. Bootstrap values (>50%) are shown above the branches. . The tree resolves monophyly of *Diplazium*, with five subclades (IA-IE).

Meanwhile another Clade grouped taxa from *Deparia*, *Athyrium*, and *Cornopteris*, reflecting complex intergeneric relationships. Notably, while the combination of molecular and anatomical data robustly supported the monophyly of *Diplazium* and internal subclades with high bootstrap values, the morphological phylogeny exhibited some discordance, particularly in the placement of species within Clade II. Such incongruence highlights the potential limitations of morphology-based inference and underscores the importance of integrating molecular data for accurate phylogenetic reconstruction. Overall, the combined interpretation of molecular, morphological, and anatomical data provides a more comprehensive understanding of *Diplazium* taxonomy and evolutionary history in Peninsular Malaysia. The molecular framework serves as a critical reference for interpreting morphological variation and guiding future taxonomic revisions within the group.

Spore Ornamentation

Spore morphology was examined for eight selected *Diplazium* species revealing a range of distinctive ornamentation patterns relevant to taxonomy. Consistent with prior studies (Liu *et al.,* 2000; Sledge, 1962; Tryon & Lugardon, 1991), all spores were monolete—bilaterally symmetrical with a linear aperture extending along a variable portion of the spore length (Fig. 6). Despite this shared characteristic, substantial variation was observed in spore surface ornamentation, allowing classification into five distinct groups.

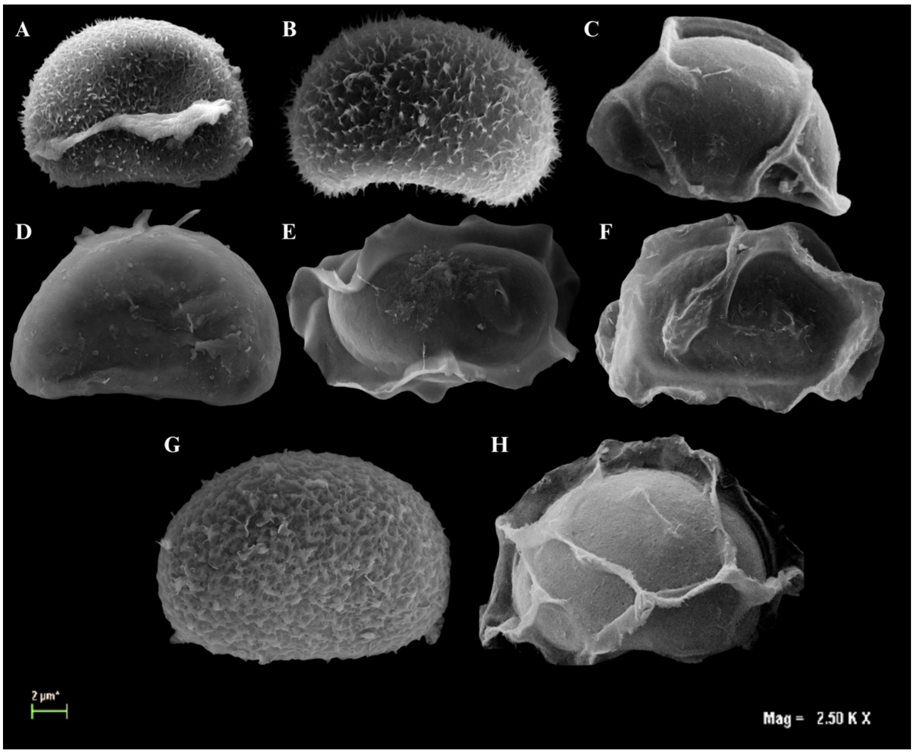

Fig. 6. Spore morphology of selected *Diplazium* species**. A** *Diplazium kunstleri.* **B** *D. latisquamatum.* **C** *D. procumbens.* **D** *D. sorzogonense*. **E** *D. malaccense,* **F** *D. tomentosum* **G** *D. esculentum* **H** *D. crenatoserratum.* All images are FESEM micrographs showing perispore ornamentation types: **A, B** echinate surface; **C, H** prominent folds; **D** low, plain folds; **E, F** long wing-like projections; **G** retate (net-like) surface; **H fenestrate folds**. Scale bars: 2 µm (all panels).

The most common pattern, observed in *D. sorzogonense* and *D. procumbens*, consisted of prominent folds with a non-fenestrate perine. In contrast, *D. crenatoserratum* exhibited spores with folds modified by sparse fenestrations, creating a mesh-like texture on the perine. Spores of *D. malaccense* and *D. tomentosum* were characterized by broadly winged folds, where expansive wing-like projections dominated the surface. *D. esculentum* spores displayed a retate micro-ornamentation type, with wide, rounded muri forming short and broad areoles. Finally, *D. kunstleri* and *D. latisquamatum* produced spores with echinate, non-fenestrate perine ornamentation, featuring narrowly conical spiny projections (echinae) differing subtly in width and density between the two species. Notable species-level distinctions include the uniformly projecting echinate folds in *D. kunstleri* and *D. latisquamatum*, where the former exhibits broader echinae compared to the narrower structures of the latter. Similarly, the folds of *D. procumbens* and *D. sorzogonense* differ in height and smoothness, illustrating fine-scale morphological diversity that may hold taxonomic significance.

DISCUSSIONS

This study presents a comprehensive integrative taxonomic framework for *Diplazium* and allied genera within Athyriaceae from Peninsular Malaysia by combining detailed morphological, anatomical, spore ornamentation, and molecular phylogenetic analyses. The multi-dimensional approach employed here substantially advances our understanding of species boundaries and phylogenetic relationships beyond previous morphology-centric studies, which often relied on a limited suite of external characters prone to homoplasy.

Morphological Variation and Taxonomic Utility

Consistent with earlier works (Nakai, 1931; Wei *et al.,* 2013), we confirm that indusium presence and sorus morphology remain critical diagnostic features within *Diplazium*. However, our morphological phylogeny indicates these characters alone do not fully resolve phylogenetic relationships, reflecting convergent evolution or morphological plasticity within the family. Scale morphology, including margin type, shape, and attachment, proved valuable for species delimitation, corroborating previous findings (Kato, 1977; Wei *et al.,* 2013), but with some species displaying unique combinations that complicate straightforward phylogenetic grouping.

Morphological Variation in Athyriaceae and Phylogenetic Implications

This study revealed considerable morphological and anatomical variation among 31 Athyriaceae taxa, with key traits providing useful taxonomic distinctions at the genus level. Notably, the presence or absence of an indusium and the shape of sori served as effective diagnostic characters. All genera examined possessed indusia protecting their sori except *Cornopteris,* confirming Nakai's (1931) earlier observations. Sorus morphology also differentiated genera: *Athyrium* and *Deparia boryana* exhibited round sori, whereas *Diplazium* and *Deparia japonica* displayed linear sori, consistent with Kuo *et al.,* (2018) and Wei *et al.,* (2013).

Despite these morphological patterns, sorus shape and indusium presence did not fully correspond to phylogenetic groupings inferred in Aliah *et al.,* (2024). For instance, *Diplazium* species with linear sori clustered alongside *Athyrium, Cornopteris*, and *Deparia* boryana, while *Deparia japonica* grouped with other *Diplazium* species bearing round sori. This disparity underscores the historical reliance on these characters in fern taxonomy but also highlights their limitations in reflecting evolutionary relationships revealed by molecular data. Rhizome architecture further illustrated evolutionary trends within *Diplazium*. Most *Diplazium* species studied from Peninsular Malaysia had erect rhizomes, except *D. procumbens*, which exhibited creeping rhizomes. Following Mickel (1974), creeping rhizomes represent a plesiomorphic trait, supported by fossil data (Stockey *et al.,* 1999), while erect rhizomes are likely derived. Historically, rhizome morphology has been a significant taxonomic trait, integrating vegetative and anatomical data in fern classification.

Scale characters exhibited notable variation and proved valuable for species delimitation within *Diplazium*. Scale margin texture (entire vs. dentate) effectively separated *Diplazium* species, complementing Kato (1977) distinction from *Athyrium*. Dentate margins are likely derived, reflecting evolutionary trends toward increased complexity (Scholch, 2003). Most species had linear scales, with *D. latisquamatum* and *D. kunstleri* distinguished by ovate to peltate scales, though these two species were not phylogenetically closely related, possibly due to differing sorus positions. Two scale attachment types—basifixed and peltate—were documented, mirroring findings by Wei *et al.,* (2013). Basifixed scales were found in *D. sorzogonense*, *D. tomentosum*, and *D. polypodioides*, while peltate scales characterized *D. crenatoserratum*, *D. malaccense, D. esculentum, D. kunstleri,* and *D. latisquamatum*.

Lamina complexity ranged from pinnate to tripinnatifid in most taxa, except for *D. subserratum* and *D. cordifolium* var. *cordifolium*, which displayed simpler lamina forms. Simplified laminae may result from paedomorphosis—retention of juvenile traits in mature fronds—as proposed by Imaichi and Kato (1992) and Wei *et al.,* (2013). However, lamina complexity did not correspond to phylogenetic groupings as in Aliah *et al.,* (2024) as species with simple laminae clustered with those having complex fronds. This highlights the limited utility of lamina morphology alone in resolving generic boundaries.

Novel Insights from Spore Ornamentation

This study presents a novel and detailed characterization of spore surface ornamentation in *Diplazium* species from Peninsular Malaysia, using field emission scanning electron microscopy (FESEM). Five distinct ornamentation patterns were identified, offering species-specific micro-morphological traits that enhance taxonomic resolution within the genus. Notably, the echinate spores with narrowly conical spines in *D. kunstleri* and *D. latisquamatum*, as well as the mesh-like, fenestrate folds in *D. crenatoserratum*, serve as diagnostic features. These findings highlight the value of spore ornamentation as a systematic tool and provide new insights into potential reproductive isolation mechanisms and cryptic diversity within *Diplazium*.

Previous studies have examined spore morphology in Athyrioid ferns from various regions, including Taiwan (Huang 1981; Liu et al. 2000), the Americas (Tryon & Tryon, 1982), and Ceylon (Sledge, 1982). However, this is the first comprehensive investigation focusing on the spores of *Diplazium* species from Peninsular Malaysia. Traditionally, *Diplazium* spores are characterized by prominent wing-like folds, either smooth or bearing irregular echinate elements emerging from a compact, perforate base (Tryon & Tryon, 1982). Intriguingly, some *Diplazium* species show surface resemblance to *Tectaria*, suggesting phylogenetic affinities between these genera—a hypothesis supported by overlapping ornamentation patterns and diversity (Tryon & Tryon, 1982).

Spore morphology, particularly the structure and ornamentation of the perine and exine, is widely recognized as a key diagnostic feature in fern systematics. Macro-ornamentation (perispore features), visible under light microscopy, and micro-ornamentation, observable through SEM, provide complementary data (Lellinger & Taylor, 1997). The taxonomic utility of *Diplazium* spores was initially proposed by Sledge (1982) and later corroborated through ultrastructural studies by Tryon and Lugardon (1991). Earlier, Tardieu-Blot (1932) also emphasized the systematic importance of spore characters and provided illustrations of various species.

General spore traits such as shape, aperture type, size, and surface architecture are valuable taxonomic markers. Fern spores are typically trilete (radially symmetrical with a triradiate aperture) or monolete (bilaterally symmetrical with a linear aperture). Trilete spores are considered evolutionarily primitive due to their earlier appearance in the fossil record (Gensel, 1980). In our study, all eight *Diplazium* species exhibit monolete spores with a linear aperture ranging from two-thirds to three-quarters of spore length, consistent with previous observations (Liu *et al.,* 2000; Tryon & Lugardon, 1991; Sledge, 1962). This monolete condition is common among large genera like *Asplenium* and within the Polypodiaceae (Tryon & Lugardon, 1991). Spore size in *Diplazium* varied across species, ranging from 35 µm to 46 µm. *D. latisquamatum* possessed the smallest spores (35.93 µm), whereas *D. procumbens* had the largest (46.53 µm). Spore size may offer indirect insights into ploidy level and spore production per sporangium, making it an informative trait for species delimitation.

The spores of *Diplazium* are generally ellipsoid, with winglike folds that vary from fimbriate or low rugate to nearly smooth; in rarer cases, they may appear stranded or echinate (Tryon & Lugardon, 1991). Retate ornamentation (rugulate sensu Tryon & Lugardon, 1991) was observed in *D. esculentum* and is formed by rounded, freely anastomosing muri that produce broad areoles—similar to inner sheath structures in *Athyrium*. Such features help distinguish *Diplazium* from closely related genera: *Athyrium* typically exhibits less elaborate, low rugate folds, while *Deparia* shows characteristic tuberculate ornamentation. Collectively, these spore morphological traits serve as reliable taxonomic characters that not only reinforce species boundaries but also provide supporting evidence for broader phylogenetic and biogeographic hypotheses within Athyriaceae. This study underscores the significance of integrating micromorphological data with traditional characters to refine fern systematics.

Anatomical Correlates and Phylogenetic Implications

Vascular anatomy, particularly stipe stele configuration, emerges as a strong phylogenetic signal supporting genus-level delimitation within Athyriaceae (Maideen *et al.,* 2021; Aliah *et al.,* 2024). The predominance of ‘U’-shaped steles in *Diplazium* contrasts with ‘V’-shaped or parallel plates in related genera, aligning well with molecular clades and providing functional and evolutionary context. Leaf venation patterns further complement these findings by revealing both free and anastomosing venation types that may represent derived states, adding nuance to species circumscription.

Comparison with Recent Molecular Phylogeny and Integrative Classification of Diplazium

Our integrative analysis, combining morphological, anatomical, and molecular data, shows strong concordance with the recent plastome-based phylogeny of *Diplazium* presented by Wei *et al.,* (2024) in Figure 7. Of the 31 *Diplazium* species examined morphologically, only 26 were represented in the molecular phylogeny (Figure 7). The remaining five species could not be included due to unsuccessful DNA extraction from older herbarium materials, consistent with the limitations noted by Aliah et al. (2024).The majority of species in our study, such as *D. crenatoserratum*, *D. sorzogonense*, *D. bantamense*, and *D. xiphophyllum*, consistently group within Subclade 7 (sect. *Anisogonium*), highlighting stable taxonomic placements across datasets.

Interestingly, species like *D. esculentum* and *D. procumbens*, which show divergent morphological traits, align more closely with Subclade 12 (sect. *Dolichostegia*) in Wei et al. (2024) a pattern also supported by their distinct spore ornamentation. Cases of discordance, such as the placement of *D. pallidum var. montanum* and *D. subserratum*, which exhibit morphological traits suggesting Clade IE but molecular data place in Subclade 7, underscore the limitations of morphology alone. These discrepancies reinforce the value of integrative taxonomy in resolving complex species relationships, particularly in morphologically convergent lineages like Diplazium. Moreover, the absence of certain species (e.g., *D. angustipinna*, *D. subintegrum*, *D. insigne*) in Wei et al. (2024) emphasizes the need for broader molecular sampling to support comprehensive systematic revisions.

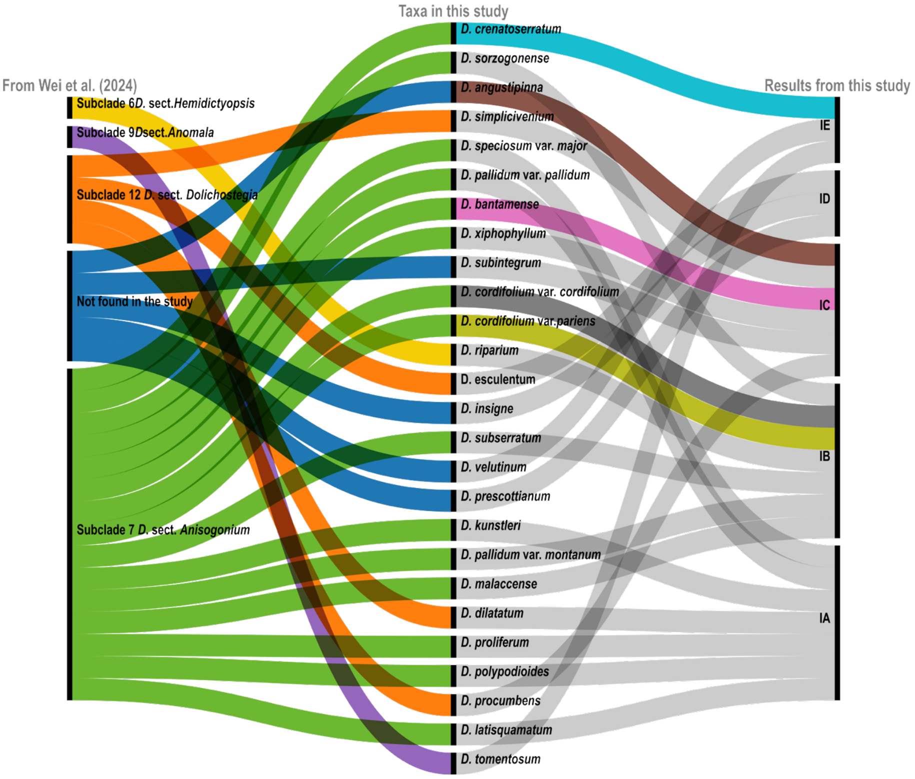

Fig. 7. Alluvial diagram illustrating the comparative classification of *Diplazium* species based on three data types: (1) subclade placement based on plastome phylogeny by Wei et al. (2024), (2) morphological evidence, and (3) integrative data combining morphology, anatomy, and molecular evidence from this study. The diagram highlights congruence and discrepancies among the three classification approaches, emphasizing the value of integrative taxonomy in resolving species boundaries and phylogenetic relationships within *Diplazium*.

Ecological and Evolutionary Considerations

Variation in rhizome habit from creeping to erect likely reflects adaptive responses to ecological niches and dispersal strategies, with creeping rhizomes facilitating clonal spread in dynamic understory habitats. The observed diversity in spore ornamentation may also relate to reproductive isolation and specialization, suggesting evolutionary divergence driven by ecological factors. These insights link morphological and anatomical variation with functional ecology, providing a holistic understanding of diversification processes in *Diplazium*.

In conclusion, the integrative taxonomy employed in this study incorporating morphological characters, spore ornamentation, and molecular phylogenetics provides a robust framework for resolving species boundaries within *Diplazium* in Peninsular Malaysia. This approach not only refines the phylogenetic relationships within the genus but also highlights key diagnostic features critical for accurate identification. To facilitate ongoing research and practical identification, we have compiled a comprehensive dichotomous key to *Diplazium* species of Peninsular Malaysia, which is provided as Supplementary Material (Supplementary Material S2). This key synthesizes morphological and molecular evidence, serving as an essential resource for taxonomists, ecologists, and conservationists working with this diverse and complex genus.

CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, this study demonstrates that combining morphological, anatomical, spore ornamentation, and molecular data yields a robust and nuanced understanding of species boundaries and phylogenetic relationships within *Diplazium* and Athyriaceae. The integration of novel micro-morphological characters, such as spore ornamentation, alongside traditional traits and molecular phylogenies, represents a methodological advance that can be applied broadly in fern systematics. Future research incorporating broader geographic sampling and genomic-scale data will further elucidate evolutionary dynamics and facilitate comprehensive taxonomic revision in this complex and diverse lineage.

Building on the integrative analyses presented above, we provide a formal taxonomic treatment that clarifies generic boundaries within Athyriaceae and resolves species delimitations within *Diplazium* in Peninsular Malaysia. This section synthesizes morphological, anatomical, and molecular evidence to offer updated diagnostic descriptions and relationships, serving as a foundation for future systematic and conservation work.

Taxonomic Treatment

Generic Delimitation within Athyriaceae in Peninsular Malaysia

The present study, combining molecular phylogenetic analyses with detailed morphological and anatomical observations, supports clear generic delimitation within Athyriaceae. *Diplazium* is strongly supported as a monophyletic genus, distinguished by its characteristic linear sori, continuous grooves on the rachis and costa, and distinctive stipe scale morphology. In contrast, *Deparia* exhibits non-continuous grooves on the rachis and costa, while Athyrium is characterized by rounded sori and generally continuous grooves. *Cornopteris* is clearly separated by the absence of an indusium, a feature consistently observed both morphologically and phylogenetically. These characters provide robust morphological markers congruent with molecular phylogenies, facilitating reliable genus-level identification within the family.

Species Delimitation and Relationships in Diplazium

Our phylogenetic framework resolved five well-supported subclades within *Diplazium*, each supported by distinct morphological traits. Notably, D. *cordifolium* var. *cordifolium* and var. *pariens* form a strongly supported sister group, sharing morphological features such as petiole length and lamina dissection, confirming their varietal status. The sister relationship of *D. dilatatum* and *D. proliferum* is corroborated by shared frond morphology and venation patterns, reflecting recent divergence. Likewise, *D. crenatoserratum* and *D. tomentosum* are closely allied both molecularly and morphologically, distinguished by stipe scale characters and rhizome morphology.

Identification Keys

To facilitate taxonomic identification and support standardized species delimitation, dichotomous keys to the genera of Athyriaceae in Peninsular Malaysia are presented here. These keys are primarily based on morphological characters that are corroborated by molecular phylogenetic data. A complete dichotomous key to the species of *Diplazium* in Peninsular Malaysia, is provided in Supplementary Material S2 to aid future identification and taxonomic revisions.

Key to Genera of Athyriaceae in Peninsular Malaysia

1. Sporangia protected by indusium 2

   Sporangia not protected by indusium .... *Cornopteris*

2. Grooves on rachis and costa not continuous .... *Deparia*

   Grooves on rachis and costa usually continuous 3

3. Sori rounded .... *Athyrium*

   Sori linear .... *Diplazium*

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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