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Comparative morpho-anatomical study in *Pyrrosia* (Polypodiaceae) from Darjeeling Himalaya, India

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ABSTRACT

The aim of the study is to compare the morpho-anatomical characters in *Pyrrosia* and create a comprehensive account to fulfil the persisting taxonomic lacuna. The genus *Pyrrosia* under Polypodiaceae is widely distributed in Darjeeling eastern Himalaya. However, detailed morpho-anatomical investigation of the species from the region is limited. The specimens were carefully observed and photomicrographed using light microscope and SEM. Principal Component Analysis have been carried out using quantitative morpho-anatomical traits and based on their differences, a taxonomic key and UPGMA dendrogram is constructed to assess the interrelatedness among the taxa. The results revealed several morphological variations in rhizome type, rhizome scales, lamina type, sori distribution *etc*. Variations in anatomical characters were also observed in laminar indument, stomata and epidermal cell type, presence or absence of sclerenchyma, stipe shape, distribution of sclerenchyma tissue strands, sclerenchymatous sheaths and spore shape and sporoderm ornamentations. The PCA results showed a variance explained by the data points on component 1(53.022%) which is greater than the variance of data points on component 2 (23.631%). Traits like number of branches in stellate hairs, size, lamina length, stipe length, rhizome scale length, rhizome scale width, number of sori in each areole shows positive loading therefore being taxonomically significant.

Keywords: morpho-anatomy, polypodiaceae, *Pyrrosia*, taxonomy

INTRODUCTION

Pyrrosia Mirbel (Polypodiaceae) is an old-world fern genus having terrestrial and epi-

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phytic habitat and nested within the sub-family Platycerioideae (Christenhusz and Chase, 2014; PPG I, 2016). *Pyrrosia* s.l. comprise of about 100 species growing across western coast of Africa to Indian Himalayas, northeast Russia to Malaysia in Asia, further to Australia and extending up to South Pacific (Hovenkamp, 1986; Shing and Iwatsuki, 1997). In recent times, 73 species present worldwide are considered accepted within the genus (WFO, 2022). Ching (1935), described around 40 species from Asia and its neighboring islands. Hovenkamp (1986), considered 10 groups on the basis of cladistics analysis while Shing (1983), segregated *Pyrrosia* into sub-genera *Niphobolus* and *Pyrrosia* which is further divided into two sections and five series. Yang (2012), based on taxonomic revision suggested two sub-genera and 6 sections. The infrageneric classification of the genera has been associated with certain controversies and arguments. Over the years, molecular phylogenetic studies established *Pyrrosia* as monophyletic and *Platycerium* as a sister group (Wei *et al*., 2017). The recent phylogenetic works based on chloroplast markers proposed sub-generic classification (Zhao *et al*., 2013; Vasques *et al*., 2017; Zhou *et al*., 2017; Wei *et al*., 2017) and explored the biogeography, evolutionary aspects, adaptations leading to drought tolerance based on nucleotide sequence of chloroplastic DNA. Taxonomically, *Pyrrosia* possess simple leaves with various outlines (Stuart, 2008). The presence of stellate hairs on both surfaces and connective venation pattern seems to be the two distinguishing characters in decoding the evolutionary aspects of the family. The common name being 'Felt fern' is due to dense mat formed by the laminar hairs (Hennipman, 1990). The taxa however, are apparently similar in appearance and often lead to complexity in distinguishing upto species-level classification (Wei *et al*., 2017). Morphological studies play a pivotal role in taxonomical approaches in ferns (Steševic and Berg, 2015; Xu *et al*., 2019). The significance of anatomical characters has also been well established in recent works (Kotrnon, 2007; Talip *et al*., 2012; Resmi *et al*., 2016; Haq, 2017; Koniyo *et al*., 2019; Dematteis *et al*., 2019). Subsequently, morphological characters combined with anatomical details proved to be of immense importance in case of fern systematics (Ogura, 1972; Kotrnon *et al*., 2007; Nopun *et al*., 2016).

Satija *et al*. (1983), reported that many species of *Pyrrosia* share close similarity and segregated based on rhizome scales, hairs and spores. *Pyrrosia glabra* is similar in appearance to *P. lanceolata* and differentiated on the basis of scale properties. Therefore, several workers conducted taxonomic revisions at regional and global context in relation to *Pyrrosia* with difficulty and confusion persists even now (Wei *et al*., 2017). In recent years, Kotrnon *et al.* (2007), studied the Thai *Pyrrosia* species and the characteristics established there served in understanding the genus specific to that region. Similarly, Sofiyanti *et al*. (2021) studied the taxa in Indonesia and highlighted the characters and its variability within the species. In modern context we do not have significant taxonomic account of different species of the genus from Indian regions. Over the years, it has been observed that extensive studies on fern morphology and anatomy specifically from Darjeeling Himalaya have been reported

(Mehra and Khanna, 1959), spore taxonomic studies (Pal and Pal, 1970), morpho-anatomy of *Lepisorus* (Ganguly and Mukhopadhyay, 2018), study on phytoliths (Mukherjee et al., 2019). However, not much work is available on the morpho-anatomical study of genus *Pyrrosia* from the region. Therefore, the present work is an endeavor to create a comprehensive account with an aim to analyze and compare the morpho-taxonomic and anatomical aspects in *Pyrrosia* and to fulfill the taxonomic lacuna.

MATERIALS AND METHODS

Study area

The field collections were conducted over a period of time along different altitudinal zones of Darjeeling Himalaya. The study area extends between 27° 13' N to 26° 27' N latitude and 88° 53' E to 87° 30' E longitude covering an area of around 3149 sq km with altitudinal ranges extending from 130 to 3636 m asl. Within this altitudinal range, wide microclimatic zones are available that makes the region rich and diverse. Mature samples were freshly collected from the field and for each voucher specimen, date, place of collection, collection number, altitude and habitat were noted. The identification of the taxa was made following relevant literature (Ghosh, 2004; Fraser-Jenkins, 2008; Kholia, 2010; Frazer-Jenkins *et al*., 2021) and correct nomenclature with authority was considered following Plants of the World Online (POWO, 2022).

Morpho-anatomical studies

Light Microscopy (LM)

The gross morphological observations of the species include characters of rhizome, stipe, costa, rachis, fronds, distribution and arrangement of sori, sporangia and spore features. All the representative specimens were fixed in 70% ethyl alcohol, stored in sealed containers and were later sectioned for anatomical studies. Free-hand techniques of Johansen (1940), were used in preparing temporary and permanent slides of the parts under study. The prepared transverse sections do not require staining and were observed directly under microscope. For anatomical studies, mature leaves were taken and boiled in water before being macerated in 35% NaOCl solution (Hovenkamp, 1986). The epidermal examinations were carried out from the lower and upper leaf surfaces of lamina to observe epidermis cells, stomata, laminar hairs including venation pattern. Pieces of epidermal peels were prepared and mounted in Canada balsam. The photographs of the sections were taken under a stereo microscope Wild M3 Heerbrugg and binocular microscope Leitz Laborlux D. The relevant measurements of microscopic characters were taken under ocular micrometer. The morphological terminologies were followed as per (Zhang *et al*.,1999; Lellinger, 2002; Sun and Zhang, 2009).

Scanning Electron Microscopy (SEM)

SEM study was made to understand the spore morphology and laminar hairs. Portion of mature frond and spores were stuck to aluminium stubs with double-sided tape, sputter-coated with gold, observed and photographed using scanning electron microscope Zeiss EVO 18 Special Edition. For describing the spore morphology and ornamentation, terminology as per Wang and Yu, (2003) and Tryon and Lugardon, (1991) was followed.

Data analysis

The diagnostic characters for the fern samples were assembled. For each trait, an average of three observations was made and the data were standardized. Qualitative and quantitative features were assembled separately. All the character data set was subjected to Principal components analysis (PCA) and Hierarchical Clustering through UPGMA algorithm using PAST 4.03 (Hammer *et al*., 2001). Subsequently, dendrogram based on degree of similarity of morpho-anatomical characters were constructed. Additionally, a set of artificial key to the species was also constructed in the dichotomous key format.

RESULTS

Detailed morphological and anatomical characters of the specimen were observed and traits like lamina, leaf dimorphism, sori distribution *etc*. were noted in the field. The microscopic evaluation of the taxa, *Pyrrosia costata* (Wall. ex C.Presl) Tagawa & K.Iwats., *Pyrrosia heteractis* (Mett. ex Kuhn) Ching, *Pyrrosia lanceolata* Farw., *Pyrrosia mannii* (Giesenh.) Ching and *Pyrrosia glabra* (Desv.) Fraser-Jenk. were performed and presented as follows.

Rhizome and Rhizome scales

In *Pyrrosia* species, rhizome is usually creeping, short, stout or slender and often covered by a dense tuft of roots. Among the five species examined, three species *i.e P. glabra, P. lanceolata* and *P. heteractis* have creeping rhizomes while short rhizome was observed in *P. costata* and *P. mannii*. The rhizome with widest diameter of up to 5 mm was seen in *P. costata* followed by *P. mannii* and *P. heteractis* while *P. lanceolata* and *P. glabra* possess smallest diameter of upto 2 mm (Table 1). Two types of scales were observed in *P. lanceolata*, the bigger lanceolate type along with a small peculiar shield like (Fig. 1). In *P. heteractis*, the rhizome scales and the stipe base scales differ from each other in shape and size (Table 2).

Anatomical study of the rhizome revealed shape varied from almost oval in *P. costata*, to almost circular in *P. lanceolata*, *P. glabra*, and *P. heteractis*. The stelar arrangement in all the taxa were dictyostelic, with meristeles arranged in a ring that ranged from 4–12 in number. Individual meristele is oval, with plate-like xylem surrounded by phloem and endodermis with 1–3 layers of parenchymatous pericycle. In *P. glabra*, *P. lanceolata*, and *P. heteractis*, a central sclerenchymatous strand is observed and in *P. mannii* the sclerenchymatous strand is scattered while it is lacking in *P. costata* (Fig. 2).

Figure 1. Rhizome scales: **A.** *P. mannii* B. *P. costata* **C.** *P. glabra* **D1.** *P. lanceolata* (bigger scale) **D2.** *P. lanceolata* (smaller scale) **E1.** *P. heteractis* **E2.** *P. heteractis* (stipe base scale) (scale bar = 1mm).

Table 1. Morpho-anatomical features of rhizome

Taxa	**Morphology**		**Anatomy**		
	Rhizome type	**Rhizome diameter (mm)**	**Arrangement of sclerenchymatous strands**	**No of Vascular bundles**	**Sclerenchymatous sheath**
P. costata	Short	5±0.01	Absent	5-6	Absent
P. heteractis	Long creeping	3±0.04	Several in centre	7-8	Present
P. lanceolata	Long creeping	1.2±0.50	Single central strand	5	Present
P. mannii	Short	2.8±0.01	Scattered	10-12	Present
P. glabra	Long creeping	1.5±0.05	Single central strand	5	Present

Table 2. Morpho-anatomical features of rhizome scale

Taxa	**Type**	**Apex shape**	**Base Shape**	**Color**	**Scale margins**	**Scale length (mm)**	**Scale width (mm)**
P. costata	Basifixed	Acuminate	Broad	Brown	Basal margin with teeth	9 ±0.015	2±0.07
P. heteractis *(rhizome scale)*	Peltate	Acuminate	Ovate	Light brown	Curly	7±0.011	0.8±0.023
(stipe base scale	Peltate	Acuminate	Broad	Light brown	Curly	5.6±0.033	0.9±0.1
P. lanceolata *(bigger scale)*	Peltate	Acuminate	Ovate	Light brown	Ciliate	5.4±0.007	0.6±0.05
(smaller scale)	Peltate	Acuminate	Shield like	Light brown	Ciliate	2.5±0.011	0.3±0.08
P. mannii	Pseudo peltate	Acuminate	Linear	Yellowish straw brown	Entire	5.8±0.055	0.6±0.018
P. glabra	Peltate	Acuminate	Ovate	Brown	Ciliate	6±0.053	0.5±0.01

Stipe

The stipe length varies among the species. However, the color remains similar as straw brown in all the studied taxa. The transverse section of the stipe shows outline as globose in *P. heteractis* and *P. costata*, heart-shaped in *P. lanceolata* and *P. glabra* while wing-shaped *in P. mannii*. There is a presence of stomata in the lower epidermis and sclerenchymatous sheath under upper epidermal cells formed by 3-10 layers. The arrangement of vascular bun-

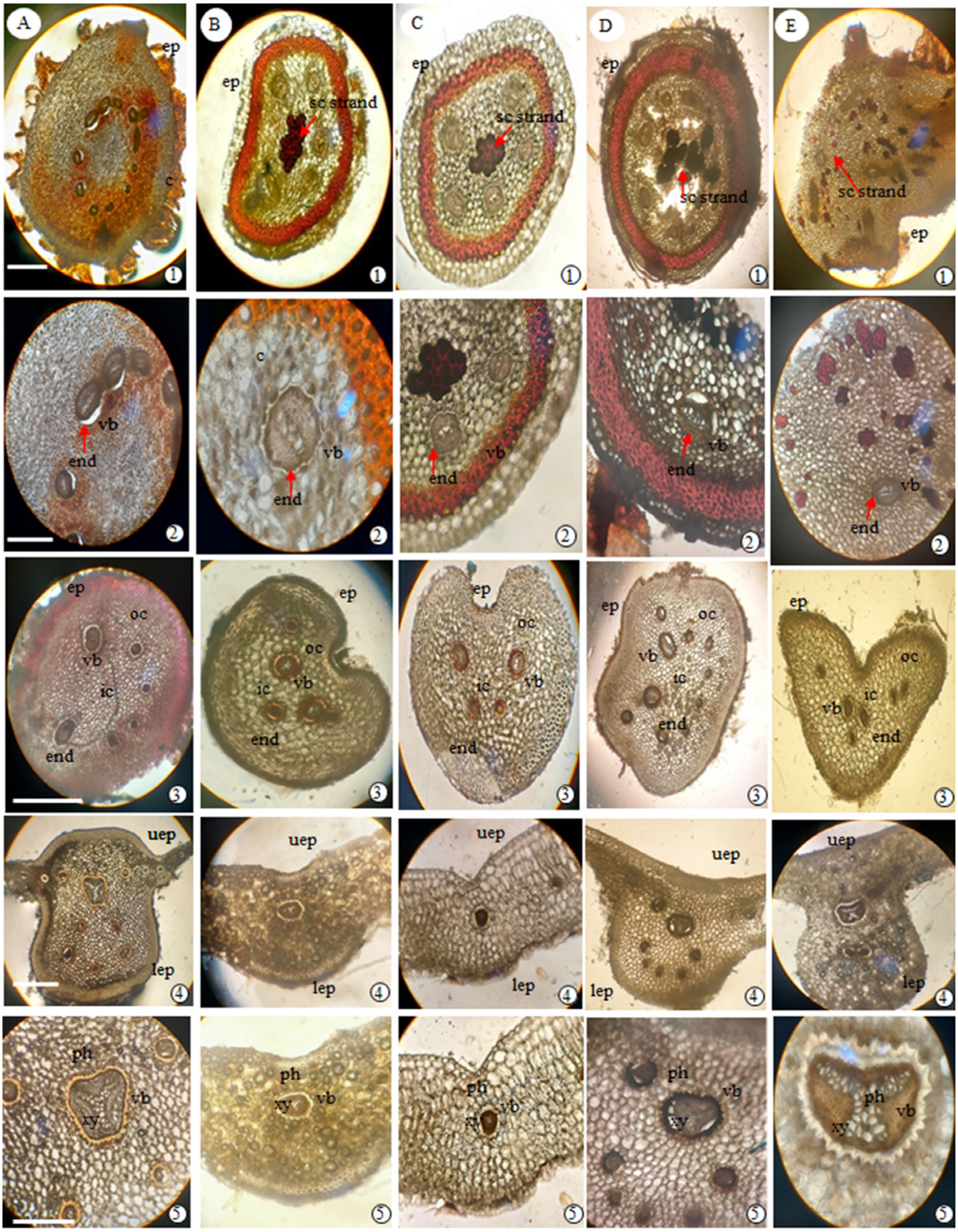

Figure 2. A. *P. costata* **B.** *P. lanceolata* **C.** *P. glabra* **D.** *P. heteractis* **E.** *P. mannii*; 1- T.S of rhizome 2- enlarged view of T.S of rhizome 3- T.S of stipe 4- T.S of midrib 5- vascular bundles of the midrib; Note: ep-epidermis; sc-sclerenchyma; vb-vascular bundles; end-endodermis; oc-outer cortex; ic-inner cortex; c-cortex; uep-upper epidermis; lep-lower epidermis; ph-phloem; xy-xylem (scale bar: 1=500μm; 2=250μm; 3=100μm; 4=100μm; 5= 50μm).

dles in the stipes have been observed as U-shaped in *P. lanceolata*, *P. heteractis*, *P. costata* and *P. mannii* and V-shaped in P. glabra (Table 3). The vascular tissue system consists about 3-15 vascular bundles with extended ends. The bundles are round or oval in transverse section with plate-like xylem and phloem on both sides having some layers of pericycle and surrounded by the endodermis (Fig. 2).

Table. 3 Morpho-anatomical features of stipe

Taxa	Morphology			Anatomy			
	Stipe color	Stipe length (mm)	Stipe width (mm)	Shape of TS	Shape of VB	No of VB	Layers of sclerenchymatous sheath
P. costata	Straw colored	5±0.05	0.3±0.01	Globose	U	5-6	5-6
P. heteractis	Straw colored	15±0.033	0.5±0.005	Globose	U	8-10	6-8
P. lanceolata	Straw colored	0.8±0.05	0.1±0.05	Heart-shaped	U	5-6	4-6
P. mannii	Straw colored	3±0.06	0.4±0.03	Heart-shaped	U	4-6	4-5
P. glabra	Straw colored	3±0.01	0.2±0.05	Heart-shaped	V	3-5	4-5

Lamina

In this study, we observed monomorphic and moderately dimorphic frond type among the examined species. However, when the fertile and sterile fronds are not very distinct in terms of shape and size, they were considered moderately dimorphic as seen in *P. glabra*, *P. lanceolata*, and *P. heteractis* (Table 4). In light microscopic observation, the cells of the upper epidermis of the lamina of most species are 5-6 sided and longitudinally orientated (Fig. 3). However, *P. mannii* have irregularly-shaped sinuous walls while straight, anticlinal walls were observed in *P. lanceolata* and *P. glabra*. The cells of the lower epidermis are similar to those of the upper in having sinuous walls except in *P. lanceolata*, the epidermal cell walls were straight (Table 5). The venation pattern showed first primary vein dichotomized and later pinnate. Lateral veinlets are simple, forked unequal as a result of branches of successive veins with regular row of areoles on both side of the midrib and the lateral veinlets. In larger leaves, as *P. costata*, the lateral veins are pinnately branched and regularly fused leading to formation of series of areoles (Fig. 3). The sori are superficial, apically distributed on the free tertiary veinlets of *P. costata*, *P. heteractis*, and *P. mannii* while in some cases, the sori were deeply sunken, located in a small depression on the lamina as seen in *P. glabra* and *P. lanceolata*. Most species have pericytic stomata restricted to the abaxial surface with very narrow guard cells encircling the stoma with polocytic in *P. mannii* (Fig. 3). Deeply sunken stomata occur in *P. lanceolata* and *P. glabra* while superficial stomata are seen in *P. costata*, *P. heteractis* and *P. mannii* (Table 5). The hypodermis is present on leaf blade and on the upper surface in *P. glabra* and *P. heteractis*. The hypodermal cells are larger with distinctly thinner wall than the epidermal cells. Mesophyll is dorsiventral in most species with chloroplast in the palisade and spongy cells of the fronds, the ground tissue in frond midrib is parenchymatous. The sclerenchymatous sheath is beneath the upper and lower epidermis of the midrib in all species.Vascular bundles in the midrib region comprises of concentric T-shaped xylem surrounded by phloem and pericyclic cells with 1-3 layers of parenchyma. The number of VBs ranges from 2-6 along the width of the midrib and is surrounded by endodermis.

Table 4. Morphological features of the Lamina

Taxa	Shape		Length (cm)	Width (cm)	Type
	Apex	Base			
P. costata	Acuminate	Gradually narrowed	44±0.065	5±0.001	Monomorphic
P. heteractis	Acuminate	Gradually attenuate	20±0.038	6±0.091	Moderately dimorphic
P. lanceolata	Acute	Cuneate	7±0.055	0.6±0.035	Moderately dimorphic
P. mannii	Acute	Gradually narrowed	12±0.055	2±0.065	Monomorphic
P. glabra	Caudate	Attenuate	10±0.071	1.5±0.014	Moderately dimorphic

Table 5. Anatomical features of the Lamina

Taxa	Epidermis				Stomata		Hydathodes	Midrib		
	Shape		Anticlinal wall		Type	Level				
	Up	Lo	Up	Lo				Hypodermis	Sclerenchyma of margin	Sclerenchymatous sheath
P. costata	QU	QU	ST	SI	PR	SUP	+	-	-	+
P. heteractis	QU	IR	SI	SI	PR	SUP	+	+	+	+
P. lanceolata	QU	QU	ST	ST	PR	DE	-	-	+	+
P. mannii	QU	IR	ST	SI	PO	SUP	+	-	-	+
P. glabra	QU	IR	ST	SI	PR	DE	-	+	-	+

Note: Up-Upper; Lo- Lower; IR-Irregular; QU-5-6 sides; ST-straight; SI-Sinuous;DE-Deep (sunken); SUP-Superficial stomata;PR-Pericytic stomata; PO-Polocytic stomata;+ = presence; - =absence

The stellate hairs are observed on both surfaces of the lamina, but are denser on the lower surface. The stellate hairs are intermixed with the sporangia and can be distinguished by their color from brown to light brown, greyish brown to whitish brown. From the SEM observations, it is evident that the stellate hairs consist of a central disc where a number of arms are attached to it. The diameter of the central disc ranged from 25±0.11µm to 44±0.13µm with smallest in *P. mannii* to largest in *P. glabra*. Three forms of stellate rays are observed, narrow boat-shaped rays in *P. costata*, boat-shaped in *P. glabra*, *P. lanceolata*, and acicular with woolly rays in *P. heteractis* and *P. mannii* (Fig. 3). Stellate hairs ranged from 500±0.33µm in *P. heteractis* to largest in *P. costata* with size of 2000±0.05µm (Table 6). The branch number in each species varied from 5 to 15 with highest in case of *P. glabra* and *P. lanceolata* having more than 10 branches and lowest in *P. mannii*. Presence of hydathodes has also been observed in *P. costata*, *P. heteractis*, and *P. mannii* on the adaxial laminar surface.

Table 6. Characterization of laminar stellate hairs

Taxa	Type	Color	Size (µm)	Branch number	Branches length (µm)	Branches width (µm)	Diameter of central disc (µm)
P. costata	Narrow boat-shaped	Light brown to greyish	2000±0.05	5-9	400±0.02	25±0.14	33±0.13
P. heteractis	Boat - shaped	Greyish brown	500±0.33	7-9	300±0.01	37±0.56	40±0.16
P. lanceolata	Boat-shaped	Whitish brown	1200±0.13	9-15	200±0.08	28±0.66	35±0.71
P. mannii	Acicular	Brown	2000±0.61	5-6	500±0.12	40±0.15	25±0.11
P. glabra	Boat-shaped	Brown	1200±0.21	10-12	250±0.15	35±0.11	44±0.13

Soral arrangement and Sporangia

Sori are distributed mostly from apical portion of frond to all over the lamina. The sori are of orbicular type with superficial in *P. costata*, *P. heteractis*, and *P. mannii* while sunken in *P. glabra* and *P. lanceolata* (Fig. 3). The diameter of the sori is maximum in *P. mannii* with 1.5±0.10mm followed by *P. heteractis* with 1.0±0.16mm, while the other species showed smaller diameter. The number of sori in each areole is variable with highest number in *P. costata* and *P. heteractis* followed by *P. mannii* with only 3-5 sori per areole. In *P. glabra* and *P. lanceolata*, it is around 1-2 sori per areoles. The sporangium is globose in shape and brown in color with longest in *P. glabra* with 310±0.15µm and smallest in *P. heteractis* with length of 210±0.13µm. The number of annular cells vary in each sporangium with about 22-25 cells in *P. mannii* while in other taxa it ranged from 16-22 annular cells. However, the sporangial stalk is markedly shorter with almost sessile in *P. mannii* (Table 7). The spores are monolete, bilateral, oblong when viewed in polar axis and lacks a perine. The spore size is variable among the species. In *P. costata*, the spores are lunar in shape with colour varying from greyish to yellowish with smooth surface ornamentation and mean size of 62.5±0.55µm in polar axis (PA) and 44±0.10µm in equatorial axis (EA). In *P. glabra*, spores are spherical in shape, light yellowish in color with smooth surface ornamentation and mean size of 41±0.33µm (PA) and 32.5±0.61µm (EA). *P. lanceolata* spores are lunar in shape with greenish yellow in color having spinose surface ornamentation and mean size of 40±0.15µm and 25±0.11µm along polar and equatorial axis respectively. In *P. heteractis*, the spores are reniform in shape with yellowish green in color and tuberculate surface with mean size of 62.5±0.01µm (PA) and 42±0.33µm (EA) while in *P. mannii*, the spores are lunar in shape, pale brown in color with sparsely verrucate surface having mean size of 67.9±0.17µm and 48.4±0.31µm along polar and equatorial axis respectively (Table 8).

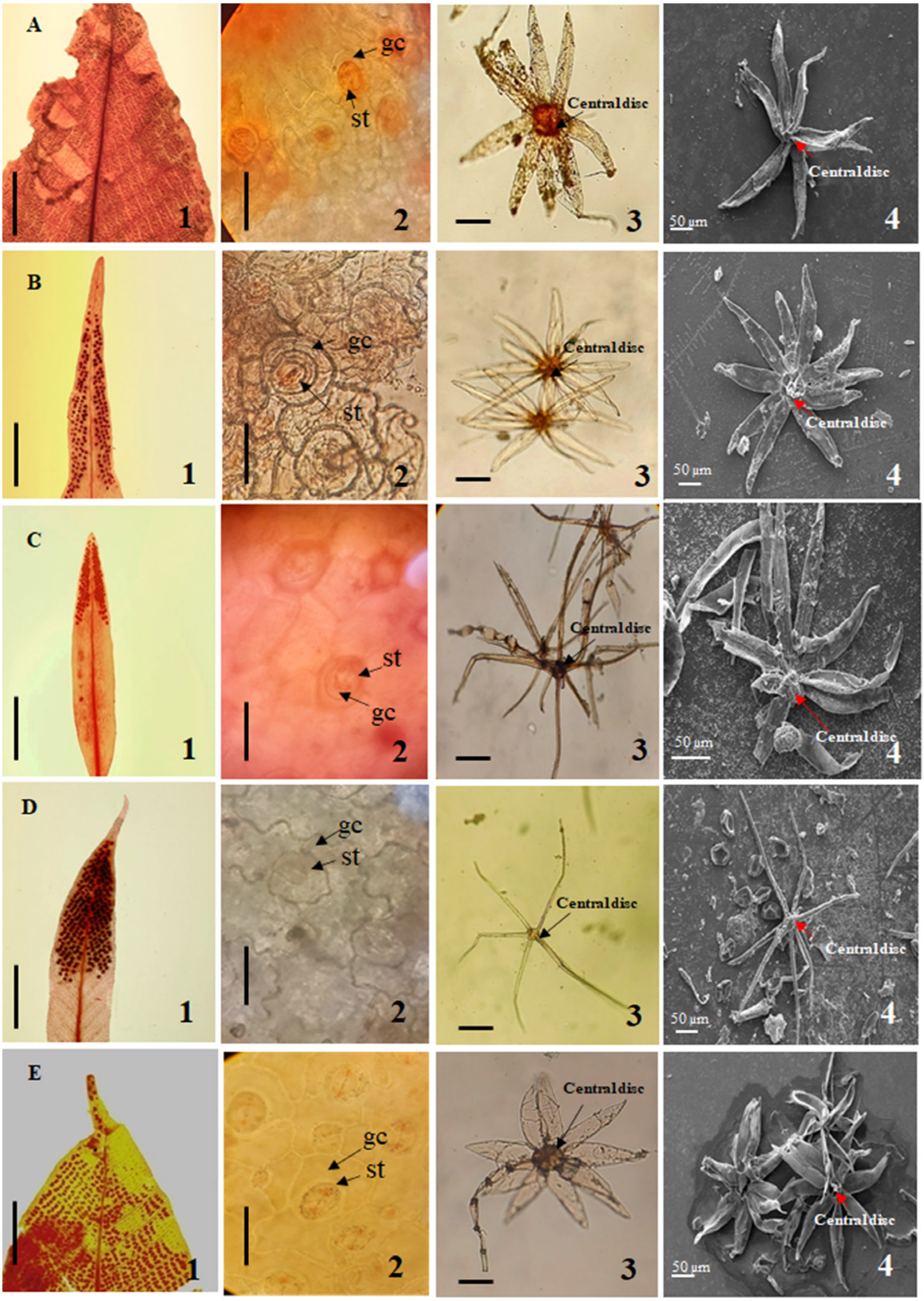

Figure 3. A. *P. costata* **B.** *P. glabra* **C.** *P. lanceolata* **D.** *P. mannii* **E.** *P. heteractis*; 1-arrangement of sori; 2- epidermis with stomata; 3- stellate hairs under LM; 4- stellate hairs under SEM; Note: gc-guard cell, st-stomata (scale bar: 1=1cm; 2=100µm; 3=50 µm).

Table 7. Morpho-anatomical characterization of sori and sporangium

Taxa	Sori				Sporangium		
	Type	Position	Diameter (mm)	No of sori in each areole	Length of sporangium (µm)	Length of stalk cells (µm)	No. of annular cells
P. costata	Sunken	Apical	0.5±0.11	10-16	225±0.07	0.2±0.01	12-14
P. heteractis	Superficial	Apical to all over	1±0.16	7-10	210±0.13	0.75±0.1	16-22
P. lanceolata	Sunken	Apical to all over	0.5±0.05	1-2	245±0.10	1.5±0.13	13-18
P. mannii	Superficial	Apical to all over	1.5±0.10	3-5	240±0.12	-	20-25
P. glabra	Sunken	Apical to all over	0.5±0.005	1-2	310±0.15	1.5±0.21	12-18

Table 8. Spore morphology and surface ornamentation

Taxa	Spore Type	Shape	Color	P.A (µm)	E.A (µm)	Surface
P. costata	Monolete	Lunar	Greyish to yellow	62.5±0.55	44±0.10	Smooth
P. heteractis	Monolete	Reniform	Yellowish green	62.5±0.01	42±0.33	Tuberculate
P. lanceolata	Monolete	Lunar	Greenish yellow	40±0.15	25±0.11	Spinose
P. mannii	Monolete	Lunar	Pale brown	67.9±0.17	48.4±0.31	Sparsely verrucate
P. glabra	Monolete	Spherical	Light yellow	41±0.33	32.5±0.61	Smooth

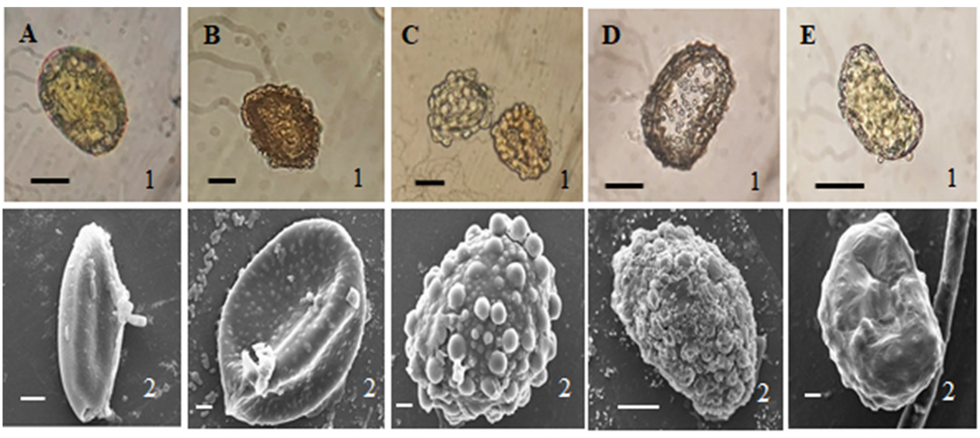

Figure 4. A. *P. costata* **B.** *P. glabra* **C.** *P. lanceolata* **D.** *P. heteractis* **E.** *P. mannii*; 1- LM images of spores; 2- SEM images of spores (scale bar = 10µm).

Principal Component Analysis

We combined morpho-anatomical characters and analyzed using PCA with a total of 11 quantitative traits. PCA on the morpho-anatomical characteristics of *Pyrrosia* species revealed that the first two principal components (PCs) were used to visualize the structure of the data in a PCA bi-plot. The variance explained by the data points on Component

1 (53.022%) is more than the variance of data points on Component 2 (23.631%). In bi-plot graph, traits like number of branches in stellate hair, size, lamina length, stipe length, rhizome scale length, rhizome scale width, number of sori in each areole shows positive loading whereas spore diameter and number of sclerenchymatous strands in rhizome show negative loading (Fig. 5). The amount of variation retained by each principal component can be understood by the eigenvalue.

Taxonomic interrelatedness

The interrelationships of the studied taxa were delineated via two methods; first, a dichotomous key to species was developed on the basis of the qualitative and quantitative morpho-anatomical data. Additionally, a hierarchical clustering based on the quantitative data and visualization through dendrogram based on degree of similarity was observed.

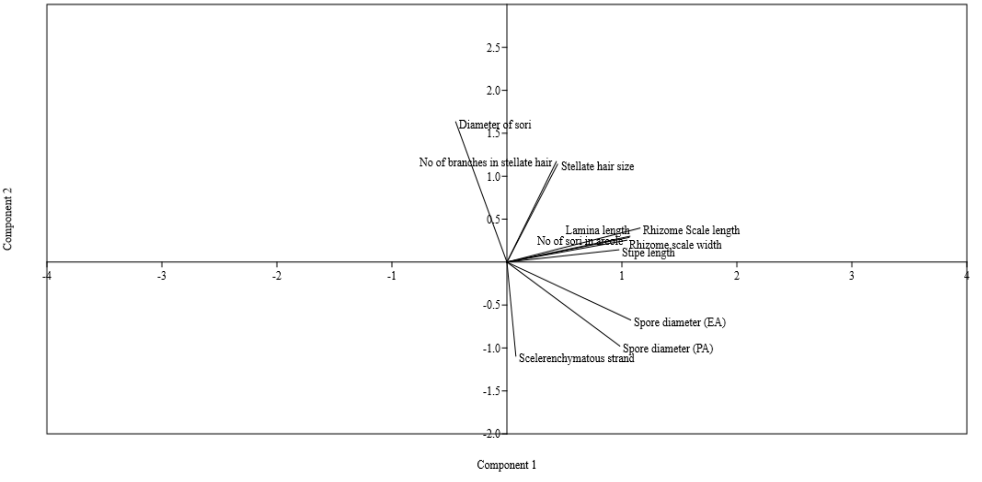

Figure 5. Principal component analysis (PCA) performed with quantitative traits.

Hierarchical clustering and dendrogram

The above discussed variations in the considered morphological characteristics have led to the grouping of 5 species under study into two major clades based on their degree of similarity. The UPGMA cluster analysis based on the quantitative and qualitative characters allowed us to group the studied species. *P. heteractis* and *P. mannii* are closely related to

Key to the selected *Pyrrosia* species

1a. Fronds monomorphic, less than 20 cm ……………………………..............*P. lanceolata*

b. Fronds monomorphic or slightly dimorphic, more than 20 cm …………..……...……..…2

2a. Fronds monomorphic……………..……..…………………………………….....………3

b. Fronds slightly dimorphic……………………………..……………………………….4

3a. Rhizome without sclerenchymatous strand…….……………………..…...............*P. costata*

b. Rhizome with scattered sclerenchymatous strand……………………….…………*P. mannii*

4a. Lamina ovate, rhizome with (many) central sclerenchymatous strand.............*P. heteractis*

b. Lamina lanceolate, rhizome with a single central sclerenchymatous strand…….*P. glabra*

each other with more than 96% similarity. It is also observed that *P. costata*, *P. glabra*, and *P. lanceolata* are related with more than 85% similarity. However, *P. heteractis* and *P. mannii* differs from *P. lanceolata* and *P.glabra* and falls under separate clade (Fig. 6).

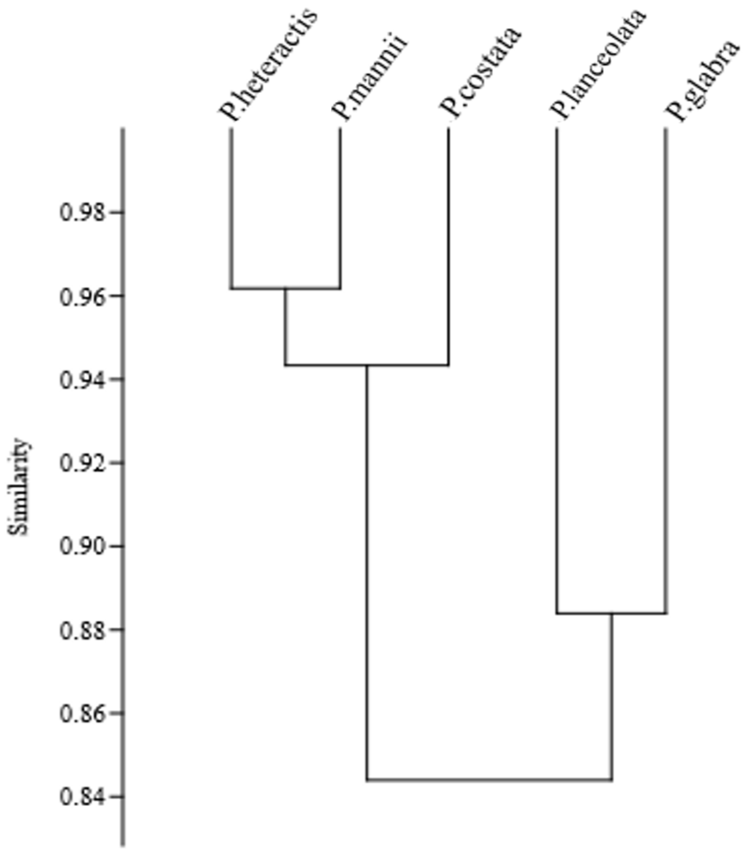

Figure 6. UPGMA dendrogram based on cluster analysis

DISCUSSION

The results show that a combination of morphological and anatomical characteristics seems useful in the identification of *Pyrrosia* species. Hovenkamp (1986), observed the scale rhizome as basifixed, peltate and pseudopeltate. In this study, *P. mannii* and *P. costata* possess pseudopeltate and basifixed rhizome scales respectively. All other examined species have peltate scales. Basifixed type scales lack stalk, attached by broad bases while peltate and pseudopeltate scales consists of a stalk and shield (Tsutsumi and Kato, 2008). The two closely related *P. glabra* and *P. lanceolata* is often confusing, on investigation we see that *P. lanceolata* has two distinctly different scales intermingled in its rhizome. They both differ in terms of size and shape. *P.glabra* only has linear-lanceolate type of scale. The stipe base scales in *P. heteractis* are very different in appearance from the scales generally present in the rhizome. The rhizome covered with scales is a distinct characteristic of genera in Polypodiaceae family (Zhang *et al*., 2013). Scales of the rhizomes have been demonstrated to be conservative and yield phylogenetic information (Christenhusz and Chase, 2014). Other characters of scales such as the insertion type and the margin morphology have been considered as a diagnostic feature in infrageneric studies (Nayar and Chandra, 1967; Hovenkamp, 1986; Shing and Iwatsuki, 1997; Lin *et al*.,2013). In the present study, the variations in the insertion type leading to the shape difference in the scale are observed. *P. costata* scales have a specific shape owing to its basifixed nature. The variations in rhizome margin exhibited mostly ciliate and toothed in the studied specimen. The PCA reveals the rhizome scale length and width as positively loaded and correlated with lamina length, stipe length characters among the species. The loading plot shows how strongly each investigated character influences a principal component (David and Jacobs, 2014). Frond dimorphism in *Pyrrosia* however ranges from monomorphic, slightly dimorphic to strongly dimorphic. In some species, the sterile and the fertile fronds occur together. The dual purpose of such organization is photosynthesis and reproduction (Chiou *et al*., 2005). The importance of fern anatomy in species identification and classification has been highlighted earlier (Ogura, 1972; Kotrnon, 2007; Talip *et al*., 2012; Resmi *et al*., 2016; Haq, 2017). Hovenkamp (1986), suggested that the arrangement of sclerenchymatous strands and their position in parenchyma is of taxonomic significance. The sclerenchymatous strands in rhizomes may be divided into four groups based on their position (Kortnon *et al*., 2007). They may be scattered irregularly over inner parenchyma (*P. mannii*) or restricted to the periphery, singular or central in the inner parenchyma (*P. glabra*, *P. lanceolata*, *P. heteractis*) whereas absent in *P. costata*. The importance of stipe anatomical details has been recorded by Kotrnon *et al*. (2007). In our study we observe the variations in the stipe and their vasculature in terms of shape and number. The frond midrib section of the taxa revealed a distinct T-shaped xylem vascular supply of the leaf midrib which is exclusive to family Polypodiaceae. Laminar anatomical features like the occurrence of a thick cuticle on the epidermis; sunken stomata; sunken sori; or distinct hydathodes are all xerophytic adaptations (Wei *et al*., 2017). The stomata observed in the specimens are pericytic and polocytic which supports the works by Sen and Hennipman (1981). They recorded nine different morphotypes of mature stomata in different genera of Polypodiaceae and derived their ontogenetical interrelationships. According to them pericytic type of stoma is an advanced type of stoma and is a secondarily derived mesogenous type of stoma, originated from polocytic type through desmocytic type. Polocytic type is the basic or primitive type of stoma in ferns (Mehra and Soni, 1983; Sen and De, 1992; Mukhopadhyay, 2021). However, polocytic stomata type occurs in *P. mannii* which may be due to reversion or secondary development (Wei, 2017). The character of trichome or stellate hairs is an important tool for the identification of *Pyrrosia* (Hovenkamp, 1986; Cheng *et al.*, 2014; Sofiyanti and Isda, 2018). Three forms of stellate rays are observed in our study viz. narrowly boat shaped rays, acicular boat shaped and acicular mixed woolly rays in *P. heteractis* and *P. mannii*. The number of rays in the stellate is often variable among the species and so do the colors that vary from brown, greyish-brown to light brown. Such variations in terms of hair shapes and color are taxonomically significant in *Pyrrosia* (Hovenkamp, 1986). The use of soral and indusial shape to delineate fern genera dates long back (Linnaeus, 1753; Smith, 1793), and has since been relied upon heavily as a diagnostic character in eupolyploid ferns (Tryon, 1952). *Pyrrosia* species have round, naked sori just as other polypodiaceae members. Multiple sori arranged mostly from apical to all over lamina in a long chain give rise to coenosori. Holttum (1954), reinstated the need of work on spores of pteridophytes and made an evaluation on the role of spores in fern taxonomy. The monolete spores in *Pyrrosia* have varied type of surface ornamentations which are revealed with the aid of SEM. A single reliable character cannot be used as an apomorphy to distinguish *Pyrrosia* from each other. However, some of the anatomical characters, such as the distribution pattern of collenchyma, sclerenchyma and parenchymatous cells in rhizomes, might be helpful in defining traits for monophyletic group recognition and species identification in *Pyrrosia*. Thus, the combination of two or more characters is necessary to identify all group (Wei *et al*., 2017). The principal component analysis captures the most variation in a data set (Jolliffe and Cadima, 2016). The score of the principal component will group together all the taxa with high similarity. The UPGMA cluster analysis based on the qualitative and qualitative characters allowed us to understand that, *P. heteractis* and *P. mannii* are closely related to each other with more than 96% similarity and these two species share above 92% similarity with *P. costata*, *P. glabra*, and *P. lanceolata*. However, *P. heteractis*, *P. mannii*, and *P. costata* form a separate clade which is separate from the *P. lanceolata* and *P. glabra* clade. This was also suggested by Nayar and Chandra (1967), as they segregated *Pyrrosia* into 6 groups on the basis of morphological data where, *P. heteractis* was considered within the second (Heteractis group), *P. mannii* within third (Mannii) group and lastly *P. lanceolata* in sixth (Varia group). Through the lens of evolution of the *Pyrrosia*, the Mannii group appeared to be more primitive condition while the Varia group represents more advanced, although differing in line of evolution (Nayar and Chandra, 1967). *P. heteractis* is quite common in the Darjeeling and Sikkim himalayan region. It resembles and is often confused as the Japanese fern *Pyrrosia lingua* (Thunb.) Farw. However, the two species possess different type of rhizome scales and *P. heteractis* have oblong-lanceolate frond with long acuminate apex (Mehra and Bir, 1964). Medicinally, the Chinese Pharmacopoeia included 3 species of *Pyrrosia*, namely *P. petiolo-*

sa, *P. sheareri*, and *P. lingua* (Fan *et al*., 2020). *Pyrrosia lingua* finds its immense importance in recent times of Covid 19 pandemic. Babich *et al*. (2020), highlighted its importance as secondary metabolites and compounds like chlorogenic acid, flavonoids and xanthones are abundant. The frond extracts also reportedly display antiviral activity (Li *et al*.,2005; Lin *et al*.,2014). The plant decoction of *P. lanceolata* has been utilized all over South Africa for treating cold and sore throat. In Mexico it is used as an itchguard (Manickam and Irudayaraj, 1992). Moreover, *P. lingua* and *P. nuda* (syn. *P. glabra*) has been used for cystic treatment (Ho *et al*., 2011). Several other species of these genera like *P. heterophylla*, *P. peltiosa*, *P. davidii* are medicinally significant (Cheng *et al*., 2014). Therefore, it is important to rightfully identify upto specific level for better efficacy (Bennett and Balick, 2008). Morphological references of medicinally important species are of utmost importance, so that the samples can be correctly identified especially when there are chances of morphologically similar species leading to confusion and improper use of those particular taxa (Oliveira *et al*., 2017).

CONCLUSION

The morph-anatomical characters considered in the present study under LM and SEM have added valuable information at family, generic and specific levels. In the present investigation, qualitative and quantitative morpho-anatomical data proved to be a pivotal taxonomic tool for distinguishing *Pyrrosia* species. It was possible to discern among the species and notice their shared characteristics as well as their dissimilarities. It can be established that morphological and anatomical traits are still relevant tool in fern systematics. Spore morphology provides a diagnostic value at generic as well as specific levels. The anatomical approach as well as PCA presented in this study is the first of its kind performed for *Pyrrosia* from Darjeeling Himalaya. The UPGMA dendrogram, PCA cladistic analyses based on morpho-anatomical traits can be used in the taxonomic discourses for species delineation of fern groups.

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