Discovery of a new species of Adder’s tongue fern from India with comparative analysis of morphological and molecular attributes

B. L. Yadav*, Mukesh K. Meghvansi, Kanta Meena & C. B. Gena

Eusporangiate fern genus *Ophioglossum* L. is commonly known as Adder’s tongue fern as its fertile frond gives the appearance of snake tongue. A new species in this fern genus, *O. trilokinathii* belonging to Ophioglossaceae family has been discovered from the plateau region of Rajasthan State of northwestern India. The new species can be distinguished from other taxa of this genus by its smaller habit, subglobose-tuberous rhizome, basipetal emergence of young roots, aggregation of old decaying roots on rhizome apex, fertile stalk as well as spike short and thick, trophophylls in rosette, ovate or orbicular and a unique sporoderm sculpture pattern under SEM having broad reticulations with thick and raised muri enclosing large hexagonal or irregular areas on the distal and proximal faces of the spores hitherto unreported in any of the presently known taxa of *Ophioglossum*. In addition, comparative study of stomatal structure, foliar anatomy and nucleotide sequence data of its three chloroplast DNA markers (*trnL-F*, *rbcL* and *psbA-trnH*) was carried out. In view of all the attributes including habitat, ecology, morphology, foliar anatomy, stomatal features, palynology and molecular phylogenetic data, the present study suggests that the *Ophioglossum* specimen collected from plateau region of Rajasthan represents a hitherto undescribed species thereby warranting its establishment as *O. trilokinathii* sp. nov. A detailed comparative account of the new taxon with its allied species has also been provided.

The fern genus *Ophioglossum* L. is cosmopolitan in distribution and is peculiar in having certain non-fern characters like absence of circinate vernation in leaf, lack of sclerechyma in the entire plant body, presence of special reproductive organ—spike and presence of petiolar collateral and cauline vascular bundles. Furthermore, the occurrence of usually large chromosome numbers in *Ophioglossum* has made it more popular among the researchers studying the plant biological systems. The report of *n* = 720 (*2n* = 1440) in a population of *O. reticulatum* L. from Shevaroy Hills, south India is the highest chromosome number possessed by any plant or animal species in the biological world. *Ophioglossum* is a very challenging taxon with regard to species delimitation and identification due to its simple plant body represented by underground rhizome (except in the epiphytic species *O. pendulum* and *O. palmatum*) and an aerial complex which consists of sterile and fertile fronds. The classification of its species is based on such features of the sporophyte as leaf size, shape, venation, features of spike and spores which are not ordinarily suffice for systematic purposes in other groups. Whereas, the classification is primarily based on the leaf attributes, the other parts of the sporophyte being buried under the ground thus, not available for systematic purposes can potentially lead to a greater taxonomic ambiguity. Furthermore, morphological plasticity is well known in species of this genus. Sometimes the intraspecific variations due to variable microclimatic conditions of the habitat or polyploidy, are so prominent that they appear to be a distinct species. This is evident from the total number of species which greatly varies from 26 to 54 under this genus.

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*Department of Life Science, Mewar University, Gangrar, Chittorgarh, Rajasthan 312901, India. Bioprocess Technology Division, Defence Research and Development Establishment, Gwalior, Madhya Pradesh 474002, India. Department of Botany, Manikya Lal Verma Government College, Bhilwara, Rajasthan 311001, India. Maharaja Ganga Singh University, Bikaner, Rajasthan 334004, India. C. B. Gena is deceased. Email: drblyadav@yahoo.com
Presently, the genus is represented by 52 species world over, of which more than 20 are reported from different bio-geographic regions of India. Rajasthan, the largest State lying in the northwestern part of India has four major physiographic regions namely, the Western Thar Desert, the Aravalli Range with Vindhyan Mountains, the Eastern plains and South-eastern plateau region. The southeastern plateau region locally called as Pathar or Upar Mall region is a humid zone with rich floristic diversity. Of the eight Ophioglossum species (O. costatum R. Br., O. gramineum Willd., O. gujaratense Patil, Kachhiyapatel, Patel & Rajput, O. indicum Yadav & Goswami, O. lusitanicum L., O. parvifolium Grev. & Hook., O. petiolatum Hook. and O. reticulatum L.) reported from this state, six have been found to occur in the Mainal area. Thus, this area happens to be a potential nano hot spot with regard to Adder’s tongue fern diversity. However, limited efforts have been made on extensive survey and detailed investigation for generating the comprehensive data pertaining to species diversity, morphology, palynology, anatomy and molecular biology of this interesting fern genus Ophioglossum. In a botanical excursion carried out during 2016–2018 with an aim to study species diversity and the field behaviour of various taxa of this genus, authors came across an interesting population of Ophioglossum in Mainal area. Initially, the plant specimens of this population were looking superficially similar to O. parvifolium and O. gujaratense. However, after comparing the morphological, palynological, anatomical and molecular characters (trnl-F, rbcL and psbA-trnH chloroplast DNA markers) of collected plants with other closely allied species of Ophioglossum, it is concluded that the population of Mainal stands as an undescribed taxon. Hence the new taxon is described as O. trilokinathii sp. nov.

**Results**

**Taxonomy.** *Ophioglossum trilokinathii*. B L Yadav, M K Meghvansi, K Meena & C B Gena sp. nov. (Fig. 1 and 2).

**Type.** INDIA. Rajasthan: Chittorgarh district, Mainal 25° 5′ 15" N, 75° 10′ 01″ E, elevation ~ 507 m, 12 August, 2016, B. L. Yadav, C. B. Gena & Kanta Meena 65.
Holotype. Central National Herbarium, Kolkata (CAL!).

Isotype. Herbarium Botanical Survey of India, Arid Zone Regional Centre, Jodhpur (BSJO!); Herbarium Mewar University, Chittorgarh, Rajasthan (MUCR 0036).

Type locality. Mainal (25° 5′ 15′′ N, 75° 10′ 01′′ E, elevation ~ 507 m) Chittorgarh district, Rajasthan, northwestern India.

Etymology. The specific epithet has been chosen in honour of Professor Triloki Nath Bhardwaja (Professor T. N. Bhardwaja), Former Vice-Chancellor, V. M. Open University, Kota, Rajasthan, India for his commendable work in the field of pteridology.

Diagnosis. *O. trilokinathii* sp. nov. is unique among the species of this genus in having basipetal emergence of roots, aggregation of old decaying roots at rhizome apex, trophophylls in rosette touching the substratum, peculiar broad reticulate spore wall ornamentation with thick and raised muri enclosing large hexagonal or irregular areas, granulose perine (perispore), differentiation of mesophyll cells into palisade and spongy tissues and elliptic stomata with thin and smooth inner margin of outer stomatal ledge.

Description. Plants terrestrial, small, 1.1–2.4 cm in height (Fig. 1A); rhizome subglobose-tuberous, 0.3–0.5 cm long, 0.1–0.2 cm broad (Fig. 1B); roots thick, fleshy, brownish white, stoloniferous, young roots on basal part of rhizome, aggregation of old decaying roots on rhizome apex (Fig. 1B,C); common stalk 0.2–0.4 cm, subterranean, white (Fig. 1B); trophophylls1–4 sometimes 5, arranged in a rosette, horizontal touching the substratum, broadly ovate or ovate orbicular, thick, margin entire, apex acute or apiculate, base cuneate, lamina surface sometimes with 1–3 longitudinal shallow furrows, midrib absent, 0.6–0.9 cm long, 0.4–0.7 cm broad (Fig. 1D);
| S. no. | Characters | O. trilokinathii sp. nov. | O. hitkishorei | O. costatum | O. gujaratense | O. parvifolium |
|--------|------------|--------------------------|---------------|-------------|---------------|---------------|
| 1.     | Habitat    | Terrestrial              | Marshy        | Terrestrial | Terrestrial   | Terrestrial   |
| 2.     | Plant height (cm) | 1.1–2.4                | 3.0–8.0       | 8–10        | Less than 10.0 | Up-to 10.0    |
| 3.     | Rhizome shape | Subglobose-tuberous     | Globose–subglobose | Subglobose with apical cupule | Tuberous | Subglobose-tuberous |
| 4.     | Root       | Basipetal emergence, non stoloniferous | Acropetal emergence, non stoloniferous | Basipetal emergence, non stoloniferous | Acropetal emergence, stoloniferous | Acropetal emergence, stoloniferous |
| 5.     | Trophophyll | Number | 1–4 | 2–6 | 1–6 | 1–4 | 1–2 |
|        | Shape      | Ovate or orbicular       | Elliptic, ovate oblancoate | Elliptic- lanceolate | Ovate elliptic, elliptic- lanceolate | Ovate, elliptic, sometimes orbicular |
|        | Position   | Flat and touching to soil surface | Upward from the ground | Upward from the ground | Horizontal slightly above ground | Flat on substratum |
| 6.     | Fertile stalk Length (cm) | 0.7–1.7               | 2.5–5.8       | 4.3–5.8 | 2.0–8.0       | 1.8–3.2       |
| 7.     | Spore size | 18–30                   | 20–30         | 36–38      | 19–28         | 31–35         |
| 8.     | Exine ornamentation | Muri, form large hexagonal on distal and proximal face, proximal face not granulate, laesural arms straight, short not reaching to the margins of central cavity | Beaded strings of exine grains form hexagonal areas on distal face, Proximal face granulate, laesural arms straight up to margins of the central cavity | Exine reticulated on distal face and porate on proximal face, muri verrniculate enclosing deep polygonal lumina, surface of muri rough, Laesural arms up to margins of central cavity | Reticulated muri of uneven heights enclosing shallow areas on distal face and distinct reticulation on proximal face, laesural arms prominent sometimes wavy reaching to the margins of central cavity | Muri wide enclosing shallow conical lumina on distal face, pitted ornamentation on proximal face, Laesural arms straight up to the margins of central cavity |

Table 1. Comparison of morphological characters among O. trilokinathii sp.nov., O. hitkishorei, O. costatum O. gujaratense and O. parvifolium.

venation reticulate, marginal aeroles with free vein endings (Fig. 1D); fertile segment arises from the junction of face of lamina and common stalk, 0.7–1.5 cm long, thick, spike short, thick, 0.4–0.9 cm long, with two rows of lateral sporangia and a sterile tip, sometimes sterile tip absent; sporangia 5–10 pairs; spores trilette, globose, perinate (Fig. 2A), 18–30 μm in size, laesural arms short, straight not reaching to the margins of central cavity of proximal face, sporoderm reticulate under Light Microscope (Fig. 2A,B).

SEM imaging revealed that spores were globose and perinate. Interestingly, spores have a unique exine ornamentation pattern of broad reticulations with thick and raised muri, enclosing large hexagonal or irregular areas on distal and proximal faces, proximal face with distinct short triradiate mark and reticulations, laesural arms smooth and straight, not reaching to the margins of cavity of proximal face. To the best of our knowledge, the exine ornamentation pattern as noted in this case has not been reported so far in any other presently known species of Ophioglossum (Fig. 2C,D).

Reproductive period. July–August.

Distribution and ecology. India—Rajasthan state, Chittorgarh district, Mainal locality. The species grow in dense populations on moist soil in open terrestrial habitat at an elevation of ~ 507 m. Plants of this species sprout from the underground rhizome after a fortnight period of first showers in the month of June–July and dry up by the mid of September every year.

Conservation status. The species has been recorded from Mainal area which is known for its famous waterfall and nearby temple of Jogniyamata. It is a plateau region supporting the occurrence of O. costatum, O. gramineum, O. parvifolium, O. indicum, O. petiolatum, and O. gujaratense. Therefore it is one of the richest localities of Adder’s tongue fern where contiguous occurrence of two or more species is frequent. The species population occurs in 1.0 × 1.0 m2 area and is represented by 300–350 individuals. Future explorations are needed to get its entire range of distribution and therefore, at present the species is treated under the category “Data Deficient” (DD) of IUCN33.

Species recognition. Comparison of the new taxon with its allied species provided in Table 1 reveals that O. trilokinathii sp. nov. partially resembles morphologically with O. hitkishorei Patel & Reddy in such features as thick trophophyll, acute apex and cuneate base. However, O. hitkishorei differs from O. trilokinathii sp. nov. by its marshy habitat, larger size, roots non stoloniferous, acropetal emergence of young roots, trophophyll upward from the ground, spore exine shows beaded strings of exine grains forming polygonal or round spaces on the distal pole and granulate proximal face.

Basipetal emergence of young roots and 1–4 or 5 trophophylls are also found in O. costatum but in this species bottom part of subglobose rhizome is devoid of roots, roots non stoloniferous, trophophylls are larger with distinct midrib, not arranged in rosette form and reticulate exine with verrniculate muri, muri thin and raised enclosing deep polygonal lumina on distal face and almost pitted ornamentation on proximal face. Laesural arms of triradiate ridge straight reaching to the margins of the central cavity of spore (Fig. 3C,D) which has also been described by earlier workers whereas exine ornamentation of spores of O. trilokinathii sp. nov. is altogether...
different in having thick and raised muri enclosing large hexagonal or irregular areas on distal and proximal faces, proximal face with distinct short tri-radiate mark and reticulations, laesural arms smooth and straight, not reaching to the margins of cavity of proximal face (Fig. 3A,B).

Comparison of the new species (O. trilokinathii sp. nov.) with O. gujaratense indicates that these two taxa resemble in having stoloniferous roots, subglobose tuberous rhizome, number of trophophylls which are 1–4 sometimes 5, appressed to the ground and common stalk subterranean but O. gujaratense differs from O. trilokinathii by its larger size, acropetal emergence of roots, trophophylls lanceolate, fertile stalk thin and long and exine with muri of uneven heights along with some flat and wider areas, enclosing shallow depressions on distal face, proximal face reticulated bearing prominent triradiate ridge, laesural arms straight sometimes wavy, reaching to the margins of the central cavity of the spore (Fig. 3E,F).

Figure 3. Comparison of spore structure of some species of Ophioglossum under SEM. (A) O. trilokinathii sp. nov. proximal face. (B) O. trilokinathii sp. nov. distal face. (C) O. costatum proximal face. (D) O. costatum distal face. (E) O. gujaratense proximal face. (F) O. gujaratense distal face. (G) O. parvifolium proximal face. (H) O. parvifolium distal face.
**Figure 4.** Stomatal features of *Ophioglossum trilokinathii* sp. nov. and its allied species. (A) *O. trilokinathii* elliptic stomata with thin and smooth inner margin of outer stomatal ledge. (B) *O. costatum* elongated elliptic or lanceolate stomata with thick and hairy inner margin of outer stomatal ledge. (C) *O. gujaratense* broad elliptic stomata with thick and rough inner margin of outer stomatal ledge. (D) *O. parvifolium* elliptic stomata with thin and striate inner margin of outer stomatal ledge.

*O. trilokinathii* also has some similarities with *O. parvifolium*, as in these two species roots are stoloniferous, common stalk subterranean, trophophylls horizontal touching the soil surface, trophophyll apex sometimes apiculate but the later species differs from the new taxon in, size (upto 10 cm vs 1.1–2.4 cm), emergence of roots (acropetal vs basipetal) number of trophophylls (1–2 vs 1–4), trophophyll base (cordate vs cuneate), fertile stalk (thin and long vs thick and short). Sporoderm structure of *O. parvifolium* is entirely different from that of *O. trilokinathii*. In former species exine is reticulate, muri thin enclosing funnel shaped lumina on the distal face and on proximal face exine is pitted, laseural arms of triradiate ridge straight reaching to the margins of central cavity of the spore (Fig. 3G,H).

Another noteworthy feature of the new taxon is the mesophyll tissue of its trophophyll which shows differentiation into palisade and spongy parenchyma (Fig. S1A). Mesophyll cells towards upper epidermis are elongate and closely packed while those towards lower epidermis are rounded or ovoid and are loosely arranged with intercellular spaces. Such differentiation is not found in other species (Fig.S1B,C) except *O. nudicaule*.

The new taxon is quite different from its allied species in its stomatal shape and inner margin of outer stomatal ledge. Trophophylls are amphistomatic. Stomata are elliptic in the new taxon (Fig. 4A) in contrast to the elongated elliptic or lanceolate in *O. costatum*, broadly elliptic in *O. gujaratense* and elongated elliptic in *O. parvifolium*. Inner margin of outer stomatal ledge is thin and smooth in the new species (Fig. 4A) while thick with small hairy structures in *O. costatum* (Fig. 4B), thick and rough in *O gujaratense* (Fig. 4C) and thin and striate in *O. parvifolium* (Fig. 4D).

**Phylogenetic relationship and genetic divergence.** Comparison of *psbA-trnH, rbcL* and *trnL-F*, nucleotide sequence datasets yielded 91% (Fig. 5), 63% (Fig. 6) and 95% (Fig. 7) ML bootstrap values respectively for *O. trilokinathii* with its closest matching specimens namely *O. sp. SAD-2020a* and *O. hitkishorei*. In addition, the values of evolutionary divergence (p-distance) between *O. trilokinathii* and *O. hitkishorei* with respect to
three markers were 0.010 (psbA-trnH; Supplementary Table S1), 0.044 (rbcL; Supplementary Table S2), and 0.00 (trnL-F; Supplementary Table S3) as calculated using MEGA X. Further, the determination of percent identity matrix of nucleotide sequences belonging to rbcL and trnL-F regions revealed that O. trilokinathii exhibited 93.10% and 97.22% identity with O. hitkishorei respectively (Supplementary Table S4 & S5). With regard to psbA-trnH region, O. trilokinathii exhibited 97.15% and 98.86% identity with O. hitkishorei and O. sp. SAD-2020a respectively (Supplementary Table S6).

Discussion
Ophioglossum is a fern well known for confused state of affairs prevailing in respect of inter-specific differences\(^5\). The taxonomy of this genus is based on such morphological characters as size of plants, shape and number of leaves, size of spike, shape and number of sporangia per spike which are easily amenable to climatic factors and pose practical difficulties in species delimitation. Thus, besides morphological characters of the plant, palynological, molecular and micromorphological features may be of considerable help in solving the taxonomic tangles in this genus. A comparison of morphological features of the new taxon with O. hitkishorei, O. costatum, O. gujaratense and O. parvifolium has been provided in Table 1. Besides the shape of rhizome, acropetal and basipetal pattern of root emergence on the rhizome of Ophioglossum species has been recognized as feature of taxonomic importance and this character was used to distinguish different species of this fern\(^31,35\). O. trilokinathii sp. nov. shares the character of basipetal pattern of emergence of young roots with O. costatum but later species is larger in size with subglobose rhizome whose bottom part being devoid of roots in contrast to the former one which is smaller in size with subglobose-tuberous rhizome.

In majority of taxa, the mesophyll tissue is homogenous made up of isodiametric spongy cells. The tropophylls in the present taxon are in rosette and lie flat on the substratum resulting into the unequal illumination of the two surfaces of the blades which lead the differentiation of mesophyll tissue into palisade and spongy parenchyma. Such differentiation of mesophyll is unknown in species of Ophioglossum except O. nudicaule\(^36\). The structure and design of mesophyll tissue in the plant leaf is one of the key traits playing important role in the regulation of photosynthesis. Elongated chlorenchyma cells of palisade layer with large amount of chloroplast which tend to stay very close to the walls of the cells to harvest maximum amount of illumination enable the plants to make optimal advantage of available light. Palisade parenchyma also helps in distribution of light more uniformly to chloroplast within the cell\(^37\).

Owing to significant variation among species in stomatal features particularly the stomatal shape, shape and ornamentation of inner margin of outer stomatal ledge, they have been used taxonomically by the earlier workers\(^38\) in separating the complex taxa of Dryopteridaceae in pteridophyta. Epidermal features and mesophyll characteristics have also been suggested by various researchers as supporting evidences in differentiating some...
Figure 6. Phylogenetic tree of *Ophioglossum* species based on *rbcL* datasets as analysed using maximum likelihood method. ML bootstrap percentages (BP) are shown on branches.

Figure 7. Phylogenetic tree of *Ophioglossum* species based on *trnL-F* datasets as analysed using maximum likelihood method. ML bootstrap percentages (BP) are shown on branches.
species of genus *Ophioglossum*\(^{17,22}\). Outer stomatal ledges prevent wide opening of the stomatal pore and its lifting above leaf epidermis\(^{39}\). This lip-like structure around the stomatal pore also helps in preventing water loss by sealing the pore during physiological stress\(^{38,41}\). Thus, the differentiation of mesophyll tissue and stomatal ledges are the features which provide survival potential to the new taxon as it is a heliophyte occurring in open terrestrial habitat.

Sporederm features were treated as of taxonomic importance in distinguishing the European species of *Ophioglossum*\(^{42,43}\). Spore coat structure could be valid mark for identification of some *Ophioglossum* species\(^{43}\). Spore characters are not easily prone to environmental influences compared to vegetative features and arethus more dependable in systematic consideration\(^{44}\). Some workers\(^{45}\) have concluded for Japanese species of *Ophioglossum* that the ornamentation pattern of a particular species collected from different localities and with distinct morphological variability possesses a specific pattern of spore exine.

Spore morphology has now assumed a significant role in pteridophytic taxonomy. Several workers\(^{9,23,34,46–51}\) have treated the exine ornamentation pattern as the most reliable character for systematic purposes in *Ophioglossum*. Thus, spore studies fully justify the relevance and validity of SEM investigation in the species delimitation of *Ophioglossum*. Three types of exine ornamentation patterns namely reticulate, verrucate and scabrate are known in species of *Ophioglossum*\(^{42}\). The exine ornamentation pattern recorded in *O. trilokinathii* sp. nov. is entirely different consisting of thick and raised muri enclosing large hexagonal or irregular areas on the proximal and distal poles of the spore. Such an exine ornamentation is not known so far in any of the species of the genus *Ophioglossum*.

DNA barcoding has been used increasingly for shedding light on species delineation in many fern species including *Ophioglossum*. For a valid plant barcode, it is emphasized to include conservative coding region like *rbcL* together with rapidly evolving non-coding region like *trnl*-P\(^{3}^{3}\). Similarly, the *psbA-trnH* intergenic region is among the most variable regions in the angiosperm chloroplast genome and is a popular tool for plant population genetics and species level phylogenetics. This region has also been suggested to be suitable for DNA barcoding studies\(^{43}\). Various researchers have successfully used these DNA barcodes for molecular identification of *Ophioglossum*\(^{20–23}\).

In the present study, the comparative phylogenetic analysis of seemingly allied species of *Ophioglossum* suggests considerable degree of differences between *O. trilokinathii* and *O. hitkishorei* with respect to *rbcL* and *psbA-trnH* regions as confirmed by determination of evolutionary divergence (p-distance) and percent identity matrix. The sequence difference between *O. trilokinathii* and *O. hitkishorei* in terms of percent identity matrix was as high as 6.9% for *rbcL* region. The sequence data of *O. sp. SAD 2020a* were available in GenBank only for *psbA-trnH* region and not for the remaining two regions (*rbcL* and *trnl*-P). Although, the data on p-distance could not provide evolutionary divergence between *O. trilokinathii* and *O. sp. SAD-2020a* respectively, percent identity matrix suggested considerable differences in the nucleotide sequences of these specimens with respect to *psbA-trnH* region. Furthermore, the morphological description of *O. sp. SAD-2020a* was not available in the published literature. Based on the information available in NCBI database with respect to the sequence data of *psbA-trnH* marker, *O. SAD2020a* specimen belongs to Kerala (Southern India) while *O. trilokinathii* is reported from northwestern part of India (Rajasthan). Both the regions have altogether different bio-geoclimatic conditions.

Taking all the attributes including habitat, ecology, morphology, foliar anatomy, stomatal features, palynological and molecular phylogenetic data into consideration we conclude that the *Ophioglossum* specimen collected from plateau region of Rajasthan represents a hitherto undescribed species thereby warranting its establishment as *O. trilokinathii* sp. nov.

**Methods**

**Morphology.**  Fresh plants were collected in polythene bags from Mainail (25° 5′ 15″ N, 75° 10′ 01″ E, elevation ~ 507 m) of Chittorgarh district, Rajasthan, India. Permission for the plant sample collection was obtained from the Forest Department, Chittorgarh. Photographs of plants growing in nature were taken. Field observations such as habitat, elevation, latitude and longitudes were recorded. Material was washed with water and fixed in Formalin, Acetic acid, Alcohol (FAA, 5:5:90) solution for further examination in laboratory. Herbarium specimens were prepared following standard method of drying, pressing and mounting on sheets. The specimens were deposited in the Herbarium, Mewar University, Chittorgarh, Rajasthan (voucher ID number B. L. Yadav, C. B. Gena & Kanta Meena 65 MUCR 00036). Morphological observations were based on fresh as well as herbarium material. All the measurements were recorded in metric scale. Comparison with other allied species of *Ophioglossum* collected from Rajasthan, India and with the digital images available online from LINN and Kew Herbarium Catalogues\(^{55,56}\) was made following published literature and expert opinion. The morphological features of spores were studied without acetylation as this technique has not been recommended in family *Ophioglossaceae* because it dissolves the fine structures of the exine\(^{46}\). Formal identification of plant specimens was carried out by the authors (B. L. Yadav and C. B. Gena) which was further verified and confirmed by Professor T. N. Bhadrwaja, Pteridologist & Former Vice-Chancellor, V. M. Open University, Kota, Rajasthan, India. All the investigations pertaining to the plant species description were performed in accordance with relevant guidelines and regulations.

**Scanning electron microscopic observations.**  Spore as well as leaf samples from mature plants were used for Scanning Electron Microscopic (SEM) analysis. Spores were mounted on aluminium stubs with double-adhesive tape and were coated with thin layer (200–400 dA) of gold–palladium using Sputter Coater model Quorum-Q-150 TES. The prepared samples were then examined using the SEM (Make FEI; Model Nova NanoSEM–450) in the SEM laboratory of Material Research Centre, Malviya National Institute of Technology,
Jaipur, Rajasthan. For SEM images of stomata, samples of leaves (small piece of leaf) were taken in place of spores.

**Venation pattern.** The entire leaves were cleared in 10% sodium hydroxide (NaOH) solution for 24 h following which they were repeatedly washed and transferred to a supersaturated solution of Chloral hydrate for 24 h until rendered transparent. After thorough washing in water, leaves were stained in aqueous safranin and mounted in 50% glycerin. Venation pattern was observed from glycerin mounted leaf under light microscope (Magnus Optosystems India).

**Foliar anatomy.** Mature leaves (trophophylls) from the FAA fixed plants were taken. Hand cut sections were stained in 1% aqueous safranin before mounting on slides in 50% glycerine and then observed under light microscope. Photomicrographs of these temporary preparations were taken using camera Model E-PL 1 (Olympus).

**Molecular characterization.** Genomic DNA extraction, PCR amplification and sequencing. The genomic DNA from the sterilized plant material was extracted using the method suggested by Doyle and Doyle. Extracted DNA was quantified employing the method of Sambrook et al. Purity of the DNA was further determined by electrophoresis in agarose gel (0.8%). PCR amplification of three chloroplast DNA (cpDNA) markers (trnL-F, rbcL and psbA-trnH) was carried out using the extracted DNA as template. A single PCR protocol was recruited in respect of all three chloroplast regions. In the process, 20 μL reaction mixture containing extracted genomic DNA template (2 μL) (1:10 dilution of the extracted DNA), forward primer (1 μL), reverse primer (1 μL), 1 × final concentration of ReadyMix Taq PCR reaction mix (SIGMA-ALDRICH) (10 μL) and nuclease free water (6 μL). The PCR was carried out in Thermal cycler (BIO-RAD iCycler). PCR program settings included: 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1.30 min, and a final elongation step at 72 °C for 10 min. Amplified chloroplast markers (trnL-F, rbcL and psbA-trnH) were visualized on 1% agarose gel under UV light by staining with ethidium bromide. Amplified PCR products were purified using GenElute PCR Clean-up kit (SIGMA-ALDRICH) and sequenced at Barcode Biosciences, Bangalore.

Alignment and phylogenetic analysis. Newly generated sequence data were aligned using Gene Tool v1.0 and their contigs were prepared. NCBI-BLAST search was conducted to reveal the sequence identity. The Sequence data were then deposited to NCBI database (Accession numbers: trnL-F, MW081148; rbcL, MW081146; psbA-trnH, MW081147).

Further, nucleotide sequences of Ophioglossum species (28 sequences of rbcL, 18 sequences of trnL-F and 22 sequences of psbA-trnH) were retrieved from the GenBank and used for comparative analysis with nucleotide sequences of O. trilokinathii sp. nov. Botrychium lunaria of the same family was included in the analysis as an outgroup taxon. Nucleotide sequences of all three chloroplast markers as mentioned above were subjected to pairwise alignment and multiple sequence alignment (MSA) using Clustal-W embedded in MEGA X with default settings. Also, MEGA X was used to find out the best fit model for phylogenetic analyses. The evolutionary history was inferred by using the Maximum Likelihood method wherein the models with the lowest BIC scores (Bayesian Information Criterion) were considered to describe the substitution pattern the best and selected as best-fit model. Accordingly, K2 (Kimura-2 parameter) model was found to be the best-fit model in case of the rbcL dataset whereas T92 (Tamura-3 parameter) was the best-fit model for of psbA-trnH and trnL-F datasets. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Initial tree (s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the corresponding best-fit model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 10.3173 for psbA-trnH, 1.8565 for rbcL and 3.7709 for trnL-F datasets). All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). The trees with the highest log likelihood (−2272.44 for psbA-trnH, −2007.65 for rbcL and −1758.23 for trnL-F datasets) are shown as Figs. 5, 6 and 7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches in the trees. The evolutionary divergence (p-distance) between sequences was calculated using MEGA X. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Codon positions taken into consideration were 1st + 2nd + 3rd + Noncoding. All positions with less than 95% site coverage were removed with partial deletion option. In total, there were 453, 226 and 297 positions in the final datasets corresponding to rbcL, trnL-F and psbA-trnH regions respectively. The percent identity matrix was calculated using Clustal Omega (https://www.ebi.ac.uk).

Data availability
All the generated sequences have been submitted to the GenBank with accession number as trnL-F, MW081148; rbcL, MW081146; psbA-trnH, MW081147.

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Author contributions

B.L.Y., C.B.G. and K.M. collected the species, prepare herbarium specimens and compared the material with other allied species. M.K.M. carried out molecular data analysis. B.L.Y. and M.K.M. prepared manuscript which was critically evaluated by C.B.G. All the co-authors reviewed, edited, and approved the manuscript before submission.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to B.L.Y.

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