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Morphology, taxonomy and epitypification of *Eaprasiola yunnanica* (C.–C.–Jao) *comb. nov.* (Prasiolaceae, Trebouxiophyceae) from China using reverse and integrative approaches

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Abstract

The *Prasiola*–like Prasiolaceae includes some 35 species of monostromatic green algae found in a wide range of habitats, including marine, freshwater, and terrestrial environments, across polar, cold, temperate, and subtropical regions. They exhibit considerable phenotypic plasticity, complicating identification based on morphology alone. Here, we used a approach based on full–length 18S rDNA amplicon sequencing to identify *Prasiola*–like specimens from China. Amplicon sequence variants (ASV) belonging to Prasiolaceae were detected in material collected at W'umonshan, Yunnan Province, China, and living specimens were subsequently collected from the corresponding sampling locations. We here designate a sequenced specimen as epitype as the holotype of P*rasiola yunnanica* C.–C. Jao is a formaldehyde–fixed specimen that cannot be sequenced. By combining molecular phylogenetics and morphological observations, we provide a comprehensive assessment of three *Prasiola*–like collections, identified through integrative taxonomy, as *Eaprasiola yunnanica* (C.–C.–Jao) *comb. nov.*, a species distinct from *Eaprasiola japonica* (Yatabe) Heesch, Guiry *et* Rindi from Japan. This finding highlights the effectiveness of the reverse taxonomy approach in detecting rare algae, and emphasizes the importance of integrative methods for achieving a thorough and accurate assessment of algal taxonomy and diversity.

Keywords

Eaprasiola japonica; Environment DNA; Epitype; Integrative taxonomy; Phenotypic plasticity; *Prasiola yunnanica*; Reverse taxonomy

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Introduction

The *Prasiola*–like members of the Prasiolaceae currently includes green algae found in a variety of habitats, such as marine, freshwater, and terrestrial environments, across polar, cold temperate, and subtropical regions (Kim *et al*., 2015; Klochkova *et al*., 2015; Rindi *et al*., 2004). As a foliose photobiont, some *Prasiola*–like–group species form lichens, such as *Mastodia tessellata* (Hooker f. *et* Harvey) Hooker f. *et* Harvey, in the Antarctic and subantarctic (Garrido–Benavent *et al*., 2017; Kohlmeyer *et al*., 2004; Pérez–Ortega *et al*., 2010). *Prasiola*–like species number 35 currently accepted species. (Guiry & Guiry, 2025). Key characteristics of *Prasiola*–like species include monostromatic blades and filaments arrangement, cells of which are typically arranged in groups of either two or four. The vegetative cells are square, with stellate chloroplasts each containing a single pyrenoid. Flagellated stages occurring during reproduction and have a flagellar root system, with flagella that rotate anticlockwise. Mitosis is closed, with cytokinesis occurring through transverse

wall deposition during telophase, consistent with other trebouxiophytes (O'Kelly *et al*., 2011; Rodríguez *et al*., 2007). Many *Prasiola*–like species exhibit considerable phenotypic plasticity (Burrows, 1991; Huisman, 2008; Rindi *et al*., 1999; Rodríguez *et al*., 2007), making it difficult to classify them based on morphological traits alone and hindering accurate identification (Metz *et al*., 2019). To address these difficulties, an integration of molecular methods with traditional morphological classification and other taxonomic approaches, is needed. Such an integrative approach not only improves species identification but also increases reliability (Darienko & Pröschold, 2019; Muggia *et al*., 2018).

Many previous studies are focused on phylogeny of the Prasiolales, sometimes with morphological analysis, to support its systematic classification as a distinct group and validate phylogenetic relationships at various taxonomic levels (Heesch *et al*., 2025; Moniz *et al*., 2012, 2014; Rindi *et al*., 2004, 2007). Sherwood *et al.* (2000) provided molecular phylogenetic evidence supporting the classification of the order Prasiolales as a distinct taxonomic group based on *rbc*L and 18S rDNA sequences. Rindi *et al*. (2004) used the *rbc*L marker to assess the Prasiolales, confirming that *Rosenvingiella* and *Prasiola*–like–group were separate genera, and that *Vittaprasiola calophylla* (Carmichael ex Greville) Heesch, Guiry *et* Rindi, *Prasiola crispa* (Lightfoot) Kützing, and *Mariprasiola stipitata* (Suhr *ex* Jessen) Heesch, Guiry *et* Rindi were separate species. Moniz *et al*. (2012) further applied the *tuf*A marker in a study of the Prasiolales, confirming previous results obtained using *rbc*L and *psa*B markers. Kim *et al*. (2015) employed molecular analyses with the *rbc*L, *psa*B, and *tuf*A markers, combined with morphological identification, to confirm that their collected samples belonged to the species currently known *Eaprasiola japonica* (Yatabe) Heesch, Guiry *et* Rindi. Previous phylogenetic analyses have demonstrated that the *Prasiola*–like– group is polyphyletic (Moniz *et al*., 2012; Rindi *et al*., 2007). Heesch *et al*. (2025) confirmed this via phylogenetic analysis and proposed that it be split it into four genera, *Prasiola*, *Mariprasiola*, *Vittaprasiola* and *Eaprasiola*.

Prasiola–like species are relatively rare in nature, making both sampling and subsequent taxonomic research particularly challenging. Therefore, it is vital to employ an operative method for detect *Prasiola*–like–group species. Reverse taxonomy is a useful technique for the identification of such rare algae, and as it is based on environmental DNA (eDNA), it is an effective strategy for detecting these elusive species in natural environments (Metz *et al*., 2019). Through high–throughput sequencing technology, amplicon sequence variants of the 18S

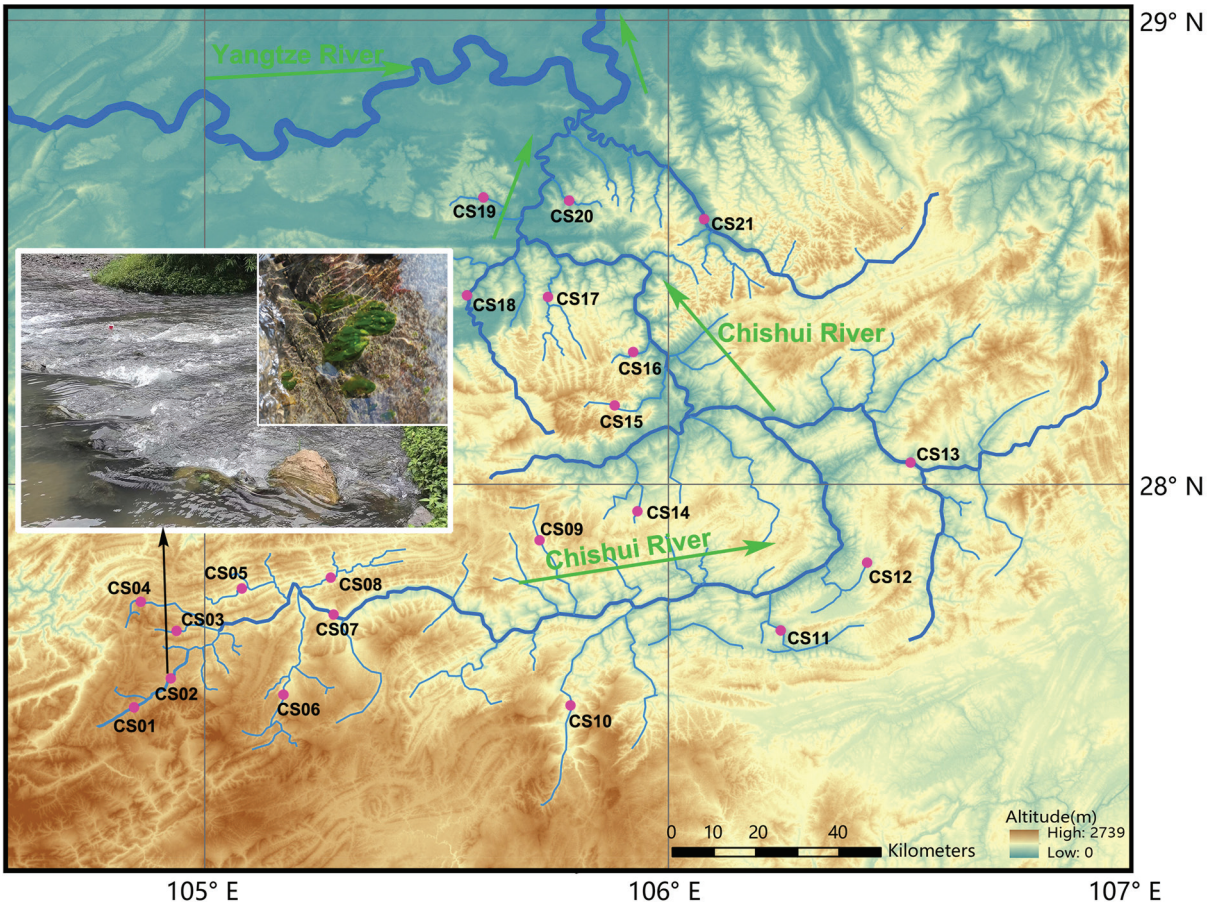

Fig. 1. The geographical distribution of 21 sites and the habitat characteristics of CS02.

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rDNA gene from eDNA samples enable the identification of algal DNA without causing extensive environmental disturbance (Hanžek *et al*., 2017). This method not only helps map the distribution of these algae but also provides valuable molecular data (Metz *et al*., 2019). By implementing this reverse taxonomy approach, researchers can significantly reduce the difficulties associated with detecting and classifying *Prasiola*–like species.

To date, eleven freshwater *Prasiola*–like–group species have been recorded in China, seven of which are endemic (Li & Bi, 2012). However, these endemic species were proposed solely based on morphological characteristics, without validation through molecular phylogenetics or other integrative approaches. To date, no molecular data from the holotypes or topotypes of these algae have been documented. Among these endemics, *Prasiola yunnanica* was first described by C.–C. Jao based upon specimens collected from the upper reaches of the Chishui River at W'umonshan, Yunnan Province, China (Jao, 1964). Here, a reverse taxonomic approach, combined with other taxonomic methods, was employed to analyze the Chishui River in the W'umonshan, with the aim of detecting material of *P. yunnanica*. The eDNA samples were collected across 21 sites over three different months, and 18S rDNA amplicon sequencing was used to investigate the presence and distribution of *P. yunnanica* in its type locality. Based on the eDNA results, targeted sampling was carried out, leading to the successful collection of mature *P. yunnanica* material. Additionally, a specimen with morphological characteristics identical to the description of the type specimen was collected from another region of Yunnan Province. As the holotype was preserved in formaldehyde solution and thus could not be sequenced, we used a designated epitype for phylogenetic analysis. Subsequent integrative taxonomic analysis of these specimens reaffirmed the validity of *P. yunnanica*.

Materials and Method

Sample collection and morphological observation: The eDNA samples were collected separately in March and December, 2023, and February, 2024. In this study, while the final analysis was performed on the eDNA samples from three field samples, sequences of *Prasiola*–like taxa were first detected using the eDNA data from the initial two collections. ASV (Amplicon sequence variants) analysis followed the protocol of Liu *et al*. (2024). Details of the 21 sampling sites are given in Table S1. After the successful identification of *Prasiola*–like–group associated amplicon sequence variants in the December 2023 and February 2024 samples, targeted collection efforts were initiated and labeled as 23CS02 and 24CS02, respectively. Additionally, we also included an air–dried sample (24MCX01) collected from Muchang town in Yunnan Province. Information on the 3 sampling sites is summarized in Table 1. Formaldehyde–fixed specimens were deposited in the Freshwater Algal Herbarium at the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan. The collection sites and habitats of the *Prasiola*–like–group specimens are shown in Fig. 1.

DNA extraction, PCR amplification, amplicon sequence variants, and Phylogenetic analysis: DNA extraction, PCR amplification, library preparation, sequencing, and subsequent Amplicon Sequence Variant analysis for the eDNA samples of 21 sites were conducted following the methods of Liu *et al*. (2024). For *Prasiola*–like–group samples from site CS02, DNA was extracted using AxyPrep genomic DNA Kit (Kangning, China) according to the manufacturer's protocols. The 18S rDNA sequences were amplified as described by Xia et al (2013), *rbc*L cpDNA sequences following Rindi et al. (2004), and *tuf*A cpDNA sequences following Famà *et al*. (2002). PCR products were sequenced by Tianyi (Hayu gene, Wuhan, China). The amplicon raw data were preserved in SRA database with accession number PRJNA1210117. The sequences were submitted to GenBank.

Phylogenetic reconstruction: To determine the phylogenetic positions of the samples, three separate phylogenetic analyses were conducted based on the 18S rDNA, *rbc*L, and *tuf*A gene sequences. Sequence alignments were performed using MAFFT v.6 (Katoh & Toh, 2008), and subsequently trimmed with TrimAl (Capella–Gutiérrez *et al*., 2009). Maximum Likelihood (ML) analysis was conducted using IQ–TREE (Nguyen *et al*., 2014), while Bayesian Inference (BI) was performed with MrBayes v3.2 (Ronquist *et al*., 2012). Node support for the ML trees was assessed using 1,000 bootstrap replicates. Bayesian posterior probabilities (PP) were estimated using four Markov Chain Monte Carlo (MCMC) chains run for a million generations, with default priors and proposal mechanisms. Convergence was verified by ensuring that the average standard deviation of split frequencies fell below 0.01, indicating stationarity. Final phylogenetic trees were visualized and edited using FigTree v1.1.1. Details of the data used to build phylogenetic trees are summarized in Table S2.

Table 1. Collection data of the studied specimens and GenBank accession numbers of rDNA sequences.

Species	Speciemen nubmer	Dates	Sites	Habitat	Accession no. of rDNA sequences		
					18S	*rbcL*	*tufA*
Prasiola yunnanica	23CS02	2023–12	105.0321 E, 27.6171 N	Surface of stone, stream, Yudong	PQ817765	PQ821649	PQ843217
Prasiola yunnanica	24CS02	2024–02	105.0321 E 27.6171 N	Surface of stone, stream, Yudong	PQ817766	PQ821650	PQ843218
Prasiola yunnanica	24MCX01	2024–04	99.2433 E 23.6790 N	Surface of stone, stream, Muchang	PV546887	PV553292	PV553293

Results

Phylogenetic analysis

For the 21 sites along the Chishui River in three distinct seasons, no ASV identified as *Prasiola*–like taxa was detected in March 2023 (Table 2). During December 2023 and February 2024, however, one ASV assigned to *Prasiola*–like taxa were identified at sites CS02 and CS03. Notably, relative abundances at both sites remained extremely low, classifying it as a rare ASV due to its relative abundance lower than 0.05%. And no *Prasiola*–like–group–related ASV was detected at any other sampling sites. This representative sequence of this *Prasiola*–like–group ASV, designated as ASV3608, was selected for further phylogenetic analysis.

Fig. 2. Phylogenetic tree of the genus *Prasiola* inferred from maximum likelihood analysis of the *rbcL* gene. Bootstrap values and Bayesian posterior probabilities are indicated at the branches. Only bootstrap values above 70% and posterior probabilities above 0.6 are shown.

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In the 18S rDNA phylogenetic tree, samples 23CS02, 24CS02, 24MCX01, and ASV3608 formed an independent clade with strong support (ML bootstrap value of 97; posterior probability of 1.00), and were clearly distinct from the *Eaprasiola japonica* clade (Fig. S1). In the phylogenetic tree constructed based on *rbc*L sequences, *Prasiola*–like taxa do not form a monophyletic grouping, mainly because *Prasiococcus calcarius* (J.B. Petersen) Vischer forms a unique branch within the polyphyletic *Prasiola*–like–group, but this is not the focus of present study. The three newly obtained *Prasiola*–like–group specimens (shaded in grey) formed a separate, fully–resolved branch, showing a close relationship with *Eaprasiola japonica* (Fig. 2). In the phylogenetic tree constructed based on *tuf*A sequences, these three specimens also cluster into a separate branch with support values of 100/1.00, and diverged from *Eaprasiola japonica* (Fig. 3). The results of these three phylogenetic analyses indicate that the three *Prasiola*–like–group specimens collected in present study are presumably not the same species as *Eaprasiola japonica*.

Morphological observations

Of the three specimens, the specimen from Muchang Town was the largest. The fronds of 24MCX01 were olive–green, growing in tufts, and were 2–14 cm in length and 0.5–3.5 cm in width (Fig. 4a). Blades were lanceolate, attenuate at the bases and apex, and undulate margins (Fig. 4a). Although specimens 23CS02 and 24CS02 were collected from the same location, their macroscopic appearances differed significantly (Fig. 4b–c). The former specimen also grew in tufts, highly undulate, 2–5 cm in height and 0.5–1.5 cm in width (Fig. 4b). The blades of latter specimen were not in tufts, and the margins of the plant body were smooth, about 0.5–3 cm in height and 0.5 – 1.0 cm in width (Fig. 4c). Both 23CS02 and 24CS02 specimens shared nearly same microscopic morphology with 2 or 4 cells forming groups in surface view, and most cells were nearly round, about 4–6 μm in diameter (Fig. 4d). However, most cells of

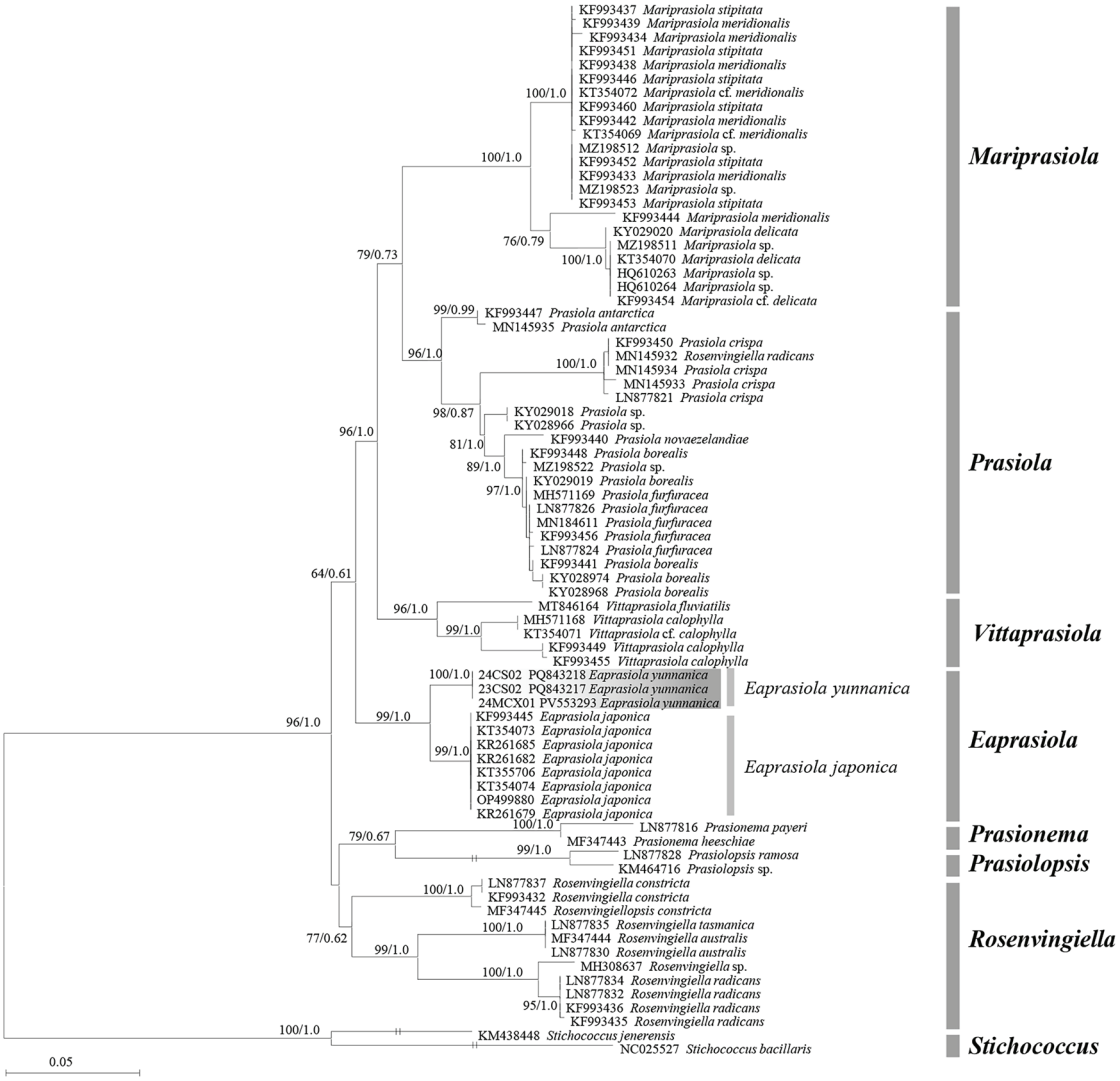

Fig. 3. Phylogenetic tree of the genus *Prasiola* inferred from maximum likelihood analysis of the *tufA* gene. Bootstrap values and Bayesian posterior probabilities are indicated at the branches. Only bootstrap values above 70% and posterior probabilities above 0.6 are shown.

24MCX01 were angular–rounded, semi–circular or other irregular in shape, with a diameter 8–10 µm, or 7–12 µm in length, 5–8 µm in width (Fig. 4e). At the base of the thallus, the cells of the three specimens gradually elongated and formed rhizoid–like holdfasts (Fig. 4f–g). In cross–section, each cell of both 23CS02 and 24CS02 were cylindrical or semi–circular, 7–10 µm in length, 3–5 µm in width (Figure 4h). However, cells of 24MCX01 were usually cylindrical, about 4–8 µm in width and 28–46 µm in length (Fig. 4i). A detailed comparison of these specimens is given in in Table 2.

Discussion

Species abundance at a given location may vary across different seasons due to environmental changes. To account for this, eDNA samples were collected separately over three months. Based on ASV information, we revisited the site and subsequently searched for living *Prasiola*–like–group specimens. Our results demonstrated that the eDNA method based on high–throughput sequencing can detect rare or ephemeral algae. Keller *et al*. (2017) used eDNA method to detect the distribution of the low–abundance diatom *Didymosphenia geminata* in North America, which helped determine if this species is an invader. This demonstrates that the eDNA approach simplifies the process of locating rare or ephemeral species, making it an ideal tool for rare–species surveys (Mahé *et al*., 2017). However, it is worth noting that the detected *Prasiola*–like–group sequences corresponded to rare ASVs, which could potentially be overlooked in some ecological analyses.

For the 35 recognized *Prasiola*–like species, sequence information remains scarce. Only 20 species have *rbc*L cpDNA sequences available for analysis, with even fewer possessing other molecular markers, leaving the phylogenetic position of over a third of species unknown and highlighting a significant molecular knowledge gap. Most of these understudied *Prasiola*–like species are recorded in China. Therefore, acquiring molecular markers from specimens collected at their type localities, such as our two specimens from the type

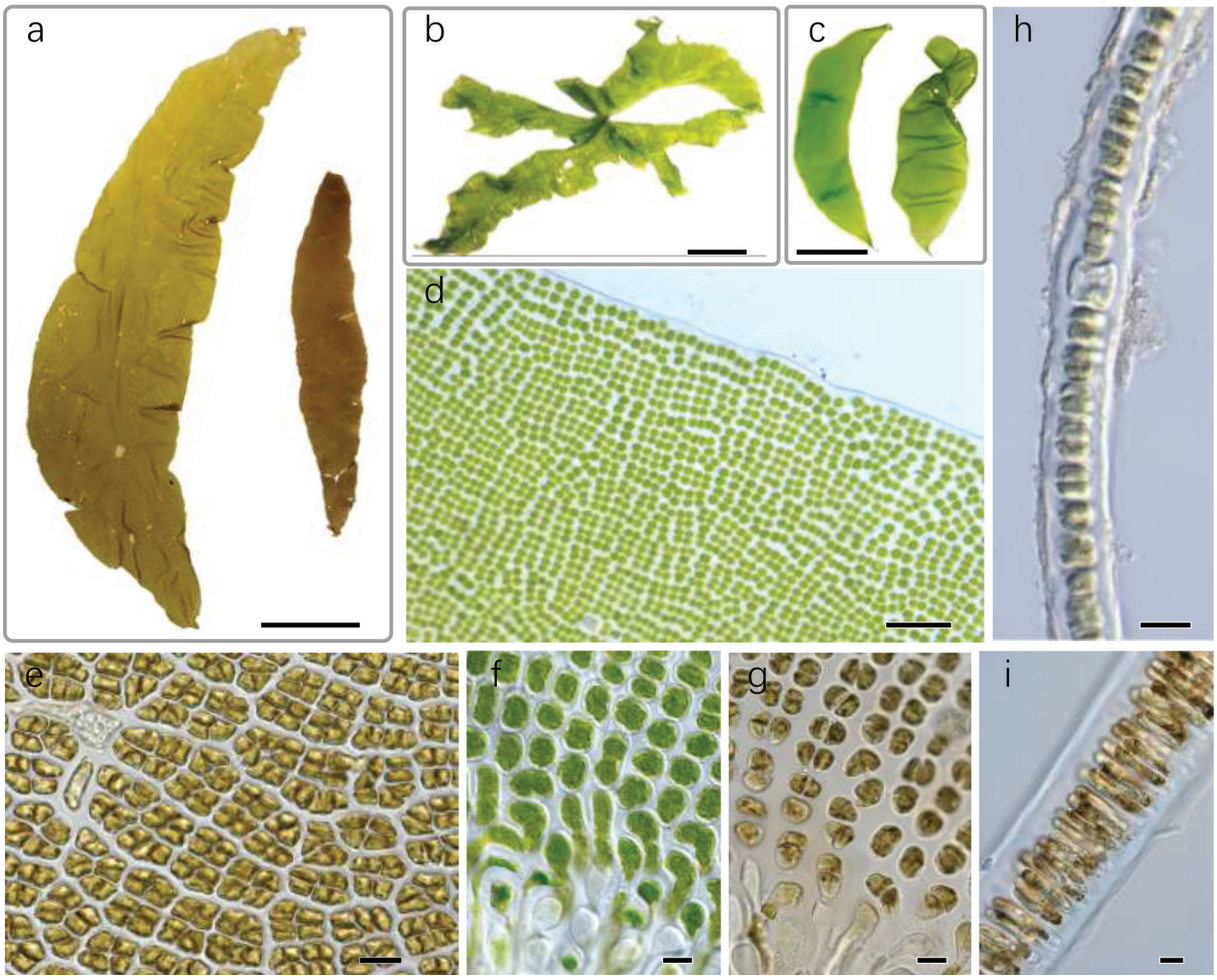

Fig. 4. (a) The morphology of sample 24MCX01. **(b)** The morphology of sample 23CS02. **(c)** The morphology of sample 24CS02. **(d)** The upper cells view of thalli 23CS02 and 24CS02. **(e)** The upper cells view of thalli 24MCX01. **(f)** The basal cells view of thalli 23CS02 AND 24CS02. **(g)** The basal cells view of thalli 24MCX01. **(h)** The transection cells view of thalli 23CS02 and 24CS02. **(i)** The transection cells view of thalli 24MCX01.

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locality of *Prasiola yunnanica*, is a critical approach to address this gap. In addition to phylogenetic analysis, a comprehensive study about the geographical distribution, morphological characteristics, and all relevant molecular data for freshwater *Prasiola*–like–group taxa in China and elsewhere was carried out, ensuring a more complete understanding of their evolutionary relationships and taxonomic diversity. Previous studies have shown that molecular markers are useful for species–level and genera–level delimitation in Prasiola–like species (Heesch *et al*., 2025; Moniz *et al*., 2012, 2014). As the holotype was preserved in formaldehyde solution and thus could not be sequenced, we used a designated epitype for phylogenetic analysis. Our phylogenetic analyses indicate that the three specimens cluster together, forming a new, well–resolved clade with extremely small genetic differences among them, clearly indicating that they are referable to the same species. The new clade (including *P. yunnanica* of our three specimens) is relatively close to *Eaprasiola japonica* in the phylogenetic tree, indicating a common origin, yet the genetic distance is sufficient to confirm they are not conspecific.

According to phylogenetic results, the 24MCX01 sample is congruent with 23CS02, 24CS02. However, the three samples show significant morphological differences. Eleven morphospecies of freshwater *Prasiola*–like–group had been recorded in China (Li & Bi, 2012). We found that three specimens exhibit significant differences in their morphological characteristics. Specimens 24MCX01 and 24CS02 were consistent with *Prasiola yunnanica*, whereas specimen 23CS02 was similar with *Prasiola elongata* (Table 2). Unfortunately, no specimens of *Prasiola elongata* were found. Due to the limited sample collection from this location, we speculated that the two morphological results are not representative. By contrast, another specimen, 24MCX01, displays more representative morphological characteristics of Jao's original description and represents a mature frond. Based on morphological observations, we concluded that both 23CS02 and 24CS02 were juvenile plants, though the specimen 23CS02 is slightly more developed than 24CS02. The significant morphological disparities among these three specimens suggest that morphological plasticity may be highly prevalent within the *Prasiola*–like species. This highlights the potential for misinterpretation when relying solely on morphological features for species identification. Thus, further collection and determination of morphologically defined species within *Prasiola*–like species are critical to address such taxonomic issues, especially for the other species supposedly endemic to China. Based on comprehensive considerations of habitat information, phylogenetic analysis, and morphological trait comparisons, we concluded that the three specimens should be identified as *Eaprasiola yunnanica* and are not referable to *Eaprasiola japonica.*

Eaprasiola yunnanica (C.–C.Jao) F.Su, Y.Liu *et* H.Zhu, *comb. nov.*

Basionym: *Prasiola yunnanica* C.–C.Jao *Botanical Bulletin of Academia Sinica* l: 110, fig. 1, 1947.

Description: Thalli tufted, with an olive–green coloration, consisting of somewhat transparent monostromatic blades that when developed fronds reach lengths of 5 cm and widths of 0.5 cm, with a discernible holdfast; fronds occasionally spiraled but usually broad–leaved and with or without undulate margins. Juvenile thalli are either tufted or individual, dark green, and smooth, measuring 5 mm in length and 1 mm in width. Chloroplasts stellate. Cells grouped in twos or fours but can form zones of 16 cells. Basal cells are predominantly oval or round, measuring 20 μm in length and 12 μm in width; upper cells are mostly oblong, measuring 10 μm in length and 5 μm in width. In section, cells consistently form an oval shape, with a height twice their width, measuring 7 μm in length and 3 μm in width.

Holotype: Freshwater Algal Herbarium, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, China, Voucher No.: HBI–SC1000 [fixed in formaldehyde solution].

Table 2. Morphological features of present three specimens.

Specimens number	Morphology of macro plants		Microscopic characteristics	
	Blade	Size	Surface view	Cross–section view
23CS02	in tuft, highly undulate margin	2–5 cm in length, 0.5–1.5 cm in width	most cells nearly round, 4–6 μm in diameter	Cells mainly cylindrical or semi–circular, 7–10 μm in length, 3–5 μm in width
24CS02	mainly in solitary, with smooth margin	0.5–3 cm in length, 0.3–1.0 cm in width	most cells nearly round, 4–6 μm in diameter	Cells mainly cylindrical or semi–circular, 7–10 μm in length, 3–5 μm in width
24MCX01	In tuft, attenuate tip at both ends, moderately undulate margin	2–14 cm in length, 0.5–1.5 cm in width	Nearly angular–round, semi–circular or other irregular shapes, 7–12 μm in length, 5–8 μm in width, or diameter about 8–10 μm	Cells mainly cylindrical, 28–46 μm in length, 4–8 μm in width

Type locality: W'umonshan Yunnan, China, leg. Y.P. Chang, 1944, in a mountain stream at 2700 m alt.
Epitype (here designated for the above holotype): Dried material deposited at the Freshwater Algal Herbarium, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, China; specimen no. HBI–24CS02 (dried material).
Epitype locality: on stones in a tributary stream of Chishui River, at Zhaotong city, Yunnan Province (105°03'21"E, 27°61'71"N), 26 ii 2024, leg. Feilong Su, Yukang Liu & Huan Zhu**.**

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