









Gigantic chloroplasts, including bizonoplasts, are common in shade-adapted species of the ancient vascular plant family Selaginellaceae

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Manuscript received 11 August 2019; revision accepted 21 January 2020.

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Citation: Liu, J.-W., S.-F. Li, C.-T. Wu, I. A. Valdespino, J.-F. Ho, Y.-H. Wu, H.-M. Chang, et al. 2020. Gigantic chloroplasts, including bizonoplasts, are common in shade-adapted species of the ancient vascular plant family Selaginellaceae. *American Journal of Botany* 107(4): 562–576.

doi:10.1002/ajb2.1455

PREMISE: Unique among vascular plants, some species of *Selaginella* have single giant chloroplasts in their epidermal or upper mesophyll cells (monoplastidy, M), varying in structure between species. Structural variants include several forms of bizonoplast with unique dimorphic ultrastructure. Better understanding of these structural variants, their prevalence, environmental correlates and phylogenetic association, has the potential to shed new light on chloroplast biology unavailable from any other plant group.

METHODS: The chloroplast ultrastructure of 76 *Selaginella* species was studied with various microscopic techniques. Environmental data for selected species and subgeneric relationships were compared against chloroplast traits.

RESULTS: We delineated five chloroplast categories: ME (monoplastidy in a dorsal epidermal cell), MM (monoplastidy in a mesophyll cell), OL (oligoplastidy), Mu (multiplastidy, present in the most basal species), and RC (reduced or vestigial chloroplasts). Of 44 ME species, 11 have bizonoplasts, cup-shaped (concave upper zone) or bilobed (basal hinge, a new discovery), with upper zones of parallel thylakoid membranes varying subtly between species. Monoplastidy, found in 49 species, is strongly shade associated. Bizonoplasts are only known in deep-shade species (<2.1% full sunlight) of subgenus *Stachygynandrum* but in both the Old and New Worlds.

CONCLUSIONS: Multiplastidic chloroplasts are most likely basal, implying that monoplastidy and bizonoplasts are derived traits, with monoplastidy evolving at least twice, potentially as an adaptation to low light. Although there is insufficient information to understand the adaptive significance of the numerous structural variants, they are unmatched in the vascular plants, suggesting unusual evolutionary flexibility in this ancient plant genus.

KEY WORDS bilobed chloroplast; chloroplast diversity; cup-shaped chloroplast; monoplastidy; shade-adapted *Selaginella*; Selaginellaceae; *Stachygynandrum*; ultrastructure.

Plants carry out photosynthesis over a huge range of environmental conditions. Although the key organelle, the chloroplast, might be expected to vary adaptively in size, number, and structure over

this range, chloroplast traits are generally highly conserved in land plants. Exceptions to this rule have the potential to be especially instructive. The monogeneric seedless vascular plant family

Selaginellaceae (sole genus *Selaginella*) are noted for having single giant chloroplasts (monoplastidy) in the dorsal epidermal cells (Banks, 2009), but as we show here, there is great diversity in chloroplast type and structure in *Selaginella* dorsal epidermal cells. Moreover, monoplastidy is also found in the upper mesophyll of some species. Although algae have high chloroplast diversity, in most groups of land plants chloroplast diversity is very low. Among nonvascular plants, hornworts are noted for giant chloroplasts, but liverworts and mosses typically have chloroplasts similar to those of the majority of vascular plants (Vanderpoorten and Goffinet, 2009). Among vascular plants, *Selaginella* stands out as an extreme exception.

Bizonoplasts are particularly striking chloroplast variants found in *Selaginella*. They are characterized by a dimorphic ultrastructure in which the upper zone consists of multiple layers of thylakoid membranes, with no grana, while the lower zone has normal grana and stroma thylakoids. Bizonoplasts have been reported relatively recently (Sheue et al., 2007; Liu et al., 2012; Reshak and Sheue, 2012; Ferroni et al., 2016) and are only known from *Selaginella*. The original finding (Sheue et al., 2007) was in *S. erythropus* (Mart.) Spring. Studies show that the bizonoplast develops from a proplastid indistinguishable from a normal vascular plant proplastid (Sheue et al., 2015). High light conditions were shown to prevent the development of the bizonoplast ultrastructure. Instead, several chloroplasts, with normal chloroplast ultrastructure, developed in each dorsal epidermal cell (Sheue et al., 2015).

In other vascular plants, there are few variants of the basic chloroplast structure. Key variants are the lamelloplast (Pao et al., 2018; originally “iridoplast”: Gould and Lee, 1996), the recently discovered minichloroplasts of Begoniaceae (Pao et al., 2018), and the bundle sheath chloroplasts of C_4 plants (Solymosi and Keresztes, 2012). Other plastids, such as chromoplasts and amyloplasts exist, but our concern here is chloroplasts *sensu stricto*, i.e., plastids with well-developed thylakoid membrane systems, chlorophyll, and photosynthetic functioning. Because chloroplasts have been studied for more than 150 years, the discovery of the bizonoplast stands out. The aims of this study were to explore the chloroplast variation in Selaginellaceae, with an emphasis on bizonoplasts (Bps), to further understand their prevalence, structural variation, environmental, morphological and phylogenetic correlates, and adaptive significance. This paper presents the results of 6 years of fieldwork on four continents to obtain fresh sample collections and habitat information on 76 species of *Selaginella*, which were identified and studied by electron microscopy. The results were then correlated with habitat features and phylogeny.

MATERIALS AND METHODS

Sources of species and environmental variables measured

In this study, plant materials from 76 species of *Selaginella* (approximately 10% of Selaginellaceae) from all seven subgenera (Weststrand and Korall, 2016) were collected worldwide from 2012 to 2018 (Table 1; Appendix S1). Among them, 64 species were collected from natural habitats, with basic environmental data recorded (location, GPS coordinates, soil type, local vegetation, light environment), and 12 species were obtained from botanical gardens. Both morphological features and DNA sequences (*rbcl*) were used for identification. Voucher specimens have been

deposited in the herbarium of National Chung Hsing University (TCB), Taiwan.

Environmental data (light intensity, temperature, humidity) were recorded in the habitats of 10 selected *Selaginella* species [*S. aristata* Spring, *S. arizonica* Maxon, *S. ciliaris* (Retz.) Spring, *S. delicatula* (Desv.) Alston, *S. devolii* H.M.Chang, P.F.Lu & W.L.Chiou, *S. doederleinii* Hieron., *S. heterostachys* Baker, *S. moellendorffii* Hieron., *S. repanda* (Desv.) Spring, and *S. tamariscina* (P.Beauv.) Spring], ranging from low to high light environments. Light intensity (photosynthetically active radiation, PAR) was measured using a portable LI-COR quantum sensor model LI-190 (Lincoln, NE, USA) for 4 years (2012–2014; 2017–2018). These data were then converted to percentage of full sunlight to facilitate comparison. Data were tested by ANOVA between species, followed with Scheffé’s post hoc test using SPSS (version 20; SPSS, Chicago, IL, USA). Environmental factors (light intensity, temperature, and humidity) of four local species (*S. doederleinii*, *S. heterostachys*, *S. repanda*, and *S. tamariscina*) native to Taiwan were continuously monitored and recorded over the year 2013 with data loggers (HOBO Pro v2 Temperature/Relative Humidity data logger and HOBO Pendant Temperature/Light 64K Data Logger; Onset Computer Corp., Bourne, MA, USA).

Preparation of plant materials for structural study

The materials (ventral leaves only for the species with leaves longer than 3 mm; otherwise segments of shoots ca. 2–3 mm long) of each of three individuals (up to five individuals in abundant populations) were used for the chloroplast study. Some branch segments were observed directly when fresh. Others were fixed in 2.5% v/v glutaraldehyde in 0.1 M sodium phosphate buffer and 70% v/v ethanol (segments of shoots ca. 5 mm long) in the field or botanical gardens, then transferred to the laboratory for structural study.

Structural study of chloroplasts and phylogenetic association

Both free hand sections (transverse view) and top views of intact microphylls (leaves) were used to observe chloroplast traits in fresh materials and some samples fixed in ethanol. Selected materials were observed with a confocal scanning laser microscope (CSLM, Leica TSC-SP5, Wetzlar, Germany) (excitation 488 nm, emission wavelength 581–756 nm) using a 63× oil immersion objective to investigate bizonoplast (Bp) morphological features. KY jelly (Johnson and Johnson, New Brunswick, NJ, USA) was used to temporarily embed freehand sections before observation.

A general electron microscopy protocol was followed (Sheue et al., 2007). Semithin sections (1 μm) were cut and stained with 1% w/v aqueous toluidine blue for observation with a light microscope (Olympus BH-2, Tokyo, Japan). Ultrathin sections (about 70 nm) were cut and stained with uranyl acetate (5% w/v in 50% methanol) and lead citrate (1% w/v in water) for examination with either a Hitachi H 600 (Tokyo, Japan) or a JEOL (JEM-2000 EXII, Tokyo, Japan) transmission electron microscope (TEM). The results on chloroplast types (with or without Bps) and plant morphology were then used to annotate the subgeneric tree of *Selaginella* published by Weststrand and Korall (2016).

Comparison of ultrastructural features of Bps

The ultrastructural features of Bps from nine selected species obtained from TEM micrographs were measured, including thylakoid

TABLE 1. Details of the 76 *Selaginella* species used in this study focusing on monoplastidy and chloroplast traits in dorsal epidermal cells of a microphyll. *Abbreviations:* A, anisophyll; bBp, bilobed bizonoplast; bCp, bilobed chloroplast; cBp, cup-shaped bizonoplast; cCp, cup-shaped chloroplast; D, typical disk-shaped chloroplast; gD, giant disk-shaped chloroplast; I, isophyll; ME, monoplastidy in the dorsal epidermal cell; MM, monoplastidy in a mesophyll cell; Mu, multiplastidy; OL, oligoplastidy; RC, reduced or vestigial chloroplasts. Chloroplast types and categories are based on dorsal epidermal cells, except for MM when monoplastidy occurs in a mesophyll cell immediately below the epidermis.

No.	Species	Subgenus (Weststrand and Korall, 2016)	Isophyll/Anisophyll	Chloroplast type	Chloroplast category
1	<i>S. anceps</i> (C.Presl) C.Presl	<i>Stachygynandrum</i> ^a	A	cCp	ME
2	<i>S. arbuscula</i> (Kaulf.) Spring	<i>Stachygynandrum</i>	A	bCp	ME
3	<i>S. arenicola</i> Underw.	<i>Rupestrae</i>	I	D	Mu
4	<i>S. aristata</i> Spring	<i>Stachygynandrum</i>	A	bBp	ME
5	<i>S. arizonica</i> Maxon	<i>Rupestrae</i>	I ^b	D	Mu
6	<i>S. arthritica</i> Alston	<i>Gymnogynum</i>	A	RC	RC
7	<i>S. articulata</i> (Kunze) Spring	<i>Gymnogynum</i>	A	cCp	MM
8	<i>S. australiensis</i> Baker	<i>Gymnogynum</i> ^a	A	cCp	MM
9	<i>S. bisulcata</i> Spring	<i>Stachygynandrum</i>	A	bCp	ME
10	<i>S. bombycina</i> Spring	<i>Stachygynandrum</i>	A	cCp	ME
11	<i>S. boninensis</i> Baker	<i>Stachygynandrum</i>	A	bBp	ME
12	<i>S. chrysoleuca</i> Spring	<i>Stachygynandrum</i> ^a	A	cCp	ME
13	<i>S. ciliaris</i> (Retz.) Spring	<i>Stachygynandrum</i>	A	D, bCp ^c	OL, ME ^c
14	<i>S. cupressina</i> (Willd.) Spring	<i>Stachygynandrum</i>	A	cCp	ME
15	<i>S. deflexa</i> Brack.	<i>Selaginella</i>	I	D	Mu
16	<i>S. delicatula</i> (Desv.) Alston	<i>Stachygynandrum</i>	A	cBp	ME
17	<i>S. devolii</i> H.M.Chang, P.F.Lu & W.L.Chiou	<i>Stachygynandrum</i>	A	bBp	ME
18	<i>S. diffusa</i> (C.Presl) Spring	<i>Gymnogynum</i>	A	cCp	MM
19	<i>S. doederleinii</i> Hieron.	<i>Stachygynandrum</i>	A	cCp	ME
20	<i>S. douglasii</i> (Hook. & Grev.) Spring	<i>Stachygynandrum</i>	A	D	Mu
21	<i>S. erythropus</i> (Mart.) Spring	<i>Stachygynandrum</i>	A	cBp	ME
22	<i>S. euclimax</i> Alston ex Crabbe & Jermy	<i>Stachygynandrum</i> ^a	A	cCp	ME
23	<i>S. eurynota</i> A.Braun	<i>Gymnogynum</i> ^a	A	RC	RC
24	<i>S. exaltata</i> (Kunze) Spring	<i>Exaltatae</i>	A	RC	RC
25	<i>S. flagellata</i> Spring	<i>Stachygynandrum</i> ^a	A	bCp	ME
26	<i>S. flexuosa</i> Spring	<i>Stachygynandrum</i> ^a	A	cCp	ME
27	<i>S. gracillima</i> (Kunze) Spring ex Salomon	<i>Ericetorum</i>	I	D	Mu
28	<i>S. haematodes</i> (Kunze) Spring	<i>Stachygynandrum</i>	A	cCp	ME
29	<i>S. heterostachys</i> Baker	<i>Stachygynandrum</i>	A	bBp	ME
30	<i>S. hieronymiana</i> Alderw.	<i>Stachygynandrum</i>	A	D	Mu
31	<i>S. horizontalis</i> (C.Presl) Spring	<i>Gymnogynum</i>	A	RC	RC
32	<i>S. huehuetenangensis</i> Hieron.	<i>Stachygynandrum</i> ^a	A	cCp	ME
33	<i>S. intermedia</i> (Blume) Spring	<i>Stachygynandrum</i>	A	cBp	ME
34	<i>S. involvens</i> (Sw.) Spring	<i>Stachygynandrum</i>	A	D	OL
35	<i>S. kraussiana</i> (Kunze) A.Braun	<i>Gymnogynum</i>	A	bCp	MM
36	<i>S. labordei</i> Hieron. ex Christ	<i>Stachygynandrum</i>	A	bCp	ME
37	<i>S. lepidophylla</i> (Hook. & Grev.) Spring	<i>Lepidophyllae</i>	A	D	Mu
38	<i>S. leveriana</i> Alston	<i>Stachygynandrum</i>	A	cCp	ME
39	<i>S. longipinna</i> Warb.	<i>Stachygynandrum</i>	A	cCp	ME
40	<i>S. lutchuensis</i> Koidz.	<i>Stachygynandrum</i>	A	bBp	ME
41	<i>S. martensii</i> Spring	<i>Stachygynandrum</i>	A	cBp/cCp ^d	ME
42	<i>S. mayeri</i> Hieron.	<i>Stachygynandrum</i>	A	cCp	ME
43	<i>S. minima</i> Spring	<i>Stachygynandrum</i> ^a	A	cCp	ME
44	<i>S. moellendorffii</i> Hieron.	<i>Stachygynandrum</i>	A	cCp	ME
45	<i>S. mollis</i> A.Braun.	<i>Stachygynandrum</i>	A	cCp	ME
46	<i>S. monospora</i> Spring	<i>Stachygynandrum</i>	A	bCp	ME
47	<i>S. nipponica</i> Franch. & Sav.	<i>Stachygynandrum</i>	A	D	OL
48	<i>S. oregana</i> D.C.Eaton	<i>Rupestrae</i>	I	D	Mu
49	<i>S. pallescens</i> (C.Presl) Spring	<i>Stachygynandrum</i>	A	D	OL
50	<i>S. picta</i> (Griff.) A.Braun ex Baker	<i>Stachygynandrum</i> ^a	A	cBp	ME
51	<i>S. plana</i> (Desv.) Hieron.	<i>Stachygynandrum</i>	A	bCp	ME
52	<i>S. poperangensis</i> Hieron.	<i>Stachygynandrum</i>	A	cCp	ME
53	<i>S. porelloides</i> (Lam.) Spring	<i>Stachygynandrum</i>	A	gD	ME
54	<i>S. porphyrospora</i> A.Braun	<i>Stachygynandrum</i> ^a	A	cCp	ME
55	<i>S. pseudonipponica</i> (Tagawa) H.M.Chang, W.L.Chiou & J.C.Wang	<i>Stachygynandrum</i>	A	D	OL
56	<i>S. pulcherrima</i> Liebm.	<i>Stachygynandrum</i>	A	bCp	ME

(Continued)

TABLE 1. (Continued)

No.	Species	Subgenus (Weststrand and Korall, 2016)	Isophyll/Anisophyll	Chloroplast type	Chloroplast category
57	<i>S. rechingeri</i> Hieron.	<i>Stachygynandrum</i>	A	bCp	ME
58	<i>S. remotifolia</i> Spring	<i>Gymnogynum</i>	A	cCp, D ^e	MM, OL ^e
59	<i>S. repanda</i> (Desv.) Spring	<i>Stachygynandrum</i>	A	D	OL
60	<i>S. revoluta</i> Baker	<i>Stachygynandrum</i>	A	cBp	ME
61	<i>S. rupicola</i> Underw.	<i>Rupestrae</i>	I	D	Mu
62	<i>S. salazariae</i> Valdespino	<i>Stachygynandrum</i> ^a	A	cCp	ME
63	<i>S. sertata</i> Spring	<i>Gymnogynum</i> ^a	A	RC	RC
64	<i>S. schaffneri</i> Hieron.	<i>Stachygynandrum</i> ^a	A	D	Mu
65	<i>S. simplex</i> Baker	<i>Stachygynandrum</i> ^a	A	cCp	ME
66	<i>S. stauntoniana</i> Spring	<i>Stachygynandrum</i>	A	D	Mu
67	<i>S. tamariscina</i> (P.Beauv.) Spring	<i>Stachygynandrum</i>	A	D	Mu
68	<i>S. uliginosa</i> (Labill.) Spring	<i>Ericetorum</i>	I	D	Mu
69	<i>S. umbrosa</i> Lem. ex Hieron.	<i>Stachygynandrum</i>	A	cCp	ME
70	<i>S. uncinata</i> (Desv.) Spring	<i>Stachygynandrum</i>	A	bCp	ME
71	<i>S. underwoodii</i> Hieron.	<i>Rupestrae</i>	I	D	Mu
72	<i>S. vogelii</i> Spring	<i>Stachygynandrum</i>	A	D	OL
73	<i>S. wallacei</i> Hieron.	<i>Rupestrae</i>	I	RC	RC
74	<i>S. wallichii</i> (Hook. & Grev.) Spring	<i>Stachygynandrum</i>	A	cCp	ME
75	<i>S. willdenowii</i> (Desv.) Baker	<i>Stachygynandrum</i>	A	bCp	ME
76	<i>S. wolffii</i> Sodiro	<i>Stachygynandrum</i> ^a	A	cCp	ME

^aInfrageneric classification is based on the key provided by Weststrand and Korall (2016).

^bThis species is slightly anisophyllous.

^cThis species may have different chloroplast types in different environments (OL, in an open grassland; ME, in a grassland shaded by trees).

^dFerroni et al. (2016) reported cBps from this species, but our material obtained from a botanic garden appears as cCps.

^eThis species may have different chloroplast types in different light environments (MM, in shade; OL, in partial shade).

group number in an upper zone, thickness of a thylakoid group, number of stacked thylakoids per thylakoid group, and stroma thickness between thylakoid groups. Only micrographs with clear thylakoid structures (sections perpendicular to membranes and lumens) in the upper zones of Bps were used to obtain data. Materials collected overseas for this comparison were more limited. For each selected species, 3–8 individuals (3 individuals of overseas species, more of local species) were used and 3–31 Bps were selected to study their ultrastructural features. To determine the number of thylakoid groups in the upper zone, micrographs at low magnification were studied for 7 to 31 Bps. Thickness of both thylakoid groups and stroma were measured with ImageJ (ImageJ 1.51s; National Institutes of Health, Bethesda, MD, USA). Data were tested by ANOVA between species, followed with Scheffé's post hoc test using SPSS version 20.

Estimating the prevalence of chloroplast types in *Selaginella*

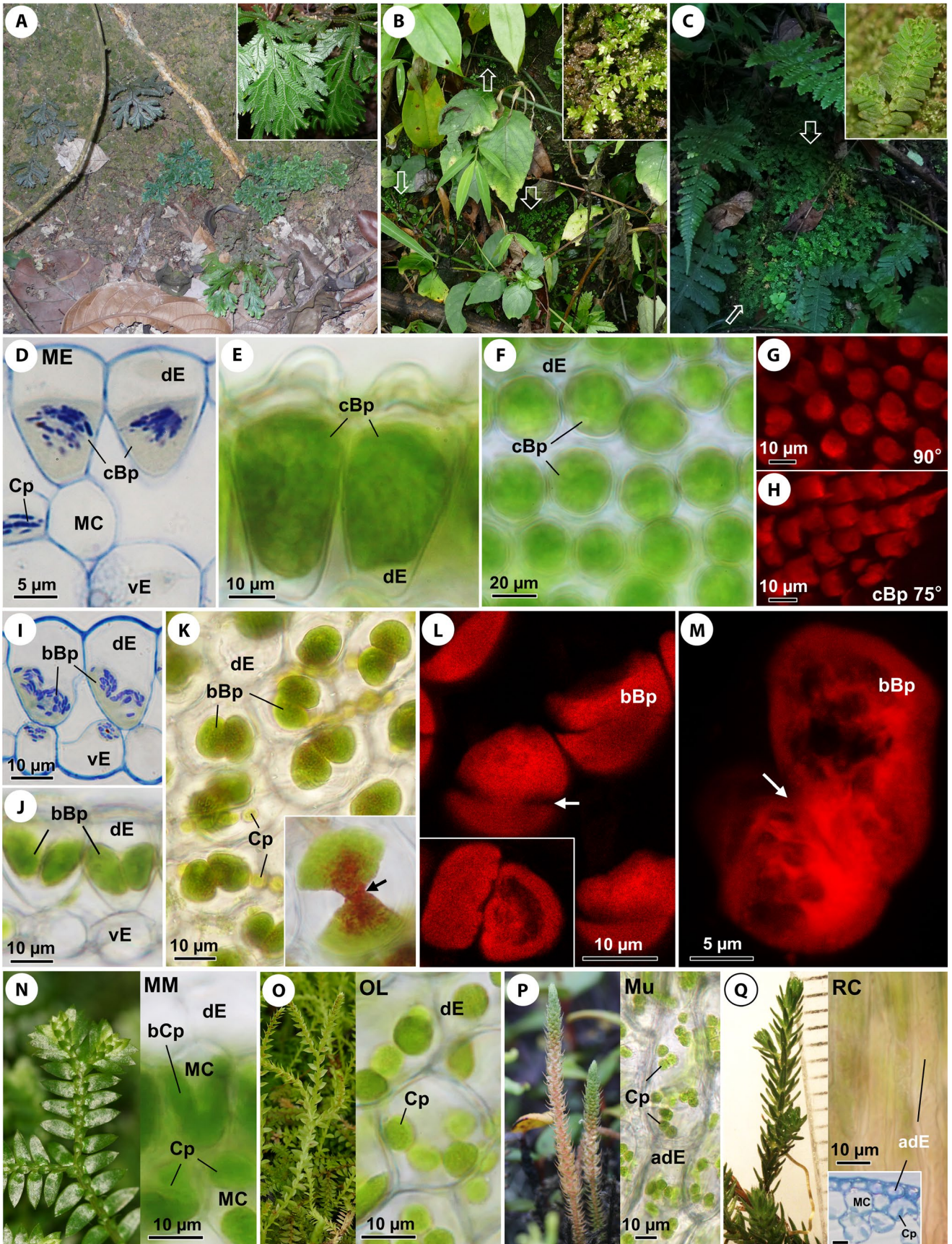
From our data, we estimated the proportion, $p_{sg,ct}$, of a chloroplast type (ct) in a subgenus (sg) simply as the number of species with

that type of chloroplast that we found in that subgenus divided by the number of species that we studied from that subgenus. To estimate the overall proportion, p_{ct} , of a given ct in the genus *Selaginella*, we took a weighted average of the proportions in the subgenera as given by the formula

$$p_{ct} = \frac{\sum_{sg} p_{sg,ct} n_{sg}}{\sum_{sg} n_{sg}},$$

where n_{sg} is the known number of species globally in the subgenus sg, and the sums are over all subgenera. This formula corrects any bias that exists in the number of species that we studied in each subgenus and gives an unbiased estimate of the proportions of each chloroplast type in the genus, subject to the assumption that our sampling of chloroplast types in a subgenus is unbiased. However, as we are unsure whether chloroplast sampling is unbiased within a subgenus, our final results can only be regarded as approximate.

FIGURE 1. Habitats and chloroplast types of *Selaginella*. (A–C) Shade-adapted *Selaginella* with monoplastids in dorsal epidermal cells (ME), which possess bizonoplast (Bp) ultrastructure. (A) *S. intermedia* with cup-shaped Bps (cBps), Singapore. (B) *S. devolii* with bBps, Taiwan. (C) *S. heterostachys*, with bilobed Bps (bBps), Taiwan. (D–F) cBps of *S. delicatula*. Transverse view: D, E; top view: F. (G, H) Confocal scanning laser micrographs (CSLM) of cBps of *S. erythropus* showing their concave tops at two different angles. (I–M) bBps of *S. heterostachys*, arrows indicating the connections between lobes. Transverse view: I, J; top view: K, with inset showing widely expanded bBp. CSLM viewed from different angles in images L and M. Three-dimensional reconstructions are shown in inset in L (top view) and in M (lateral view with a bBp tilted forward revealing the connection). (N) (Left) *S. kraussiana* and (right) its bilobed chloroplast in the first mesophyll cell layer. (O) *S. nipponica* and its oligoplastidy (OL) in dorsal epidermal cells. (P) *S. deflexa* and its multiplastidy (Mu) in adaxial epidermal cells. (Q) *S. wallacei* and its reduced or vestigial chloroplasts (RC) in adaxial epidermal cells (ruler with 1 mm divisions). The inset shows leaf structure close to the adaxial surface (10 μm scale bar). **Abbreviations:** adE, adaxial epidermal cell; bBp, bilobed bizonoplast; bCp, bilobed chloroplast; cBp, cup-shaped bizonoplast; Cp, typical chloroplast; cCp, cup-shaped chloroplast; dE, dorsal epidermal cell; MC, mesophyll cell; ME, monoplastidy in dorsal epidermal cells; MM, monoplastidy in mesophyll cells; Mu, multiplastidy; OL, oligoplastidy; RC, reduced or vestigial chloroplasts; vE, ventral epidermal cell.



RESULTS

Chloroplast diversity

Unlike seed plants with mesophyll as the main photosynthetic tissue, chloroplasts of *Selaginella* appear in both epidermal cells and mesophyll cells (Fig. 1). In most cases, *Selaginella* mesophyll cells and ventral epidermal cells have multiple chloroplasts with the typical structure of those in vascular plants generally. Among the 76 species studied, chloroplast variants were found in dorsal epidermal cells or in some species in the mesophyll cells directly below the dorsal epidermal layer. Note that only in dorsiventral species (e.g., *S. kraussiana* in Fig. 1N) are the dorsal and ventral epidermises defined. The corresponding terms for nondorsiventral species (e.g., *S. deflexa* in Fig. 1P) are adaxial and abaxial. However, only dorsiventral species were found to have unusual chloroplasts. Table 2 gives the chloroplast categories that we identified based on chloroplast size, number per cell, and tissue location. Briefly, four major categories are delineated: monoplastidy (M, one large chloroplast per cell; 49 species) (Fig. 1D–N), oligoplastidy [OL, (2)3–10 chloroplasts per cell; 7 species] (Fig. 1O), multiplastidy (Mu, more than 10 chloroplasts per cell in all photosynthetic cells; 14 species) (Fig. 1P), and reduced or vestigial chloroplasts (RC; few barely visible chloroplasts, 6 species) (Fig. 1Q). We estimate from these data that the genus *Selaginella* is 70% M, 11% OL, 9% Mu, and 4% RC. These categories of chloroplast size and number are related to microphyll morphology (anisophylly or isophylly). The isophyllous species (generally nondorsiventral) are the Mu type, but the anisophyllous species (generally dorsiventral) have more diverse chloroplast types, viz. M, OL, and RC (Table 1).

Monoplastids, M, are especially large, normally occupying a substantial fraction, up to ~80%, of the cell volume, with linear dimension up to 40 μm (Fig. 1E). Monoplastidy may appear in a dorsal epidermal cell (ME type) (Fig. 1D–M) or in a mesophyll cell immediately below the epidermal layer (MM type) (Fig. 1N), or uniquely, to date, in *S. plana* (Desv.) Hieron. where only the ventral epidermal cells are not monoplastidic. Among them, 44 species are

ME, and five are MM. Moreover, three shapes of M chloroplasts are further recognized: cup-shaped (28 ME species and 4 MM species), bilobed (15 ME species and 1 MM species), and giant disk (one ME species) (Table 1). The monoplastids in some ME species are classified as bizonoplasts based on the presence of two ultrastructure zones as reported by Sheue et al. (2007) (Table 2).

Occurrence and forms of bizonoplasts

This study found nine additional species with Bps beyond the two species previously reported (Fig. 1A–C; Table 1). Bizonoplasts may appear as either cup-shaped (6 species) (cBp, Fig. 1D–H) or bilobed (bBp, 5 species) (Fig. 1I–M). Here, *S. delicatula*, *S. intermedia* (Blume) Spring, *S. picta* (Griff.) A. Braun ex Baker, and *S. revoluta* Baker are newly reported to have cBps, and *S. aristata*, *S. boninensis* Baker, *S. devolii*, *S. heterostachys*, and *S. lutchuensis* Koidz. were found to have bBps. From a microphyll top view, a cBp appears as a circle in a dorsal epidermal cell (Fig. 1F, G), but from a lateral view the concave top is evident (Fig. 1H). Unlike cBps, bBps appear dumbbell-shaped from the top view (Fig. 1K, L). The shape of a bBp is deeply bilobed with a narrow connection at the base of each lobe (Fig. 1I–M; Appendix S2). However, the shape and number of bBps per dorsal epidermal cell are difficult to judge directly from free hand and semithin sections. Thus, confocal microscopy was applied to construct 3-dimensional images and confirm that the bBp is a monoplastid (Fig. 1L, M).

Ultrastructural variants of chloroplasts

The upper zones of cBps consist of groups of thylakoids, which horizontally traverse the entire upper part of the Bp in regular parallel arrangements (Fig. 2A). Each group of thylakoids comprises 3–5 stacked thylakoids (Fig. 2A), but appears as a thin line at low magnification TEM. The lower zones of cBps consist of normal granal thylakoids and stroma thylakoids similar to the typical chloroplasts in mesophyll cells and ventral epidermal cells (Fig. 2A). From a paradermal TEM view of a cBp, the thylakoid groups of the upper

TABLE 2. *Selaginella* chloroplast categories and characteristics. Abbreviations: cBp, cup-shaped bizonoplast; bBp, bilobed bizonoplast.

Chloroplast type	Feature ^a	Location ^a	Shape	Ultrastructure	Icon
M (monoplastidy)					
ME (monoplastid in a dorsal epidermal cell)	Monoplastid	Dorsal epidermal cell	Cupped, bilobed, or giant disk	Bizonoplast or normal; giant disks always normal	
MM (monoplastid in a mesophyll cell)	Monoplastid	Mesophyll cell	Cupped or bilobed	Normal	
OL (oligoplastidy)	(2)3–10 chloroplasts per cell	Dorsal epidermal cell	Disk	Normal	
Mu (multiplastidy)	More than 10 chloroplasts per cell	All photosynthetic cells	Disk	Normal	
RC (reduced or vestigial chloroplasts)	Reduced or vestigial chloroplasts	Dorsal or adaxial epidermal cell	Disk	Thylakoids less developed	

^aThe second and third columns define the categories stated in the first column. The other columns give more chloroplast features associated with these categories.

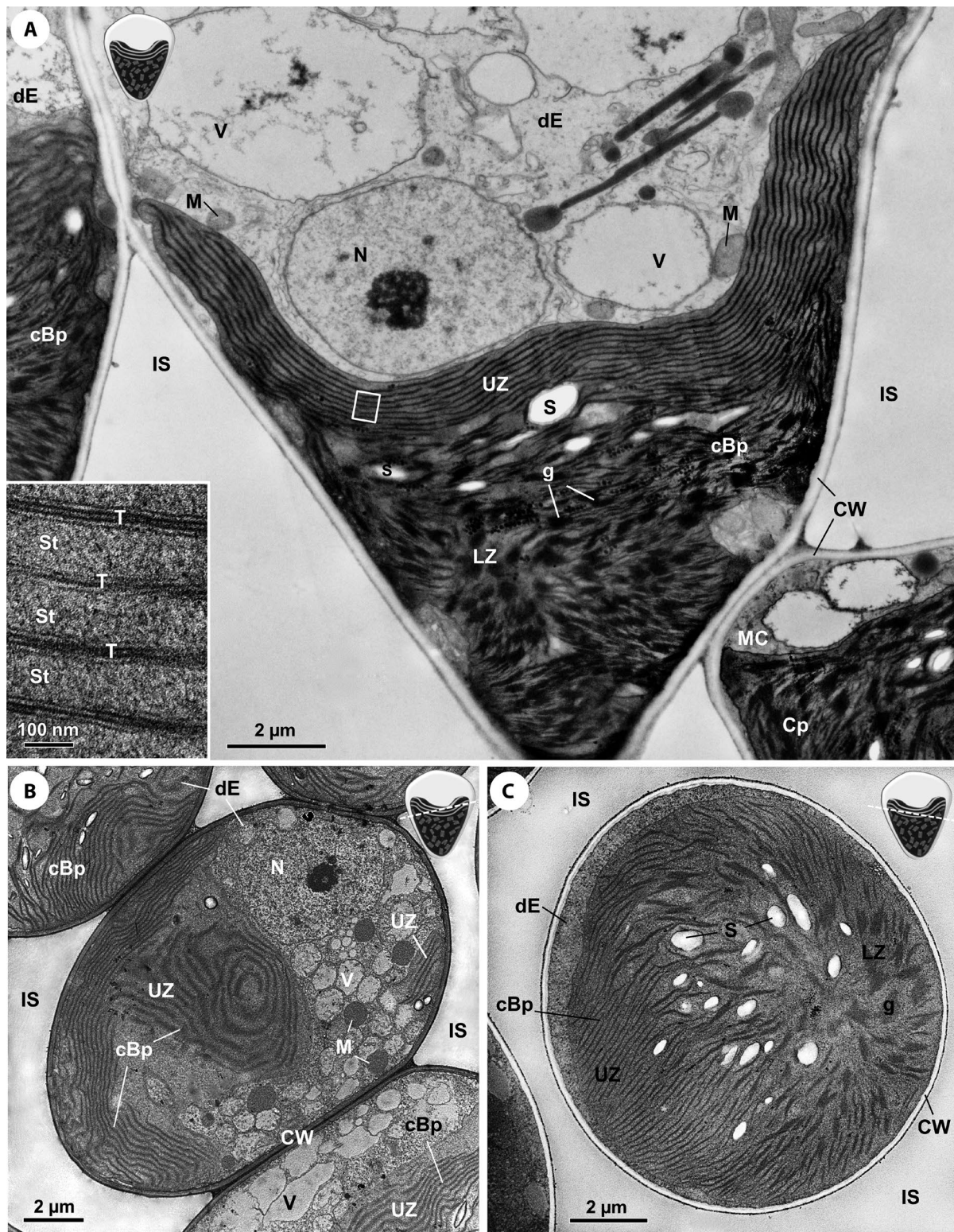


FIGURE 2. TEM of cup-shaped bizonoplasts (cBps) of *S. erythropus*. The chloroplast drawings indicate approximate section locations. (A) Transverse section of cBp in dorsal epidermal cell showing regularly layered upper zone (UZ) above lower zone (LZ). Inset, closeup of LZ, with groups of 3–5 stacked thylakoids. (B, C) Paradermal sections of cBps. (B) Top of cBp showing partial UZ with groups of thylakoids. These layered thylakoids form a pattern of concentric circles expanding toward the cell wall. (C) Boundary between two zones of cBp showing parallel thylakoids groups in UZ, grana and starch grains in LZ. *Abbreviations:* cBp, cup-shaped bizonoplast; Cp, chloroplast; CW, cell wall; dE, dorsal epidermal cell; g, grana; IS, intercellular space; LZ, lower zone; M, mitochondrion; MC, mesophyll cell; N, nucleus; S, starch grain; St, stroma; T, thylakoid; UZ, upper zone; V, vacuole.

zone appear as concentric circles, which expand regularly toward the cell wall of the dorsal epidermal cell (Fig. 2B). A section at the boundary between the two zones may show part of the upper zone (partially parallel thylakoid groups) and part of the lower zone (randomly scattered grana and stromal thylakoids) (Fig. 2C). Thylakoid groups often appear thicker and blurred in paradermal sections due to different cutting angles (Fig. 2B, C).

The bBps are usually slightly smaller than the cBps, and each occupies less than one half of a dorsal epidermal cell in a microphyll (Fig. 1J, K). At the apex of each lobe of the bBp is an upper zone, which is similar in structure to the upper zone of the cBps and lines the interior side of the lobe, becoming thinner farther from the apex, and eventually disappearing (Fig. 3A–E). Although bBps appear as various shapes when viewed with TEM, depending on the section location and angle, their ultrastructural features are similar to cBps (Fig. 3B–D). In longitudinal TEM views, bBps occasionally appear as two separate lobes even though connected at the base (Fig. 3A).

The cBps of four selected *Selaginella* species with sufficient materials for study (*S. delicatula*, *S. erythropus*, *S. intermedia*, and *S. revoluta*) show morphological similarity. There are no significant ultrastructural differences in the upper chloroplast zones of these four species (Fig. 4A–D, left group). The average number of thylakoid groups in the upper zone of a bBp is close to that of a cBp (Fig. 4B). Although there is evidence of variation between species in the thickness of the stroma between two thylakoid groups, the species differences are not well resolved (Fig. 4C, D).

Bilobed chloroplasts need not be bizonoplasts, and then they have normal ultrastructure (Fig. 3F). They are either ME (monoplastidy in dorsal epidermal cells) or MM (monoplastidy in mesophyll cells). In general, bilobed chloroplasts (bizonoplast or not) are located at the narrow base of a funnel-shaped cell, with a nucleus between its lobes and a large vacuole above (Fig. 3A, F). Normal chloroplast structure is found in OL, Mu (Fig. 3G), and RC chloroplasts (Fig. 3H, I), but an RC is relatively small (3–4 μm or smaller), with less-elaborated thylakoid membranes and rarely contains starch grains. Reduced or vestigial chloroplasts (RC) were found in epidermal cells of some isophyllous (e.g., *S. wallacei*, adaxial epidermal cells) (Fig. 1Q) and some anisophyllous species (e.g., *S. exaltata*, dorsal epidermal cells) (Fig. 3H, I). Mesophyll tissues with larger chloroplasts are the main photosynthetic tissues in the RC species studied.

The association between chloroplast traits, habitats, and phylogeny

The light habitats of 10 selected species range from extremely low-light montane forests (0.4–2.1% full sunlight), partial shade (11.2–25.5% full sunlight) to a high-light desert (40.5–53.8% full sunlight)

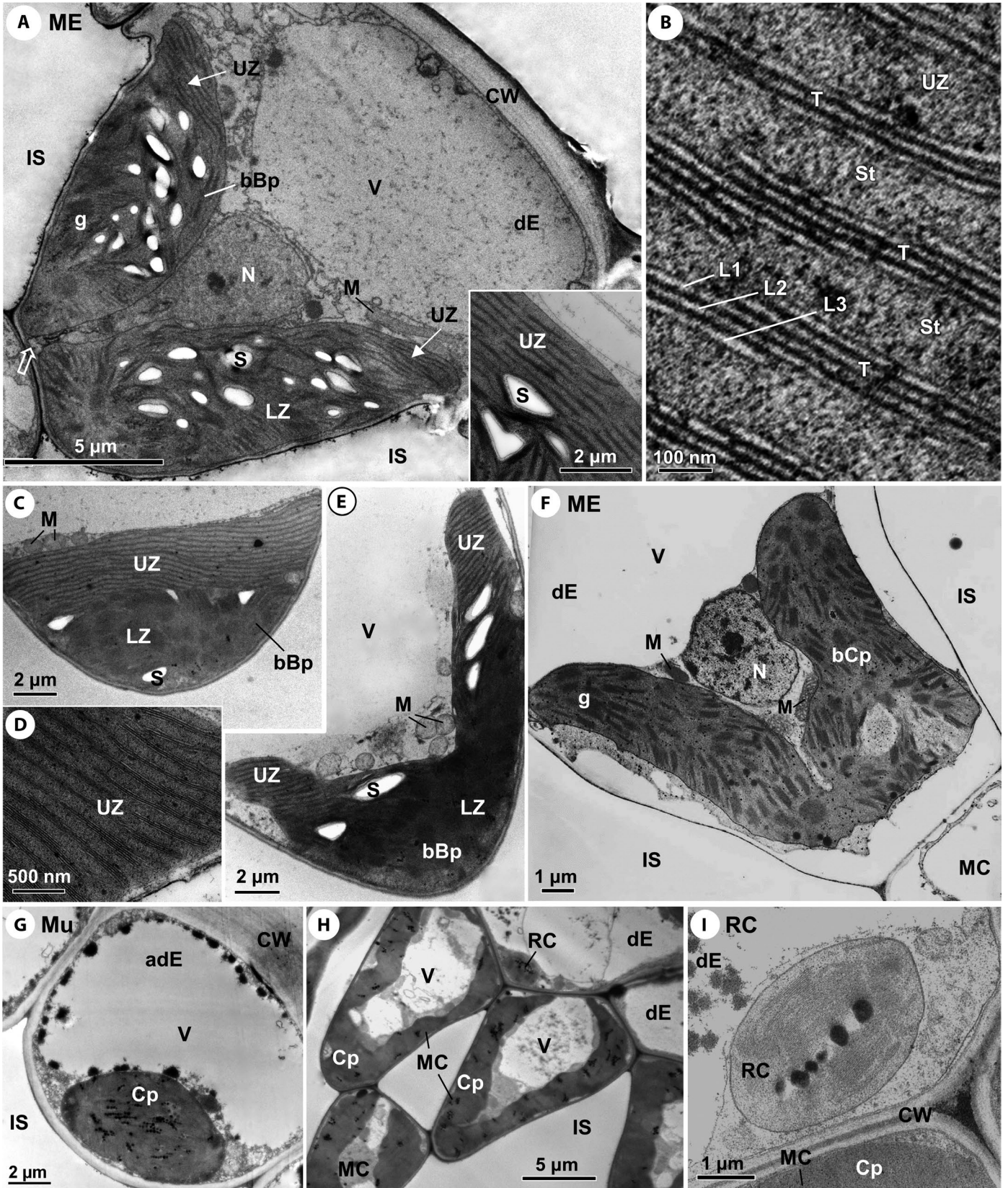
(Fig. 5A). A strong association between the chloroplast number in a dorsal epidermal cell (M, OL, and Mu) and light environment was found. The species with M (ME, Bp or not) are found in low-light environments (Fig. 1A–C). For the species with Bps, light intensity ranges from 0.4% to 2.1% full sunlight in their natural habitats. By contrast, the species with OL are found in partial-shade environments, while the species with Mu are found in high-light environments (Fig. 5A).

The annual environmental data (light, temperature, and humidity; near 24°N) of four native Taiwanese species of *Selaginella* recorded in their natural habitats provide more detailed information (Fig. 5B–D; Appendix S1). The two species with MEs and bBps are shade-adapted (*S. doederleinii* with cCps, *S. heterostachys* with bBps), growing in the forest understory in relatively low light. *Selaginella doederleinii* lives in a closed forest with evergreen trees and deep shade all year, but *S. heterostachys* (in a forest with deciduous trees) gradually receives more light when it sporulates in late summer and autumn. The species with OL (*S. repanda*, on a partially shaded slope) was found in partial shade. In contrast, the species with Mu (*S. tamariscina*, on a rocky slope) is often sun-exposed and receives large fluctuations in light due to changes in canopy openness in the deciduous forest (Fig. 5B).

The variations in mean monthly temperature of these four species from Taiwan represent three types of northern hemisphere, subtropical, montane habitats: lowland (*S. repanda*), middle elevation (*S. doederleinii* and *S. heterostachys*), and relatively high elevation (*S. tamariscina*) (Fig. 5C). In these habitats, June to September are the warmest months, but humidity generally fluctuates over the year. Only the habitat of *S. doederleinii* (with cCps) has relatively high humidity throughout the year. *Selaginella heterostachys*, a species with bBps, encounters relatively high humidity in the spring and summer growing season, but conditions are drier during sporulation and senescence in autumn and winter (Fig. 5D). Unfortunately, humidity data for *S. heterostachys* are missing for January and November to December.

Our annotation of the *Selaginella* phylogeny tree published by Weststrand and Korall (2016) (Fig. 6; Tables 1,3) shows that the basal clade of *Selaginella*, subg. *Selaginella*, features isophyllous, nondorsiventral shoots and Mu chloroplasts. Similar results were also found for subg. *Ericetorum* and *Rupestrae*, but the drought-tolerant species have apparent thick cell walls in their dorsal epidermis. Although the other four subgenera share the same features of anisophyllous and dorsiventral shoots, they have different chloroplast types in their dorsal epidermal cells. Subgenus *Exaltatae* has reduced or vestigial chloroplasts in its dorsal epidermal cells (RC) and subg. *Lepidophyllae* is Mu. Only subg. *Gymnogynum* and subg. *Stachygynandrum* possess high chloroplast diversity in

FIGURE 3. TEM of different forms of chloroplasts in the dorsal or adaxial epidermal cells of *Selaginella*. (A–F) Monoplastidy in a dorsal epidermal cell (ME), including bilobed bizonoplasts (bBps) in A–E and a bilobed chloroplast (bCp) with normal ultrastructure in F; (G) Multiplastidy (Mu); (H, I) Reduced or vestigial chloroplasts (RCs). (A) bBp of *S. heterostachys* in funnel-shaped dorsal epidermal cell. At the apex, each lobe has an upper zone, that runs along the interior side of the lobe, narrowing farther from the apex until it disappears. The open arrow (bottom left) at the base of the two lobes indicates the location of the connection, which is just out of view. Inset shows part of upper zone. (B) Close-up of upper zone of *S. aristata*. Each group consists of 4–6 thylakoids. Note that some terminal thylakoids can be seen at connections. (C–E) bBps from different section angles: *S. aristata* in (C, D) and *S. devolii* in (E). (F) bCp in dorsal epidermal cell of *S. willdenowii*. (G) Disk-like chloroplast in adaxial epidermal cell of *S. deflexa*. (H, I) RC in *S. exaltata* dorsal epidermal cells. Mesophyll cells have larger chloroplasts (Cp). **Abbreviations:** adE, adaxial epidermal cell; bBp, bilobed bizonoplast; bCp, bilobed chloroplast with normal ultrastructure; Cp, chloroplast; g, granum; IS, intercellular space; L, lumen (1–3); LZ, lower zone; M, mitochondrion; MC, mesophyll cell; ME, monoplastidy in a dorsal epidermal cell; Mu, multiplastidy; N, nucleus; RC, reduced or vestigial chloroplasts; S, starch grain; St, stroma; T, thylakoid; UZ, upper zone; V, vacuole.



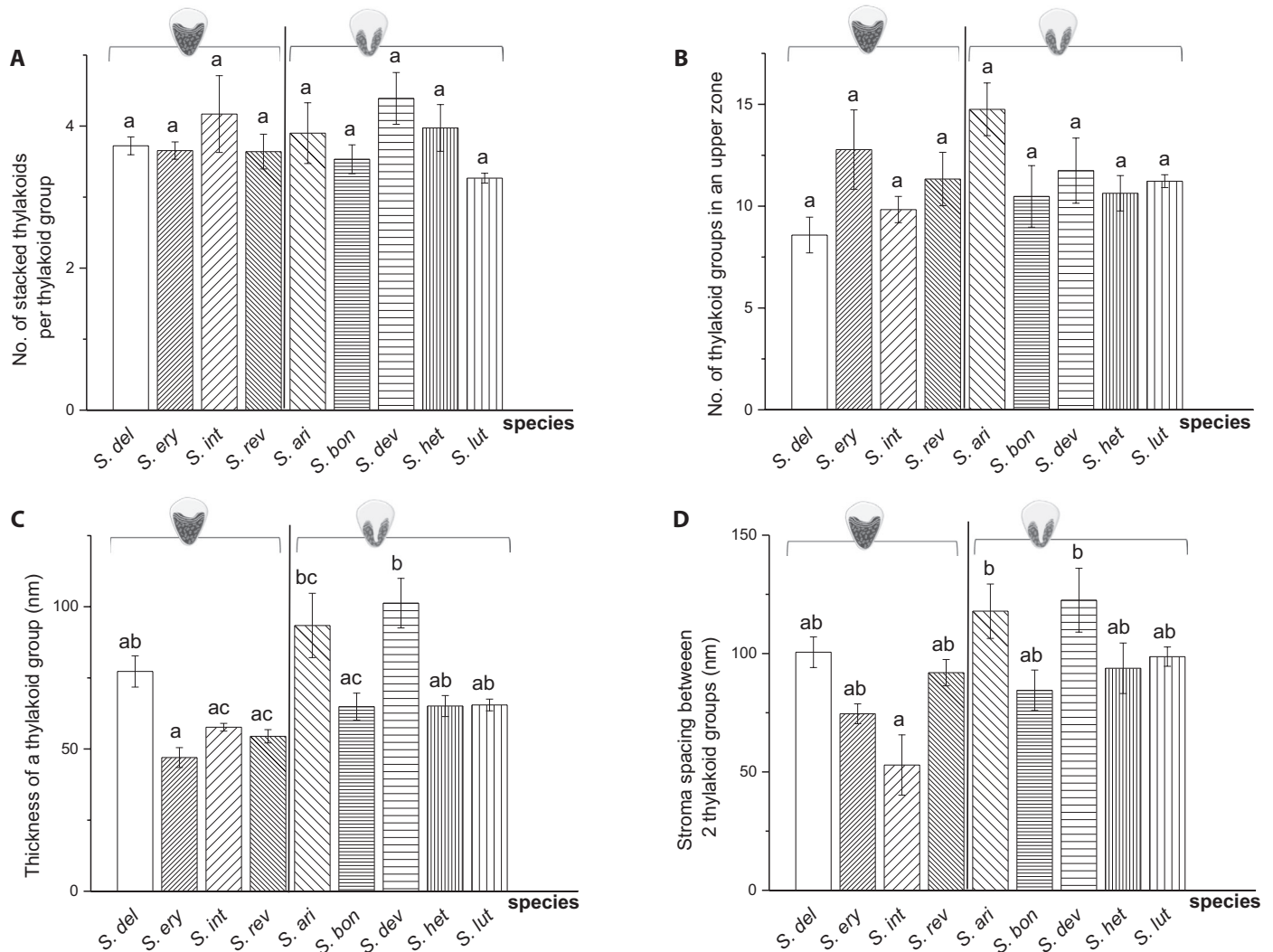


FIGURE 4. Comparative ultrastructural features of upper zones of bizonoplasts (Bps) of nine *Selaginella* species. Four species have cup-shaped bizonoplasts (cBps, left icon): *S. delicatula* (*S. del.*), *S. erythropus* (*S. ery.*), *S. intermedia* (*S. int.*), and *S. revoluta* (*S. rev.*); five species have bilobed Bp (bBps, right icon): *S. aristata* (*S. ari.*), *S. boninensis* (*S. bon.*), *S. devolii* (*S. dev.*), *S. heterostachys* (*S. het.*), and *S. lutchuensis* (*S. lut.*). (A) Number of stacked thylakoids per thylakoid group (ANOVA $F_{8,24} = 7.857, p < 0.001$). (B) Number of thylakoid groups in upper zone (ANOVA $F_{8,24} = 1.198, p = 0.341$). (C) Thickness of a thylakoid group (nm) (ANOVA $F_{8,32} = 1.707, p = 0.135$). (D) Stroma spacing between adjacent thylakoid groups (nm) (ANOVA $F_{8,24} = 4.410, p < 0.01$). Values are means \pm SE. Different letters indicate a significant difference between species within a group as determined by Scheffé’s post hoc test ($p < 0.05$).

their dorsal epidermal cells (Bp, ME, MM, Mu, OL, and RC), including monoplastidy. It is noteworthy that the ME chloroplast appears in subg. *Stachygynandrum* and the MM chloroplast appears in subg. *Gymnogynum*, respectively. However, Bps were only found in subg. *Stachygynandrum*.

DISCUSSION

Long before modern microscopy, Haberlandt (1888, 1914) reported monoplastidy, describing the chloroplasts as bowl-shaped, in several species of *Selaginella* including *S. martensii* Spring and *S. grandis* T.Moore. Later, monoplastidy was reported in a few more species of *Selaginella* (Ma, 1930; Jagels, 1970a, b). Webster (1992) highlighted monoplastidy as a major peculiarity of *Selaginella*. The results reported here, with 49 of 76

species monoplastidic, mean that monoplastidy is not an uncommon phenomenon in Selaginellaceae, but also that it is not universally found, contrary to previous reports (Webster, 1992; Banks, 2009). We estimate that approximately 70% of *Selaginella* species are monoplastidic. However, the most basal species in our study, viz., *S. deflexa* Brack., is Mu; i.e., it has multiple typical chloroplasts per cell. Based on the types of monoplastids (M) recognized in this study, previously reported monoplastids are (1) the ME type (M in the dorsal epidermal cell), e.g., *S. apus* [current name *S. apoda* (L.) Spring], *S. serpens* (Desv.) Spring, and *S. uncinata* (Desv.) Spring (Ma, 1930; Jagels, 1970a, b), and (2) the MM type (M in the mesophyll cell), e.g., *S. kraussiana* (Kunze) A.Braun (Haberlandt, 1914; Jagels, 1970a). Monoplastidy in *Selaginella* truly stands out among vascular plants. All other vascular plants with chloroplasts have a population of small chloroplasts in each photosynthetic cell (usually mesophyll cells).

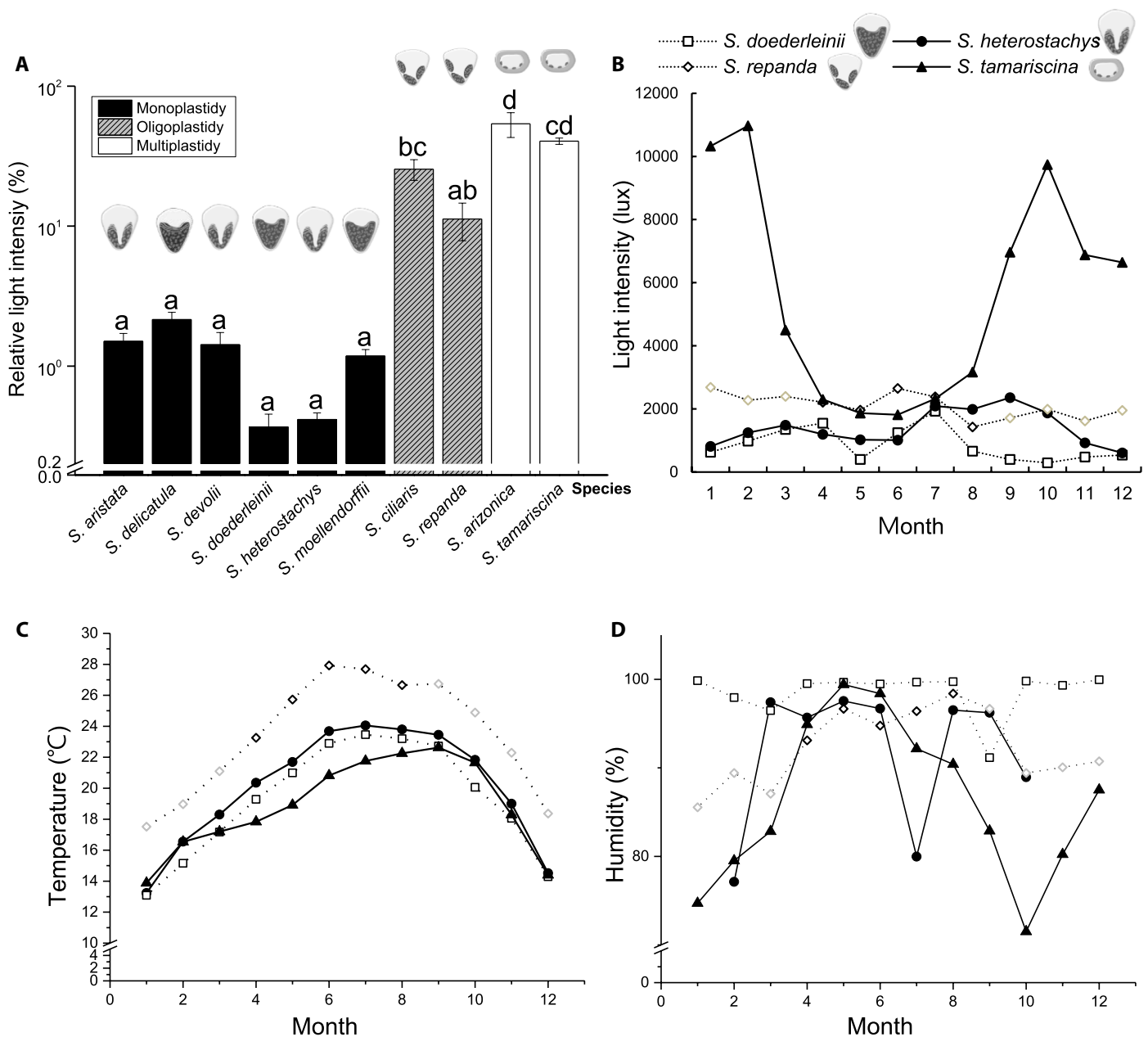


FIGURE 5. Environmental data recorded from natural habitats for selected *Selaginella*. (A) Light intensity (photosynthetically active radiation) interpreted as percentage of full sunlight for 10 species with chloroplast traits noted for comparison. Average values are based on 3–10 measurements at different locations (ANOVA $F_{9,54} = 31.035$, $p < 0.001$). The values are means \pm SE. Different letters indicate a significant difference between species as determined by Scheffé's post hoc test. (B–D) Annual environmental data for four selected *Selaginella* in Taiwan. The data for the dormant period for *S. repanda* (September–March) are indicated by lighter symbols. (B) Mean monthly light intensity. (C) Mean monthly temperature. (D) Mean monthly relative humidity. Icons show chloroplast types in the epidermal cells, which are the same as in Fig. 6.

Monoplastid chloroplasts vary not just in leaf tissue location (ME versus MM), but also in form (cup-shaped, bilobed, and giant disk) and in ultrastructure. A bizonoplast (Bp) uniquely has a dimorphic ultrastructure with distinctive and regularly layered thylakoids at the top of the single giant chloroplast. Before this study, Bps had only been reported in two *Selaginella* species, *S. erythropus* (Sheue et al., 2007, 2015) and *S. martensii* (Ferroni et al., 2016), although the material of *S. martensii* that we obtained from a botanic garden showed cup-shaped chloroplasts with typical ultrastructure

(no upper zone) in the present study. Unexpectedly, here an additional nine species of *Selaginella* were newly found to have Bps in their dorsal epidermal cells, bringing the total to 11 of 76 species with Bps. Seven of these species are from Taiwan where we studied all known native species of *Selaginella*. Because most of these species with Bps are small and cryptic, the potential exists that species with Bps are under-sampled elsewhere.

Although the chloroplast traits in the dorsal epidermal cells of *Selaginella* are species-specific and persistent, rare variations

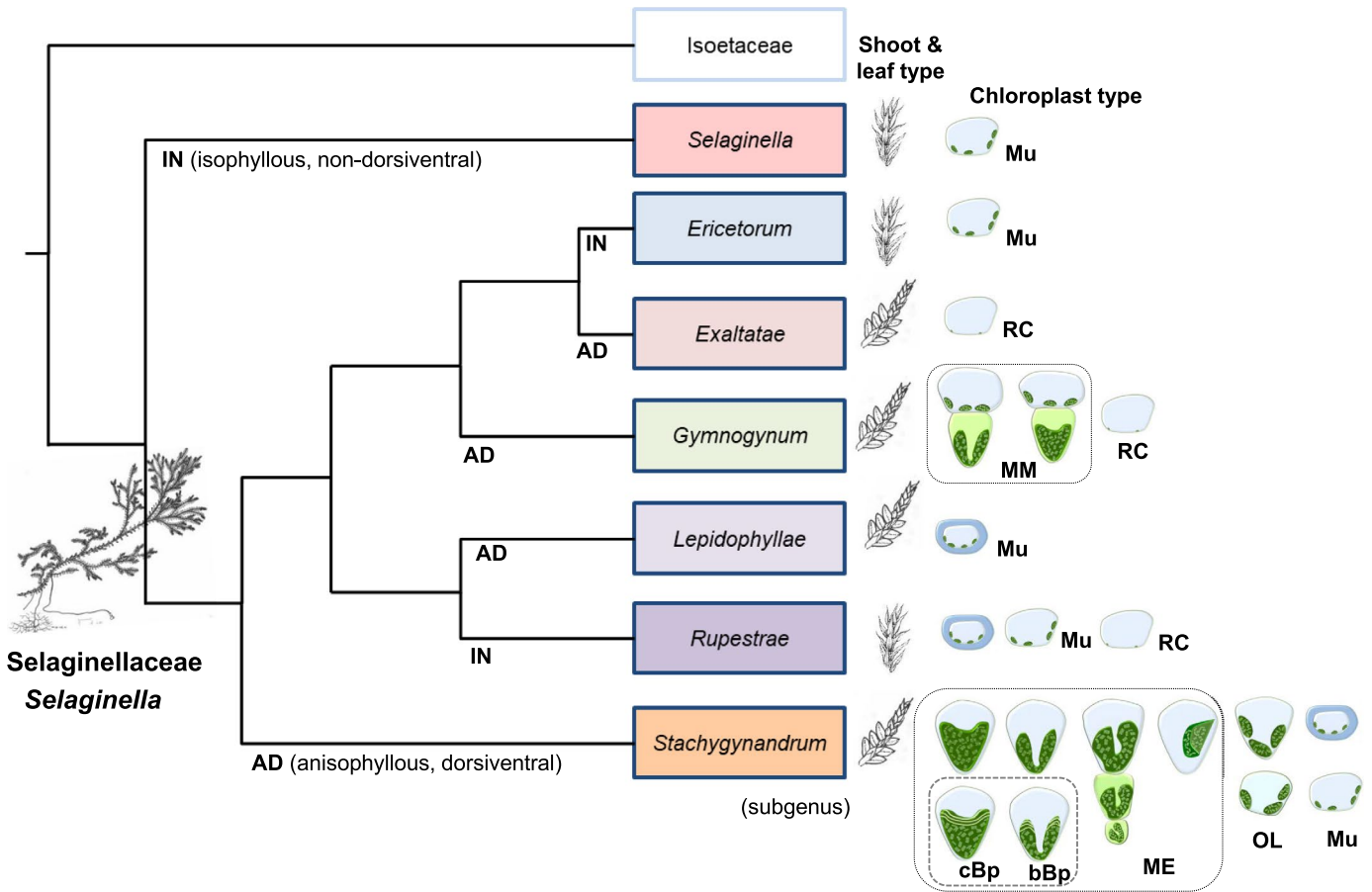


FIGURE 6. Chloroplast diversity in the dorsal epidermal cells of Selaginellaceae associated with subgenera and morphological traits. The phylogenetic tree is modified from that of Weststrand and Korall (2016). *Abbreviations:* AD, anisophyllous, dorsiventral; IN, isophyllous, nondorsiventral; Bp, bizonoplast (b, bilobed; c, cup-shaped); ME, monoplastidy in the dorsal epidermal cell; MM, monoplastidy in mesophyll cell; Mu, multiplastidy, OL, oligoplastidy; RC, reduced or vestigial chloroplasts.

on chloroplast number per cell and ultrastructure were observed. For example, most of the studied material of *S. remotifolia* has bilobed giant chloroplasts in its mesophyll cells beneath dorsal epidermal cells (MM trait, bCp), but the OL trait (2–10 chloroplasts per cell) is rare in these mesophyll cells. In *S. erythropus*, the ME trait with Bp ultrastructure, occurs in dorsal epidermal cells of microphylls, but two Bps in a cell adjacent to a stoma were observed once (C. R. Sheue, unpublished data). Ma (1930) also pointed out that in *S. apoda* (syn. *S. apus* in Ma, 1930), the dorsal epidermal cells near the tip of a microphyll usually contain one chloroplast, but the cells near the base of a microphyll may have two or more chloroplasts.

The species of *Selaginella* with monoplastidy (both ME and MM) in this study all live in deep shade, receiving only on average 0.4–2.1% of full sunlight (Fig. 5A). In strong contrast, species with multiple chloroplasts live in open places, with on average more than 40.5% of full sunlight. *Selaginella doederleinii* with ME cup-shaped chloroplasts was recorded with the lowest light intensity. Among the four species with Bps, the species with cBp (*S. delicatula*) was found at higher light intensities than the three species with bBps (bilobed Bp). More in-depth studies are needed to understand the effects of Bps on plant physiology and how species with cBps and bBps differ ecophysically.

Despite the shape differences between cBp and bBp, the ultrastructure of their upper zones of parallel thylakoid groups is similar. A bBp has bivalve, shell-like lobes with an upper zone at the apex. This upper zone runs along the interior side of the lobe, becoming thinner farther from the apex until it disappears. Our observations show that the angle between the two lobes can change in response to light, potentially optimizing its shape in a given light environment. In contrast, the concave top of a cBp is similar to a basin, yet still has some ability to change shape. The comparative data for the nine *Selaginella* species (Fig. 4) with Bps show that the upper zones typically contain about 11 parallel thylakoid groups, with each group comprising 3–5 stacked thylakoids. Their ultrastructural features are very similar even in different species. Some variations are found in the thicknesses of thylakoid groups and in the stroma thickness between two thylakoid groups, but these variations are subtle. Convergent evolution is suggested by the occurrence of these structures in distantly related species from distant parts of the Earth (from the New World to the Old World) in shaded environments.

Both bBps and cBps are located at the bases of funnel-shaped dorsal epidermal cells surrounded by intercellular space. The refractive index inside a dorsal epidermal cell ($n_{\text{cell}} = 1.425$, Gausman et al., 1974) is higher than air ($n_{\text{air}} = 1$), which means that some portion of the light striking base of the cell, depending

TABLE 3. Chloroplast categories of subgenera of the 76 studied *Selaginella*. *Abbreviations:* A, anisophylly; bBp, bilobed bizonoplast; bCp, bilobed chloroplast; cBp, cup-shaped bizonoplast; cCp, cup-shaped chloroplast; D, typical disk-shaped chloroplast; gD, giant disk-shaped chloroplast; l, isophylly; ME, monoplastidty in the dorsal epidermal cell; MM, monoplastidty in the mesophyll cell; Mu, multiplastidty; OL, oligoplastidty; RC, reduced or vestigial chloroplasts.

Subgenus	Number of species (studied no./ total no.)	Microphyll (sp. no.)	Chloroplast category (sp. no.)	Chloroplast type (sp. no.)
<i>Ericetorum</i>	2/8	l (2)	Mu (2)	D (2)
<i>Exaltatae</i>	1/3	A (1)	RC (1)	RC (1)
<i>Gymnogynum</i>	9/40	A (9)	MM (5), RC (4)	bCp (1), cCp (4), RC (4)
<i>Lepidophyllae</i>	1/2	A (1)	Mu (1)	D (1)
<i>Rupestrae</i>	6/50	l (6)	Mu (5), RC (1)	D (5), RC (1)
<i>Selaginella</i>	1/2	l (1)	Mu (1)	D (1)
<i>Stachygynandrum</i>	56/600	A (56)	ME (44), Mu (5), OL (7)	bBp (5), bCp (10), cBp (6), cCp (22), D (12), gD (1)

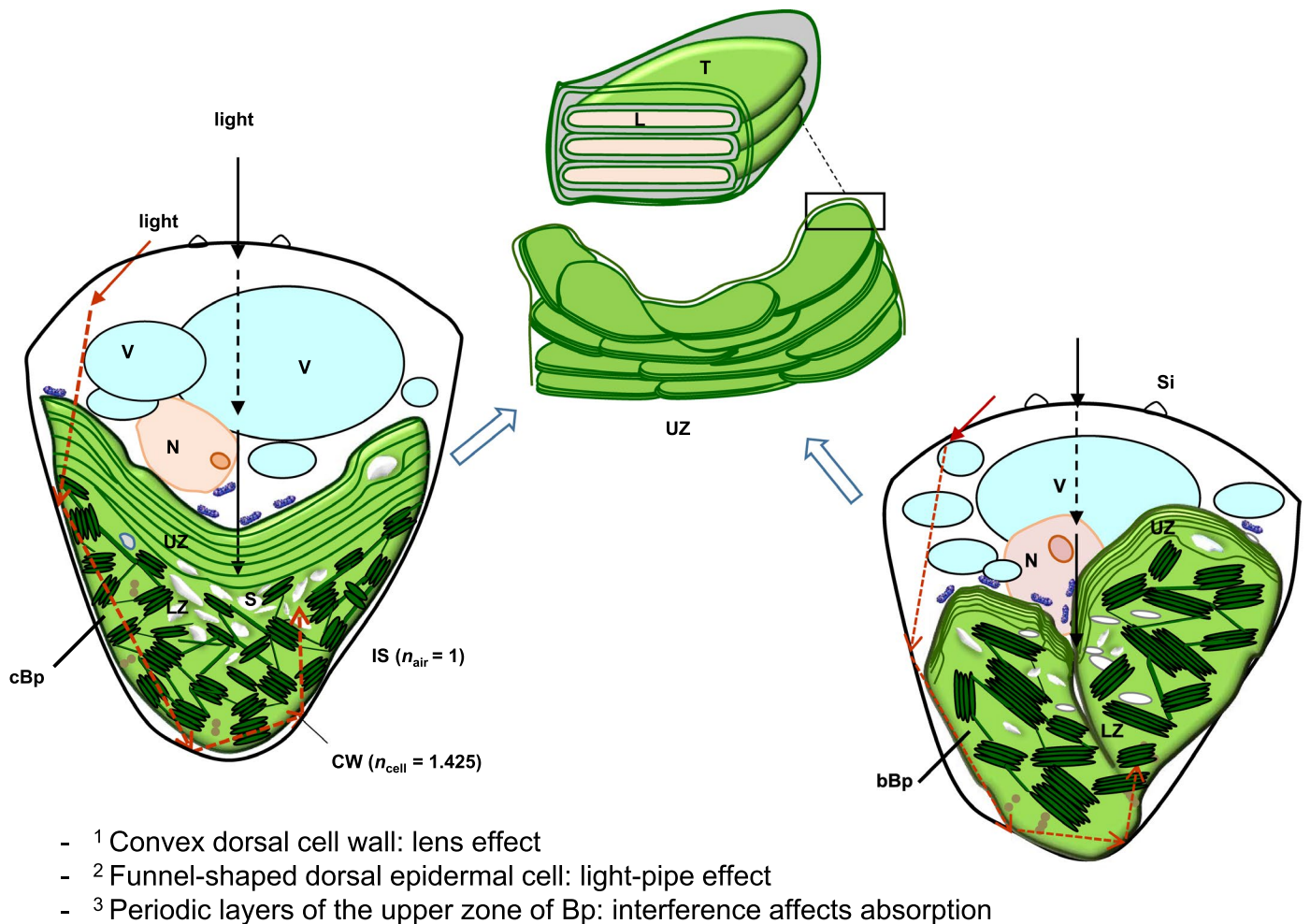


FIGURE 7. Diagrams of bizonoplasts (Bps) in dorsal epidermal cells, with a proposed 3-dimensional structure for their upper zones. Potential light paths are marked to show optical properties. The cup-shaped Bps (cBp) and bilobed Bps (bBp) have regularly arranged groups of thylakoids in their upper zones, which are located above lower zones that have typical chloroplast ultrastructures. The concave top of a cBp is similar to a basin, while a bBp is similar to a bivalve shell with a narrow connection at the base. Both types of Bp have the potential to open and close as light conditions change, and both are located at the base of funnel-shaped dorsal epidermal cells surrounded by intercellular space. The refractive index inside cells ($n_{\text{cell}} = 1.425$) is higher than air ($n_{\text{air}} = 1$) which may cause multiple reflections when light paths hit the cell-air boundaries obliquely, potentially increasing the opportunity for light absorption as the light passes through the Bp multiple times. *Abbreviations:* Bp, bizonoplast (b, bilobed; c, cup-shaped); CW, cell wall; IS, intercellular space; L, lumen; LZ, lower zone; N, nucleus; S, starch grain; Si, silica body; T, thylakoid; UZ, upper zone; V, vacuole.

on its angle, will be reflected back into the Bp (Liu et al., 2012) increasing the amount of light absorbed (Fig. 7). This particular feature of a Bp, however, is shared with other monoplastids that

do not have a layered structure in the upper zone. The layered structure of the upper zone of a Bp may additionally interfere with the light waves, with the potential to enhance absorption

and reflection depending on the wavelengths and light angles (Jacobs et al., 2016). The presence of light interference is evident from blue iridescent features found on microphylls of some species (*S. erythropus*, *S. heterostachys*, and *S. delicatula*) during development (C. R. Sheue, unpublished data). Recently, Masters et al. (2018, pp. 1, 6) successfully characterized iridescence in *S. erythropus* and determined that it is from “one-dimensional photonic multilayers” (the upper zone of the Bp). These light interference effects of the upper zone, together with internal reflections at the cell boundary potentially make the Bp a unique photonic system deserving further study. This iridescence from a Bp is in contrast to the iridescence caused by layered lamellae on the outer cell wall of dorsal epidermal cells (Héban and Lee, 1984) in *S. willdenowii* (Desv.) Baker and *S. uncinata* (Desv.) Spring.

Among the seven subgenera, subg. *Selaginella* (erect with helically arranged isophylls [equal-sized leaves] and stems lacking rhizophores) is the most basal group of Selaginellaceae (Zhou et al., 2015; Weststrand and Korall, 2016). Because Mu chloroplasts are found in subgenus *Selaginella*, we may infer that the ancestors of genus *Selaginella* were Mu. The Mu trait (7 species) and the RC trait (1 species) are characteristic of the other two subgenera (*Ericetorum* and *Rupestrae*) that share similar morphological traits with subg. *Selaginella* (but with rhizophores, and a few species of *Ericetorum* with anisophylls) (Weststrand and Korall, 2016). However, the majority of Selaginellaceae with dorsiventral shoots and anisophylls (usually with smaller dorsal leaves and larger ventral leaves) (ca. 91%) belong to four subgenera (*Exaltatae*, *Gymnogynum*, *Lepidophyllae*, and *Stachygynandrum*) (Table 3). These subgenera have the highest chloroplast diversity in this family, exhibiting all chloroplast types (Mu, OL, ME, MM, and RC). However, M occurs only in the two subgenera with the highest species diversity, *Gymnogynum* (40 spp., 5 of 10 studied spp. have MM chloroplasts) and *Stachygynandrum* (600 spp., 44 of 56 studied spp. with ME type). About 79% and 55% of studied species of *Stachygynandrum* and *Gymnogynum*, respectively, have the M trait. Notably, the 11 species with Bps all belong to subg. *Stachygynandrum*, the largest subgenus of *Selaginella*, comprising about 600 species and occurring in both the Old World and the New World (Weststrand and Korall, 2016). Eight species with Bps are from the Old World, and three species are from the New World (*S. erythropus*, *S. martensii*, and *S. revoluta*). Although monoplastidy occurs in two subgenera, *Stachygynandrum* (ME type, 44 species) and *Gymnogynum* (MM type, 5 species), all Bps (cBp and bBp) are the ME type. Thus, Bp appears to be an apomorphy of subg. *Stachygynandrum* in *Selaginella* because the members of other subgenera have only typical chloroplast ultrastructures regardless of chloroplast size and number.

In contrast with most plants with multiple chloroplasts per photosynthetic cell, algae often contain only a few or a single giant chloroplast in a cell (Solymosi, 2012). In algae, monoplastidy is common in green algae and some species of Rhodophyta (unicellular or multicellular), but multiplastidy also occurs in both groups (Solymosi, 2012; de Vries and Gould, 2018). Chloroplasts of algae have diverse shapes, including cup-shaped, reticulate, ring-shaped, helical, cuboidal, star-shaped, and bilobed (Solymosi, 2012). However, in land plants multiplastidy and small chloroplasts, appearing as disk-shaped, spherical, ellipsoidal, or lens-shaped, are prevalent, with few exceptions. Monoplastidy in mature photosynthetic cells of land plants is restricted to hornworts and *Selaginella*. However, hornwort monoplastids vary in shape from spherical to ellipsoidal, to lens-shaped, spindle-shaped, and star-shaped (Renzaglia et al.,

2007), unlike *Selaginella* monoplastids. Moreover, many species of hornwort have pyrenoids in their chloroplasts and grana consisting of stacks of short thylakoids, lacking end membranes, and surrounded by channel thylakoids (Vaughn et al., 1992; Villarreal and Renner, 2012). These major differences in monoplastidy between hornworts and *Selaginella* are suggestive of independent evolution of this trait in these distantly related plant groups. This inference is further supported by the absence of monoplastidy in basal *Selaginella* species.

The high diversity of chloroplast types in *Selaginella* revealed here greatly expands the known chloroplast diversity in vascular plants. Selaginellaceae are a widely distributed species-rich family (Jermy, 1990), which arose more than 370 million years ago (Banks, 2009) and successfully adapted to environments ranging from tropical forests to hot deserts. Their strikingly different chloroplasts, especially the bizonoplast with its special internal structure and potential optical integration with its containing cell, imply that much is to be learned from these otherwise unprepossessing plants, especially from multidisciplinary approaches combining physics, physiology, systematics, and ecology.

ACKNOWLEDGMENTS

This article is dedicated to the memories of the late Professor V. Sarafis and the late Dr. C. C. Tsai, both of whom greatly assisted with this study. The authors thank Prof. M. S. B. Ku and two anonymous reviewers for providing help and valuable comments; the University of Bristol Botanic Garden (UK), Cairns Botanic Gardens (Australia), Sun Yat-sen University (China), South China Botanic Garden (China), The Dr. Cecilia Koo Botanic Conservation Center (KBCC, Taiwan), Smithsonian Tropical Research Institute, and Mr. Z. X. Chang for providing materials or field trip help; and Dr. W. N. Jane (Academia Sinica, Taiwan) and Miss P. C. Chao (Precision Instruments Center, National Chung Hsing University) for assisting with TEM. This study was supported by the Ministry of Science and Technology, Taiwan (awards MOST-101-2621-B-005-002-MY3; MOST 104-2621-B-005-002-MY3; MOST 107-2621-B-005-001).

AUTHOR CONTRIBUTIONS

J.W.L. participated in structural studies, plant collection and identification, collection of environmental data, preparation of figures, and drafting the manuscript. S.F.L. participated in structural and molecular studies, plant collection and identification, and collection of environmental data. C.T.W. participated in structural studies, plant collection, and identification. I.A.V. participated in plant collection and identification, preparation of a table, and editing of the manuscript. J.F.H. and Y.H.W. participated in structural studies. H.M.C. participated in plant collection, identification, and molecular studies. T.Y.G. participated in plant collection and identification and collection of environmental data. W.H.Y. participated in plant collection and identification and editing of the manuscript. N.S.A. participated in planning expeditions, preparing specimens, and editing the manuscript. M.F.K., C.C., S.D., H.O., A.B., P.S., B.A., R.K., and N.N. participated in plant collection and identification. C.L.H. supervised the molecular identifications and preparing data files. P.C. organized expeditions, participated in plant collection and statistical analyses, and edited the manuscript. C.R.S. coordinated

the entire project, supervised the structural studies, participated in plant collection and identification, writing the manuscript, and preparing data files and figures.

DATA AVAILABILITY

All data underlying the study are included in the manuscript, its supplementary materials, or access information is provided in the supplementary tables.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Materials of *Selaginella* used in this study, with information on distribution, voucher specimens, and habitat.

APPENDIX S2. Video of bilobed bizonoplast of *Selaginella heterostachys* viewed with a confocal laser scanning microscope.

LITERATURE CITED

- Banks, J. A. 2009. *Selaginella* and 400 million years of separation. *Annual Review of Plant Biology* 60: 223–238.
- de Vries, J., and S. B. Gould. 2018. The monoplastidic bottleneck in algae and plant evolution. *Journal of Cell Science* 131: jcs203414.
- Ferroni, L., M. Suorsa, E. M. Aro, C. Baldissarroto, and S. Pancaldi. 2016. Light acclimation in the lycophyte *Selaginella martensii* depends on changes in the amount of photosystems and on the flexibility of the light-harvesting complex II antenna association with both photosystems. *New Phytologist* 211: 554–568.
- Gausman, H. W., W. A. Allen, and D. E. Escobar. 1974. Refractive index of plant cell walls. *Applied Optics* 13: 109–111.
- Gould, K. S., and D. W. Lee. 1996. Physical and ultrastructural basis of blue leaf iridescence in four Malaysian understory plants. *American Journal of Botany* 83: 45–50.
- Haberlandt, G. 1888. Die Chlorophyllkörper der Selaginellen. *Flora* 71: 291–308.
- Haberlandt, G. 1914. *Physiological plant anatomy*. MacMillan, London, UK.
- Héban, C., and D. W. Lee. 1984. Ultrastructural basis and developmental control of blue iridescence in *Selaginella* leaves. *American Journal of Botany* 71: 216–219.
- Jacobs, M., M. Lopez-Garcia, O. P. Phrathep, T. Lawson, R. Oulton, and H. M. Whitney. 2016. Photonic multilayer structure of *Begonia* chloroplasts enhances photosynthetic efficiency. *Nature Plants* 2: 16162.
- Jagels, R. 1970a. Photosynthetic apparatus in *Selaginella*. I. Morphology and photosynthesis under different light and temperature regimes. *Canadian Journal of Botany* 48: 1843–1852.
- Jagels, R. 1970b. Photosynthetic apparatus in *Selaginella*. II. Changes in plastid ultrastructure and pigment content under different light and temperature regimes. *Canadian Journal of Botany* 48: 1853–1860.
- Jermy, A. C. 1990. Selaginellaceae. In K. Kubitzki [ed.], *The families and genera of vascular plants*, vol. 1, K. U. Kramer and P. S. Green [eds.], Pteridophytes and gymnosperms, 39–45. Springer, Berlin, Germany.
- Liu, J. W., C. T. Wu, Y. H. Wu, C. C. Tsai, P. Chesson, and C. R. Sheue. 2012. Novel adaptation to deep shade environments in basal vascular plants: bizonoplasts of *Selaginella*. 97th Annual Meeting of the Ecological Society of America, 2012, Portland, OR, USA.
- Ma, R. M. 1930. The chloroplasts of *Selaginella*. *Bulletin of the Torrey Botanical Club* 57: 277–284.
- Masters, N. J., M. Lopez-Garcia, R. Oulton, and H. M. Whitney. 2018. Characterization of chloroplast iridescence in *Selaginella erythropus*. *Journal of the Royal Society Interface* 15: 20180559.
- Pao, S. H., P. Y. Tsai, C. I. Peng, P. J. Chen, C. C. Tsai, E. C. Yang, M. C. Shih, et al. 2018. Lamelloplasts and minichloroplasts in Begoniaceae: iridescence and photosynthetic functioning. *Journal of Plant Research* 131: 655–670.
- Renzaglia, K. S., S. Schuette, R. J. Duff, R. Ligrone, R. J. Shaw, B. D. Mishler, and J. G. Duckett. 2007. Bryophyte phylogeny: advancing the molecular and morphological frontiers. *Bryologist* 110: 179–213.
- Reshak, A. H., and C. R. Sheue. 2012. Second harmonic generation imaging of the deep shade plant *Selaginella erythropus* using multifunctional two-photon laser scanning microscopy. *Journal of Microscopy* 248: 234–244.
- Sheue, C. R., V. Sarafis, R. Kiew, H.-Y. Liu, A. Salino, L.-L. Kuo-Huang, Y.-P. Yang, et al. 2007. Bizonoplast, a unique chloroplast in the epidermal cells of microphylls in the shade plant *Selaginella erythropus* (Selaginellaceae). *American Journal of Botany* 94: 1922–1929.
- Sheue, C. R., J. W. Liu, J. F. Ho, A. W. Yao, Y. H. Wu, S. Das, C. C. Tsai, et al. 2015. A variation on chloroplast development: the bizonoplast and photosynthetic efficiency in the deep-shade plant *Selaginella erythropus*. *American Journal of Botany* 102: 500–511.
- Solymosi, K. 2012. Plastid structure, diversification and interconversions I. Algae. *Current Chemical Biology* 6: 167–186.
- Solymosi, K., and Á. Keresztes. 2012. Plastid structure, diversification and interconversions II. Land plants. *Current Chemical Biology* 6: 187–204.
- Vanderpoorten, A., and B. Goffinet. 2009. *Introduction to bryophytes*. Cambridge University Press, Cambridge, UK.
- Vaughn, K. C., R. Ligrone, H. A. Owen, J. Hasegawa, E. O. Campbell, K. S. Renzaglia, and J. Monge-Najera. 1992. The anthocerot chloroplast: a review. *New Phytologist* 120: 169–190.
- Villarreal, J. C., and S. S. Renner. 2012. Hornwort pyrenoids, carbon-concentrating structures, evolved and were lost at least five times during the last 100 million years. *Proceedings of the National Academy of Sciences, USA* 109: 18873–18878.
- Webster, T. R. 1992. Developmental problems in *Selaginella* (Selaginellaceae) in an evolutionary context. *Annals of the Missouri Botanical Garden* 79: 632–647.
- Weststrand, S., and P. Korall. 2016. A subgeneric classification of *Selaginella* (Selaginellaceae). *American Journal of Botany* 103: 2160–2169.
- Zhou, X.-M., and L.-B. Zhang. 2015. A classification of *Selaginella* (Selaginellaceae) based on molecular (chloroplast and nuclear), macromorphological, and spore features. *Taxon* 64: 1117–1140.