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Cultivation Techniques for Terrestrial Clubmosses (Lycopodiaceae): Conservation, Research, and Horticultural Opportunities for an Early-Diverging Plant Lineage

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ABSTRACT.—Clubmosses (Lycopodiaceae) represent the closest living counterparts to early vascular plants, but inability to culture terrestrial taxa has made much of the clade inaccessible to *ex-situ* conservation, experimental research, and horticulture. In an attempt to identify conditions conducive to repeatable *ex vitro* culture, the utility of clayey and sandy loams amended with pumice as a medium was observed for 20 terrestrial species spanning all three subfamilies of Lycopodiaceae. Using this media class, a series of effective sporophyte cultivation and propagation techniques were developed for all 9 North American genera. Strategies are described for selection of appropriate propagules, establishment, propagation, and long-term maintenance for each genus. Sporophytes of all taxa established readily in oligotrophic, largely inorganic media under high humidity. Large clonal colonies were maintained with frequent application of weak fertilizer solution under bright light, high humidity, and year-round moderate temperatures in two greenhouses, a growth chamber, and laboratory.

KEY WORDS.—Cultivation, Lycopodiaceae, lycophytes, *Lycopodium*, propagation

Lycopodiaceae, commonly referred to as clubmosses, are extant representatives of an ancient lycophyte clade, retaining striking morphological similarities to Late Silurian-Early Devonian lycopsids and their putative allies (Hueber, 1992; Xue, 2011; Kerp *et al.*, 2013). As the only homosporous plants with meristems reminiscent of seed plants (Philipson, 1990; Imaichi and Hiratsuka, 2000), members of this group provide the closest plausible functional and developmental counterparts to basal eutracheophytes (Imaichi and Hiratsuka, 2000: Fig. 6). Aside from their importance phylogenetically, a number of clubmosses (*Huperzia* and *Phlegmariurus* spp.) have medicinal properties, and have yielded huperzine A used to treat neurodegenerative disorders such as Alzheimer's Disease (for review, see Cheng *et al.*, 1996; Tang and Han, 1999). Biologically active and structurally complex '*Lycopodium*' alkaloids have also been isolated from members of other genera (e.g. Ma and Gang, 2004; Mandal *et al.*, 2010).

While several paleotropical epiphytic *Phlegmariurus* spp. are widely grown, the rest of the family consists of terrestrial genera virtually absent from

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cultivation. These terrestrial lineages are not only morphologically diverse but also encompass growth habits more similar to early vascular plants than the few epiphytic taxa accessible to researchers (Kerp *et al.*, 2013). Despite their importance and diversity, little has been published on successful cultivation of terrestrial Lycopodiaceae since Barrows, (1935) aside from slow and difficult pharmaceutical *in vitro* tissue culture and gemma establishment for *Huperzia* (Szyputa *et al.*, 2005; Wang *et al.*, 2011) and *in vitro* sporophyte generation from nodular callus tissue in *Lycopodiella* (Atmane *et al.*, 2000). This dearth of published work stems from a long history of failed to partially successful *ex vitro* culture attempts for terrestrial clubmosses (Jones, 1987; Mickel, 1994; Hoshizaki and Moran, 2001; Byfield and Stewart, 2007; Ma *et al.*, 2007; Cullina, 2008). What literature that does exist on the subject (e.g. Barrows, 1935; Jones, 1987; Heim, 1988; Mickel, 1994; Hoshizaki and Moran, 2001; Cullina, 2008; McAuliff, 2008; Copeland, 2010) is either limited in detail or describes results difficult to replicate in other facilities (Benca, *pers. obs.*).

Several obstacles impeding conservation efforts for terrestrial clubmosses could be overcome if reliable cultivation techniques were made available. Among these are dependence on largely unsuccessful site-to-site transplantation practices of regionally imperiled populations (Byfield and Stewart, 2007) as well as unsustainable wild harvest for pharmaceutical, homeopathic, and floral industries (Matula, 1995; Nauertz and Zasada, 1999; Ma *et al.*, 2006; Ma *et al.*, 2007).

Unlike many ferns, relying upon spore banks and *in vitro* germination are not the most effective means for short-term *ex-situ* conservation of clubmosses, particularly for those with subterranean (non-photosynthetic) gametophytes. Spores of these taxa have a low germination percentage (Whittier, 1977, 1981; Whittier and Webster, 1986; Whittier, 1998) and are slow to germinate (Whittier, 1998). Fortunately, most terrestrial clubmosses are excellent candidates for asexual sporophyte propagation due to their largely clonal growth habit.

Described here are sporophyte *ex-vitro* cultivation techniques and strategies developed across different facility types over several years. Using these techniques while controlling several environmental parameters, cultivation success was replicated for all North American genera of Lycopodiaceae, including several species of conservation concern (Table 1).

Classification.—Lycopodiaceae is divided into 16 morphologically-distinct genera (Vasconcellos and Franco, 1967; Holub, 1975; 1983; 1991; Øllgaard, 1979a; 1987; Haines, 2003; Field and Bostock, 2013) encompassing three subfamilies: Lycopodioideae, Lycopodiellioideae, and Huperzioidae (Wagner and Beitel, 1992; Øllgaard, 2012b). Fossil records indicate an Early Jurassic minimum divergence time for these subfamilies (Wikström, 2001) and their genera represent monophyletic groupings not known to hybridize (see cladograms of Wikström and Kenrick, 2000; 2001; Øllgaard, 2012b).

For simplification, the two subfamilies Lycopodioideae and Lycopodiellioideae are termed ‘strobilate’ clubmosses herein, similarly to Wikström and Kenrick, (2001). Members of these two subfamilies display shoots with

TABLE 1. Sources of material and success in establishment under greenhouse cultivation: UWBG = University of Washington Botany Greenhouse, UCBG = University of California Botanical Garden; UCB Lab = UC Berkeley laboratory; (+) = Cultivation success at the facility; (-) = cultivation failure at facility; (N/A) = Not applicable i.e., not tested at facility. * denotes *threatened species* or *exploitably vulnerable* (at state level); ** denotes *endangered species* (at state and/or national level); and ^(?) denotes possibly new species whose known distribution is highly restricted (Larson, *per. comm.*) State and national conservation status available on *USDA website*. <http://plants.usda.gov/threat.html> .

Species	Collector	Locality	UWBG	UCBG	UCB Lab
<i>Dendrolycopodium hickeyi</i> (Wagner, Beitel, Moran) Haines	D. Foglia	Rensselaer Co., NY	+	+	+
<i>Diphasiastrum digitatum</i> * (Dill. ex Braun) Holub	D. Foglia	Rensselaer Co., NY	+	+	N/A
<i>Diphasiastrum sitchense</i> ** (Ruprecht) Holub	J. Benca	Snohomish Co., WA	+	-	N/A
<i>Huperzia haleakalae</i> (Brack.) Holub	J. Benca	Snohomish Co., WA	+	N/A	N/A
<i>Huperzia lucidula</i> * (Michx.) Trevis.	H. Bicher	NC	+	+	+
<i>Huperzia miyoshiana</i> (Makino) Ching	J. Benca	Snohomish Co., WA	+	+	+
<i>Huperzia occidentalis</i> (Clute) Beitel	J. Benca	Snohomish Co., WA	+	+	+
<i>Lycopodiella alopecuroides</i> ** (L.) Cranfill	P. Sheridan	VA	+	+	+
<i>Lycopodiella appressa</i> ** (Chapm.) Cranfill	P. Sheridan	VA	+	-	+
<i>Lycopodiella inundata</i> 'robust form' (L.) Holub	J. Larson	Muskegon Co., MI	+	-	+
<i>Lycopodiella margueritae</i> ** Bruce, Wagner, Beitel	J. Larson	Muskegon Co., MI	+	-	N/A
<i>Lycopodiella prostrata</i> (Harper) Cranfill	R. Carter	Camden Co., GA	+	+	+
<i>Lycopodiella subappressa</i> ** Bruce, Wagner, Beitel	J. Larson	Muskegon Co., MI	+	-	N/A
<i>Lycopodiella</i> sp. 2 ^{*(?)}	J. Larson	Muskegon Co., MI	+	+	+
<i>Lycopodiella</i> sp. 3 (or hybrid) ^(?)	J. Larson	Muskegon Co., MI	+	+	+
<i>Lycopodium clavatum</i> ** L.	J. Benca	Thurston Co., WA	+	+	N/A
<i>Lycopodium clavatum</i> ** L.	J. Benca	Snohomish Co., WA	+	+	N/A
<i>Lycopodium cf. clavatum</i> ** L.	C. Husby	Dominican Republic	N/A	+	N/A
<i>Lycopodium venustum</i> * ^(?) Gaudich	K. Kowelo	Oahu, HI	+	+	N/A
<i>Lycopodium cf. venustum</i> ^(?)	J. Benca	Hawaii, HI	N/A	N/A	N/A
<i>Palhinhaea cernua</i> (L.) Franco & Vasc.	K. Kowelo	Oahu, HI	+	+	N/A
<i>Palhinhaea cernua</i> (L.) Franco & Vasc.	C. Husby	Hawaii, HI	N/A	+	+
<i>Palhinhaea cernua</i> * (L.) Franco & Vasc.	C. Alford	Indian River Co., FL	+	-	+
<i>Palhinhaea cernua</i> (L.) Franco & Vasc.	M. Grantham	South Africa	+	+	+
<i>Phlegmariurus reflexus</i> (Lam.) Øllg.	C. Wunderlich	Peru	+	+	+

TABLE 1. Continued.

Species	Collector	Locality	UWBG	UCBG	UCB Lab
<i>Pseudolycopodiella cf. caroliniana</i>	P. Sheridan	Surry Co., VA	+	-	+
<i>Pseudolycopodiella caroliniana</i> ** (L.) Pic. Serm.	P. Sheridan	Surry Co., VA	-	N/A	N/A
<i>Pseudolycopodiella caroliniana</i> ** (L.) Pic. Serm.	T. Matthews	Broxton Co., GA	N/A	N/A	+
<i>Spinulum annotinum</i> ** (L.) Haines	E. Duffield	Snohomish Co., WA	+	N/A	N/A
<i>Spinulum annotinum</i> ** (L.) Haines	C. Carmichael	Antrim Co., MI	N/A	-	N/A

*All specimens (unless noted in acknowledgements) were collected as cuttings from exposed road embankments outside city, county, or state parks. Cuttings were collected from private property only with prior permission of landowners. Only several cuttings were sampled from any wild specimens to ensure minimal impact on natural populations.

anisotomous dichotomous branching, giving rise to determinate, orthotropic aerial branches bearing strobili and indeterminate prostrate to arching trailing stems (referred to colloquially here as “runners”) with which they spread vegetatively. While in many cases, runners grow above-ground (e.g. *Lycopodium*), in several genera they are obligately, facultatively, or seasonally subterranean (Fig. 1C,E,F). Collectively, the two subfamilies encompass seven recognizable genera in North America (Lycopodioideae: *Dendrolycopodium*, *Diphasiastrum*, *Lycopodium*, and *Spinulum*; Lycopodielloideae: *Lycopodiella*, *Palhinhaea*, and *Pseudolycopodiella*) (Fig. 1C–I; Haines, 2003; Øllgaard, 2012a; Øllgaard, 2012b).

In contrast, North American members of subfamily Huperzioidae do not spread via indeterminate runners, and instead shoots undergo isotomous dichotomous branching. The huperzioid growth habit consists of tight clusters of erect to recumbent/pendant shoots. Two genera represent this group in North America (*Huperzia* and *Phlegmariurus*). *Huperzia* sensu stricto have erect shoots that are gemmiferous, while those of *Phlegmariurus* are erect to pendulous but not gemmiferous (Fig. 1A,B) (Øllgaard, 2012b; Field and Bostock, 2013). *Phlegmariurus* is ancestrally epiphytic (Wikström and Kenrick, 1997; Wikström *et al.*, 1999) and its members readily form adventitious shoots from the stem base while *Huperzia* is ancestrally terrestrial, (Wikström *et al.*, 1999) lacks adventitious branching, and instead forms clonal “fairy-rings” as older portions of the dichotomizing shoot system are buried in leaf litter and subsequently degrade (Reutter, 1987).

MATERIALS AND METHODS

Cultivation techniques are divided into nine chronologic sections for clarity: I) Plant Materials: Selection of Appropriate Propagules, II) Preparation of Cuttings and Root Initiation, III) Greenhouse, Laboratory, and Growth Chamber Conditions, IV) Medium, V) Pot assembly, VI) Planting and Establishment, VII) Greenhouse Acclimation and Specimen Culture, VIII) Propagation and Exceeding Colony Climax Stage, and IX) *Huperzia* Gemmae Propagation.

I. Plant Materials: Selection of Appropriate Propagules.—All taxa were originally obtained as wild-collected cuttings or divisions (Table 1), then established at the University of Washington to supply culture attempts at facilities in California. For *Lycopodium*, *Lycopodiella*, and *Pseudolycopodiella*, actively growing shoots with one or more undamaged, newly-emerged roots and/or root primordia near their shoot apices were separated from the parent plant using pruning shears (Fig. 2A,E; 7A,B). *Lycopodium* cuttings ranged in length from 20 to 40 cm while *Lycopodiella* and *Pseudolycopodiella* cuttings were 14 to 20 cm.

In *Palhinhaea*, a single dichotomizing root initiates at each point the arching runner establishes contact with the ground (Figs. 1H; 2C,D; 7C). Arching runner apices that had recently developed root primordia (indicated by abaxial swelling at the shoot apex) were separated from the parent colony using

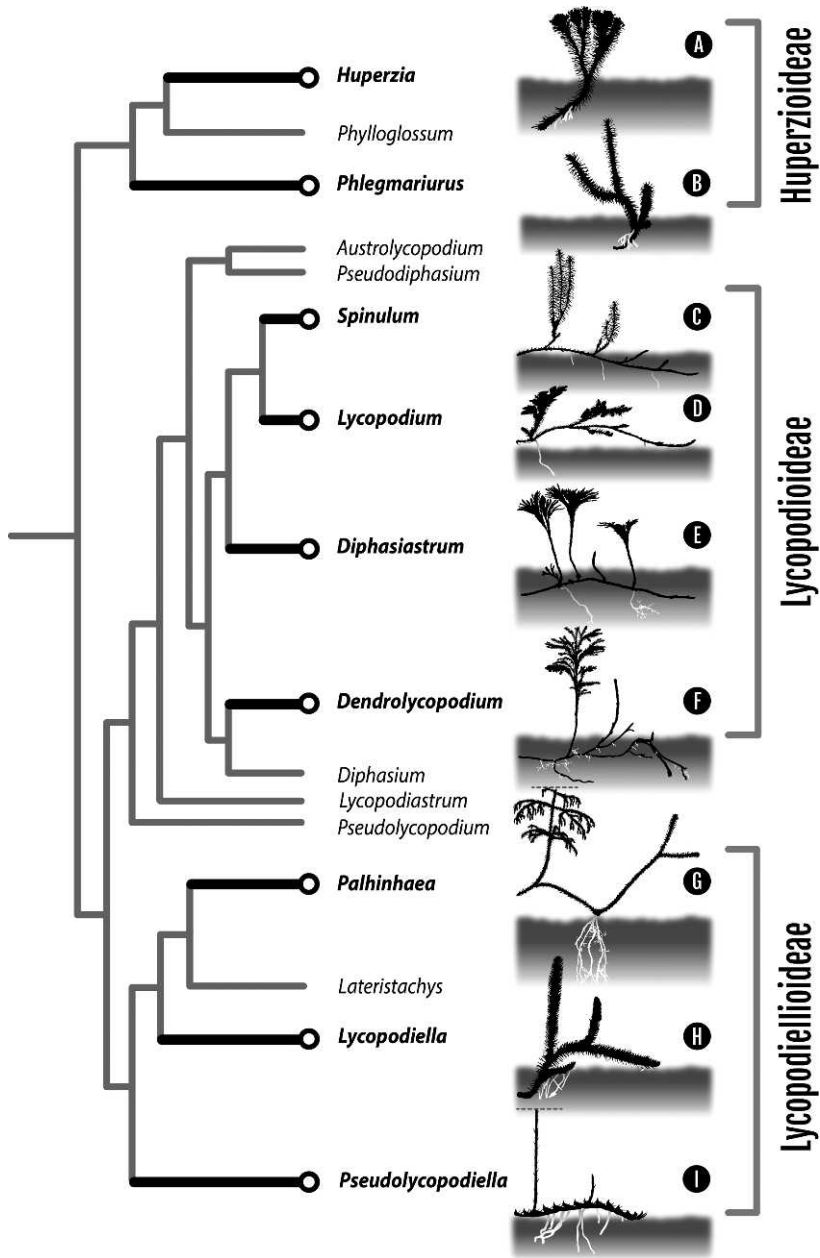


FIG. 1. Cladogram of the Lycopodiaceae *sensu* Vasconcellos and Franco, (1967), Holub, (1975; 1983; 1991), Haines, (2003), and Øllgaard, (2012a,b). Tree based on Fig. 4 of Wikström (2001). Subfamilies designated *sensu* Wagner and Beitel, (1992) and Øllgaard, (2012b). A–I: Aerial and subterranean profiles of growth habits for representatives of the nine North American genera cultivated at UWBG and UCBG (names bolded): A) *Huperzia* – *H. miyoshiana*. B) *Phlegmariurus* – *P. reflexus*. C) *Spinulum* – *S. annotinum*. D) *Lycopodium* – *L. venustum*. E) *Diphasiastrum* – *D. digitatum*. F) *Dendrolycopodium* – *D. hickeyi*. G) *Palhinhaea* – *P. cernua*. H) *Lycopodiella* – *L. alopecuroides*. I) *Pseudolycopodiella* – *P. caroliniana*.

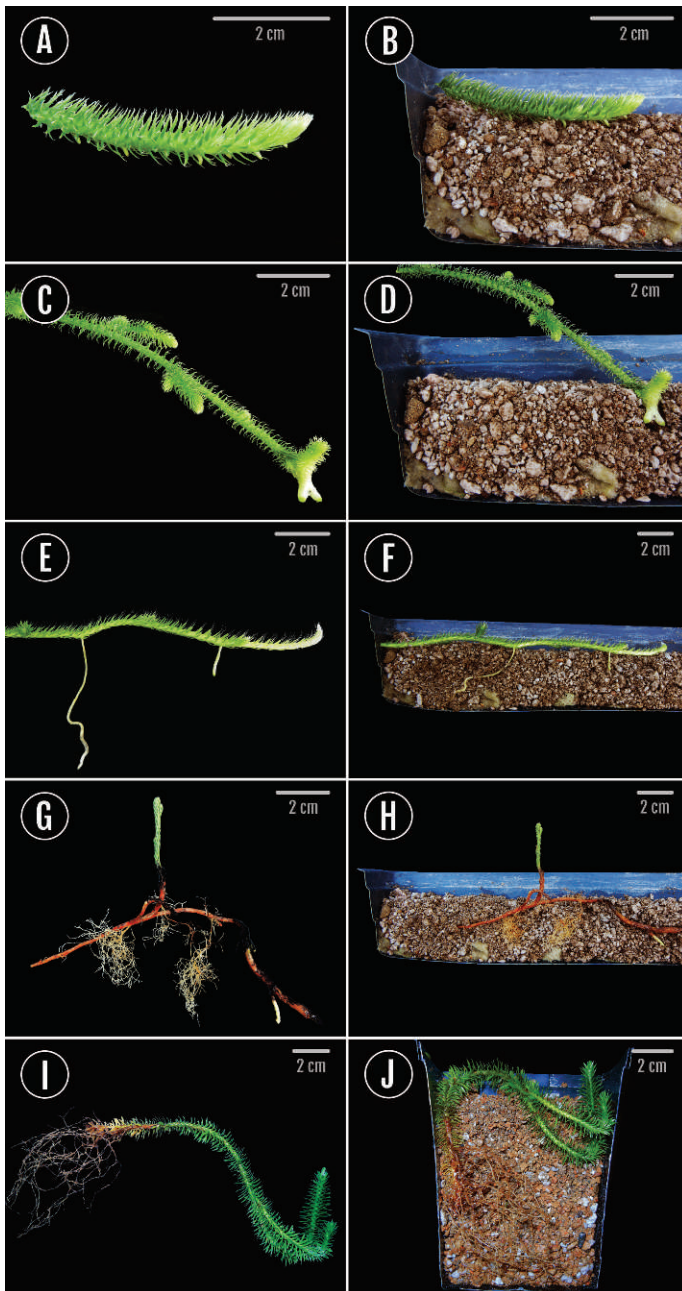


FIG. 2. A,C,E,G,I: cutting material used for establishing clubmosses with contrasting growth habits. B,D,F,H,J: Cross sectional views of pot and substrate column demonstrating orientation of planted propagules for each growth habit. A–B) *Lycopodiella alopecuroides*. C–D) *Palhinhaea cernua*. E–F) *Lycopodium clavatum*. G–H) *Dendrolycopodium hickeyi*. I–J) *Huperzia lucidula*.

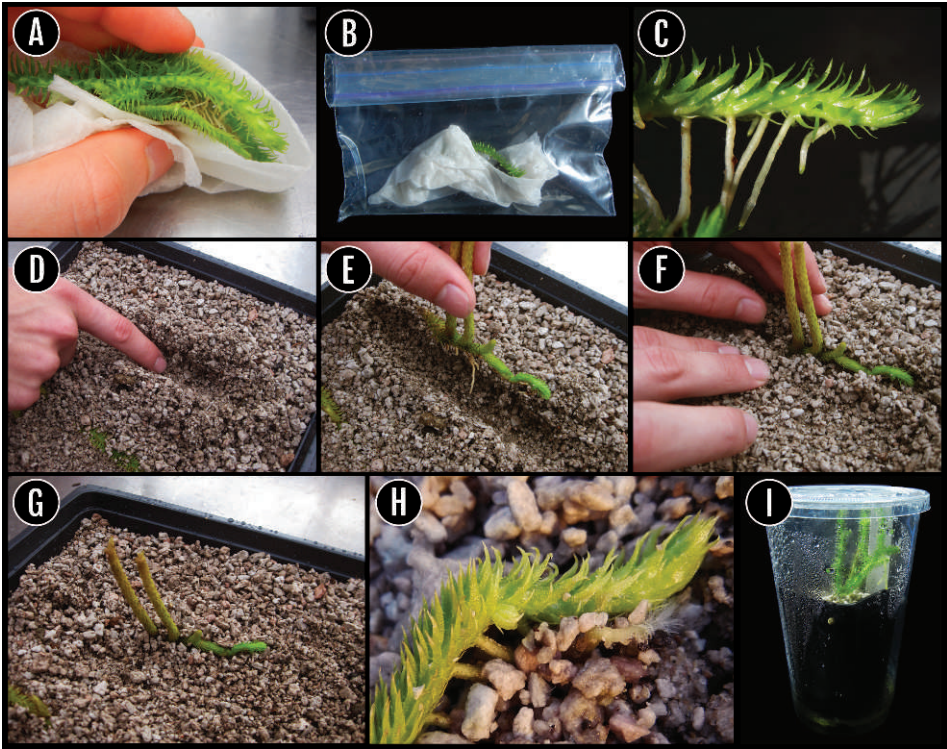


FIG. 3. A–C: Preparation of cuttings and root initiation. A) *Lycopodiella alopecuroides* cuttings wrapped in a ‘funnel’ of moistened paper towel. B) Wrapped cuttings [A] placed in 1-gallon Ziploc® freezer bag and misted. C) *Lycopodiella appressa* cutting ready for planting: showing root elongation, root hair production, and formation of mucilage sheaths at the root apices. D–G: Planting procedure for a strobilate clubmoss with above-ground runners (*Lycopodiella prostrata*). H) Rooting/establishment of *Lycopodiella prostrata* on medium one week after planting (note production of root hairs on extending root below shoot apex). I) Planted *Palhinhaea cernua* cutting inside sealed clear plastic cup with lid.

pruning shears and measured 10 to 20 cm in length (Fig. 2C). Collection of firmly-rooted apices in the field was avoided as root systems were difficult to extract undamaged. Cuttings collected with damaged dominant root apices seldom established and were susceptible to rot.

For *Dendrolycopodium*, *Diphasiastrum*, and *Spinulum*, actively growing subterranean runners were excavated using a trowel and clipped from the parent plant. Divisions included one or more aerial branches, newly-produced roots/root primordia, and branching of the subterranean runner system. Cuttings ranged between 20 to 30 cm in length (Fig. 2G).

For *Huperzia* and terrestrial *Phlegmariurus*, entire plants were collected if possible. When not feasible, stems were clipped from the parent plant towards the shoot base so that each retained at least one or more adventitious roots (Fig. 2I). In *Huperzia*, whenever present, gemmae were collected and

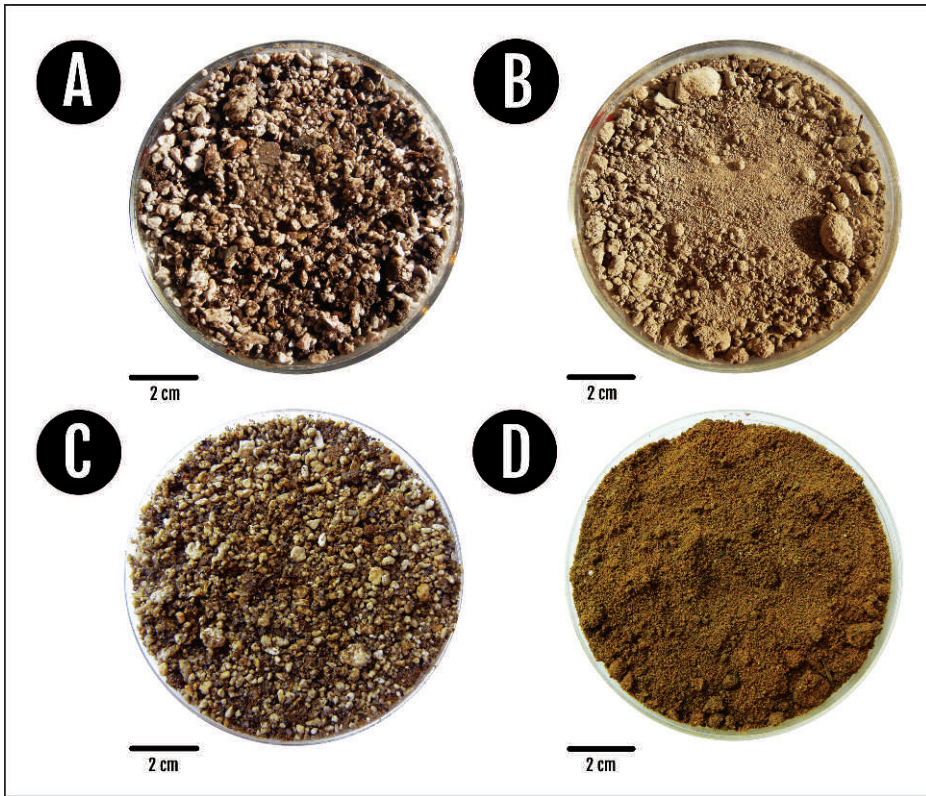


FIG. 4. A) Clayey loam/pumice mix used for growing terrestrial clubmosses at UWBG and in growth chambers. B) Clayey loam used in medium at UWBG and in growth chambers. C) Sandy loam: pumice mixture used at UCBG and in the laboratory. D) Sandy loam used in medium at UCBG and in the laboratory.

established using the protocol outlined in the “*Huperzia Gemmae Propagation*” section (Fig. 7E).

II. Preparation of Cuttings and Root Initiation.—After collection, cuttings were rinsed with cool soft tap water to remove adhered sediments and senescent tissues. Once cleaned, a moist paper towel was wrapped in a loose spiral around each individual cutting, ensuring that roots and root primordia (but not shoot apices) were placed in contact with the towel (Fig. 3A). For taxa with ‘fleshy’ turgor-supported stems (e.g. *Lycopodiella*, *Pseudolycopodiella*, *Huperzia*, and *Phlegmariurus*) the paper towel used was nearly saturated with water (Table 2). For taxa with more structurally-reinforced, rigid or wiry stems (e.g. *Dendrolycopodium*, *Diphasiastrum*, *Lycopodium*, *Palhinhaea*, and *Spinulum*) the towel was only lightly misted (Table 2). Lower moisture levels were necessary for the latter taxa due to their susceptibility to rot under damp conditions.

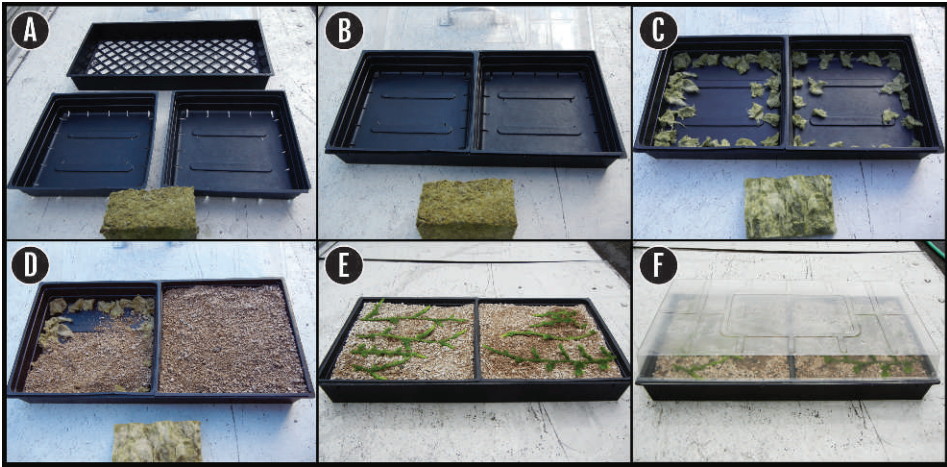


FIG. 5. Procedure for tray assembly and planting cuttings. A) Materials: two 11×11" half-trays [used in place of one 1020 greenhouse tray], a web flat, and pre-soaked hydrophilic rockwool block (clear propagation dome not shown). B) Both half-trays placed into the web flat. C) Plugging drainage holes at the bottom of half trays with hydrophilic rockwool. D) Filling trays with pre-mixed loam/pumice medium. E) *Lycopodium clavatum* cuttings planted in medium and watered with soft tap water. F) Clear propagation dome placed on top of flat.

Wrapped cuttings were then placed in sealed clear plastic bags (1 to 2 gallon Ziploc® freezer bags) on shelves under T-5 grow lights in a laboratory for two to three weeks at 20° to 22°C (Fig. 3B). Rooting in ‘fleshy’ taxa occurred within 3 to 14 days, and 7 to 20 days in those with wiry stems. During the establishment phase, any regions of cuttings that began senescing were promptly disposed. Cuttings were removed from bags following root elongation, production of root hairs, and exudation of mucilage sheaths (Fig. 3C).

III. Greenhouse, Laboratory, and Growth Chamber Conditions.—Observations were made in greenhouse and growth chamber facilities at the University of Washington (UW), three greenhouses at the University of California Botanical Garden (UCBG), and a laboratory at the University of California, Berkeley (UCB). The source of tap water at the UWBG was naturally neutral to slightly basic; pH ranging from 7.5 to 7.8 without fertilizer solution and slightly acidic (6.8) with fertilizer solution. At the UCBG, the source of tap water was basic (pH: 9); while at UW and UCB, departmental tapwater used to water plants was usually neutral (pH: 6.5–7.5).

At UWBG, observations lasted from 2007 to 2013. Plants were grown on open greenhouse benches in two research rooms constructed of clear-glazed glass. Temperatures in the greenhouse rooms annually ranged from 16.5° to 27°C but were maintained at 22° to 24°C for much of the year. The research rooms both had overhead misting systems that were activated by increasing temperature. This meant that during warmer (~23° to 27°C) summer days, the misting systems operated continuously from 6:00 am to 9:00 pm whereas in October through March, they did not operate at all throughout the day due

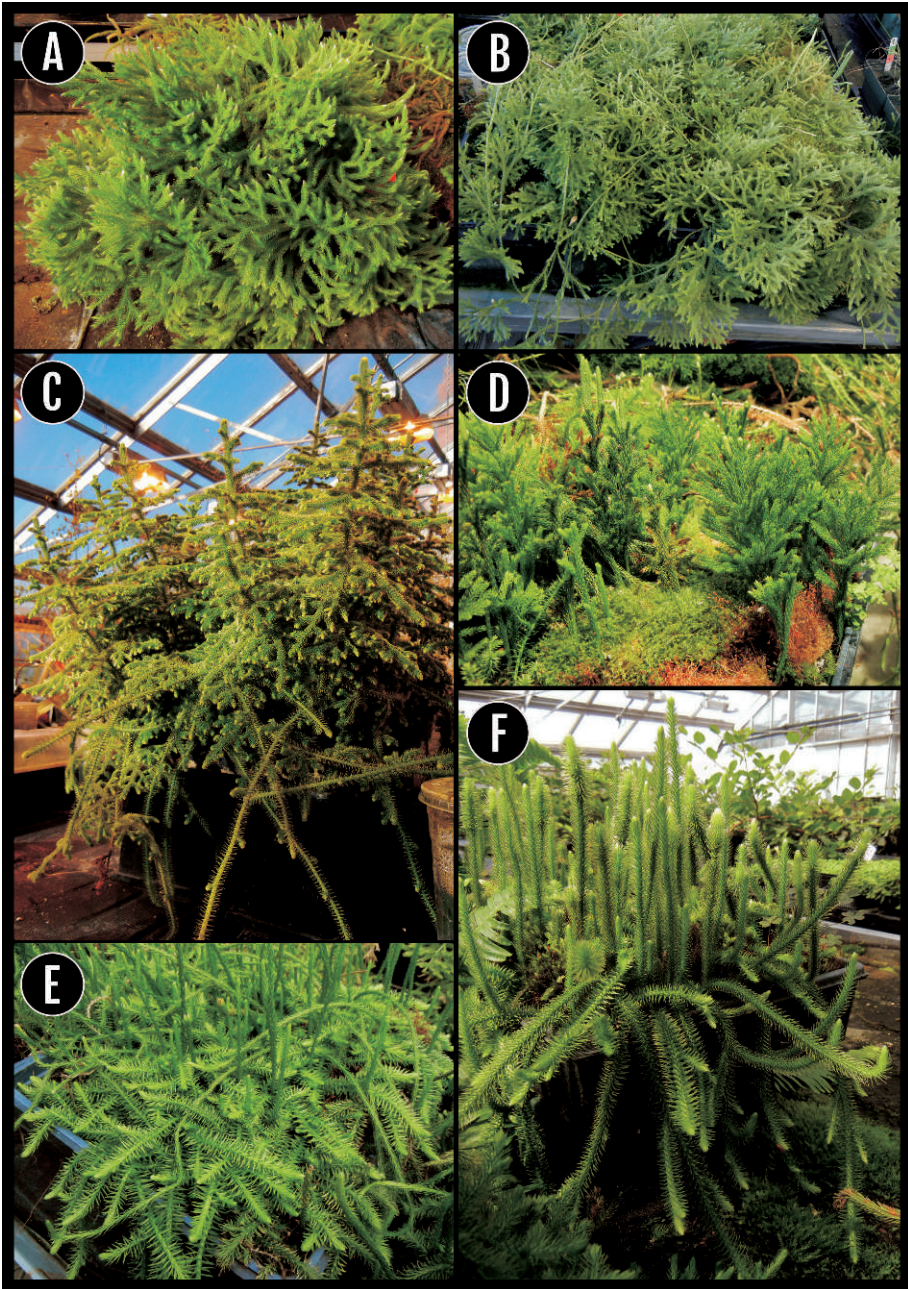


FIG. 6. Terrestrial clubmoss colonies at climax stage: ready for propagation. A) *Lycopodium venustulum* covering greenhouse tray. B) *Lycopodium* cf. *venustulum* in greenhouse trays. C) *Palhinhaea cernua* (South African form) in greenhouse tray elevated upon four perennial pots. D) *Dendrolycopodium hickeyi* in greenhouse tray. E) *Lycopodiella prostrata* clonal mat covering raised greenhouse tray. F) *Lycopodiella alopecuroides* producing strobili while extending beyond confines of a raised greenhouse tray.

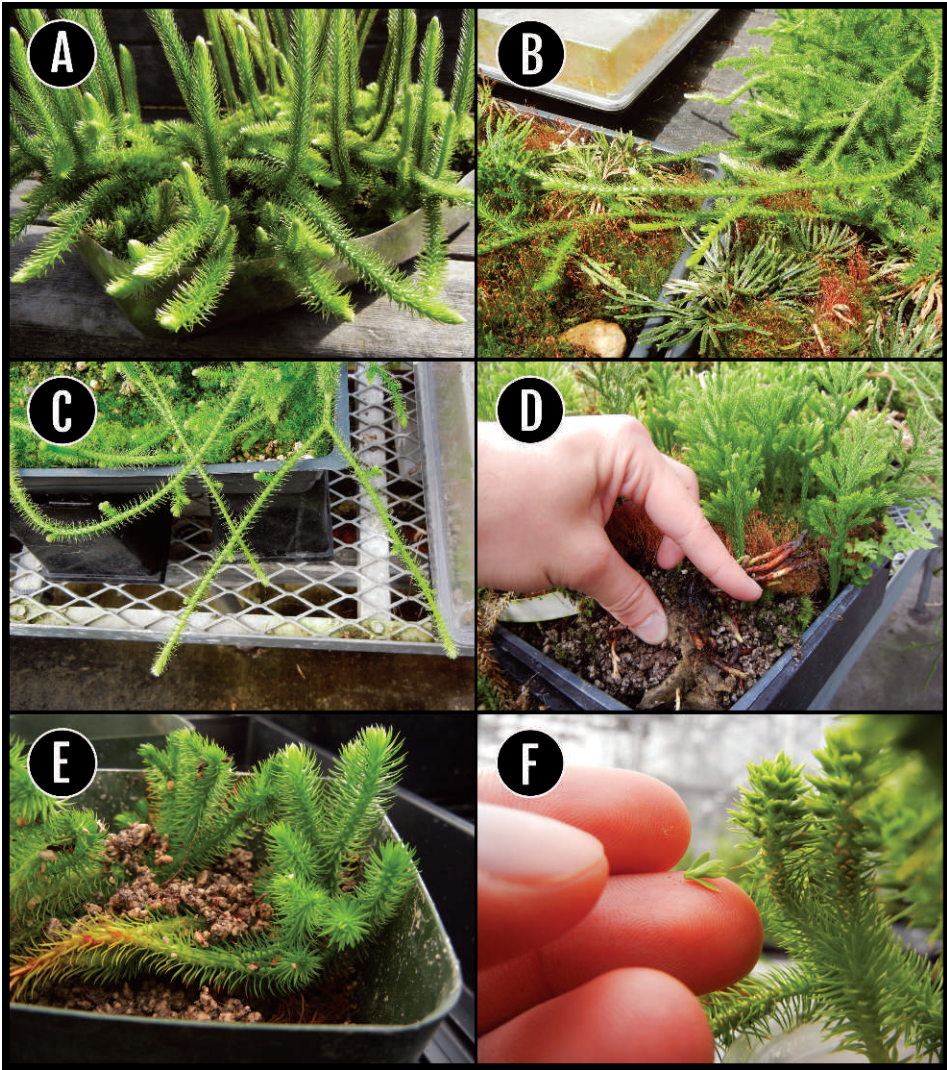


FIG. 7. A–D): Examples of regions of several clubmosses selected for cutting-based propagation. A) Runners of *Lycopodiella alopecuroides* extending beyond pot rim with root primordia. B) *Lycopodium clavatum* runner growing over greenhouse bench initiating roots. C) Shoot region of *Palhinhaea cernua* (South African form) used for cuttings or layering. D) Subterranean shoot system of *Dendrolycopodium hickeyi*. E–F): Huperzioid maintenance and propagation: E) Repotted *Huperzia miyoshiana* in the process of burial for layering (note medium partially covering lower portion of stem), roots initiating towards base. F) *Huperzia miyoshiana* gemma on finger adjacent to branch with intact gemmaphore-gemma complexes.

TABLE 2. Media and preparation phase moisture level by genus.

Subfamily	Genus	Medium	Paper towel moisture level (rooting stage)
Huperzioideae	<i>Huperzia</i>	3:1:1 pumice/peat/sandy or clayey loam	nearly saturated
Lycopodioideae	<i>Phlegmariurus</i>	3:1 pumice/sandy or clayey loam	nearly saturated
	<i>Dendrolycopodium</i>	3:1 pumice/sandy or clayey loam	lightly misted
	<i>Diphasiastrum</i>	3:1 pumice/sandy or clayey loam	lightly misted
	<i>Lycopodium</i>	3:1 pumice/sandy or clayey loam	lightly misted
Lycopodielloideae	<i>Spinulum</i>	3:1 pumice/sandy or clayey loam	lightly misted
	<i>Lycopodiella</i>	3:1:1 pumice/peat/sandy or clayey loam	nearly saturated
	<i>Palhinhaea</i>	3:1 pumice/sandy or clayey loam	lightly misted
	<i>Pseudolycopodiella</i>	3:1:1 pumice/peat/sandy or clayey loam	nearly saturated

to cool weather. Fertilizer solution was applied every other day via hose systems, alternating weekly between 17:5:17 N-P-K and 20:10:20 N-P-K solution.

At UCBG, observations lasted from 2012 to 2014. Divisions were obtained from specimens cultivated at the UWBG and grown on open benches in three different greenhouses at UCBG. The first greenhouse, House 1, ranged from 11° to 32°C and had an overhead misting system operating for one minute every two hours in summer and one minute every three hours the rest of the year. House 1 was built of opaque fiberglass and cooled via two swamp coolers. The second greenhouse, Research House, was built of white-washed glass panes and ranged from 26° to 29°C with no overhead misting system. The third greenhouse, Propagation House, was also constructed of white-washed glass panes and ranged from 15° to 25°C with no overhead misting system. In each house, 18-18-18 N-P-K soluble fertilizer [Romeo Packing Company, Half Moon Bay, CA] was applied bimonthly via hose systems at 1/4th strength.

Growth chamber observations at UW occurred in 2011. Greenhouse trays of established *Lycopodiella alopecuroides*, *L. appressa*, and *Lycopodium clavatum* propagated from UWBG specimens were placed in a walk-in Conviron growth chamber for two 5-month temperature treatments: 15°C and 25°C. During this time, cuttings were watered every other day alternating between pure soft tap water from UW departmental facilities and dilute fertilizer solution provided by the UWBG.

Laboratory culture conducted at UCB occurred from spring 2013 through winter, 2014. Taxa listed in Table 1 were obtained as cuttings from cultivated specimens at the UWBG and UCBG and planted in a variety of shallow Glad® and Ziploc® clear plastic food storage containers half-filled (2 to 3 cm deep) with peat-amended medium (Table 2). Planted containers were placed without lids into sealed gallon Ziploc® freezer bags and housed under T-5 growlights set to a 12-hour photoperiod (Fig. 8C). During establishment, cuttings were misted weekly with pure soft tap water. Following establishment

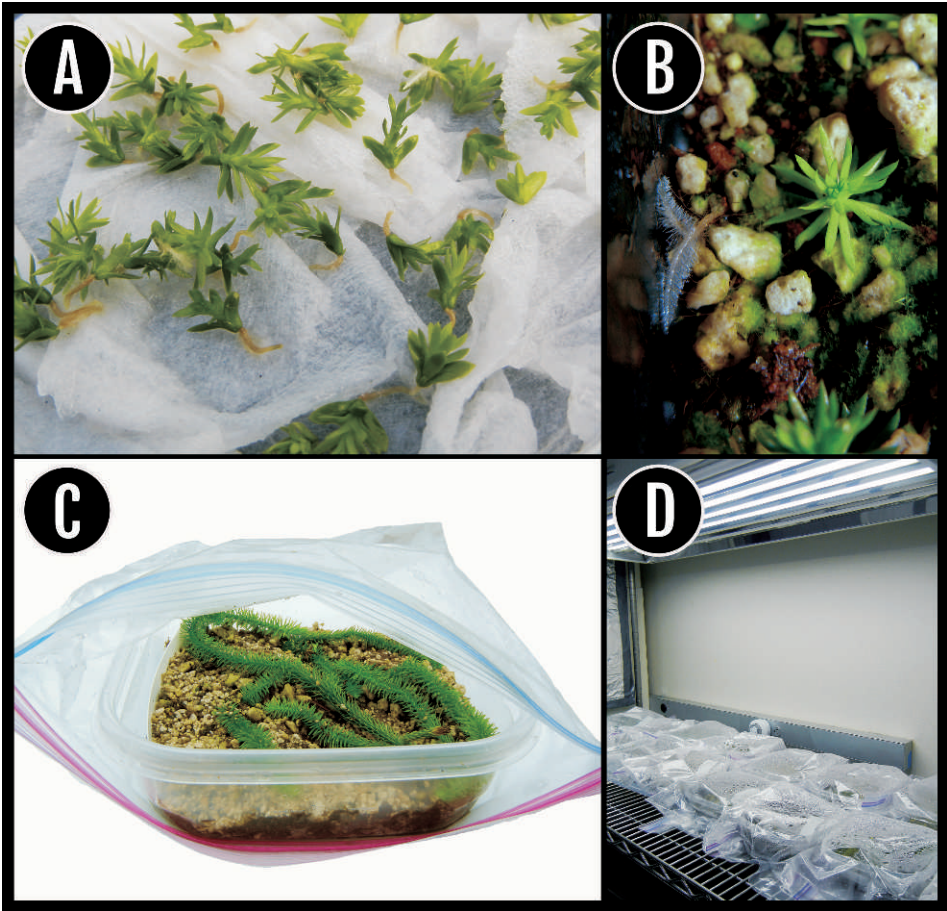


FIG. 8. A) Gemmae of *Huperzia occidentalis* rooting onto damp paper towel several weeks after removal from the parent plant. B) Established gemmiling of *H. occidentalis* showing signs of establishment: shoot and root growth. C) *Lycopodiella prostrata* growing in open Glad® food storage container containing medium sealed about to be sealed in a freezer bag. D) Bagged food storage containers containing terrestrial clubmosses on wire shelving unit under a T-5 light bank in the laboratory.

[4 months after planting], containers were watered weekly alternating between one week tap water and one week diluted 7-8-6 N-P-K liquid fertilizer solution (Orchid-Pro; Dyna-Gro, Richmond, CA). Any excess water pooled at the bottom of containers was drained prior to resealing cultures in bags.

IV. Medium.—For a condensed genus-by-genus listing of media, see Table 2. For strobilate members and terrestrial *Phlegmariurus*, a mix of 3:1 fine-grained (~2–5 mm diameter) pumice to a heavy sandy or clayey loam was used (Fig. 4A,C). *Huperzia*, *Lycopodiella*, and *Pseudolycopodiella* were grown in a 3:1:1 mix of fine-grained pumice, heavy sandy or clay loam, and *Sphagnum* peat. At UWBG/UW facilities, clayey loam (Fig. 4B) was collected from an

exposed hillside adjacent to the greenhouse facilities. At UCBG/UCB facilities, the sandy loam used (Fig. 4D) was pre-mixed by American Soil and Stone, Richmond, CA using sand from San Francisco and sandy clay loam from San Rafael with no organic amendments. Specifically, this sandy loam was 78.4% sand, 10.4% silt, and 11.1% clay (pH: 7.4, no lime present, ECe: 1.6 dS/m, 0.26 ppm boron, Sodium Absorption Ratio: 1.4). The sand consisted of the following grain sizes and ratios when sieved through a 2 mm screen: 2.4% 1–2 mm diameter, 6.2% 0.5–1 mm diameter, and 69.8% 0.05–0.5 mm diameter.

V. Pot Assembly.—For strobilate taxa, containers were prepared by placing standard 1020 greenhouse trays [11" W × 21.37" L × 2.44" D; #11-3000-1, T.O. Plastics; Clearwater, MN] into heavy-weight web flats [10.75" W, 21.26" L, 2.40" D; #15-9340-1, Belden Plastics; St. Paul, MN] (Fig. 5A–B). Web flats were used to increase structural integrity of greenhouse trays. Drainage holes within trays were then plugged with pre-soaked hydrophilic rockwool (Fig. 5C) to prevent fine-grained sediment loss while still permitting drainage. Trays were subsequently filled with medium, watered with pure soft tap water, then manually patted to a firm state (Fig. 5D–E).

For *Huperzia gemmae* and *Palhinhaea* cuttings, square rose pots [2¼" W × 3¼" D; #1680: Anderson Die and Manufacturing; Portland, OR] were prepared in a similar manner to greenhouse trays. For larger specimens of *Huperzia* and terrestrial *Phlegmariurus*, the same procedure was used with respective media (Table 2) in plastic perennial pots [4.25" W × 4.87" D; #SQL0450, Kordlok™, Brantford, Ontario].

VI. Planting and Establishment.—For *Lycopodium*, *Lycopodiella*, *Palhinhaea*, and *Pseudolycopodiella*, cuttings were planted so that the entire shoot system remained above ground while roots/root primordia were either buried or directly contacting the medium (Fig. 2B,F; 3D–G). For taxa with subterranean runners, (*Dendrolycopodium*, *Diphasiastrum*, and *Spinulum*), cuttings were planted so that only aerial branches remained aboveground (Fig. 2H). For all taxa, a depression the size and shape of the respective cutting was made in the new medium, the cutting was then lowered into this depression and ‘pinched’ with substrate when at a desired height (Fig. 3D–G). For wiry cuttings, stones were used to weigh down rooting portions of runners. Once planted, greenhouse trays were covered by clear vinyl propagation domes [2.5" H × 11" L × 21.25" W; #11-3348-1, Hummert International™; Earth City, MO] (Fig. 5F), then placed upon four inverted perennial pots on an unshaded greenhouse bench. *Palhinhaea* cuttings planted in rose pots were misted with pure tap water then placed in clear plastic cups [24 oz.; #SCC PXT24, SOLO® brand; USA]. These cups were then filled to 1.0 cm deep with tap water, sealed, and placed on greenhouse benches in full to partial sun or under growlights (Fig. 3I). Greenhouse trays remained covered by propagation domes and *Palhinhaea* cuttings maintained in cups for 6 to 8 months after planting. Covered trays for all taxa were watered with unfertilized soft tap water on alternating days and capillary pools at the bottom of *Palhinhaea* cups were maintained as necessary to ensure constant elevated substrate moisture and humidity. After this stage *Palhinhaea* cuttings were transferred into greenhouse trays covered by tall

propagation domes [6" H × 21" L × 11" W; #6HD, Hummert International™, Earth City, MO] and treated similarly to the above taxa for an additional month.

For *Huperzia* and terrestrial *Phlegmariurus* cuttings/specimens, only basal regions of shoots (along with any intact roots) were buried under medium (Figs. 2J; 7E). Once planted, pots containing cuttings were placed in clear plastic tote containers with clamping lids [22.75 gallon; #292020; Container Store®] filled to 1.0 cm deep with pure soft tap water for subirrigation and elevated humidity. Totes were placed on greenhouse benches under bright light to partial shade for 6 months.

VII. Greenhouse Acclimation and Specimen Culture (UWBG).—For strobilate clubmosses, establishment was indicated by noticeable shoot elongation and/or branching, generation of new roots, and resistance to uprooting. Once established, propagation domes were removed from trays, which from then on were watered daily, alternating between one day tap water and one day dilute fertilizer solution. Whenever stray runners grew beyond the confines a tray, they were manually flexed and reoriented to colonize regions with open medium. In repositioned arching or wiry runners, rooting portions were pinned to the medium surface using stones or bent skewers to encourage establishment.

In huperzioids, establishment was indicated by terminal shoot growth and division, production of gemmae (in *Huperzia*), and resistance of the plant to uprooting attempts. Once established, specimens were removed from plastic tote containers and placed in partial shade on a greenhouse bench. Plants were watered and fertilized in the same manner as strobilate taxa.

VIII. Propagation and Exceeding Colony Climax Stage.—Two to three years following establishment, most strobilate taxa formed thick, clonal mats covering the entire tray surface (Fig. 6). Despite appearing robust and vigorous in growth, colonies at this ‘climax stage’ consistently began senescing within 6 to 8 months of covering the tray surface. Senescence (indicated by loss of turgor, discoloration, and rotting or drying of actively growing shoots) was abrupt but often followed degradation of older, underlying regions of the clonal network. Colony die-off therefore may have been influenced by a combination of factors such as media degradation/eutrophication and physical obstruction to rooting of younger runners by thickets of older branches.

To prevent colony loss at climax stage, three techniques were employed (one technique per colony). The first involved splitting colonies into several actively growing divisions, removing senescent portions and aged media, and establishment using protocols outlined for cuttings (sections VI–VII). The second involved taking and establishing cuttings from actively growing runners (Fig. 7) using the above protocols. The third technique was to encourage stray runners growing beyond tray confines to root into adjacent trays filled with fresh medium. In such cases, stones or skewers were used to pin rooting portions of runners against the medium surface. Once rooted into and spreading across the new tray, the stray runner was severed from the parent colony and maintained similarly.

When older portions of huperzioid shoots began showing signs of aging or senescence (slight yellowing or browning towards the branch base) plants were unpotted and basal senescent tissues removed, leaving one or more adventitious roots intact towards the shoot base. In a pot containing fresh medium, the oldest (basal) portions of the shoot were laid prostrate in a shallow depression, then buried under 0.5 to 1.5 cm of medium (Fig. 7E). Within 2 to 3 months of layering, adventitious roots emerged from the cortex of newly buried regions. Through time, vegetative propagation resulted from layering of progressively degrading dichotomizing axes.

IX. *Huperzia Gemma Propagation*.—In *Huperzia*, gemmae are either produced throughout the year (e.g. *H. miyoshiana*, *H. occidentalis*, *H. haleakalae*) or annually (*H. lucidula*; Reutter, 1987). For collection, gemmae were detached from gemmaphores by gently brushing shoots from the apex to base (Fig. 7F). Detached gemmae were then scattered onto moist, crumpled paper towels placed inside sealable plastic sandwich bags and misted with pure tap water (Fig. 8A). Bags containing gemmae were sealed and placed under T-5 grow lights set to a 12-hour photoperiod at 20° to 22°C in laboratory facilities. Root initiation occurred proximal to the basal abscission region of gemmae within 3 to 14 days, followed closely by shoot elongation and leaf production (Fig. 8A; Reutter, 1987; Wang *et al.* 2011). Rooting gemmlings were transplanted to rose pots containing *Huperzia* medium (Section IV; Table 2) ensuring that both root and abscission zone were buried while the shoot apex remained aboveground. Planted rose pots were then placed in clear 1 gallon Ziploc® freezer bags, sealed, and kept for 6 to 8 months on shaded greenhouse benches or under grow lights at 20° to 22°C. Weekly dilute fertilizer application began one month after planting. After 4 months, pots containing well-established gemmlings (those that rooted and displayed noticeable shoot growth (Fig. 8B)) were then placed on open greenhouse benches either in shallow troughs filled to 0.5 cm of water or on capillary mats to ensure constant moisture of the medium.

Gemmae of annual producers (*H. lucidula*) were treated in the same manner as other *Huperzia*, but provided a cool stratification treatment in a refrigerated storage room under T-5 growlights at 15°C for two months prior. For additional considerations on establishing gemmae, see Wang *et al.*, (2011).

DISCUSSION

The most challenging aspects of growing terrestrial Lycopodiaceae are initial establishment, acclimation to greenhouse conditions, and long-term maintenance of colonies. Success in all three of these stages requires six environmental conditions to be provided: I) constant high humidity and substrate moisture, II) a year-round source of bright light, III) year-round moderate temperatures, IV) fertilizer following establishment, V) well-aerated, largely inorganic substrates with a heavy loam component, and VI) reduced competition, herbivory, and pathogen exposure. Here, each of these six conditions will be discussed.

HUMIDITY AND WATERING.—Most lycophytes are highly susceptible to desiccation due to their passive stomatal control and reduced capacity to respond to atmospheric and soil water deficits, endogenous abscisic acid, and epidermal cell turgor pressure (Brodribb and McAdam, 2011; McAdam and Brodribb, 2012; 2013). Due to these ancestral physiological constraints, high humidity and constant substrate moisture together are requirements for sporophyte survival and growth in contained pots. These conditions are particularly important to maintain during cutting preparation and establishment stages, though the medium should not be kept water-logged nor shoot apices remain overly damp. Although established colonies are less susceptible to humidity fluctuations, they can succumb to dehydration quickly (sometimes within a day) during periods of hot, dry weather if watering regimes are not adjusted accordingly. Susceptibility to drying is most pronounced in root-bound specimens with large volumes of foliage at climax stage (e.g. *Palhinhaea*; D. Ewing, *pers. comm.*). At UWBG and UCBG, few plants showing symptoms of turgor loss (limp or brittle stems and shriveled leaves) recovered when subsequently hydrated. Clubmosses therefore do not appear capable of recovering from dehydration and cannot be permitted to dry at any stage.

Substrate water pH may also impose challenges on clubmoss culture in regions with naturally hard (pH > 7.0) tap water. Under such situations, using sodium-softened or distilled water may be necessary to provide neutral to acidic conditions (pH ≤ 7.0) (Husby, *pers. comm.*).

LIGHT.—Contrary to prior suggestions of Barrows, (1935), bright light is an important component of successful terrestrial clubmoss culture. Many terrestrial taxa are considered stress-tolerating pioneers, forming robust colonies in frequently disturbed open habitats such as erosional scars, bunch grass páramo, forest margins, talus slopes, road embankments, heaths, and peatlands (e.g. Øllgaard, 1979b; Rasmussen and Lawesson, 2002; Muller *et al.*, 2003; Gilman and Marshfield, 2004; Wilfried Bennert *et al.*, 2007; Horn *et al.*, 2013). In Ecuador; where Lycopodiaceae has especially diverse representation, few, if any terrestrial species grow in shaded forest understories while all (more than 50 spp.) occur in cool, open, upland habitats (Øllgaard, 1979b). In evergreen conifer forest understories of western North America, specimens of numerous species are noticeably more diminutive in stature than their conspecifics growing at well-lit forest margins (Benca, *pers. obs.*). Most commonly-encountered hardwood forest understory taxa in the northern hemisphere have orthotropic aerial branches that provide ample light-harvesting opportunities when deciduous canopies are absent (Nauertz and Zasada, 1999). Similarly, Svensson *et al.*, (1994) observed that high latitude *Spinulum annotinum* cuttings produced significantly longer horizontal runners and more aerial branches when grown under deciduous rather than evergreen *Vaccinium* canopies. These observations coupled with presence of such taxa in open habitats at high latitudes and elevations suggest understory clubmosses are more likely seasonally shade-tolerant than shade-dependent. Additionally, maintaining open habitats via frequent disturbance regimes (e.g.

grazing, mowing, or controlled burning) has been a foundational practice in conservation efforts for dwindling populations of prostrate genera in Europe and North America (Byfield and Stewart, 2001; Rasmussen and Lawesson, 2002; Muller *et al.*, 2003; Gilman and Marshfield, 2004; Shackelford, 2004).

TEMPERATURE.—Clubmosses are difficult to establish and maintain if the facility itself is not capable of providing moderate temperatures in conjunction with bright light and high humidity throughout the year. For example, at UCBC, high mortality rates occurred during the establishment phase in many taxa due to prolonged dormancy induced by persistently cold temperatures, low light availability, and short photoperiod in winter (House 1), and high temperatures/drying in summer (Propagation and Research houses). Such losses did not occur during the establishment phase in temperature and humidity-controlled research rooms of the UWBG or in growth chamber and laboratory facilities.

FERTILIZER.—In *S. annotinum*, extended periods of root growth and phosphate uptake along with efficient phosphorous utilization suggest adaptations for growing in oligotrophic substrates (Headley *et al.*, 1985). This condition is likely widespread across the strobilate clades considering the habitats they associate with and the observation that *Diphasiastrum digitatum* cutting establishment is impaired by fertilizer application in the field (Railing and McCarty, 2000). In addition, premature rot of newly planted cuttings treated with fertilizer was observed across several genera at UWBG. However, cutting growth rate of most clubmosses observed at UWBG diminished if fertilizer solution was not provided within several months following establishment. Thus, previous accounts suggesting fertilizer has little (or even detrimental) effects on terrestrial clubmosses (Jones, 1987; Railing and McCarty, 2000) apply only to the rooting/establishment stage. Moreover, increased productivity in established cuttings of all taxa following weak fertilizer application indicates that fertilizer is necessary for culture success following establishment. This response also suggests clubmoss sporophytes do not require mycorrhizal or cyanobacterial associates for successful culture, contrary to prior speculation (e.g. Mickel, 1994; Byfield and Stewart, 2007; McAuliffe, 2008).

MEDIUM.—Well-aerated inorganic substrates amended with mineral loams promote establishment in terrestrial clubmosses more readily than organic-based mixes possibly because these components take longer to degrade while increasing water use efficiency and nutrient buffering capacity (Owen *et al.*, 2008). Mineral loams may also provide trace minerals necessary for growth. Furthermore, decomposing organic soil components can impede plant growth by decreasing aeration capacity of the medium through increased water retention and compaction (Bildnerback *et al.*, 2005; Calonje *et al.*, 2010), while releasing harmful concentrations of CO₂ in the rhizosphere (Whitcomb, 2003). Fermenting organic media components also release organic acids that can reduce root growth, increase root oxygen loss, and induce die-back in wetland plants (Nichols, 1981; Armstrong and Armstrong, 1999, 2001).

Prior accounts emphasizing the importance of using acidic peat- and sand-based substrates for clubmoss culture (Heim, 1988; Cullina, 2008) acknowledge such mixes are less productive for strobilate taxa than substrates obtained from their natural habitats. Transplantation and greenhouse cultivation success have also been noted for several understory taxa of the Northeastern United States using well-aerated “native soils” (Barrows, 1935). Since nutrient-poor, heavy mineral substrates are prevalent across many productive habitats (Page, 1979; Øllgaard, 1979b; Jones, 1987; Matula, 1995; Page, 1997, 2004; Gilman and Marshfield, 2004; Rusea *et al.* 2009), clayey and sandy loams are likely an important contributor to the enhanced performance of clubmosses grown in ‘native soils.’ Establishment of all North American genera in two different types of mineral loam (clayey in Washington and sandy in California) indicates that terrestrial Lycopodiaceae can readily acclimate to a variety of loams in culture. Regardless of type, loam-amended inorganic media consistently sustain more productive colonies than fired clay products (e.g. Turface®).

COMPETITION, PESTS, AND PATHOGENS.—Being slow-growing and generally low in stature, terrestrial clubmosses are especially vulnerable to over-shading from competing vegetation (Øllgaard, 1979b; Page, 1997; Rasmussen and Lawesson, 2002). Once fertilizer application begins, competition from encroaching algal mats and bryophyte colonies becomes a persistent threat, particularly for taxa with prostrate runners such as *Lycopodiella* and *Pseudolycopodiella*. Competition can be reduced by frequent weeding followed by adding new medium, ‘mowing’ mosses with scissors, or discontinuing fertilizer solution application for several-week increments while flushing the substrate daily with pure soft tap water.

Slugs and snails are the most prevalent pests of terrestrial clubmosses in greenhouses, but can be hampered by elevating trays/web flats above the greenhouse bench upon inverted perennial pots (e.g. Figs. 6C,E,F; 7C) along with monthly slug bait application. Alternatively, a strip of copper wrapped around each tray may eliminate snail and slug ingress altogether (C. Husby, *pers. comm.*).

Under shaded, humid conditions with limited air circulation, colonies of some clubmosses can succumb to fungal infection and rot. Symptoms of infection include loss of turgor in hydrated plants accompanied by discoloration (orange, yellow, graying, or blackening coloration) followed by fungal fruiting body emergence and rotting shoot apices. Such symptoms did not occur in plants grown under bright light, high humidity, and ample air circulation. Infected specimens should be disposed of quickly to prevent spread of pathogens to neighboring plants.

Conclusion.—Providing the environmental conditions, media, and suggested maintenance herein, cultivation success can be achieved for members of all three subfamilies of clubmosses in greenhouse, laboratory, and growth chamber facilities. Given the success observed across much of the phylogenetic span of Lycopodiaceae, these techniques can be used to increase

accessibility of the entire clade to *ex-situ* conservation, experimental research, and horticulture.

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