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Reproductive Biology of *Aglaomorpha cornucopia* (Copel.) M.C. Roos, a Rare and Endemic Fern from the Philippines

YAO-MOAN HUANG[1]
Taiwan Forestry Research Institute, 53 Nan-Hai Rd., Taipei 10066, Taiwan
VICTOR B. AMOROSO[1] and FULGENT P. CORITICO
Central Mindanao University, Musuan, Maramag, Bukidnon, Philippines
CHIA-WEN KO and TZU-TONG KAO
Taiwan Forestry Research Institute, 53 Nan-Hai Rd., Taipei 10066, Taiwan
JOHN REY CALLADO
Philippine National Herbarium, Manila 2659, Philippines
WEN-LIANG CHIOU[2]
Taiwan Forestry Research Institute, 53 Nan-Hai Rd., Taipei 10066, Taiwan

ABSTRACT.—*Aglaomorpha cornucopia* (Copel.) M.C. Roos is an endemic and rare epiphytic fern from the Philippines. *Ex situ* germplasm storage and growth are important complementary tools for conserving this rare fern. This study was conducted to document the reproductive biology of this species. Mature sporophylls of *A. cornucopia* were collected in May, 2012 from Mt. Apo, the Philippines. Each sporangium bore 64 yellow, monolete spores. The average spore size was 49.3±3.7 μm. Fresh spores germinated 100% within one week of sowing (mean germination time (MGT) <1 week). Air-dried mature spores remained completely viable even after one year of storage at 3°C, although mean germination time was somewhat delayed (MGT=1.4 weeks). Spore germination was of the *Vittaria*-type, whereas gametophyte development was of the *Drynaria*-type. Adult gametophytes were cordiform-annual and gametangia were of the leptosporangiate type. Unicellular papillate hairs appeared on marginal, dorsal, and ventral surfaces of the gametophytes. Gametophytes first produced antheridia and archegonia after seven weeks of culture. Gametophytes began to sexually produce sporophytes after 13 weeks of culture. The rate of sporophyte production reached 64% after 26 weeks culture. Results of this study suggest that cold temperature spore storage and *in vitro* culture offer reliable techniques for conserving this rare fern.

KEY WORDS.—gametophyte, sexual expression, spore viability, young sporophyte

Aglaomorpha Schott (Polypodiaceae) is a genus of large-sized ferns of tropical Asia, comprising about 13–14 species (Janssen and Schneider, 2005; Roos, 1986). Four species of *Aglaomorpha* are native to the Philippines, including *A. cornucopia* (Copel.) M.C. Roos, *A. pilosa* (J. Sm.) Copel., *A. meyeniana* Scott, and *A. splendens* (J. Sm.) Copel. (Copeland, 1960). *Aglaomorpha cornucopia*, once classified as *Thayeria cornucopia* Copeland (Copeland, 1960), is an endemic epiphyte in the Philippines. It is distributed in the mountains of Mindanao and Luzon (Barcelona, 2005; Copeland, 1960)

[1] Authors contributed equally.
[2] Corresponding author: chiouwl@gmail.com.

and regarded as a rare species due to its limited distribution (Amoroso, 1987; Amoroso *et al.*, 2011; Barcelona, 2005; Copeland, 1960).

Although *in situ* conservation should be the primary focus for conserving this fern, germplasm storage and *in vitro* culture are also important complementary conservation tools (Pence, 2004). Fern spore banking has been implemented for both research and germplasm storage (Huang *et al.*, 2003b; Ko *et al.*, 2006; Lloyd and Klekowski, 1970; Pence, 2000) and can preserve thousands of genotypes in much less space and lower cost than is needed to grow one plant (Pence, 2008). The use of cold storage temperatures may expand spore longevity in many ferns (Pence, 2008). In addition to spore banking, gametophytes and sporophytes also offer opportunities for *ex situ* conservation by *in vitro* cultures (Chao *et al.*, 2010; Chiou *et al.*, 2006; Chou *et al.*, 2007; Huang *et al.*, 2001; Pence, 2004, 2008).

The goal of this study was to explore the reproductive biology of the endangered *Aglaomorpha cornucopia* and to evaluate the efficacy of cold storage as a method for prolonging spore longevity. The viability of spores of *A. cornucopia* that had been stored for one year was monitored. Gametophyte development and reproductive biology were observed.

Materials and Methods

Two sporophylls of *Aglaomorpha cornucopia* were collected from one individual on May 2012 in the Energy Development Corporation (EDC) forest, Mt. Apo, Mindanao, the Philippines (N 7° 0′ 53″ E 125° 13′ 14″; elev. 1344m).

The sporophylls were air-dried in the laboratory for seven days to release spores. The frequency of normal spores and the length of the longest axis of 100 randomly sampled spores were measured. Spores were stored in a refrigerator at 3°C. Voucher specimens (*Kuo 2792*, *Amoroso et al. CMUH0007821*) were deposited in the herbaria of the Taiwan Forestry Research Institute (TAIF) and of Central Mindanao University (CMUH).

Fresh spores and one-year-old spores, which had been stored at 3°C, were sown onto four separate membrane filters (Pall Supor®-450, Michigan, USA) in each of four plastic boxes (PHYTATRAY II™ No. P5929, Sigma, USA) under LED white fluorescent illumination of 6.3±0.3 μmole m^{-2} s^{-1} for 10 h d^{-1} (LI-COR, light meter, LI-250A). The daily temperature ranged from 20–28°C. Humidity was monitored to avoid desiccation of the cultures. To count the germination rate and the mean germination time (MGT), 100 randomly sampled spores (25 spores per membrane filter) were observed at 1-week intervals after sowing for a total of 4 weeks (until the germination rate did not increase). The MGT was calculated based on the equation of Ellis and Roberts (1981):

$$MGT = \sum(fx) \Big/ \sum x$$

where x is the number of spores germinated in week f, and f is the number of weeks counted from the beginning of culture.

To observe the development and sexual expression of gametophytes, fresh spores were sown into the four boxes (PHYTATRAY II™ No. P5929, Sigma, USA) filled with medium (vermiculite:peat:perlite, 4:4:2). The densities were 180–220 spores cm^{-2}, with culture conditions as above. One hundred randomly sampled gametophytes (25 per box) were removed (and not returned) and their sexual expression, age, and size were recorded during weeks 6, 7, 9, 11, and 13 after spore sowing. The percentages of gametophytes bearing sporophytes were recorded until the percentage remained stable. The mean sporophyte production time (MSPT) was calculated similar to the MGT, as:

$$\mathrm{MSPT} = \sum(\mathrm{fs}) \Big/ \sum \mathrm{s}$$

where s is the number of sporophytes produced in week f, and f is the number of weeks counted from the beginning of culture.

To assess sexual reproduction, sporophyte formation was observed by making paraffin sections. Three gametophytes, which bore sporophytes, were fixed in FPGA (Formalin:Propionic acid:Glycerol:95% Ethanol:distilled water = 1:1:3:7:8) overnight, dehydrated in an alcohol-TBA (*tert*-butanol) series for one day, infiltrated with paraffin (Paraplast Plus*, Kendall) at 60°C for one day, embedded in paraffin, and sectioned (10 μm thick serial sagittal sections of sporophyte) with a rotary microtome (RM2255, Leica).

Relative DNA levels between gametophytes and sporophytes were estimated to assess if sporophytes were produced sexually or apogamically. A total of about 5 cm^2 of gametophyte tissue from a total of 10 gametophytes and a total of 5 cm^2 from 5 fronds of one sporophyte were collected, respectively, minced in a chopping buffer (Epics-XL, Beckman) on ice, filtered through nylon mesh (20 μm, Peak Technology, Taiwan), cultured at 37°C for 15 minutes, and stained with 2.5% propidium iodide solution at 4°C in the dark for 15 minutes. The histogram peaks of genome size determined from the flow cytometer (FACScan, BD Technologies) were used to estimate relative ploidy levels of gametophytes and sporophytes (Cousin *et al.*, 2009).

Morphological observations were made with light microscopes (Leica, Wild M8; Leitz, Dialux 20) or a tabletop scanning electron microscope (SEM) (Hitachi, TM3000).

Results

There were 64 spores per sporangium. All fresh mature spores of *Aglaomorpha cornucopia* were yellow, bilateral, and monolete, with simple laesura. The average length of the spores was 49.3±3.7 μm. The perine surface was tuberculate to ornate, or somewhat vermiculate on the proximal side. The marginal processes were flat to rounded-verrucate, ornamented with echinate and papillate projections and the exospore was smooth (Fig. 1).

Both fresh and one-year-old spores attained 100% germination; however, germination time differed. Fresh spores germinated within one week after they

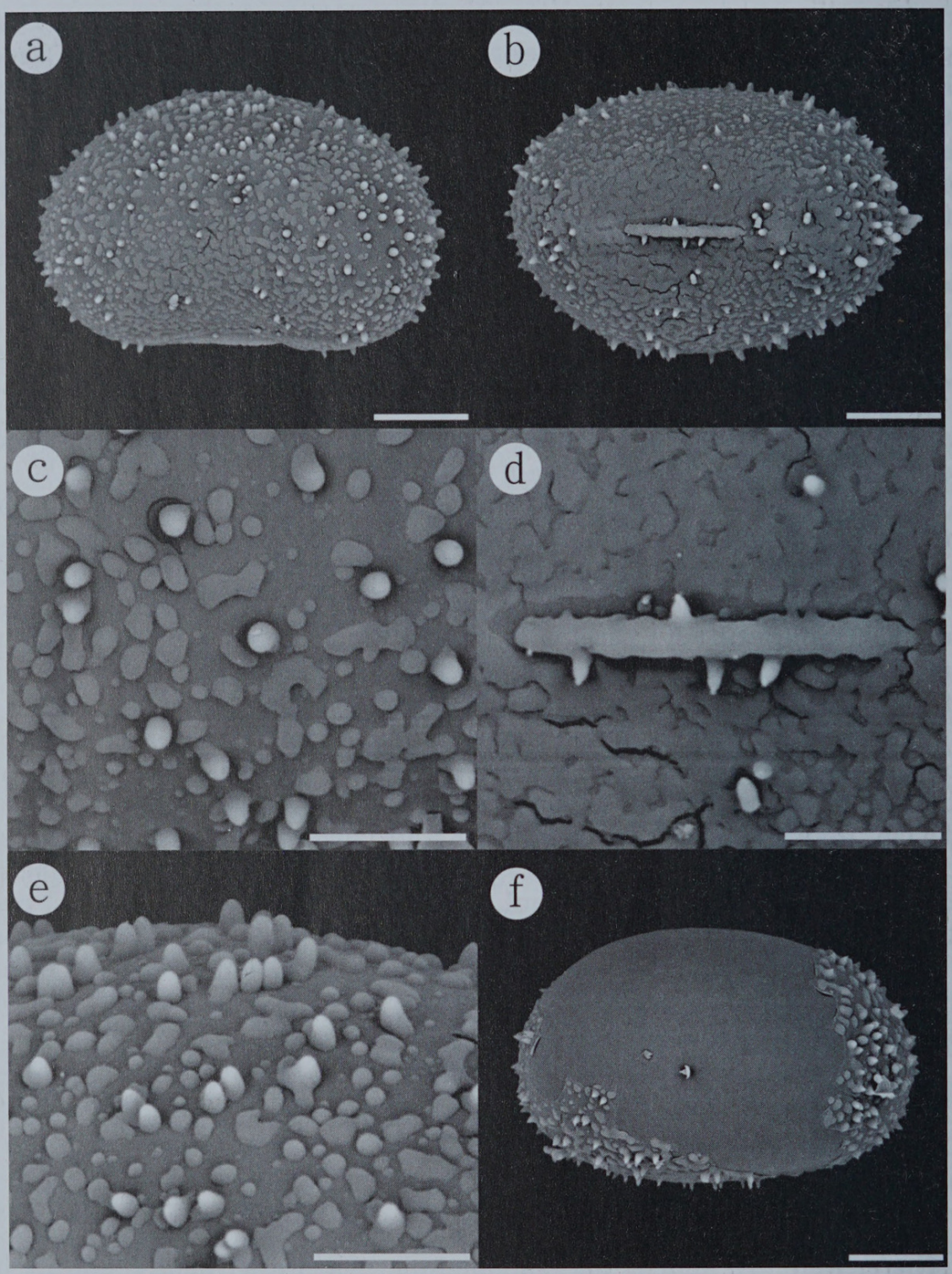

FIG. 1. SEM image of *Aglaomorpha cornucopia* spore. a: lateral view. b: proximal view. c: tuberculate to ornate perine sculpture. d: laesura and vermiculate perine sculpture at proximal end. e: flat to rounded verrucate marginal processes ornamented with echinate and papillate projections on perine. f: abraded spore, showing the plain exospore below. a, b, f: bars = 10 μm; c, d, e: bars = 5 μm.

were sown. The mean germination time (MGT) was less than 1.0 week. After one-year cold storage (3°C), spore germination was somewhat delayed compared to that of fresh spores with a MGT of 1.4 weeks, which was not significantly different from the former (*t*-test, $p>0.05$).

Following spore germination, the spore wall ruptured from the laesura on the proximal surface (Fig. 2a). A rhizoidal cell and a basal cell were formed by the first cell division parallel to the equatorial plane of the spore (Fig. 2b). Then the first rhizoid elongated (Fig. 2c), and a uniseriate filament was formed parallel to the polar axis of the spore by a series of transverse divisions (Figs. 2d–f).

When gametophytes were 3 to 5 cells long, filament growth was terminated. The middle cells divided parallel to the axis of the filament. Usually all the filament cells except the apical cell and basal cell divided longitudinally. Next, broad spathulate plates were formed (Figs. 2g–j). A wedge-shaped, meristematic cell formed in the apical region when the spathulate gametophytes were ca. 5 cells wide (Fig. 2j). The apical meristematic cell underwent repeated oblique divisions until it was replaced by a pluricellular meristem, which divided actively and formed an apical notch (Figs. 2k–m). Eventually, symmetrical (or nearly) cordate gametophytes were formed (Figs. 2n–o). Unicellular papillate hairs were produced on the margins when gametophytes were 3–5 cell wide, and were scattered on surfaces and margins with age.

When gametophytes were about 1 mm wide, a cushion with >one cell layers formed behind the meristem. The wings of young gametophytes were one cell thick and were usually flat, but became more curved, ruffled, and irregularly crenate with age.

Gametophytes first produced antheridia during early heart-shaped stages, when they were more than 0.5 mm wide, after 7 weeks of culture. However, some filamentous or spathulate male gametophytes less than 0.5 mm wide were found after 9 weeks. Antheridia appeared on the ventral surfaces and/or margins of the gametophytes where they were only one cell layer thick. They often intermingled with rhizoids (Fig. 3a). The hemispherical to subglobose antheridium wall was composed of a basal cell, a lower ring cell, an upper ring cell, a crescent-shaped cell, and an elliptical opercular cell (Fig. 3b). When mature antheridia were watered, the opercular cell and crescent-shaped cell were shed, and 32 spermatozoids were released from each antheridium.

Archegonia formed on the ventral surfaces of cushions approaching the notch (Fig. 4a). They did not appear until gametophytes were about 2 mm wide, after 7 weeks of culture. The necks of archegonia were composed of 4 tiers of cells, with 4 cells per tier (Fig. 4b).

Hermaphroditic gametophytes formed at ca. 4 weeks after male and female gametophytes were formed. The sizes of male gametophytes were significantly smaller than those of female and hermaphroditic ones (*t*-test, $p<0.05$). Female and hermaphroditic gametophytes were similar in size (Table 1). In most hermaphroditic gametophytes, antheridia were usually produced prior to cushion formation while the archegonia occurred only after the cushion

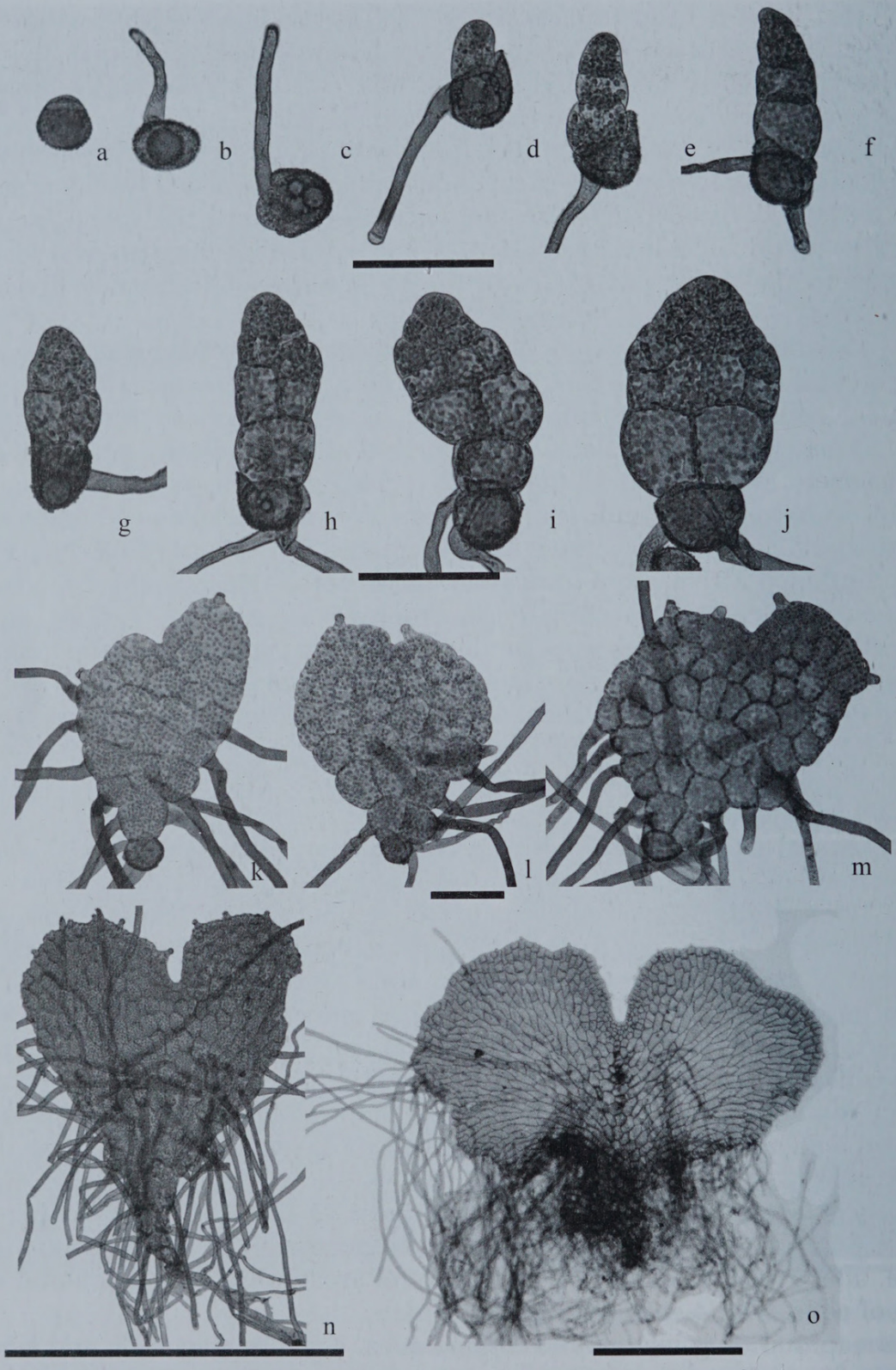

FIG. 2. Morphology of the gametophytes of *Aglaomorpha cornucopia*. a–b: rupture of spore, upper side denotes the position of the proximal surface. c: elongation of the first rhizoid. c–f: uniseriate gametophyte. g–j: spathulate gametophyte. k–o: cordate gametophyte. a–m, bar = 100 μm; n–o, bar = 1 mm.

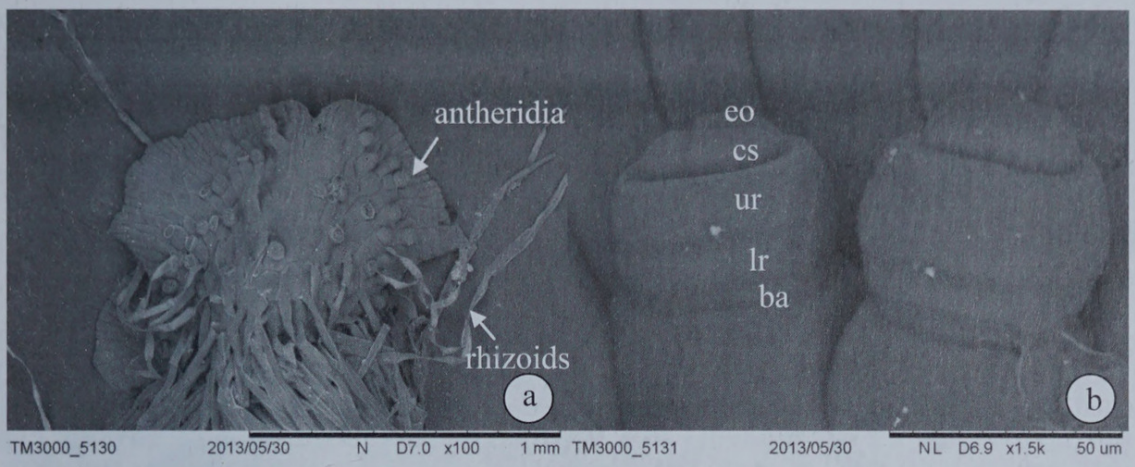

FIG. 3. Antheridia of *Aglaomorpha cornucopia*. a: on the ventral surface of gametophyte. b: cells of antheridium, ba=basal cell, ur= upper ring cell, lr=lower ring cell, cs= crescent-shaped cell, eo=elliptical opercular cell.

formed. When hermaphroditic gametophytes were old, they possessed only empty antheridia.

Female or hermaphroditic gametophytes did not produce sporophytes until 13 weeks. Sporophyte production reached a stable and maximum rate (64%) after 23 weeks. The mean time to sporophyte production was 17 weeks. Each young sporophyte consisted of a foot that separated the tissue from the gametophyte (Fig. 5).

The C-values (genome size) of gametophyte cells were half those of sporophyte cells (Fig. 6).

The first several sporophyte fronds were simple, cuneate at base, with single or forked free veinlets. Subsequent fronds had oblong blades, with pinnately free veinlets, followed by the production of leaves with areolate venation (Fig. 7).

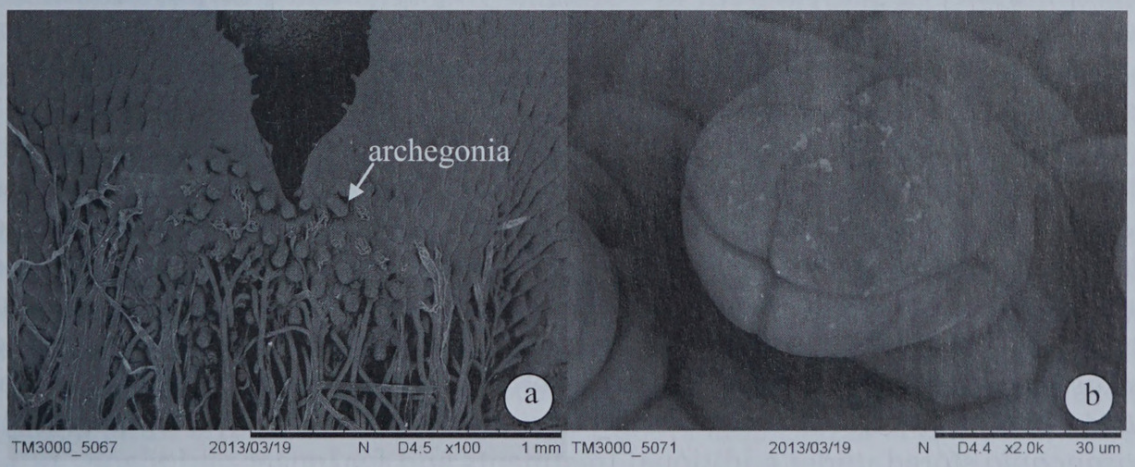

FIG. 4. Archegonia of *Aglaomorpha cornucopia* a: on the anterior cushion of gametophyte. b: archegonium.

TABLE 1. Sexual expression (%) and gametophyte size (mm) of *Aglaomorpha cornucopia* at different times in culture. Number of gametophytes bearing sporophytes in parentheses. A: asexual; M: male; F; female; H: hermaphroditic.

Size	6wk				7wk				9 wk				11 wk				13 wk			
(mm)	A	M	F	H	A	M	F	H	A	M	F	H	A	M	F	H	A	M	F	H
<0.5	41	-	-	-	40	-	-	-	18	10	-	-	3	8	-	-	-	13	-	-
0.5-1.0	49	-	-	-	31	2	-	-	14	11	3	-	2	12	-	-	-	13	-	-
1.1-2.0	10	-	-	-	17	5	1	-	10	5	7	-	1	15	4	2	-	17	-	1
2.1-3.0	-	-	-	-	-	-	4	-	1	-	12	-	1	3	16	3	-	9	2	4
3.1-4.0	-	-	-	-	-	-	-	-	-	-	8	-	1	-	6	2	-	-	5 (1)	5 (1)
4.1-5.0	-	-	-	-	-	-	-	-	-	-	1	-	-	-	8	1	-	-	7 (1)	-
>5.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	2	-	-	20 (8)	4 (1)
Sum	100	-	-	-	88	7	5	-	43	26	31	-	8	38	44	10	-	52	34 (10)	14 (2)

DISCUSSION

Conservation of rare species is an important aspect in the global maintenance of biodiversity, yet, little experimental work has been completed on the prospects of spore banking in rare tropical ferns. The goal of this study was to explore the reproductive biology of the endangered *Aglaomorpha cornucopia* and to evaluate the efficacy of cold storage as a method for prolonging spore longevity.

Spore number and reproduction mode.—Reproductive systems vary in ferns. Whereas most taxa are sexual outcrossers, a large number of species reproduce asexually through apogamy or other means. Spore number per sporangium is generally considered a primary indicator of reproductive mode for most leptosporangiate ferns. Sexual taxa usually have 64-spored sporangia, and apogamous taxa have 32-spored sporangia (Huang *et al.*, 2006; Knobloch, 1967). This pattern also fits *Aglaomorpha cornucopia*, which had 64 spores per sporangium and reproduced sexually, as evidenced by its sporophyte formation (the clear separation between gametophyte and sporophyte) and the genome size difference observed between the sporophyte and gametophyte. Another congener, *A. meyeniana*, also has 64-spored sporangia and reproduces sexually (Ko *et al.*, 2004; Nayar, 1965).

Spore viability.—Although both fresh spores and spores that had been in cold storage for one-year achieved 100% germination, the one-year-old spores displayed delayed germination relative to fresh spores. These results indicate that spores of this species can be stored at cold temperature for at least up to one year. The dried spores of many ferns can remain dormant for several years, and longevity may be improved by reducing storage temperature. Spore banking holds the potential for long-term *ex situ* germplasm storage (reviewed by Pence, 2004, 2008). Additional work should investigate the viability of spores of *A. cornucopia* stored under additional treatments and for longer periods.

Spore germination and gametophyte development.—Nayar and Kaur (1971) defined several types of spore germination and gametophyte development.

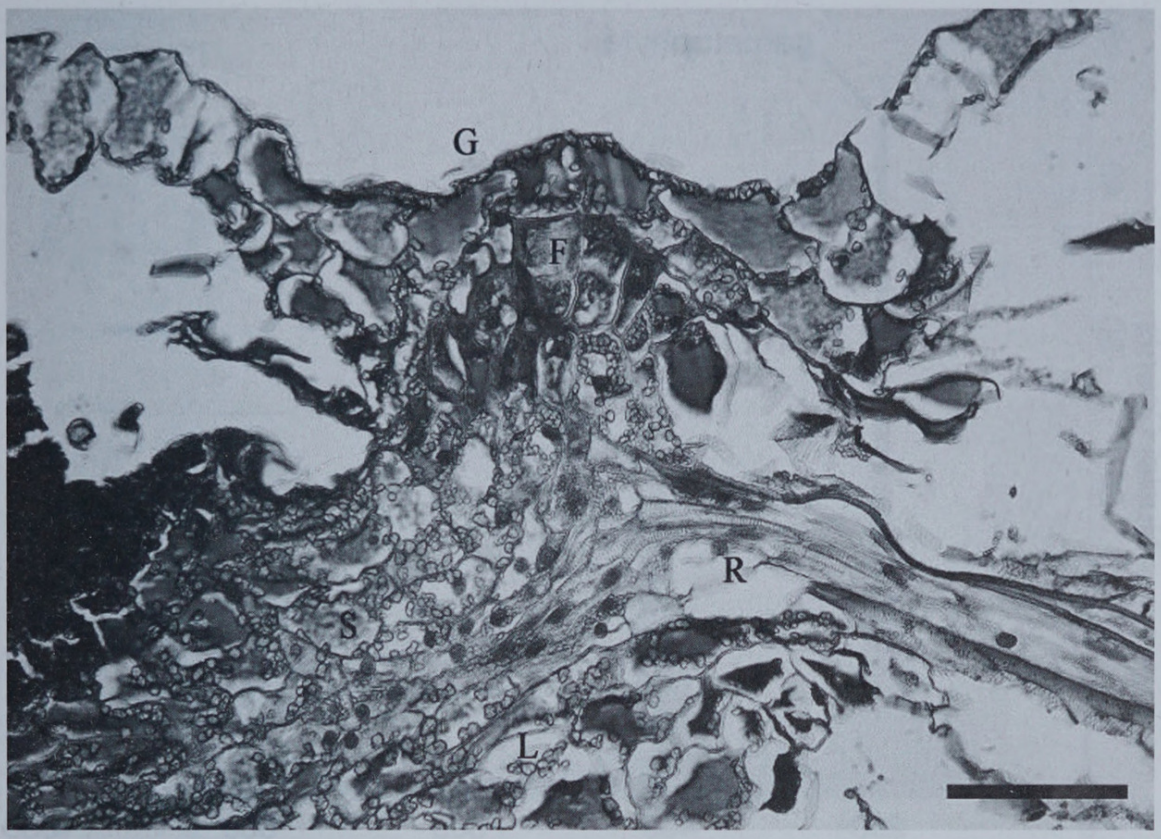

FIG. 5. Section of a gametophyte bearing young sporophyte (lower part) of *Aglaomorpha cornucopia*. A clear boundary between gametophytic tissue (G) and the foot (F) of young sporophyte were observed. (L) primary leaf; (R) primary root; (S) shoot. Bar = 100 μm.

Spore germination in *A. cornucopia* is of the *Vittaria*-type, and gametophyte development follows the *Drynaria*-type. These are the most common germination and development types in the Polypodiaceae. Other than the *Drynaria*-type of gametophyte development, other development types have been observed in some epiphytic members of the Polypodiaceae (Chiou and Farrar, 1997). We found that unicellular papillate hairs were scattered on gametophytes, and this is likely a stable characteristic of the Polypodiaceae (Nayar and Kaur, 1971).

The mature gametophyte of *A. cornucopia* is a typical cordate type. Once the cordate gametophyte sexually produced a young sporophyte, the gametophyte began necrosis. The life span of observed gametophytes of *A. cornucopia* was less than 6 months and could be classified as cordiform-annual following the definition by Farrar *et al.* (2008). The cordiform-annual gametophyte has been reported to be typical of terrestrial fern species (Farrar *et al.*, 2008); however, it has also been reported in many other epiphytic ferns of the Polypodiaceae (Chiou and Farrar, 1997; Ganguly *et al.*, 2009; Pérez-García *et al.*, 1998; Reyes *et al.*, 2003) as in *A. cornucopia*.

Gametangia.—Nayar and Kaur (1971) reported that the leptosporangiate type of antheridium commonly produces only 16 to 32 spermatozoids.

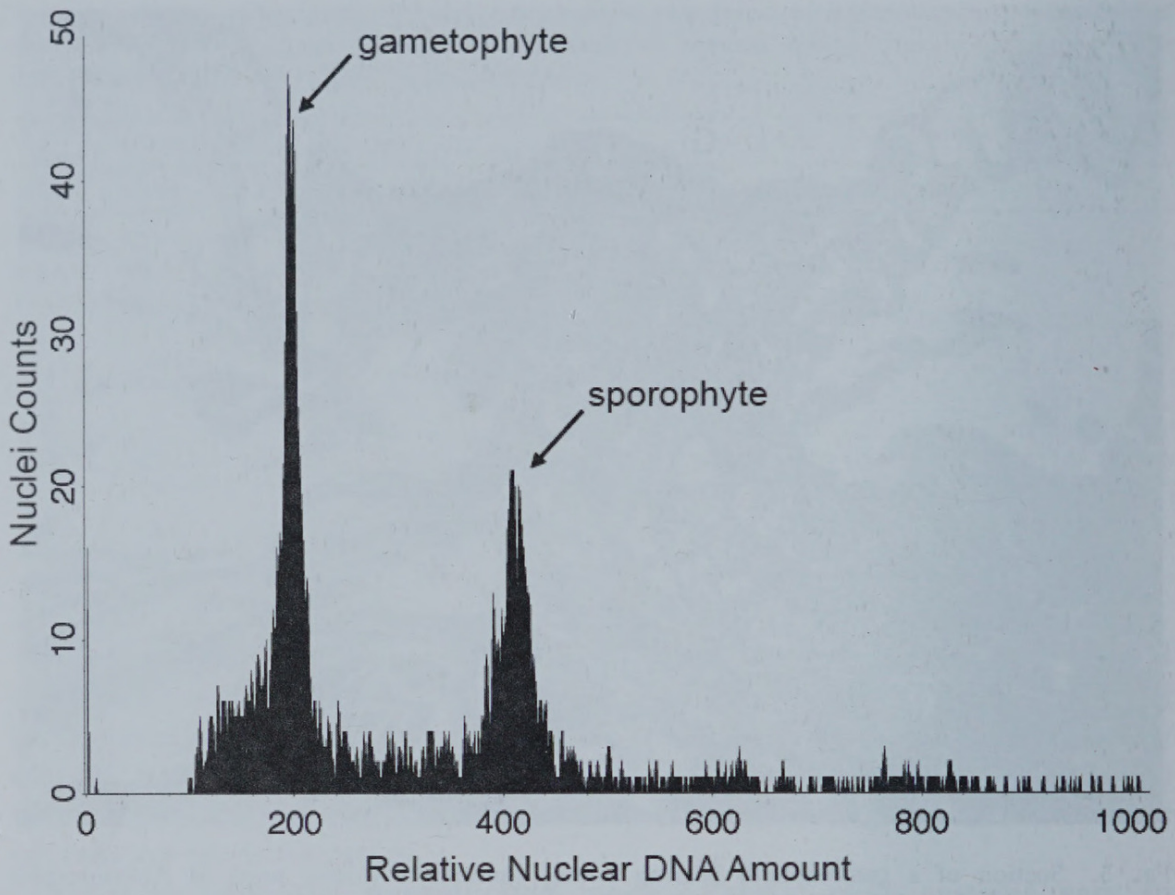

FIG. 6. Relative C-value (genome size) of gametophyte and sporophyte in *Aglaomorpha cornucopia*.

Gametophytes of *A. cornucopia* produced 32 spermatozoids/antheridium in this study. It is worth noting that sexual haploid and apogamous triploid gametophytes of *Pteris fauriei* Hieron. produced 64- and 32-spermatozoid antheridia, respectively (Huang *et al.*, 2006). In addition, the gametophytes of three apogamous *Pteris* (*P. cretica* L., *P. pellucidifolia* Hayata, and *P. wulaiensis* C. M. Kuo) were also observed to produce 32-spermatozoid antheridia (Huang *et al.*, 2011). However, no such data have been reported in other species of Polypodiaceae. More work is needed to determine if the spermatozoid number/antheridium in ferns is related to phylogeny and/or indicative of reproductive mode.

Apart from the central spermatogenous cell which eventually develops into spermatozoids, an antheridium possesses a set of jacket cells that envelope this central spermatogenous zone (Leung and Näf, 1979). The number of antheridial jacket cells varies among leptosporangiate ferns. Antheridia with jackets of ≥5 cells have been described in most early leptosporangiate ferns, such as Osmundaceae, Hymenophyllaceae, and some tree ferns (Chen *et al.*, 2008; Huang *et al.*, 2003a; Momose, 1967; Nayar and Kaur, 1971). In contrast to early leptosporangiate ferns, the typical antheridium of polypods has a jacket of three cells, a basal cell, a ring cell, and a cap cell (Momose, 1967; Nayar and

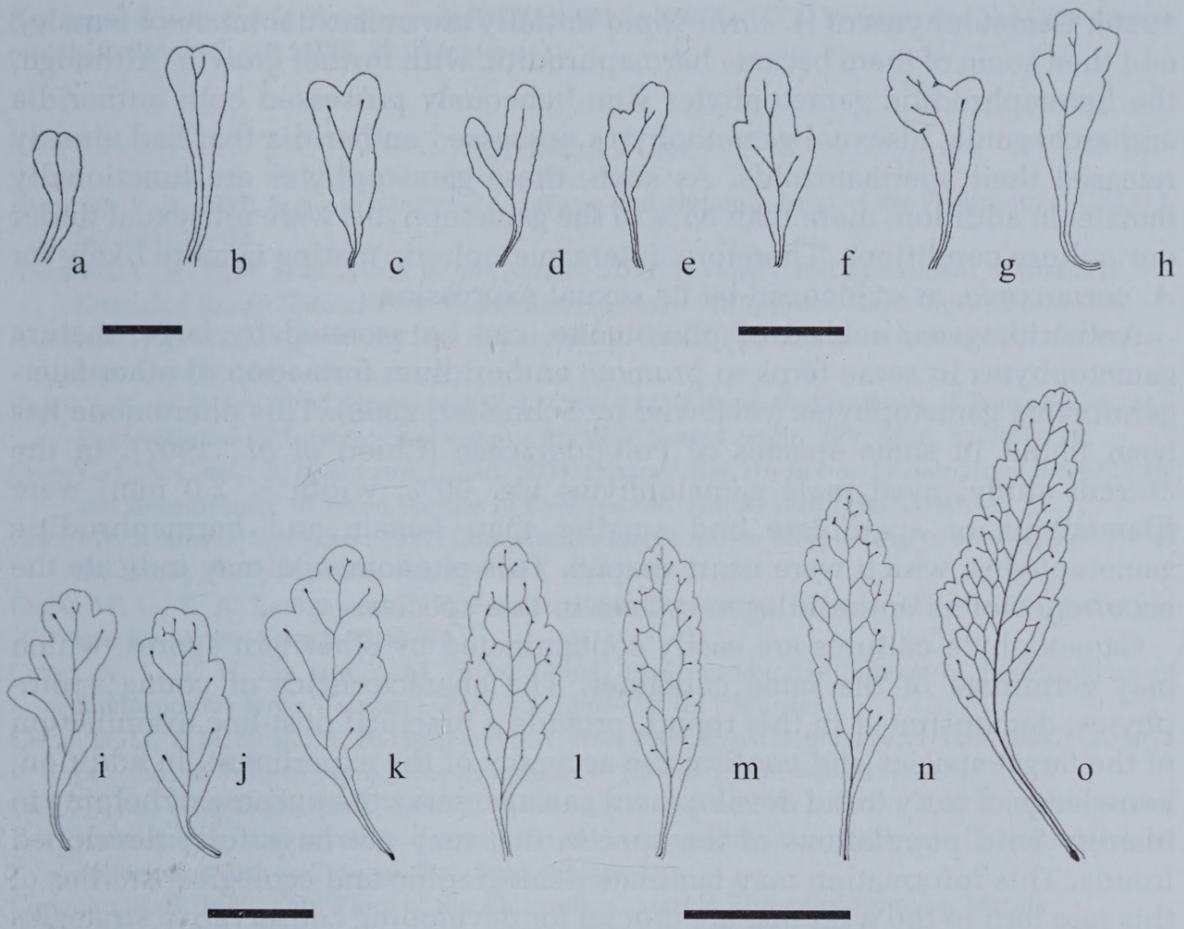

FIG. 7. Frond profiles of young sporophyte of *Aglaomorpha cornucopia*. a–d: first frond with free single to forked veinlet. e–k: subsequent fronds with free pinnate veinlets, l–o: subsequent fronds with areolae. a–b, bar = 2 mm; c–k, bar = 5 mm, l–o, bar = 1 cm.

Kaur, 1971; Pérez-García and Mendoza-Ruiz, 2004, 2005, 2006; Raghavan, 1989; Zhang *et al.*, 2011). In *A. cornucopia* we found that jackets of the antheridia were composed of 5 cells: an opercular cell and a crescent-shaped cell that made up the cap cell, and the ring cell divided into upper ring cell, lower ring cells, and basal cell. This is unlike the typical 3-celled-jacket per antheridium previously reported in other species in the Polypodiaceae by Nayar and Kaur (1971) and Leung and Näf (1979). Indeed, we believe this to be the first report of such antheridia within polypods.

The 4-celled neck of the archegonium of *A. cornucopia* is similar to other species of Polypodiaceae. According to Momose (1967), the cell numbers of each tier of the archegonium neck in polypod ferns are 3–7, whereas they are 4–9 in other leptosporangiate ferns.

Sexual expression and antheridiogen.—Homosporous ferns have the potential to produce hermaphroditic gametophytes. Some mechanisms have been documented to avoid the occurrence of intragametophytic selfing, such as high genetic load, and/or temporal separation of male and female gametangia on a single gametophyte (Chiou *et al.*, 2002, 2003; Klekowski, 1982; Peck *et al.*,

1990). Gametophytes of *A. cornucopia* initially are unisexual (male or female), and then some of them became hermaphroditic with further growth. Although, the hermaphroditic gametophytes simultaneously possessed both antheridia and archegonia, bisexual gametophytes possessed antheridia that had already released their spermatozoids. As such, these gametophytes are functionally female. In addition, more than 85% of the gametophytes were unisexual under our culture conditions. Therefore, intergametophytic mating is more likely for *A. cornucopia*, as evidenced by its sexual expression.

Antheridiogens, a kind of pheromone, can be secreted by large, mature gametophytes in some ferns to promote antheridium formation of other later-germinated gametophytes (reviewed by Schneller, 2008). This pheromone has been found in some species of Polypodiaceae (Chiou *et al.*, 1997). In the current study, most male gametophytes (ca. 90%, width < 2.0 mm) were filamentous or spathulate and smaller than female and hermaphroditic gametophytes, which were heart shaped. This phenomenon may indicate the occurrence of an antheridiogen system in this species.

Gametophyte cultures are easily contaminated by other fern spores, which may germinate in the same container. The characteristics of young sporophytes, demonstrated in this report, provide a practical post-hoc examination of the target species and confirm the accuracy of the experiment. In addition, knowledge of early frond development can also serve the purpose of helping to identify wild populations of the species that may not have fully developed fronds. This information may facilitate demographic and ecological studies of this rare fern in the wild that are crucial for developing conservative strategies *in situ* (Testo and Watkins, 2013).

Conclusions.—In this study, *Aglaomorpha cornucopia*, a rare endemic fern from the Philippines, was cultured from spores. Spore germination, gametophyte development, and the formation of young sporophytes were closely observed and illustrated in detail. Results show that the sporophytes reproduced sexually, as evidenced by spore number/sporangium, the genome sizes of sporophytes were double those of gametophytes, and there was a clear separation between the tissues of the gametophyte and sporophyte. Intergametophytic mating is more likely than intragametophytic selfing as inferred by sexual expression and indirect evidence of an antheridiogen system in this species. Spores remained viable after one-year of storage at 3°C. These results suggest that spores of *A. cornucopia* could be stored under cold storage for at least one year for ultimate use in *ex situ* conservation programs.

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