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Rescue, ecology and conservation of a rediscovered island endemic fern (*Anogramma ascensionis*): *ex situ* methodologies and a road map for species reintroduction and habitat restoration

KATIE BAKER[1]†, PHIL LAMBDON[2]†, EDWARD JONES[1], JAUME PELLICER[1], STEDSON STROUD[3], OLIVIA RENSHAW[3], MATTI NIISSALO[1], MARCELLA CORCORAN[1], COLIN CLUBBE[1] and VISWAMBHARAN SARASAN[1]*

[1]*Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, UK*
[2]*St Helena Nature Conservation Group, Jamestown STHL 1ZZ, St Helena*
[3]*Conservation Department, Ascension Island Government, George Town ASCN 1ZZ, Ascension Island*

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Last seen in 1958, the Ascension Island endemic fern, *Anogramma ascensionis*, was listed as extinct on the 2003 IUCN Red List. However, a 2009 survey rediscovered four plants on Green Mountain. Spores were collected and cultured *in vitro* at the Royal Botanic Gardens, Kew, where a living collection of thousands of gametophytes and hundreds of sporophytes has been developed. To gain further insights into the biology of this species and the potential implications of *in vitro* multiplication for conservation purposes, samples were characterized from the karyological point of view. Chromosome analysis of root tips has confirmed that the species is tetraploid, and flow cytometry assessments have revealed that haploid gametophytes produce diploid sporophytes, which confirms natural fertilization. In addition, an *rbcL* sequence from *A. ascensionis* has been generated and compared with those published for other *Anogramma* spp., suggesting a close relationship with *A. chaerophylla* from Brazil. Further surveys of Green Mountain have reported the presence of 40 *A. ascensionis* sporophytes in total. Vegetation community analyses have suggested that the present population may be confined to suboptimal habitats. We therefore propose that, prior to the dramatic transformation of the vegetation on the island as a result of the invasion of alien species (particularly *Adiantum* spp.), *A. ascensionis* may have flourished in more humid and shaded parts of the mountain. A multidisciplinary approach involving *in vitro* culture, invasive species clearance and controlled translocation is discussed as the future roadmap for the conservation of this critically endangered fern. Our experiences have also highlighted lessons more broadly applicable to the conservation of extremely rare species elsewhere in the world, especially on remote island systems. *Botanical Journal of the Linnean Society*, 2014, **174**, 461–477.

ADDITIONAL KEYWORDS: *Adiantum* – Ascension Island – endangered species reintroduction – invasive alien species – translocation – UK Overseas Territory.

INTRODUCTION

The UK Overseas Territory Ascension Island (7°57′S, 14°22′W) is a small (97 km^2), young (*c.* 1 Myr), volcanic land mass with a predominantly hot, arid climate and a low native biodiversity (Ashmole & Ashmole, 2000). Ten endemic vascular plant species have been recorded, most of which are confined to the moist, central upland area known as Green Mountain (Cronk, 1980). Although a comparatively small endemic flora, this density of unique species remains much higher than over much of the continental land masses, and the simplified ecosystems have much to tell us about the processes of evolution, colonization and the assembly of ecological communities (Wilkinson, 2004). Three of the endemic species are now thought to be extinct, and the others are critically endangered (Lambdon *et al.*, 2010).

*Corresponding author. E-mail: v.sarasan@kew.org
†These authors contributed equally to this work.

Many of the most pressing problems of threatened island endemics are caused by invasive species, which are generally considered to have particularly severe impacts on small island ecosystems. Target 10 of the *Global Strategy for Plant Conservation* (GSPC) has been developed to address this issue. In the case of Ascension, invasive plants and grazing animals undoubtedly represent the most severe threats to native habitats. Ninety-five per cent of the 249 wild species of vascular plant on the island have been introduced by humans, and these cover > 99.8% of the vegetated habitats (Lambdon & Darlow, 2008). As a result, endemics have been reduced to a few, isolated refuges which are likely to suffer from further losses without immediate action. In extreme cases, the remaining populations may already have become non-viable because of their small size and fragmentation.

Here, we report the case of one such endemic species which has suffered an extreme reduction in population size over the past century. *Anogramma ascensionis* (Hook.) Diels (Adiantaceae) is a small fern which was considered to be extinct until a small population was rediscovered in 2009. The event sparked both an immediate problem of securing the short-term survival of the species and a longer term problem of restoring a species to the wild in an environment in which almost all habitats have been severely modified. This latter situation was compounded by the fact that little information was available to understand the full ecology of a species thought to be now clinging on only in a marginal habitat.

Using an interdisciplinary approach, we piece together our derived understanding of the biology of the species and use it to formulate an integrated conservation plan. Our approach includes the *in vitro* recovery of gametophytes from viable spores, propagation and sporophyte development and transplantation to form a living collection, now established at the Royal Botanic Gardens, Kew (RBG Kew). We also reconstruct details of the natural ecology of the species from the limited available data and use this to develop a habitat-led approach for reintroduction and long-term monitoring. We discuss the lessons which can be learned from this extreme case in terms of wider conservation issues, especially associated with remote island biodiversity hotspots. Finally, we analyse and compare nucleotide sequences with those of other published *Anogramma* spp. to provide a preliminary insight into the evolutionary history of *A. ascensionis*.

MATERIAL AND METHODS

STUDY SPECIES

Anogramma ascensionis (Hook.) Diels is commonly known as the Ascension Island parsley fern. Only a few records of the diminutive sporophytes have ever been made, mostly when Joseph Hooker visited the island in 1842. A later sighting from 1958 by Eric Duffey was the last for many years (Duffey, 1964). Intensive searches failed to find any survivors after this report. Key areas of the island are searched on a yearly basis by the Ascension Island Conservation Department, and a detailed survey of the island was conducted in 2008 as part of the EU-funded South Atlantic Invasive Species Project (Lambdon & Darlow, 2008). This is in addition to occasional visits by other botanists in search of extinct species, e.g. Cronk and Benjamin in 1980 (see Cronk, 1980), and an expedition from Edinburgh University in 2007 (Gray *et al*., 2008). The species was eventually red listed as extinct in 2003 (Gray, 2003). However, in 2009, four plants were discovered by Conservation Officer, Stedson Stroud, during the routine annual plant census, and it has now been re-assessed as critically endangered (Lambdon *et al*., 2010).

Anogramma gametophytes are considered to be perennial and the sporophytes function as annuals (Bolle, 1863; Goebel, 1877), which is an unusual situation in ferns. *Anogramma* spp. are unique among ferns in having pea-like structures called tubercles, except in the case of *A. osteniana* Dutra (Nakazato & Gastony, 2003). The tubercles have the capacity to stay dormant during environmentally stressful periods and to generate new gametophytic lobes when favourable environmental conditions resume (Baroustis, 1976; Pangua, Pérez-Ruzafa & Pajarón, 2011). However, as *A. ascensionis* had not been studied and the rediscovered plants were growing on a dry, unstable and inhospitable cliff face, which did not appear to be ideal for further recruitment (Fig. 1A, B), there was a danger that, without immediate action, the population may have faced extinction.

IN VITRO PROPAGATION

It was judged by a group representing botanical expertise on Ascension Island (comprising the present authors, further members of the Ascension Island Conservation Department and Alan Gray from the Centre for Ecology and Hydrology, Edinburgh, UK) that intensive *in vitro* cultivation provided the best chance of preserving the *A. ascensionis* population in the short to medium term. *In vitro* spore germination is a reliable method for the propagation of ferns, and this procedure is being carried out widely, often in conjunction with cryopreservation, for the conservation of many species (Ford & Fay, 1999; Goller & Rybczynski, 2007; Pence, 2008; Barnicoat *et al*., 2011).

In 2007, attempts had been made by the authors to cultivate *A. ascensionis* spores from historical herbarium sheets, but without success. However, fresh

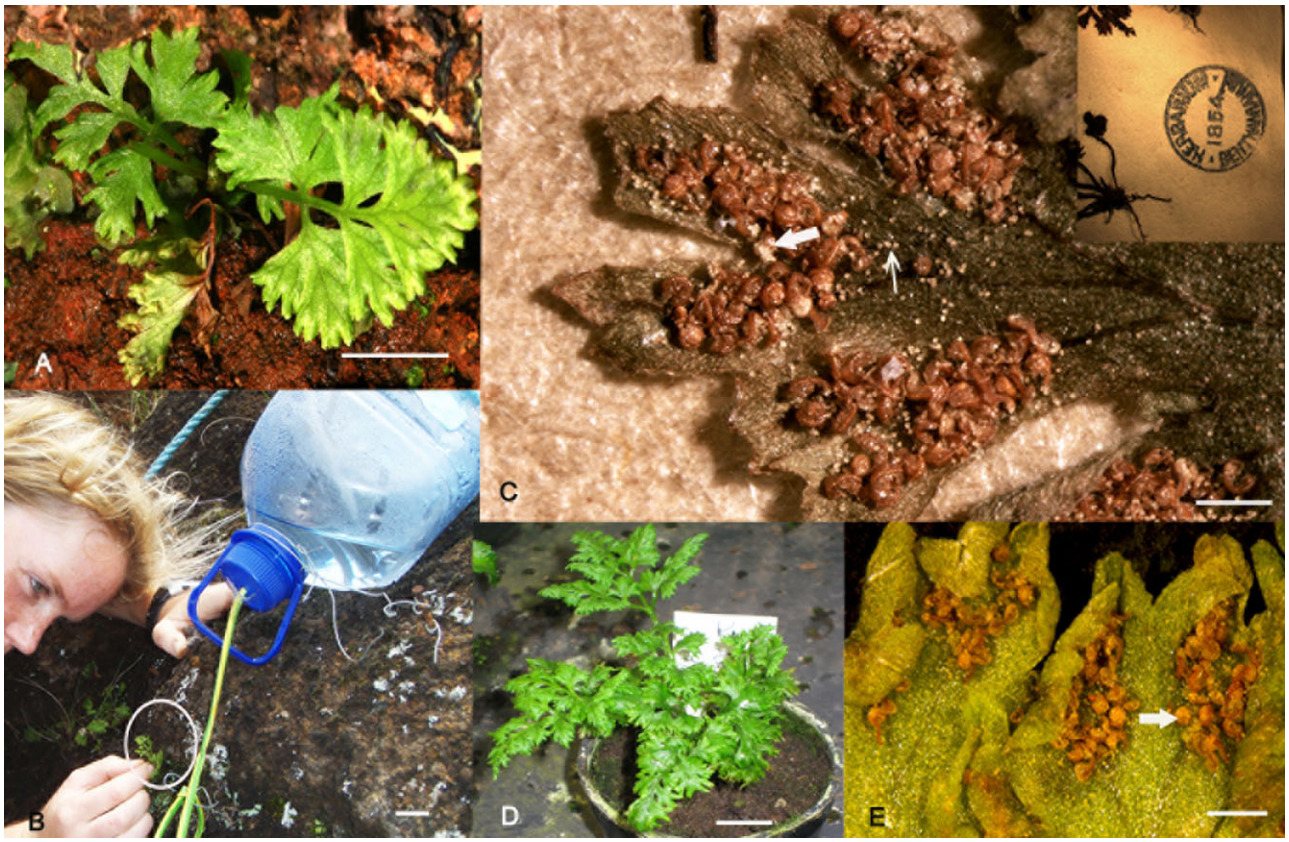

Figure 1. *Anogramma ascensionis*. A, Plant in the wild; bar, 1 cm. B, Steep rock face where the first population was rediscovered; bar, 4 cm. C, Frond of plant collected by Joseph Hooker in 1848; bar, 1 mm. D, *In vitro*-raised sporophyte under glasshouse conditions; bar, 1 cm. E, Frond of photoautotrophically grown sporophyte; bar, 1 mm.

fern spores typically germinate fairly freely (Aragon & Pangua, 2004), which led us to expect a reasonable chance of successfully establishing plants *in vitro*. It was therefore critical to achieve a successful sterilization method to remove all surface contaminants from spores to obtain and maintain cultures in a sterile condition. In *Cyathea delgadii* Sternb., the treatment of spores using calcium hypochlorite and the antifungal agent nystatin was essential for the maintenance of spores under sterile conditions, following the two-step method of Simabukuro, Dyer & Felippe (1998). However, in *Platycerium bifurcatum* (Cav.) C.Chr., spores lost viability after sterilization and, with increasing age, gave rise to plants with fewer and shorter rhizoids (Camloh, 1999). In the present project, the available fronds were small with only a limited number of spores, and so it was important to keep as many as possible in a viable state in the sterile germination medium. Sodium dichloroisocyanurate (SDICN) has proved to be an effective sterilant when dealing with small and difficult samples (Sarasan *et al*., 2006). Other methods were also adopted to maximize the prospect of success. Spores were separated based on their density and, hence, viability. This will be a useful practice for future cryopreservation projects as high-quality spores can be selectively stored. Media containing activated charcoal (AC) often promote the healthy growth and multiplication of gametophytes. This positive effect has been reported for a number of ferns, during both spore germination and vegetative propagation (Thakur, Hosoi & Ishii, 1998; Barnicoat *et al*., 2011).

Fronds were initially collected from two of the four rediscovered plants onto 9-cm filter paper and sealed in a Petri dish before being transported to Conservation Biotechnology, RBG Kew, within 24 h of collection (Gill, 2010). Spores were extracted from fronds and bleached for 20 min in a 10-mL centrifuge tube containing 0.5% (w/v) SDICN solution, and centrifuged at 168 × g initially to collect 'heavy' spores and at 670 × g to collect 'light' spores. Both 'heavy' and 'light' spore pellets were diluted to 1000 spores mL^{-1} in sterile water. The spore suspensions were cultured as described by Barnicoat *et al*. (2011), except that 2 mL L^{-1} of Plant Preservative Mixture (PPM; Apollo

Table 1. *Anogramma ascensionis* sporophyte development on different media. Effect of sucrose, supporting media and activated charcoal (AC) (MS, Murashige and Skoog medium; HS, Hoagland's solution)

Culture medium	Sucrose (%)	Supporting medium	AC (%)	Percentage of gametophyte clumps* with sporophytes
1/2MS	2	0.8% agar	0.15	3 ± 1.1[a]
1/4MS	2	0.8% agar	0.15	21.8 ± 3.1[a]
1/2MS	0	1 vermiculite : 1 coir	Nil	75.2 ± 8.9[b]
1/4MS	0	1 vermiculite : 1 peat	Nil	7.4 ± 6.1[a]
1/2HS	0	1 vermiculite : 1 coir	Nil	71 ± 12.7[b]
1/4HS	0	1 vermiculite : 1 peat	Nil	18 ± 9.4[a]

*Each clump has a minimum of 10 actively growing gametophytes; the same letters indicate no significant variation between treatments. Agar medium supplemented with 0.15% AC, but vermiculite + coir and vermiculite + peat mixes were devoid of AC.

Scientific Ltd, Stockport, UK) was included in the culture medium, hereafter called the germination medium (GM). Once the cultures had been demonstrated to be sterile 1 week after culture initiation, the filter paper with 'heavy' and 'light' spores was transferred to GM ± 0.15% (w/v) AC.

The same protocol was repeated for the germination of spores collected from six randomly selected second-generation sporophytes from photoautotrophic culture and glasshouse. Germination counts were made over a period of 3 months. Unless stated otherwise, all the cultures were grown in the growth room at a temperature of 21 ± 2 °C and a photoperiod of 16 ± 5 μmol m^{-2} s^{-1} at 16 h light : 8 h dark. Gametophytes were cultured in half-strength Murashige and Skoog medium (1/2MS) (Murashige & Skoog, 1962) ± AC to identify the medium suitable for multiplication. Three clumps of multiplied gametophytes with normal morphology from 1/2MS medium containing 2% sucrose and 0.15% AC were transferred to 30 mL of different concentrations of MS medium and Hoagland's solution (HS; Hoagland & Arnon, 1938) in Magenta jars (Table 1). Five replicates were included in each treatment and the performance of the plants in each was assessed.

Weaning

Single second-generation sporophytes on media as described in Table 1 were moved to a CO_2-enriched (1000 ppm) photoautotrophic system. They were grown at a temperature of 23 ± 2 °C in a photoperiod of 50 ± 5 μmol m^{-2} s^{-1} in 16 h light : 8 h dark cycles. Twenty sporophytic plants were tested in each medium. Sixty sporophytes were transferred to two glasshouse growing areas; half were grown inside a propagator at high humidity (vents closed all the time for the entire growth period) and the other half outside on a propagation bench in the Tropical Nursery at RBG Kew at 23 ± 2 °C (day) and 18 °C (night), and the plants were watered every day. All sporophytes were grown in 6-cm plastic pots (Fig. 1D) on a capillary mat in RBG Kew General Potting Mix 3, which contained 10% sterilized loam, 45% coir and 45% Silvafibre (Green-tech Horticulture, Nun Monkton, York, UK).

Sporangial distribution on the fronds

As the rediscovered plants appeared stunted and of poor quality relative to historical collections, morphological comparisons were made between *in vitro*-raised plants and plants on herbarium sheets at Kew Herbarium. Sporangial distribution over the frond was assessed by taking images under a Leica MZ 16 stereomicroscope. More than 6-week-old fronds, close to peak spore production, from six randomly selected glasshouse-grown plants and photoautotrophic cultures, were compared with fronds of sporophytes from Ascension Island. The Ascension sporophytes included those collected recently and historical specimens from Hooker 1842 (K000212932, K000367616) and Moseley 1876 (K000367614) at RBG Kew (Fig. 1C, E). Plant heights were also measured. These tests were conducted as a means of assessing the relative sporophyte quality between historical and modern wild plants, and laboratory-grown individuals.

Ploidy determination: chromosome counting and nuclear DNA content

Polyploidy might be associated with shifts in the reproduction mode and has the potential to influence the levels of genetic variation of a given population. With such a premise and in order to evaluate the formation of sporophytes (clonal vs. sexual), the chromosome number of *A. ascensionis* was determined in root-tip meristems obtained from propagated sporophytes growing in pots. The procedure followed was

that of Pellicer, Fay & Leitch (2010) with minor modifications, including a 3 h pre-treatment with 0.05% aqueous colchicine and staining with 2% aqueous aceto-orcein for 20 min. The DNA ploidy allocation of gametophytes and sporophytes was assessed by flow cytometry using propidium iodide (PI). Young sporophytic fronds and gametophytes were analysed individually and combined to calculate ratios of relative fluorescence. Samples were processed following the procedure described in Ebihara *et al*. (2005). Briefly, tissues from each specimen were chopped using a new razor blade in 1.5 mL of isolation buffer supplemented with PI (50 μg mL^{-1}) and 2-mercaptoethanol (2 μg mL^{-1}). The crude suspension was kept on ice for 5 min, filtered through a 30-μm nylon mesh and incubated for 15 min at 37 °C. To estimate the absolute nuclear DNA content, sporophyte fronds were processed with an internal standard (*Petroselinum crispum* 'Champion Moss Curled'; Obermayer *et al*., 2002; 1C = 2.25 pg). Three individuals were used for genome size estimation and, for each, three replicates were prepared, recording 5000 particles. Samples were analysed using a Partec Cyflow SL3 flow cytometer fitted with a 100-mW green solid state laser (Cobolt Samba). The resulting histograms were analysed with FlowMax software (v. 2.7, Partec GmbH).

DNA extraction, sequencing and phylogenetic analysis

To circumscribe and shed light on the taxonomic status of *A. ascensionis*, a phylogenetic tree was constructed. With the exception of *A. ascensionis*, DNA sequences used for phylogenetic analysis were downloaded from GenBank, using the same accessions as in Nakazato & Gastony (2003). Genomic DNA from *A. ascensionis*, previously collected at the original locality on Ascension Island, was obtained from the RBG, Kew DNA bank. The *rbcL* region was amplified by polymerase chain reaction (PCR) using the primer combinations described in Nakazato & Gastony (2003). PCR products were purified using DNA purification columns according to the manufacturer's protocol (QIAquick; Qiagen Ltd, Crawley, UK) and cycle sequenced using Big Dye terminator v3.1 chemistry (ABI, Warrington, Cheshire, UK) following the protocol recommended by the manufacturer.

Nucleotide sequences were assembled and edited using BioEdit version 7.0.9 (Hall, 1999). Alignments were made with ClustalW (Thompson *et al*., 1997) using default settings implemented in BioEdit. The single-partition dataset (*rbcL*) was employed to build phylogenetic trees. Phylogenetic reconstruction using Bayesian inference (BI) was carried out with MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). The most appropriate nucleotide substitution models for each partition were chosen with MrModeltest v. 2.3 (Nylander, 2004). The best-fitting model based on both the Akaike information criterion (AIC) and hierarchical likelihood-ratio test (hLRT) was GTR + I + G. Four Markov chains were run simultaneously for 30×10^6 generations and sampled every 1000 generations. Data from the first 7.5×10^5 generations were discarded as the 'burn-in' period, and the remaining trees were used to construct 50% majority-rule consensus trees. The posterior probabilities (PPs) of the nodes were calculated from the pooled samples.

Statistical analysis of laboratory studies

SPSS 16.0 was used for statistical analysis. For photoautotrophic micropropagation, a one-way analysis of variance (ANOVA) with a Games–Howell *post-hoc* test was used to test for differences in sporophyte production between the mixes. Comparisons of means were evaluated by protected least-significant differences (LSDs) at the 5% level of significance. Sporophyte production data conformed to an approximately normal distribution with equal variances.

Mapping and background ecological data

After the initial discovery, further plants were found after extensive searches. The locations of the populations were recorded by GPS and the numbers of plants were noted at regular intervals (every 3–4 months or less).

An assessment of the distribution of the highly invasive maidenhair ferns (*Adiantum raddianum* C.Presl and *Adiantum capillus-veneris* L.) was conducted in March 2011. The ferns occupy cliffs and banks on the upper parts of Green Mountain and are thus in direct competition with *A. ascensionis*. The habitats are difficult to survey as much of the area is almost inaccessible, but a constructed path known as Elliott's Pass extends around the mountain on a level contour at *c*. 700 m altitude; this was used as a transect as its cut banks provide almost continuous areas of vertical cinder face. The path was divided into 23 sections, each spanning 200–300 m (see Fig. 2). In each section, the lowest 2 m of bank along the inner border of the path was carefully inspected, and the abundance of maidenhair fern (both species together) was noted according to a DAFOR (dominant, abundant, frequent, occasional or rare) abundance scale (Table 2).

In addition, more detailed vegetation surveys were conducted from a series of point quadrats scattered along Elliott's Path. The locations of the quadrats were selected at random, except for the criterion that they should be representative of areas which were either

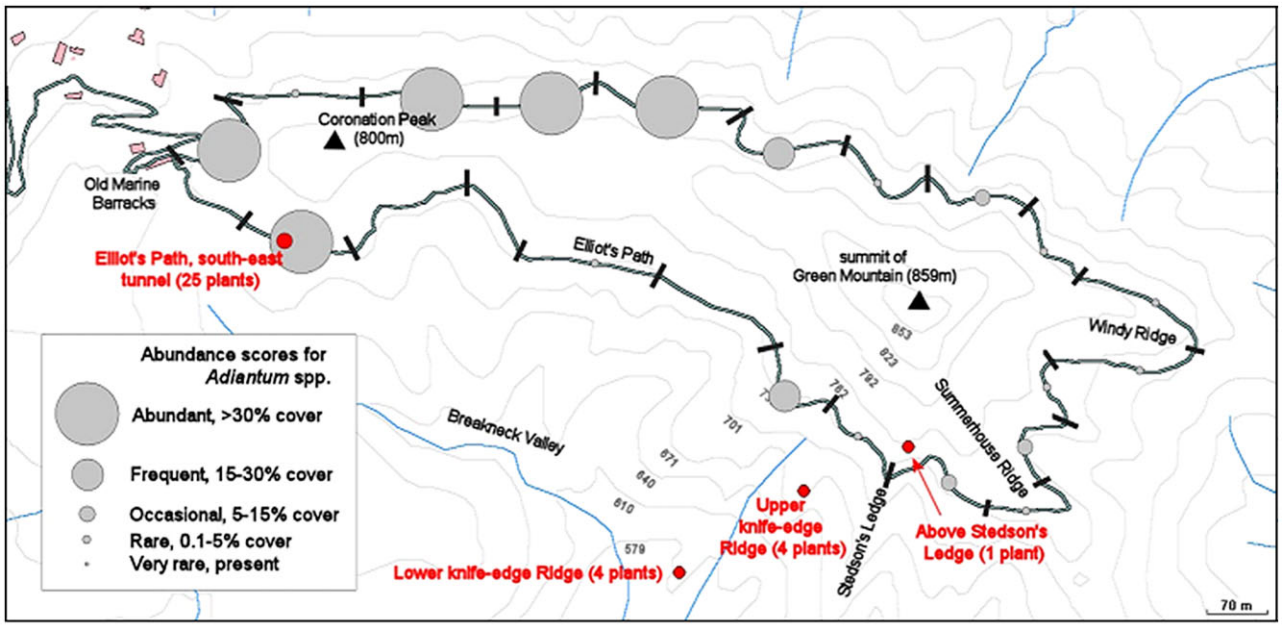

Figure 2. Relative distributions of *Anogramma ascensionis* and *Adiantum* spp. on Green Mountain. Locations of *Anogramma ascensionis* populations are indicated in red, with the number of individuals at each site noted. *Adiantum* abundances were recorded from a series of transects recorded along Elliot's Path. Abundance scores are marked at the centre of each transect.

Table 2. The DAFOR (dominant, abundant, frequent, occasional or rare) scale used to assess abundance during vegetation monitoring exercises

Category	Title	Cover (%)
1	Dominant	≥ 60
2	Abundant	≥ 30
3	Frequent	≥ 15
4	Occasional	≥ 5
5	Rare	≥ 1
6	Very rare	A few plants only

reasonably heavily invaded by maidenhair fern (with at least 20% cover) or areas with well-developed vegetation communities containing little maidenhair fern (< 5% cover). The former group comprised ten and the latter 21 quadrats. However, some quadrats were located close together (within 40 m of each other), and were aggregated into a single 'site': nine sites for the *Adiantum*-invaded group and seven for the uninvaded group. Sites tended to have similar vegetation communities and, for certain analyses, averages were taken, or a site was used as a blocking factor to avoid pseudo-replication. A further three quadrats were assessed at locations of *A. ascensionis* individuals. In this group, sample size was limited because of the inaccessibility of the locations.

Quadrats were always located on areas of near-vertical bank < 2 m above the path, and were 1 × 1 m^2 in size. The following data were collected from each quadrat: aspect, according to an eight-point compass direction; percentage shade, from the percentage of hemisphere in which sky was visible when looking directly upward from the centre of the quadrat (estimated manually); percentage bare cinder; mean vegetation height (cm); percentage total bryophyte cover; percentage cover of maidenhair fern (both species together); and a list of all species present in the quadrat, including vascular plants, bryophytes and lichens, with a DAFOR score to indicate the abundance of each (Table 2).

Statistical analysis of plant community composition

The quadrat data identified three types of plant community on the cinder banks of Green Mountain, depending on whether the habitat was uninvaded, heavily invaded by *Adiantum* spp. or contained *A. ascensionis*. To investigate differences between these groups, the following analyses were conducted.

The average species richness of each site was calculated (number of species per quadrat). The species present in the quadrat were divided into natives (those arriving on the island without the aid of humans) and aliens (those introduced by humans in

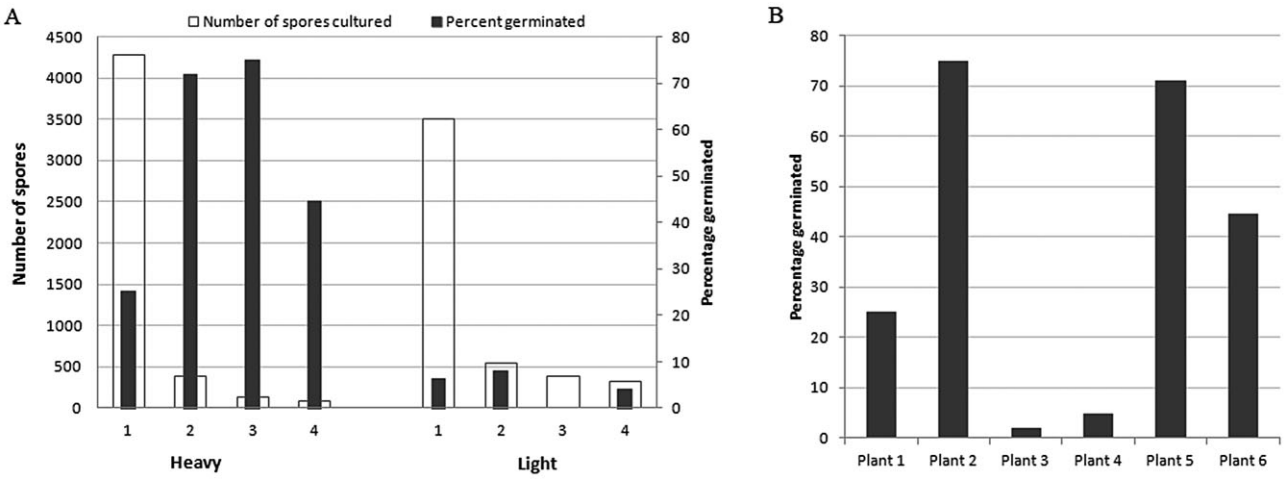

Figure 3. A, Percentage of *Anogramma ascensionis* spores germinated from four plates with 4671 'heavy' spores and four plates with 4684 'light' spores over a 4-week period. Spores were plated at high or low density in both categories. B, Percentage of *Anogramma ascensionis* spores germinated from different sporophytes grown under glasshouse conditions at the Royal Botanic Gardens, Kew (RBG Kew) (approximately 200 spores from each plant were cultured).

the last two centuries). The proportion of the quadrat surface covered by each group was assessed and mean values were calculated for each site.

To identify major trends in the complex community structure, de-trended correspondence analysis (DCA) was conducted on the original quadrat data. This ordination technique identifies a series of composite variables which explain the maximum amount of joint variation between the abundances of all the original species. Each composite variable is termed an 'axis', and ranked in order of the variance explained. DCA was conducted using CANOCO for Windows 4.5 (Biometris, Wageningen, the Netherlands). The technique is based on the assumption that the abundances display a unimodal distribution along the axes of community composition, with the form of the curve estimated by segmental de-trending. Abundance was estimated from the DAFOR score (approximately proportional to the logarithm of the percentage cover) and rare species were down-weighted to reduce the influence of outliers on the results.

Relationships between the variables describing community structure (diversity, percentage cover of natives and the DCA axes) and the major explanatory variables (percentage shade, percentage bare ground, etc.) were explored by solving generalized linear models. The analyses conducted in percentage data were arcsine-transformed. A normal distribution was assumed and Wald confidence intervals were applied. Site was included as a random explanatory variable and the models were solved by maximum-likelihood iteration using the GLIMMIX procedure in SAS (SAS Institute Inc., Cary, NC, USA, 2002).

RESULTS

IN VITRO PROPAGATION

The first spores germinated after 1 week, but some took up to 3 months. The spore viability test showed a range of 0–75% viability. The percentage germination of 'heavy' spores was 29.4% and this percentage was higher than the mean for 'light' spores at 3.3% (Fig. 3A). No 'light' spores from first-generation glasshouse-grown sporophytes germinated under similar conditions. Overall, 52.2% of spores collected from sporophytes grown in the glasshouse germinated under *in vitro* conditions (Fig. 3B).

GAMETOPHYTE MULTIPLICATION

Gametophytes were initially filamentous but, after 2 months, became heart shaped and started to multiply on the same culture medium, as reported previously (Nayar & Kaur, 1969, 1971). AC-containing medium was selected as the maintenance and multiplication medium. From the vegetative multiplication stage of the gametophytes, it took 8–18 weeks for sporophytes to appear on either 1/2MS with 2% sucrose, 1/4MS or HS (both without sucrose). The presence of sugar in the medium was not conducive to sporophyte development. However, both MS and HS media supported sporophytic development when supplemented with a mixture of vermiculite and coir. Sporophytes first developed on vermiculite and coir mix 8 weeks after gametophytes were transferred, but, on other media, the timing was in the range 10–16 weeks. The Games–Howell *post-hoc* test revealed that vermiculite and coir medium was significantly

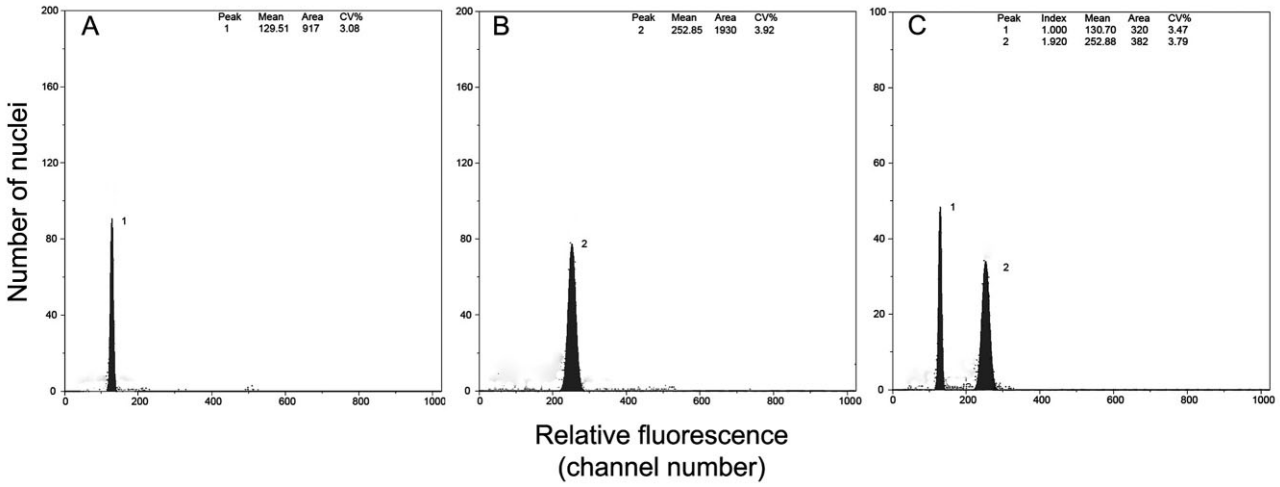

Figure 4. Representative flow histograms of relative fluorescence obtained after isolation of nuclei of *Anogramma ascensionis*. A, Gametophyte (peak 1, 1C-value). B, Sporophyte (peak 2, 2C-value). C, Gametophyte (peak 1) and sporophyte (peak 2). Statistical data of peaks is given: Mean, mean channel number; Area, number of nuclei in peak; CV%, coefficient of variation of peak.

better at inducing sporophyte development than vermiculite and peat medium under similar conditions (Table 1). All the sporophytes had the same morphology and gradually developed healthy root systems.

The survival rate of sporophytes 6 weeks after transplantation to the glasshouse was 90%. All photoautotrophically raised fronds continued to grow and produce sporangia, except for the fronds which developed on agar. Spores produced from some sporophytes germinated on the same pot and produced healthy gametophytes.

The maximum height of the four original rediscovered wild plants averaged 33.5 ± 5.3 mm (n = 4; mean ± standard error). Plants collected by Hooker (1842) and Moseley (1876) were significantly larger (mean = 67.7 ± 10.7 mm; n = 15; two-group Student's t-test with unequal variances: P = 0.014). Plants grown in high humidity under glasshouse conditions at RBG Kew (mean = 63.2 ± 6.8 mm; n = 12) were almost as large as the specimens on Hooker's and Moseley's herbarium sheets (Fig. 1C). Glasshouse-grown sporophytes had darker green fronds than plants re-found in the wild, which were pale green (Fig. 1C–E). Sporangial distribution (the proportion of the frond covered by sori) was highest in the Hooker (1842) and Moseley (1876) collections, but glasshouse-grown and photoautotrophic plants were comparable (Fig. 1C, E). Plants in the wild had markedly lower sporangial distribution. As a result of the problems in comparing fresh and dried plants, quantitative data are not presented for this parameter.

Cytogenetics and phylogenetic analysis

Flow cytometry revealed that ratios between the peak position of the isolated nuclei from gametophytes and sporophytes were almost 1 : 2 (Fig. 4), indicating that the nuclear DNA content of the gametophyte was half that of the sporophytes, confirming that sporophytes had developed via fertilization. The nuclear DNA content in sporophytes of *A. ascensionis* was estimated to be 1C = 4.72 ± 0.04 pg. The sporophytic chromosome number of *A. ascensionis* was $2n$ (= $4x$) = 116 (see Supporting Information Fig. S1).

A Bayesian phylogram displaying the evolutionary relationships in *Anogramma* is presented in Figure 5. The specimen of *A. ascensionis* studied here (GenBank accession KF680664) appeared to be embedded in a strongly supported clade, confirming the close relationship between this species and *A. chaerophylla* (Desv.) Link, especially with one of the accessions from Brazil. *Anogramma ascensionis* and the Brazilian accession of *A. chaerophylla* had identical nucleotide sequences.

Population survey and distribution of competing *Adiantum* species

After the discovery of the initial four plants in July 2009, targeted searches in similar, remote habitats revealed a more extensive distribution. The known population of *A. ascensionis* was approximately 40 plants in the wild in 2010, with individuals scattered along the south side of Green Mountain between 600 and 750 m altitude (Fig. 2), although not all were

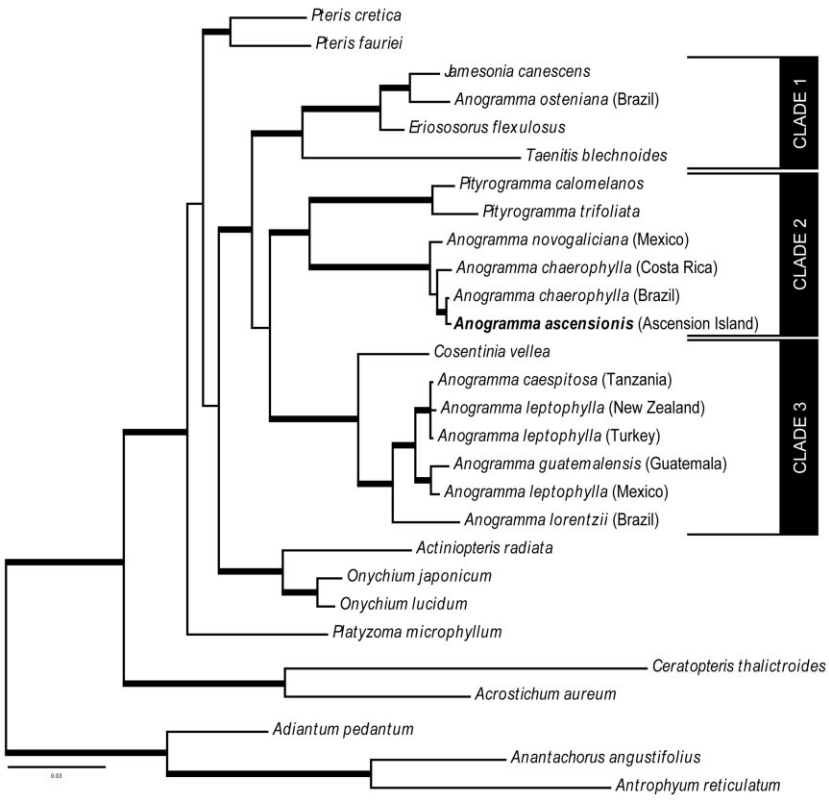

Figure 5. Bayesian phylogram (50% consensus tree) based on *rbcL* sequences of *Anogramma* and related genera. The three major lineages containing *Anogramma* spp., already reported by Nakazato & Gastony (2003), are highlighted as clades 1, 2 and 3. Bold branches indicate posterior probability (PP) ≥ 0.95.

present simultaneously and 25 of the individuals were found in a single patch occupying just 1 m^2. As the population is extremely dispersed and the sporophyte plants are small and short lived, there may be further undetected individuals elsewhere. It is also probable that the numbers will fluctuate between years and that the sporophyte count could thus come close to zero in unfavourable seasons. The persistence of the gametophytes has yet to be understood, as they are generally not easily observed in their natural micro-habitats of deep crevices, but, even if capable of perennating in the wild, there is no guarantee that they survive the desiccating conditions periodically experienced at the current known sites. It is likely that regeneration occurs at least partly from a spore bank, and plants were germinated from soil scraped from around the original site when mixed with peat compost in the local endemic nursery (S. Stroud, unpubl. data, 2011). However, the longevity of the spores is unknown.

Among the most serious competitors of *A. ascensionis* are the maidenhair ferns, *Adiantum* spp. The two species introduced to Ascension Island were not distinguished during the transect survey as they can be difficult to identify and local trainees were involved in the fieldwork. However, *A. raddianum* dominates the north side of the mountain and *A. capillus-veneris* is dominant on the south side. Collectively, they are often extremely abundant on the north and west slopes, but become much more local in the south and east. The main reason for this may be that the prevailing winds are south-easterly. These are often strong and bring cool, moist air to the exposed banks and cliffs along this side of the mountain. *Adiantum* spp. do not appear to cope well with these conditions and are mainly restricted to sheltered corners and gullies in such areas. The most sheltered parts of the south face tend to be overgrown with taller, much more vigorous weeds, where all ferns are rare.

Analysis of cinder bank vegetation communities on Green Mountain

Although the native flora of Green Mountain is now degraded, localized patches of native communities still persist on the cinder banks (Appendix). These tend to be diverse, low and open, with a high proportion of bare ground. There is a strong negative relationship

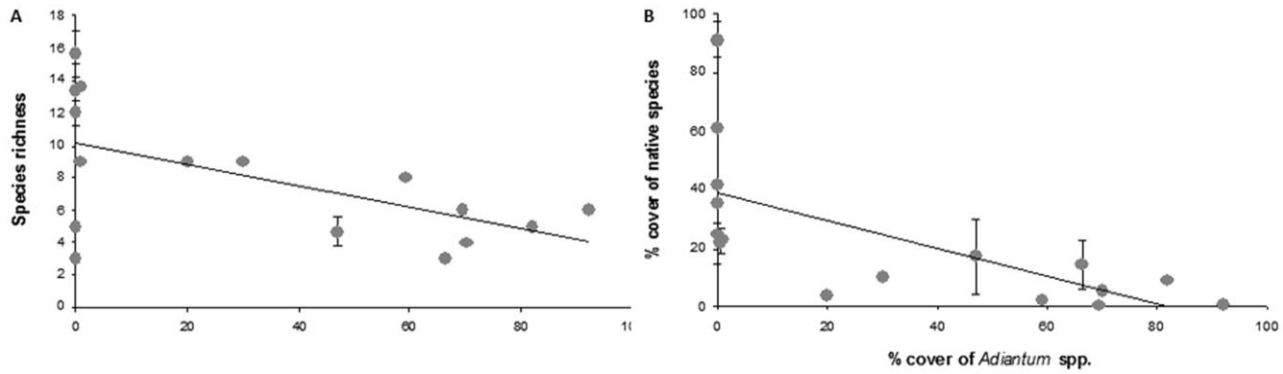

Figure 6. Relationship between percentage cover of *Adiantum* spp. and species richness (A) and percentage cover of native species (B). Data were collected from quadrats scattered along Elliot's Path. Where more than one quadrat was assessed at a given site, the mean for the site is displayed with error bars representing the standard error.

between the presence of these native communities and the presence of *Adiantum*. Although species richness is influenced by a number of factors, such as shading and accessibility to grazing animals (introduced sheep or rabbits), the number of species per quadrat declined markedly with percentage cover of *Adiantum* (Fig. 6A; $r^2 = 0.34$, $P < 0.001$). The native community component was particularly heavily affected, with the percentage cover of native species declining strongly (Fig. 6B; $r^2 = 0.44$, $P < 0.0001$). At high levels of cover, the dense hanging fronds smother almost all competition.

Adiantum invasion is only one of a number of complex factors determining community composition on the cliffs of Green Mountain, and DCA was employed to analyse these relationships in more detail. The first DCA axis explained 15.5% of the variation in community composition, and the second axis explained 8.7%. Although these two axes still left a substantial proportion of variation unexplained, they described major trends which were clearly important. Axis 1 was correlated strongly and positively with species richness ($r^2 = 0.60$, $P < 0.0001$) and negatively with percentage shade ($r^2 = 0.59$, $P < 0.0001$). However, it did not describe the simple transition from invaded to native communities, as there was no strong correlation with percentage cover of native species ($r^2 = 0.14$, $P = 0.093$) or percentage bare ground ($r^2 = 0.10$, $P = 0.67$). Axis 2 was not strongly correlated with any of the variables for which we had data (for all, $P > 0.05$). It appeared to be broadly related to the degree of exposure, with low values representing unexposed sites and high values representing sheltered sites. Exposure is a difficult factor to measure directly and, although the species present at the most exposed sites were mostly native, there were also scattered native species present in other habitats [principally bryophytes and the opportunist fern *Christella dentata* (Forssk.) Holttum].

The relationship between axis 1 and axis 2 became apparent when mean scores for each species were plotted against each other (Fig. 7). Scores for the two axes were not entirely independent, but species responded to the driving factors behind them according to a 'U'-shaped relationship. To summarize, three groups could be identified.

Group 1: low axis 1/high axis 2 scores: These species were characteristic of heavily shaded, sheltered communities which were dominated by relatively few, predominantly alien, species. Unsurprisingly, *Adiantum* belonged firmly in this group, as did aggressive and problematic invaders, such as *Clidemia hirta* (L.) D.Don (Melastomaceae) and *Begonia hirtella* Link (Begoniaceae), and shade-tolerant native bryophytes, such as *Sematophyllum* sp. (Hypnaceae) and *Bryoerythrophyllum* sp. (Pottiaceae).

Group 2: high axis 1/high axis 2 scores: These species were characteristic of sunny, moderately sheltered habitats which represent good growing conditions. Such sites hosted a diverse range of species, but were usually dominated by vigorous aliens, such as *Rubus rosifolius* Sm. (Rosaceae), *Spermacoce verticillata* L. (Rubiaceae), *Ageratum conyzoides* (L.) L. (Asteraceae) and *Sporobolus africanus* (Poir.) Robyns & Tournay (Poaceae). Most of the native components were small bryophytes which inhabit the gaps, e.g. species of *Metzgeria* (Metzgeriaceae), *Cololejeunea* (Lejeuneaceae) and *Frullania* (Frullaniaceae).

Group 3: low axis 1/negative axis 2 scores: This smallest group included species which frequented exposed, not particularly shaded, habitats amongst open areas of bare ground. They were almost entirely native, represented by the endemic grass *Sporobolus caespitosus* Kunth (Poaceae), the endemic fern *Asple-*

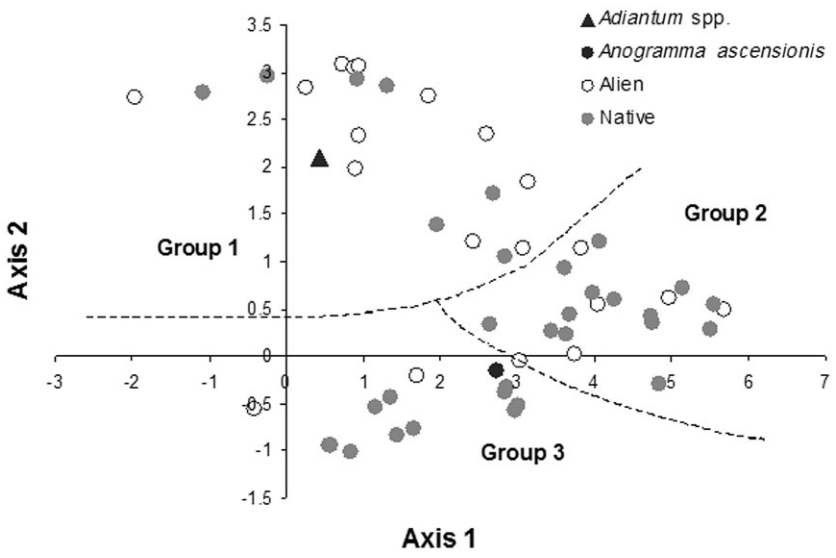

Figure 7. Mean species scores obtained from the first two axes of a de-trended correspondence analysis (DCA) of community composition, derived from 34 quadrats situated on and around Elliott's Path. Native species are indicated by grey symbols and alien species by open symbols. The points representing *Adiantum* spp. and *Anogramma ascensionis* are highlighted.

nium adscensionis Bernh. (Aspleniaceae), the native bryophytes *Entosthodon* sp. (Funariaceae), *Isotachis* sp. (Balantiopsaceae), *Plagiochasma rupestre* (G.Forst.) Stephani (Aytoniaceae) and *Calymperes erosum* Müll. Hal. (Calymperaceae), and the few lichen species noted, e.g. *Teloschistes flavicans* (Sw.) Norman (Teloschistaceae). This community was highly characteristic and almost confined to the south-easterly ridges of the mountain. Most of its components are rare.

This three-group framework is useful in understanding the conservation challenges faced on Green Mountain. Most of the surface area is now heavily dominated by non-native species which are more vigorous and competitive than the natives in reasonably favourable environments. A few, such as *Adiantum* spp., which are tolerant of heavy shade, have also come to dominate the sheltered sites. However, native refuges still persist on the most wind-exposed ridges. As the entire mountain was, until 200 years ago, almost certainly clothed mainly in low, open vegetation comprised heavily of fern and bryophyte swards, the native species have evolved a high tolerance of wind exposure and can survive on thin soils prone to periodic drought. Subsequent colonization by invasive vegetation has improved soil quality and increased shelter, limiting suitable open habitat to extreme sites.

The position of *A. ascensionis* in the ordination analysis (Fig. 7) is particularly significant. It clearly belongs to Group 3, but lies somewhere near the periphery, close to the edge of Group 2. This suggests that it is not particularly tolerant of exposed conditions. Before the dramatic transformation of the vegetation, it is likely that *A. ascensionis* was a characteristic component of the more sheltered parts of the mountain, perhaps flourishing in humid and shaded places. The fact that such areas have been heavily invaded would explain why it has come so close to extinction. The current locations indicate that it can tolerate dry, open situations, but such sites may perhaps represent suboptimal habitat, at the range edge. A requirement for open banks in sheltered situations highlights the extreme threat posed by *Adiantum* spp., which have vigorously colonized similar niches.

DISCUSSION

Surveys conducted on Green Mountain have estimated the wild population of *A. ascensionis* to be approximately 40 sporophytes. Spores collected from the field have now been successfully cultured *in vitro*, leading to a current living collection of hundreds of sporophytes at RBG Kew. Here, efforts have been made to combine information from biological and ecological variables that might have had a direct influence on the dynamics of this species, and have supplied detailed information of application to the conservation of extremely rare species. Flow cytometry analysis revealed that gametophytes produced sporophytes with twice the genome size as a result of natural fertilization. A chromosome analysis of root tips confirmed that the species is tetraploid. Comparison of *rbcL* sequences with those published for other *Anogramma* spp.

indicated a close relationship with *A. chaerophylla*. Vegetation community analyses suggested that the present population may be confined to suboptimal habitats. We therefore hypothesize that, prior to the dramatic transformation of the vegetation as a result of the invasion of alien species (particularly *Adiantum* spp.), *A. ascensionis* may have flourished in more humid and shaded parts of the mountain.

Nuclear DNA content and karyological data in *A. ascensionis*: ploidy allocation and sporophyte formation

Our tests showed that the DNA content falls within the range of C-values compiled in pteridophytes (Plant DNA C-values, release 5.0; Bennett & Leitch, 2010). As data at the generic level are scarce, with only one additional C-value report in diploid ($2n = 2x$) sporophytes of *A. leptophylla* (1C = 2.64 pg; J. Pellicer, unpubl. data, 2011), it is difficult to infer any evolutionary trend in this parameter in the genus. Previous karyological studies conducted in *Anogramma* showed a certain degree of variability in the basic chromosome number, with records of $n = 26$, 27 and 29 (e.g. Gastony & Baroutsis, 1975; Baroutsis & Gastony, 1978; Rasbach & Reichstein, 1990). The existence of $n = 26$ is restricted to *A. leptophylla* and, according to Lovis, Rasbach & Reichstein (1993), this secondary number could have originated via Robertsonian fusion. Regardless of the karyotypic mechanisms leading to the existence of multiple basic chromosome numbers in the genus, our new count ($2n = c.$ 116) confirmed a tetraploid cytotype in this species. Furthermore, it was also demonstrated that gametophytes have n chromosomes, so that it is safe to assume that sporophytes have $2n$. This is not the first time that the presence of tetraploid cytotypes has been reported in this genus [e.g. in *A. lorentzii* (Hieron.) Diels and *A. chaerophylla*; Gastony & Baroutsis, 1975]. Genome size values and DNA ploidy analysis estimated by flow cytometry showed that sporophytes were likely to have originated via gamete fusion and developed naturally under *in vitro* conditions, which is critical for the successful rescue and recovery of species that are on the edge of extinction. Asexual clones have more limited genetic recombination and lower mutation rates, which can lead to a narrowing of genetic diversity in cultivated accessions, reduced capacity to respond to changing environmental conditions and, under certain conditions, increased levels of homozygosity which may uncover genetic abnormalities.

Phylogenetic and phylogeographical remarks on *A. ascensionis*

Previous morphological studies conducted by Tryon & Tryon (1982) in *Anogramma* suggested that the group is polyphyletic, and already indicated a close relationship between *A. ascensionis* and *A. chaerophylla*, which could eventually require their transfer to a new genus. The phylogenetic results presented here agree with this former treatment, and confirm recent findings of G. Gastony, T. Nakazato and G. Yatskievych (unpubl. data) based on plastid and amplified fragment length polymorphism (AFLP) data. Focusing on the lineage that includes *A. ascensionis* and its relatives (Fig. 5, clade 2), a potential long-dispersal colonization event from the Neotropics to Ascension could be invoked to explain such a relationship. Human introduction is extremely unlikely in this case, because of the recent colonization of Ascension by humans. The island was not settled until 1815 and, although a small farm was established on part of Green Mountain a few years later (at first, mainly stocked with European crops), the bulk of the area remained virtually unexplored until Joseph Hooker visited in 1842 (Hart-Davis, 1972). From the number of his collections (more than any other Ascension endemic), it is likely that he already found *A. ascensionis* to be relatively widespread.

Given the young age of Ascension (*c.* 1 Myr) and the lack of sequence divergence from other *Anogramma* spp., the initial colonization would have been a comparatively recent event in evolutionary terms. The nucleotide sequence obtained for the *rbcL* region was identical to that published for one Brazilian accession of *A. chaerophylla* by G. Gastony, T. Nakazato and G. Yatskievych (unpubl. data). This suggests that intraspecific genetic variation requires more study in the genus, and further hypervariable markers should be used to shed light on phylogenetic relationships. *Anogramma chaerophylla* is a morphologically variable species but, generally, *A. ascensionis* differs from it in the consistent small size, the degree and shape of dissection of the pinnules, the paler colour and the only slight differentiation between the earlier sterile leaves and the later fertile leaves. Gametophyte biology has yet to be fully elucidated, and we cannot confirm to what extent the differences are a result of environmental factors. Rapid ecological and morphological differentiation has also been noted within endemic species of *Elaphoglossum* Schott ex J.Sm. from a 'neighbouring' oceanic island, St Helena, where identical sequences were recovered from *E. nervosum* (Bory) Christ and *E. dimorphum* (Hook. & Grev.) T.Moore, even though the pair can be clearly distinguished in appearance and habitat (Eastwood *et al.*, 2004). Indeed, according to the results of Vasco, Moran & Rouhan (2009), *Elaphoglossum* would have also experienced an eastward dispersion from the Neotropics towards St Helena, with subsequent diversification on the island.

The predominant winds and ocean currents have an important role in facilitating west to east colonization

routes from Africa to the mid-Atlantic islands (e.g. Cronk, 1987). In agreement with this, many native and endemic fern species on Ascension Island and St. Helena (e.g. *Marattia purpurascens* deVries and *Asplenium ascensionis* Watson) are believed to have an African evolutionary origin (Murdock, 2008; Lambdon, 2012). However, despite these islands being closer to continental Africa, recent phylogenetic studies have revealed other probable long-dispersal events from the Neotropics in genera such as *Microgramma* C.Presl and *Pleopeltis* Willd. (Janssen, Kreier & Schneider, 2007).

Ecological reconstruction of habitats of *A. ascensionis*

The possible presence of functionally perennial gametophytes makes it difficult to assess the true remaining wild population of *A. ascensionis*. Gametophytes are virtually impossible to locate as they occur in deep crevices and are almost indistinguishable from those of other ferns with the naked eye. However, the extant population is undoubtedly small. There is little habitat left for annual ferns to thrive, and the few suitable locations are under constant threat from invasive species. Compared with wild populations on the Green Mountain cliffs, plants grown in high humidity under glasshouse conditions at RBG Kew were larger, of a deeper green and of good quality, reminiscent of the specimens collected by Hooker in the 19th century. This finding supports the idea that the wild habitats occupied today are suboptimal, on the edge of the natural range of the species, and suggests that the species has been outcompeted in its favoured damper and more shady locations. The ordination analysis of community structure provides further evidence consistent with this hypothesis, and Hooker originally described the habitat as 'wet rocks and banks' (in a note accompanying herbarium specimen K000212932). Habitat restoration and reintroduction programmes can only be effective if correctly informed by ecological observation, although, in the case of a species which has been extremely rare throughout botanical history, this can only be assessed by the painstaking piecing together of the smallest of clues.

If the above inference is correct, extant sites for *A. ascensionis* are probably not particularly suitable target locations, and more extensive effort is needed to clear invasive species from more sheltered habitats on Green Mountain. General restoration guidelines adopted on Ascension Island include: the use of barriers of controllable, dense thicket (even if composed of non-native species) to slow the recolonization rate of aggressive invaders from distant sources; ensuring a high ground cover of native species, to outcompete and suppress seedling establishment of invasive species; and planting restored habitats over as large an area as possible, creating numerical advantages which favour the establishment of natives from an annual seed or spore crop.

On Ascension, where parts of the mountain slopes were probably always covered in a mosaic of bare ground, low vegetation and open fern swards, dense ground cover is unlikely to be appropriate. However, the role of the rich native bryophyte flora needs to be examined in more detail. Not only are bryophyte communities highly endangered in their own right, but they also appear to provide good germination sites for native vascular plants (P. Lambdon, unpubl. data, 2009).

Successful and sustainable habitat restoration is a long-term goal. Thus, *in vitro* culture to ensure the survival of *ex situ* populations is essential in the medium term. Indeed, at RBG Kew, spontaneous germination of spores from some laboratory-grown sporophytes resulted in a second generation within weeks in the same pot. This suggests that, under ideal conditions, natural regeneration will not be a problem.

Implications for the future of rare species conservation

Tropical montane ecosystems, such as cloud forests, are globally important for their biodiversity, wealth of utilizable resources and carbon storage. Ferns represent a significant component of such floras, particularly on remote islands in which the minute, wind-blown spores have permitted much better long-distance colonization than observed for flowering plants (Muñoz *et al*., 2004). Fern preservation is thus important in maintaining the integrity of such ecosystems. Cryopreservation of spores, rather than conventional freezer storage, provides a safer alternative for long-term conservation of fern germplasm (Ballesteros *et al*., 2012). The low representation of pteridophytes in germplasm banks reflects the difficulties of their long-term storage, and more research is greatly needed in this area (Godefroid *et al*., 2011). Cryopreservation of spores or gametophytes, where sufficient genetically diverse material is available, could help the *ex situ* conservation of many ferns that are on the verge of extinction. Other threatened fern species from Ascension and St Helena are now being targeted for cryopreservation at RBG Kew.

Although small, remote islands are critical centres of threatened global biodiversity, they are paradoxically amongst the areas least equipped to deal with urgent conservation problems. A lack of access to training is one common issue. A second problem is that laboratory facilities are often poor or non-existent. In this case,

the rescue would have foundered were it not for the help of the Royal Air Force in ensuring swift passage of fertile material over 1000 miles to the UK. Ideally, the development of on-island facilities to deal with basic micropropagation would be the best answer to the problem, but, in most cases, this goal is unlikely to be realised in the short term.

The *A. ascensionis* project has provided a number of insights into the process of rare species conservation, particularly concerning 'last-ditch' rescue efforts. Limited access to technology, expertise and facilities in biodiversity hotspot areas can contribute substantially to the risk of species loss in critical situations. When global populations of a species are restricted to just a few individuals, intensive and prompt conservation efforts are likely to be required in order to effect a rescue, which is often not possible because of a lack of funds and manpower. Understanding the ecology of critically rare species may be difficult, as the last surviving populations may not be truly representative of the norm: they may be found in suboptimal locations where threats are less intense, and could be suffering from inbreeding effects because of loss of genetic diversity. The logistics of restoring *A. ascensionis* and other ferns to the wild on Green Mountain has highlighted the fact that, although the maintenance of long-term conservation collections in botanic gardens and nurseries is invaluable in preserving some biodiversity, it may become impractical in the longer term. Large numbers of plants often cannot be sustained in cultivation because of a lack of space and resources, and it can be difficult to keep genetically distinct populations separate.

Our experiences suggest that the solutions could include the following. As a rule, it may be more cost-effective to 'bring species back from the brink' before their plight becomes critical, despite a reluctance to spend scarce funds when it is not perceived as immediately essential. The presence of overseas 'centres of excellence', with expertise, capacity and funding to take on emergency conservation projects on behalf of struggling nations, is an invaluable resource in the global struggle to halt biodiversity loss. In the case of very rare species, rapid action may be needed to overcome crisis situations. This is only possible if close partnerships between the hosts and the centres of excellence are already in place, with coordinated funding streams to enable swift, integrated programmes which can tackle propagation and reintroduction in one package. Spore-, gametophyte- and seed-banking measures are vital in helping to preserve genetic diversity. However, they are only one of the necessary tools and should not be seen as a panacea. Plants in the wild, reproducing naturally and adapting to changing environmental conditions where they can be studied in detail, represent the safest conservation option. Rapid return to the wild also avoids excessive long-term reliance on living collections, with its potential problems (e.g. Lauterbach, Burkart & Gemeinholzer, 2012). For species which have been extremely rare for some time, careful analysis of all available information, including historical records, and indirect inference from ecological data may be needed in order to gain a better reflection of the likely optimum ecology. As an example of indirect inference, it could be asked: 'can we deduce anything if the surviving plants are growing in association with other species on the edge of their typical range?'.

These principles apply not just to islands, but also to species which are on the edge of extinction in global biodiversity hotspot countries throughout the world, where much of the threatened biodiversity exists and resources are limited. In the specific case of *A. ascensionis*, there remains a realistic prospect of re-establishing viable wild populations on Ascension Island. Rehabilitation of areas suitable for reintroduction is an important step. Invasive species will be eradicated from potential sites on Green Mountain as part of the preparation. Work is now underway to produce around 1000 sporophytes of *A. ascensionis* for this purpose from freshly collected spores of plants in the wild.

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REFERENCES

Aragon CF, Pangua E. 2004. Spore viability under different storage conditions in four rupicolous *Asplenium* L. taxa. *American Fern Journal* **94:** 28–38.

Ashmole P, Ashmole M. 2000. *St Helena and Ascension Island: a natural history*. Oswestry: Anthony Nelson.

Ballesteros D, Estrelles E, Walters C, Ibars AM. 2012. Effects of temperature and desiccation on *ex situ* conservation of nongreen fern spores. *American Journal of Botany* **99:** 721–729.

Barnicoat H, Cripps R, Kendon J, Sarasan V. 2011. Conservation *in vitro* of rare and threatened ferns – case studies of biodiversity hotspot and island species. *In Vitro Cellular and Developmental Biology – Plant* **47:** 37–45.

Baroustis JG. 1976. *Cytology, morphology and developmental biology of the fern genus* Anogramma. PhD Thesis, Indiana University, Bloomington.

Baroutsis JG, Gastony GJ. 1978. Chromosome numbers in the genus *Anogramma*, II. *American Fern Journal* **68:** 3–6.

Bennett MD, Leitch IJ. 2010. Plant DNA-values database (release 5.0, December 2010). Available at: http://data.kew.org/cvalues/

Bolle C. 1863. Die Standorte der Farrn auf den canarischen Inseln. I. *Zeitschrift für Allgemeine Erdkunde* **14:** 289–334.

Camloh M. 1999. Spore age and sterilization affects germination and early gametophyte development of *Platycerium bifurcatum*. *American Fern Journal* **89:** 124–132.

Cronk QBC. 1980. Extinction and survival in the endemic vascular flora of Ascension Island. *Biological Conservation* **17:** 207–219.

Cronk QCB. 1987. The history of endemic flora of St Helena: a relictual series. *New Phytologist* **105:** 509–520.

Duffey E. 1964. The terrestrial ecology of Ascension Island. *Journal of Applied Ecology* **1:** 219–254.

Eastwood A, Cronk QCB, Vogel JC, Hemp A, Gibby M. 2004. Comparison of molecular and morphological data on St Helena: *Elaphoglossum*. *Plant Systematics and Evolution* **245:** 93–106.

Ebihara A, Ishikawa H, Matsumoto S, Lin SJ, Iwatsuki K, Takamiya N, Watano Y, Ito M. 2005. Nuclear DNA, chloroplast DNA, and ploidy analysis clarified biological complexity of the *Vandenboschia radicans* complex (Hymenophyllaceae) in Japan and adjacent areas. *American Journal of Botany* **92:** 1535–1547.

Ford MV, Fay MF. 1999. Spore-derived axenic cultures of ferns as a method of propagation. In: Hall RD, ed. *Plant cell culture protocols. Methods in molecular biology, Vol. 111*. Totowa: Humana Press, 159–168.

Gastony GJ, Baroutsis JG. 1975. Chromosome numbers in the genus *Anogramma*. *American Fern Journal* **65:** 71–75.

Gill V. 2010. Experts rediscover plant presumed extinct for 60 years. Available at: http://www.bbc.co.uk/news/10402534 [accessed 31 March 2011].

Godefroid S, Rivière S, Waldren S, Boretos N, Eastwood R, Vanderborght T. 2011. To what extent are threatened European plant species conserved in seed banks? *Biological Conservation* **144:** 1494–1498.

Goebel K. 1877. Entwickelungsgeschichte des Prothalliums von *Gymnogramma leptophylla* Desv. (Fortsetzung). *Botanische Zeitung (Berlin)* **35:** 671–711.

Goller K, Rybczynski JJ. 2007. Gametophyte and sporophyte of tree ferns *in vitro* culture. *Acta Societatis Botanicorum Poloniae* **76:** 193–199.

Gray A. 2003. *Anogramma ascensionis*. IUCN Red List of Threatened Species. Version 2010.1. International Union for Conservation of Nature. Available at: http://www.iucnredlist.org [accessed 30 May 2010].

Gray A, Gardner S, Kirk L, Robinson P, Smolka Z, Webster L. 2008. The status and distribution of the endemic vascular flora of Ascension Island. Undergraduate report, University of Edinburgh.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41:** 95–98.

Hart-Davis D. 1972. *Ascension. The story of a South Atlantic Island*. London: Constable & Co. Ltd.

Hoagland DR, Arnon DI. 1938. *The water-culture method for growing plants without soil*. Berkeley, CA: University of California College of Agriculture Experimental Station, 347–353.

Janssen T, Kreier H, Schneider H. 2007. Origin and diversification of African ferns with special emphasis on Polypodiaceae. *Brittonia* **59:** 159–181.

Lambdon P. 2012. *Flowering plants and ferns of St Helena*. Newbury: Pisces Publications.

Lambdon PW, Darlow A. 2008. Botanical survey of Ascension Island and St Helena, 2008. A report on the current state of plant invasions, and their implications for conservation and management. Unpublished report, Royal Society for the Protection of Birds, Sandy, Bedfordshire.

Lambdon PW, Stroud S, Gray A, Niissalo M, Renshaw O, Sarasan V. 2010. *Anogramma ascensionis*. IUCN Red List of Threatened Species. Version 2010.4. International Union for Conservation of Nature. Available at: http://www.iucnredlist.org/apps/redlist/details/43919 [accessed 31 March 2011].

Lauterbach D, Burkart M, Gemeinholzer B. 2012. Rapid genetic differentiation between *ex situ* and their *in situ* source populations: an example of the endangered *Silene otites* (Caryophyllaceae). *Botanical Journal of the Linnean Society* **168:** 64–75.

Lovis JD, Rasbach H, Reichstein T. 1993. The chromosome number of *Anogramma leptophylla* (Adiantaceae, Pteridophyta) from New Zealand & South Africa. *Fern Gazette* **14:** 149–154.

Muñoz J, Felicisimo AM, Cabezas F, Burgaz AR, Martinez I. 2004. Wind as a long-distance dispersal vehicle in the Southern Hemisphere. *Science* **304:** 1144–1147.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue culture. *Physiologia Plantarum* **15:** 473–497.

Murdock AG. 2008. Phylogeny of marattioid ferns (Marattiaceae): inferring a root in the absence of a closely related outgroup. *American Journal of Botany* **95:** 626–641.

Nakazato T, Gastony J. 2003. Molecular phylogenetics of *Anogramma* species and related genera (Pteridaceae: Taenitidoideae). *Systematic Botany* **28:** 490–502.

Nayar BK, Kaur S. 1969. Types of prothallial development in homosporous ferns. *Phytomorphology* **19:** 179–188.

Nayar BK, Kaur S. 1971. Gametophytes of homosporous ferns. *Botanical Review* **37:** 295–396.

Nylander JAA. 2004. *MrModeltest v2. Program distributed by the author*. Uppsala: Evolutionary Biology Centre, Uppsala University.

Obermayer R, Leitch IJ, Hanson L, Bennett MD. 2002. Nuclear DNA C-values in 30 species double the familial representation in Pteridophytes. *Annals of Botany* **90:** 209–217.

Pangua E, Pérez-Ruzafa I, Pajarón S. 2011. Gametophyte features in a peculiar annual fern, *Anogramma leptophylla*. *Annales Botanici Fennici* **48:** 465–472.

Pellicer J, Fay MF, Leitch IJ. 2010. The largest eukaryotic genome of them all? *Botanical Journal of the Linnean Society* **164:** 10–15.

Pence VC. 2008. Cryopreservation of bryophytes and ferns. In: Reed BM, ed. *Plant cryopreservation: a practical guide*. New York: Springer, 117–140.

Rasbach H, Reichstein T. 1990. The chromosome number of *Anogramma leptophylla* (Adiantaceae Pteridophyta) from Europe. *Fern Gazette* **13:** 341–348.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Sarasan V, Cripps R, Ramsay MM, Atherton C, McMichen M, Prendergast G, Rowntree JK. 2006. Conservation *in vitro* of threatened plants – progress in the past decade. *In Vitro Cellular and Developmental Biology – Plant* **42:** 206–214.

Simabukuro EA, Dyer AF, Felippe GM. 1998. The effect of sterilization and storage conditions on the viability of the spores of *Cyathea delgadii*. *American Fern Journal* **88:** 72–80.

Thakur RC, Hosoi Y, Ishii K. 1998. Rapid *in vitro* propagation of *Matteuccia struthiopteris* (L.) Todaro – an edible fern. *Plant Cell Reports* **18:** 203–208.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25:** 4876–4882.

Tryon RM, Tryon AF. 1982. *Ferns and allied plants with special reference to tropical America*. New York: Springer-Verlag.

Vasco A, Moran RC, Rouhan G. 2009. Circumscription and phylogeny of the *Elaphoglossum ciliatum* group (*E.* sect. *Lepidoglossa*, Dryopteridaceae) based on cpDNA sequences. *Taxon* **58:** 825–834.

Wilkinson DM. 2004. The parable of Green Mountain: Ascension Island, ecosystem construction and ecological fitting. *Journal of Biogeography* **31:** 1–4.

APPENDIX

Full community composition of the original *Anogramma ascensionis* rediscovery site. Data are means of three 1-m² quadrats

Physical parameters of site	
Slope (deg)	75
Aspect	SW
Bare ground (%)	85
Mean herb height (mm)	50
Litter	None

Species list

Major group	Family	Species	Cover (%)
Lichens	Teloschistaceae	*Teloschistes flavicans* (Sw.) Norman	1
Lichens	Parmeliaceae	sp.	5
Lichens		Other	10
Marchantiopsida	Aytoniaceae	*Plagiochasma rupestre*† Lehm. & Lindenb.	10
Marchantiopsida	Fossombroniaceae	*Fossombronia* sp.†	10
Bryopsida	Calymperaceae	*Calymperes erosum* C.Müll.	5
Bryopsida	Dicraniaceae	*Campylopus* sp. (aff. *pilifer* Brid.)*	5
Bryopsida	Bryaceae	*Splachnobryum gracile*† Broth. & Paris	1
Bryopsida	Meesiaceae	*Leptobryum pyriforme* Wilson*	5
Pteridophyta	Adiantaceae	*Adiantum capillus-veneris* L.	5
Pteridophyta	Psilotaceae	*Psilotum nudum* (L.) P.Beauv.	+
Pteridophyta	Thelypteridaceae	*Christella dentata* (Forssk.) Brownsey & Jermy	1
Eudicots	Apiaceae	*Centella asiatica* (L.) Urb.	+
Eudicots	Asteraceae	*Ageratum conyzoides* L.	1
Eudicots	Asteraceae	*Gnaphalium purpureum* L.	+
Eudicots	Begoniaceae	*Begonia hirtella* Link	1
Eudicots	Melastomataceae	*Clidemia hirta* D.Don	1
Eudicots	Myrtaceae	*Psidium guajava* L.	+
Eudicots	Oxalidaceae	*Oxalis corniculata* L.	5
Eudicots	Rosaceae	*Rubus rosifolius* Sm.	1
Eudicots	Plantaginaceae	*Veronica javanica* Blume	1
Eudicots	Verbenaceae	*Lantana camara* L.	+
Monocots	Cyperaceae	*Cyperus polystachyos* Rottb.	+
Monocots	Poaceae	*Digitaria ciliaris* (Retz.) Koeler	+
Monocots	Poaceae	*Polypogon tenuis*† Brongn.	+
Monocots	Poaceae	*Paspalum scrobiculatum* L.	1
Monocots	Poaceae	*Sporobolus africanus* (Poir.) A.Robyns & Tournay	1

*Identification tentative.

†Species likely to be particularly associated with microhabitats suitable for *A. ascensionis*. As so few independent sample points were available, this assessment is not based on numerical analysis.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Mitotic chromosomes from sporophyte of *Anogramma ascensionis* ($2n = c.$ 116). Scale bar, 10 μm.