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# Characterization of ten polymorphic microsatellite loci for the threatened species *Grimmia curviseta* Bouman (Grimmiaceae, Musci)

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*Grimmia curviseta* Bouman is a moss species endemic to the Canary Islands in the North Atlantic Ocean, which has a very restricted distribution across the summit areas of Tenerife and La Palma islands. Using massive sequencing, we developed ten polymorphic microsatellite markers for this species. The pattern of microsatellite alleles per locus provides preliminary evidence that *G. curviseta* is allodiploid. The average number of alleles per locus ranged from 2 to 7, and observed heterozygosities varied from 0.022 to 1.000. A significant genetic differentiation was observed between the Tenerife and La Palma populations, suggesting that there are some limitations to dispersal. This set of microsatellites constitutes a valuable tool to investigate patterns of genetic diversity across the distribution range of *G. curviseta*, information that may eventually be used to establish conservation strategies for the species. Owing to the cross-amplification with the closely related species *Grimmia montana* Bruch & Schimp., our study also demonstrates the utility of these markers for population-level genetic analyses in the genus *Grimmia*.

**Keywords:** Canary Islands, Conservation biology, Endemism, Genetic variation, Microsatellite

## Introduction

Oceanic islands are ideal natural laboratories for the study of ecology and evolution because of their geographical isolation, large numbers and relatively limited geological ages, and because they are initially populated by species that have dispersed from elsewhere (Whittaker & Fernández-Palacios, 2007). Despite their relative small areas and strong geographic isolation, oceanic islands are typically known for their high percentage of endemic species but only moderate levels of species richness (Whittaker & Fernández-Palacios, 2007; but see Patiño *et al.*, 2015), prompting the question of their relative conservation value (Vanderpoorten *et al.*, 2011). However, bryophytes on oceanic islands exhibit one of the lowest levels of endemism among land plant groups, which has been related to their high long-distance dispersal capabilities and niche conservatism (Patiño *et al.*, 2014). Endangered bryophyte species restricted to a single archipelago or even to a few islands should, thus, be featured prominently in global and regional conservation policies (Olsson *et al.*, 2009; González-Mancebo *et al.*, 2011; Patiño *et al.*, 2013).

The endangered moss species *Grimmia curviseta* Bouman constitutes a rare high mountain species restricted to the oceanic islands of Tenerife and La Palma (Canary Islands). Recent surveys showed that *G. curviseta* seems to have declined across its distribution range, particularly on La Palma, remaining as Vulnerable on the Spanish Atlas of Threatened Bryophytes (González-Mancebo *et al.*, 2011). A conservation program recently initiated to protect the species (Garilleti & Albertos, 2012) includes the evaluation of genetic variation across its distribution range.

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Such information is essential for the future management of this endangered species, including the potential re-introduction of native genotypes into the depauperate populations of La Palma. Given that molecular tools for addressing such population-level conservation issues remain scarce in bryophytes, here we aimed to discover microsatellite loci to achieve this objective.

Microsatellites are codominant and selectively neutral markers with high levels of polymorphism and are one of the most reliable molecular tools in population genetic studies. Both the identification and development of microsatellite markers represent significant challenges (Santana *et al.*, 2009) and microsatellite libraries in bryophytes have only been developed occasionally (e.g. Leonardia *et al.*, 2006; Hutsemékers *et al.*, 2008; Kophimai *et al.*, 2013) or in particular genera such as *Sphagnum* (Karlin *et al.*, 2010 and references therein). In the present study, we describe the methodology used to obtain microsatellite markers for *G. curviseta* by using massive sequencing (Allentoft *et al.*, 2008; Bentley *et al.*, 2008; Santana *et al.*, 2009). By using microsatellite-enriched genomic DNA, massive sequencing allows the possibility of investigating hundreds of gigabases in a single sequencing run, resulting in a very cost-effective tool to identify hypervariable microsatellites for studying populations of endangered (non-model) organisms (Ekblom & Galindo, 2011; Yu *et al.*, 2011). Finally, following previous studies (e.g. Karlin *et al.*, 2009, 2010; Kophimai *et al.*, 2013), we used the designed microsatellites as proxies to predict the ploidy level of *G. curviseta*'s gametophytes.

## Materials and Methods

Forty-eight samples of *Grimmia curviseta* were collected across the distribution range on Tenerife and La Palma islands in 2012, between 2140 and 2950 m.a.s.l. A total of 15 localities were studied across the distribution area. A few shoots were sampled from two to four cushions per locality, which were separated by a minimum distance of 1 m. Samples were stored in silica gel and total DNA was extracted from the green tissues of shoots of each sample after removing the sediment particles and dead tissues. The extraction of genomic DNA was carried out using the SP Plant DNA kit (OMEGA bio-tek), following the manufacturer's instructions. DNA was finally eluted in 200 µl elution buffer from extraction kit. The final concentration of the DNA dilutions was quantified with a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, USA).

We employed a next generation sequencing (NGS) approach to design microsatellite sequences starting from 20 µl DNA (300 ng/µl) of one single specimen from Tenerife (sample 12C; see Table S1, Supplementary Material). DNA integrity was first assessed by electrophoresis through 1% agarose gel containing RealSafe (1:20,000) (REAL, Durviz s.l.), and then 2 µl DNA was subjected to enzymatic digestion using the Ion Shear™ Reagent Kit (Life Technologies, CA, USA) at 37°C for 15 min. Fragmented DNA was purified with Agencourt® AMPure® XP Reagent kit (Beckman Coulter) and adapters (A and P1) were ligated using Ion Xpress™ Fragment Library Kit (Life Technologies CA, USA), in accordance with the manufacturer's instructions. After ligation, 150–210 bp fragments were selected and, then, purified with Expin™ PCR SV kit (GeneAll) under the manufacturer's recommendations.

Both Platinum® PCR SuperMix High Fidelity and Library Amplification Primer Mix from the Ion Xpress™ Fragment Library Kit (Life Technologies CA, USA) were used to amplify the library. Cycling conditions were 15 s at 95°C, 15 s at 58°C and 1 min at 72°C during 9 cycles. Amplification products were valued at Qubit® 2.0 fluorometer (Life Technologies, CA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA) using the Agilent High Sensitivity DNA kit (Agilent Technologies, USA).

Final concentration of DNA was 23 pM. Emulsion PCR carried out using 5 µl of eluted library with Ion One Touch™ Template kit (Life Technologies, CA, USA) and further enriched with the same kit. Efficiency of enrichment was valued following manufacturer's recommendations. Sequencing was carried out on a PGM (Personal Genome Machine, Life Technologies CA, USA) using both an Ion Sequencing kit and Ion 314 Chip kit (Life Technologies CA, USA).

The NGS reads were de novo assembled using SeqMan NGen (version 4.1.0; DNAStar, Madison, WI), with the default settings. Subsequently, search for microsatellites in the contigs obtained was carried out with SciRoKo 3.4 (Kofler *et al.*, 2007). Ten microsatellites were selected to analyze patterns of microsatellite alleles in *G. curviseta*, which had to fulfill the following criteria: (a) to have suitable flanking regions to design primers; (b) to have a repetition number of the motif higher than 6 bp; and (c) to have amplification products with a size ranging from 100 to 250 bp. Primer design was performed with Primer3 v. 4.0.0 software (http://primer3.ut.ee/), whereas we used Mfold software (Zuker, 2003) to check possible secondary structures on microsatellite fragments.

PCR reactions on the 48 specimens of *G. curviseta* (see Table S1, Supplementary Material) were carried in 20 µl of genomic DNA using 150 µM each dNTP, 0.2 µM each primer, 40 ng of DNA, 0.6 M of bovine

serum albumin (BSA) and 1U of Green Taq DNA polymerase (GenScript). In all cases the forward primer was labeled with VIC or FAM fluorescent dyes. The PCR reactions were carried out in a T100™ Thermal Cycler (BioRad) with the following thermal touchdown protocol: one cycle at 94°C for 2 min, followed by 40 cycles at 94°C for 20 s, 50–60°C for 20 s, 72°C for 30s and a final step of 10 min at 72°C. PCR products were confirmed by electrophoresis in 1.7% agarose gels containing RealSafe (1:20,000) (REAL, Durviz s.l) and visualized at Molecular Imager® ChemiDoc$^{TM}$ XRS+ Imaging System (BIO-RAD) using Image Lab$^{TM}$ v. 4.0 software (BIO-RAD).

Cervus (Marshall *et al.*, 1998) was used to calculate allele frequencies, expected and observed heterozygosity, polymorphic information content (PIC), non-exclusion probability (NE-I) and Hardy–Weinberg equilibrium for each locus. Wright's Fixation index (FST) value was estimated using Arlequin version 3.5 (Excoffier *et al.*, 2005) and the FST significance was obtained after 10,000 random permutations. Finally, we used Micro-Checker version 2.2.3 (Van Oosterhout *et al.*, 2004) to test for null alleles.

To check whether the new markers described amplify in different species of the same genus, we tested them on 48 specimens of *Grimmia montana* Bruch & Schimp (see Table S1, Supplementary Material) collected across 13 localities on both Tenerife and La Palma. This native species is from comparable habitats in the high mountain areas of Gran Canaria, Tenerife and La Palma islands and is a relatively closely related species (phylogenetically) to *G. curviseta* (Hernández-Maqueda *et al.*, 2008; Rodríguez-Romero *et al.*, unpublished data). Sequence data are deposited on GenBank under accession n° LN998999-LN999008 (see Table 2).

## Results and Discussion

Massive sequencing produced a total of 635 Mbp of sequence data and 3,720,851 total reads in the final library (73% of usable sequences), which contained 535,273 assembled contigs for *Grimmia curviseta*. Mean read length was 170 bp. From these, only 36,851 contigs of the total assembled number contained perfect microsatellite sequences. Dinucleotide repeats were 5.3 times commoner than tri-repeats. Tetra-, penta- and hexanucleotide repeats set corresponds to 1.5% of the total number of repeats. GC content was higher in di- and tri-repeats than tetra- and penta-repeats. The most frequent dinucleotide was $[CG]_n$ (143.76/Mbp), followed by $[AG]_n$ (31.4/Mbp), $[AC]_n$ (22.15/Mbp) and $[AT]_n$ (14.31/Mbp). Of the trinucleotide repeats, [CCG] was the most frequent (11.68/Mbp). Most other repeat motifs appeared at much lower frequencies (Table 1). These findings suggest that the NGS method employed here is fast, simple and eliminates a number of technical difficulties, compared to the traditional 'hybrid capture method' (Ostrander *et al.*, 1992). While traditional microsatellite development requires the development of a number of libraries and probes for each type of repeat, massive sequencing efficiently searches and detects all kinds of repeats taking advantage of specific bioinformatic tools (Kofler *et al.*, 2007).

Following the first search step, the amplification of ten microsatellites in all the specimens of *G. curviseta* was positive and specific. We analyzed the genetic variation for these ten loci across 48 specimens of *G. curviseta* covering its distribution range (Table 2). These ten microsatellites showed polymorphism across *G. curviseta*'s geographic distribution and were at Hardy–Weinberg equilibrium, except *CUR5* and *CUR13* that showed homozygous excess, and *CUR15* that had an excess of heterozygous. Although *CUR5* and *CUR13* showed evidence of null alleles, this result could not be relevant as bisexual species, like *G. curviseta*, are potentially capable of intragametophytic selfing (i.e. merging of gametes produced by gametophytic shoots grown from the same protonema, and hence, originating from the same spore).

After analyzing the pattern of microsatellite alleles of each sample, we observed that each specimen had one or two alleles at a given locus, represented by one or two high (strongly) intense fluorescent peaks, respectively. Indeed, 3 out 10 loci had two alleles per locus per specimen. In the cases where we detected two peaks, the distance between them was a multiple of the microsatellite repeat motif (results not shown). No specimen showed single peaks for all microsatellites, and the number of alleles per locus ranged from 2 to 7 (Table 2). Thus, the observed pattern of microsatellite alleles indicates that *G. cursiveta* has diploid gametophytes. Indeed, near or total fixation for two alleles per locus per individual has been long considered evidence of allopolyploidy, although not without limitations (for review see Shaw, 2009). Consequently, the observed heterozygosities across loci ranged from 0.043 to 0.957, with heterozygosity appearing to be fixed or nearly fixed in one out of ten markers (Table 2). Although the levels of fixed heterozygosity observed in *G. curviseta* are relatively low compared to the levels detected in other species (Karlin *et al.*, 2009, 2010; Kophimai *et al.*, 2013), we hypothesize that this Canarian endemic moss might have an allopolyploid origin, as has been previously suggested for other species based on fixed heterozygous patterns in isozyme (e.g. Wyatt *et al.*, 1988) and microsatellite expression (e.g. Karlin *et al.*, 2010). Despite the fact that the present study does not allow us to formally identify the putative ancestors of *G. curviseta*, a strong correspondence in leaf shape, cell

**Table 1 Complete list of microsatellites detected using massive sequencing and motif statistics. Contain standardized motif (Motif), total counts (Counts), the average length of microsatellites having the corresponding motif (Average length), average counts per million base pairs of simple sequence repeats having the corresponding motif (Counts/Mbp) and standard deviation of the microsatellite length (StdDeviation AverageLength).**

| Motif | Counts | Average length | Counts/Mbp | StdDeviation AverageLength |
|---|---|---|---|---|
| CG | 20721 | 8.49 | 143.76 | 0.84 |
| AG | 4526 | 8.71 | 31.40 | 1.91 |
| AC | 3192 | 8.74 | 22.15 | 2.05 |
| AT | 2062 | 8.65 | 14.31 | 1.60 |
| CCG | 1684 | 12.83 | 11.68 | 1.36 |
| AGC | 1121 | 13.18 | 7.78 | 2.74 |
| ACG | 868 | 13.02 | 6.02 | 2.22 |
| AAG | 597 | 13.48 | 4.14 | 3.15 |
| ACC | 445 | 12.95 | 3.09 | 1.67 |
| ATC | 337 | 13.50 | 2.34 | 5.12 |
| AGG | 285 | 12.94 | 1.98 | 1.27 |
| AAC | 245 | 14.73 | 1.70 | 7.24 |
| AAT | 151 | 14.28 | 1.05 | 9.12 |
| AAAG | 64 | 21.98 | 0.44 | 11.82 |
| AAAT | 53 | 21.75 | 0.37 | 13.01 |
| ACGG | 51 | 19.69 | 0.35 | 4.35 |
| ACT | 47 | 12.7 | 0.33 | 1.24 |
| AAAC | 38 | 28.42 | 0.26 | 20.71 |
| AAAAT | 25 | 22.44 | 0.17 | 3.02 |
| AACC | 17 | 18.71 | 0.12 | 5.13 |
| AAAAAT | 16 | 26.56 | 0.11 | 3.10 |
| AATC | 13 | 19.38 | 0.09 | 2.76 |
| AATG | 11 | 32.82 | 0.08 | 18.86 |
| ATAC | 10 | 21.40 | 0.07 | 5.83 |
| AACTC | 10 | 34.70 | 0.07 | 19.07 |
| ATCGCC | 9 | 29.33 | 0.06 | 3.68 |
| AGCG | 8 | 18.75 | 0.06 | 2.54 |
| AGGC | 6 | 17.33 | 0.04 | 1.49 |
| AAAAG | 6 | 23.33 | 0.04 | 3.14 |
| AACG | 6 | 22.83 | 0.04 | 8.82 |
| AAAAC | 5 | 21.20 | 0.03 | 0.75 |
| AAGAG | 5 | 23.80 | 0.03 | 3.49 |
| CCCG | 5 | 16.40 | 0.03 | 0.80 |
| AAGC | 5 | 26.40 | 0.03 | 12.86 |
| ACCG | 5 | 20.00 | 0.03 | 5.62 |
| ACTC | 4 | 24.50 | 0.03 | 11.93 |
| ATAG | 4 | 38.50 | 0.03 | 28.18 |
| ACGC | 4 | 21.00 | 0.03 | 6.44 |
| AAAGG | 3 | 22.67 | 0.02 | 1.70 |
| ACGAGC | 3 | 32.00 | 0.02 | 2.16 |
| ACCACG | 3 | 30.33 | 0.02 | 3.09 |
| AGAGC | 3 | 22.00 | 0.02 | 1.63 |
| ATGC | 3 | 18.00 | 0.02 | 2.16 |
| ACGGGC | 3 | 28.67 | 0.02 | 3.30 |
| ACACCG | 3 | 34.00 | 0.02 | 1.63 |
| AACGGC | 3 | 27.00 | 0.02 | 3.56 |
| AAGG | 3 | 31.67 | 0.02 | 20.07 |
| ACCTC | 3 | 26.33 | 0.02 | 1.25 |
| AATT | 3 | 16.00 | 0.02 | 0.00 |
| CCGCG | 3 | 20.00 | 0.02 | 0.00 |

shape in leaf base and costal architecture (Bouman, 1991; Maier, 2010), points to *G. orbicularis* Bruch *ex* Wilson as one of the progenitors. Further population genetic and phylogenetic research is essential to infer the evolutionary origin of *G. curviseta*, specifically whether its polyploid gametophytes are of autoploid or alloploid origin.

The *CUR5*, *CUR13* and *CUR15* loci were highly informative (PIC ≥ 0.5; Table 2), whereas *CUR6*, *CUR11* and *CUR20* were only moderately informative (PIC = 0.5–0.25). Average non-exclusion probability for the identity of two unrelated individuals ranged between 0.166 (*CUR15*) and 0.891 (*CUR18*). The analysis of molecular variance showed that 88% of total variation accounted for individuals within populations and 12% of the variation was among populations from Tenerife and La Palma ($F_{ST} = 0.1209$; $P < 0.001$), pointing to a significant differentiation among populations. These findings show that the microsatellite loci identified here display the appropriate level of variation for further demographic studies.

The test of cross-amplification with *Grimmia montana* resulted in five amplified polymorphic loci (*CUR5*, *CUR7*, *CUR15*, *CUR19*, *CUR20*). *Grimmia*

**Table 2 Characterization of ten polymorphic microsatellites for *Grimmia curviseta*.**

| Locus | Primer sequences 5′–3′ | Repeat motif | Length | Accession n° | $T_a$ °C | k | Size range (bp) | Ho | He | PIC | NE-I | HW |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CUR5 | *F: ACTTCTCCAAAGCGGTACGA<br>R: CGTTAGGATTGTTTTCCACGA | $(GTCG)^7$ | 184 | LN998999 | 50 | 5 | 160–184 | 0.114 | 0.512 | 0.466 | 0.284 | NS |
| CUR6 | *F: TTTTCTCCTCCCCGCTATGT<br>R: ATCAAGGCCCGCTAGTGAAG | $(GTCC)^6$ | 176 | LN999000 | 60 | 5 | 176–196 | 0.300 | 0.446 | 0.403 | 0.350 | NS |
| CUR7 | $^+$F ATTCCCCGCACCTCTGAC<br>R: CAGCCGAGTCACTGATCCTT | $(CGCT)^7$ | 178 | LN999001 | 52 | 2 | 146–178 | 0.043 | 0.043 | 0.042 | 0.918 | NS |
| CUR11 | *F: ACCCTAACTGTGATCGAATGCA<br>R: GGTCCGCTTCCAAATCTCTT | $(CA)^{11}$ | 164 | LN999002 | 53 | 4 | 154–164 | 0.271 | 0.433 | 0.387 | 0.369 | NS |
| CUR13 | $^+$F: AGCTCCAAGCGAGAGAAAGG<br>R TTTGTTGTCTTCGCAGATCG | $(GA)^{15}$ | 195 | LN999003 | 55 | 4 | 187–199 | 0.184 | 0.621 | 0.534 | 0.229 | * |
| CUR15 | $^+$F: CACACTAATAGCCGCTGCAC<br>R: AATGATCTTCGTTCCTGTTTGC | $(CA)^{13}$ | 168 | LN999004 | 50 | 6 | 156–168 | 0.957 | 0.691 | 0.641 | 0.143 | * |
| CUR17 | *F: AGATGAGGTCTTGGGGAGGT<br>R: AGGCGTCTTCAGCCTTAGAA | $(AG)^9$ | 227 | LN999005 | 52 | 2 | 200–220 | 0.149 | 0.175 | 0.158 | 0.699 | NS |
| CUR18 | *F: TCTCTGTTGACACCCACTTCC<br>R: TCTGACTTCATCGCCACAAC | $(TC)^{12}$ | 201 | LN999006 | 52 | 7 | 187–209 | 0.362 | 0.691 | 0.637 | 0.146 | * |
| CUR19 | *F ACACTCCACCCCAGAGAAAG<br>R: CTCACTCCACCAACAAAGCA | $(GA)^9$ | 187 | LN999007 | 56 | 7 | 187–189 | 0.130 | 0.240 | 0.228 | 0.591 | NS |
| CUR20 | $^+$F: ATCGGACGTTGAAGAAGTGG<br>R: CGCGTTGAAATAGCAATCTG | $(AG)^9$ | 222 | LN999008 | 52 | 2 | 218–222 | 0.396 | 0.321 | 0.267 | 0.516 | NS |

Notes: ($^+$) Primers 5′-labelled with VIC, (*) 6-FAM-labelled primers. Number of alleles per locus (k); observed (Ho) and expected (He) heterozygosity; polymorphic information content (PIC); average non-exclusion probability for identity of two unrelated individuals (NE-I); significance of deviation from Hardy–Weinberg equilibrium (HW; * = $p < 0.001$, NS = not significant).

**Table 3 Characterization of five polymorphic microsatellites for *Grimmia montana* designed from *G. curviseta*.**

| Locus | Primer sequences 5′–3′ | Repeat motif | Accession n° | $T_a$ °C | K | Size range (bp) | He | PIC | NE-I |
|---|---|---|---|---|---|---|---|---|---|
| CUR5 | F: ACTTCTCCAAAGCGGTACGA<br>R: CGTTAGGATTGTTTTCCACGA | (GTCG)7 | LN998999 | 50 | 3 | 164–180 | 0.082 | 0.079 | 0.847 |
| CUR7 | F ATTCCCCGCACCTCTGAC<br>R: CAGCCGAGTCACTGATCCTT | (CGCT)7 | LN999001 | 52 | 3 | 181–203 | 0.120 | 0.115 | 0.780 |
| CUR15 | F: CACACTAATAGCCGCTGCAC<br>R: AATGATCTTCGTTCCTGTTTGC | (CA)13 | LN999004 | 52 | 17 | 155–187 | 0.910 | 0.891 | 0.018 |
| CUR19 | F ACACTCCACCCCAGAGAAAG<br>R: CTCACTCCACCAACAAAGCA | (GA)9 | LN999007 | 52 | 12 | 195–227 | 0.631 | 0.608 | 0.157 |
| CUR20 | F: ATCGGACGTTGAAGAAGTGG<br>R: CGCGTTGAAATAGCAATCTG | (AG)9 | LN999008 | 52 | 2 | 205–215 | 0.041 | 0.040 | 0.921 |

Notes: Number of alleles per locus (k); expected (He) heterozygosity; polymorphic information content (PIC); average non-exclusion probability for identity of two unrelated individuals (NE-I). All forward primers were FAM-labeled.

*montana* showed a single peak per specimen for the five selected microsatellites, confirming the haploid level proposed, based on chromosome counts ($n = 13$; Anderson & Crum, 1958; Fritsch, 1991). The number of alleles per locus ranged from 3 to 17. *CUR15* and *CUR19* loci were highly informative (PIC $\geq 0.5$), whereas *CUR5*, *CUR7* and *CUR20* were found to be uninformative (PIC $< 0.25$). Average non-exclusion probability for the identity of two unrelated individuals ranged from 0.018 (*CUR15*) to 0.921 (*CUR20*) (Table 3).

Maintaining the genetic diversity of natural populations is important in conservation because it strongly determines the ability of taxa to adapt to changing environments. Our study presents ten polymorphic microsatellite markers designed *de novo* from *G. curviseta*, which will permit detection of the amount of genetic variation necessary to reinforce the depauperate populations (i.e. low expected effective population size) from La Palma through the re-introduction of appropriate local genotypes. In addition, these new markers are available for future population-level studies of genetic diversity, historical demography and conservation biology of not only *G. curviseta* but also relatively closely related species. Furthermore, our findings reinforce the idea that massive sequencing (Allentoft *et al.*, 2008; Santana *et al.*, 2009; Ekblom & Galindo, 2011) permits the rapid generation of a huge amount of sequence data in a very cost-effective way, being potentially applicable to other threatened plant species on islands which need urgent conservation policies to prevent their extinction.

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