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Standard Paper

New microsatellite loci to quantify genetic diversity of the photosymbiodeme-forming lichen *Sticta canariensis*

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Abstract

Sticta canariensis is a lichen which is rare in all parts of its range in Atlantic Europe and Macaronesia, where it occurs in laurisilva forests, a habitat highly threatened by global change. Thus, this species is of high priority for inclusion in conservation programmes where genetic diversity should be considered. We have established new microsatellite loci and generated a dataset that demonstrates the genetic diversity of the lichen-forming fungus *S. canariensis* from eight locations across its disjunct range, in Macaronesia, Norway and England. We genotyped 25 microsatellite loci for 65 specimens and detected five genetic clusters which resemble major geographical divisions, specifically among the Macaronesian archipelagos. The total number of observed alleles ranged from 2 to 22. These are the first microsatellite markers developed for *S. canariensis* and they will be useful for population genetic studies and for conservation assessments.

Keywords: England; genetic diversity; Macaronesia; Norway; *Peltigeraceae*; population structure

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Introduction

Due to their high polymorphism and Mendelian inheritance, microsatellites are a useful marker type for population genetic studies (Schlötterer 2000; Abdelkrim *et al.* 2009) and are an inexpensive alternative to whole genome sequencing. Microsatellite markers are also a widely used tool for fungal genetic studies, specifically for plant pathogens, mycorrhizal fungi and lichens (Tenzer *et al.* 1999; Werth 2010; Minasiewicz *et al.* 2022; Velasco-Anacona *et al.* 2022). In lichens they are highly species-specific and have proved useful for assessing genetic variation within species at different spatial scales (Walser *et al.* 2003; Werth 2010). For example, microsatellites have been used in studies of the lichen *Lobaria pulmonaria* (L.) Hoffm. at all levels: across 27 European countries (Widmer *et al.* 2012), at the regional level within Europe (Scheidegger *et al.* 2012), between continents and from a single tree (Walser *et al.* 2003), to name a few. There are a limited number of studies investigating the relatedness of Macaronesian lichen populations. For example, Alors *et al.* (2017) found populations of *Parmelina carporrhizans* (Taylor) Poelt & Vezda to be highly diverse but lacking spatial structure. Conversely, Werth *et al.* (2021) found substantial divergence between geographical regions in populations of three *Lobaria* species. Data on additional species are needed to better understand the genetic relationships between lichen populations on the Macaronesian islands and elsewhere in Europe.

Sticta (Schreb.) Ach. is a monophyletic fungal genus belonging to *Lobarioideae* (*Peltigeraceae*, *Peltigerales*, *Lecanoromycetes*, *Ascomycota*) (Miadlikowska & Lutzoni 2004; Miadlikowska *et al.* 2014; Lumbsch & Leavitt 2019). *Sticta* species have colonized every continent except Antarctica, as well as islands throughout the world, indicating that species of this genus are capable of long-distance dispersal (LDD), a potential driver of speciation (Widhelm *et al.* 2018).

Here, we study the charismatic lichen-forming fungus *Sticta canariensis* (Bory) Delise, a foliose macrolichen with the unusual ability to form photosymbiodemes, that is to form symbioses with photosynthetic partners belonging to two different kingdoms (Magain & Sérusiaux 2015). In the case of *Sticta canariensis* (Fig. 1), the partners are unicellular green algae (‘*Symbiochloris* sp. 3′, *Trebouxiales*) (Dal Grande *et al.* 2014; Škaloud *et al.* 2016) and cyanobacteria (*Nostoc*) (Magain & Sérusiaux 2015). Association with these different partners results in dramatically different morphologies and reproductive strategies in *S. canariensis*, as similarly reported for other photosymbiodeme-forming lichens (Armaleo & Clerc 1991).

The bright green chloromorph has elongate and dichotomously branched lobes, 5–15 mm in diameter, forming thalli 5–15 cm in diameter. Apothecia are brown, 1–2 mm, commonly forming both laminally and marginally. The chloromorph grows on deciduous trees (e.g. *Laurus*, *Fraxinus*, *Quercus*) (A. Goss and S. Werth, personal observations).

The cyanomorph is dark blue-grey, often with white mottling. Lobes, 1–2 cm in diameter, are overlapping, irregularly incised with phyllidiate margins. Phyllidia, 0.5–0.8 mm, are flattened, ascending, dichotomously and several times branched, forming dense clusters at thallus margins, becoming laminal in older individuals. Thalli, up to 10 cm diameter, often form extensive colonies. The cyanomorph also grows on deciduous trees, but additionally on the soil and rocks of cliff sides and boulders in

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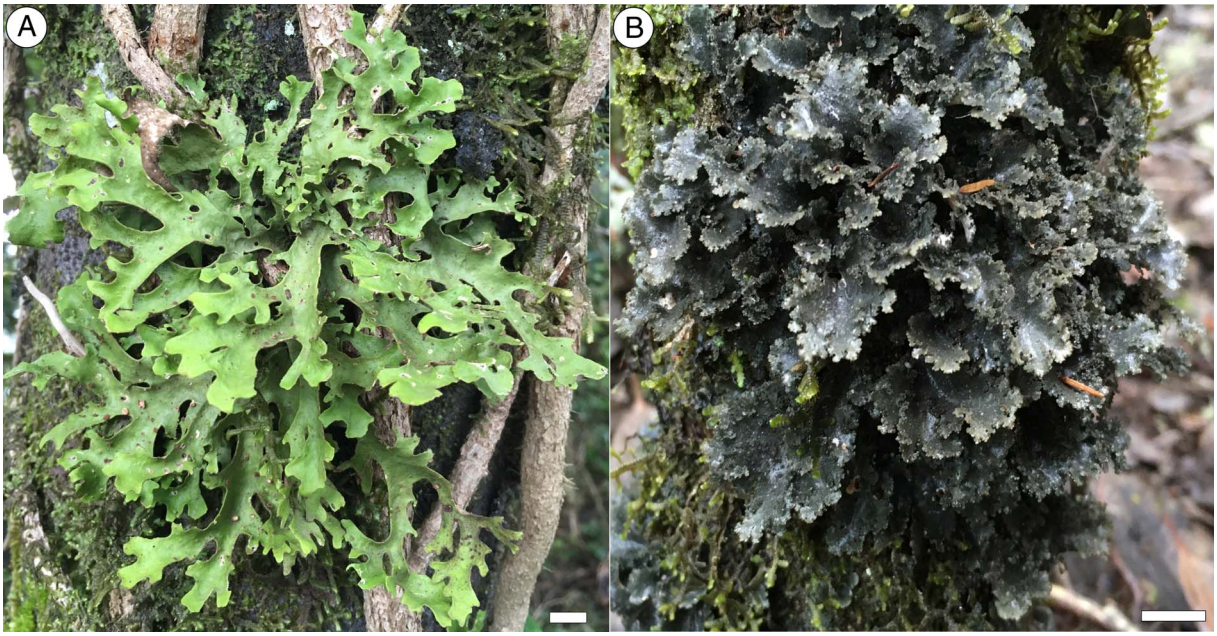

Figure 1. Photosymbiodemes of the lichen-forming fungus *Sticta canariensis*, showing dramatically different morphologies. A, chloromorph with small brown apothecia. B, cyanomorph with phyllidia (asexual propagules). Scales = 1 cm. In colour online.

close proximity to water sources, and in cool and shady forests (A. Goss and S. Werth, personal observations) (Moncada & Lücking 2012). Composite thalli consist of cyanomorph and chloromorphs occurring together, as small bright green leaflets arising from the dark cyanomorph thalli.

Sticta canariensis is rare and threatened in all parts of its disjunct range in temperate and subtropical habitats of Macaronesia and Atlantic Europe, and is thus of great conservation concern (Moncada & Lücking 2012). The cyanomorph is by far the most common morph, while the chloromorph distribution is extremely limited. The cyanomorph and composite thalli are considered of least concern (LC), but the independent chloromorph has been listed as vulnerable (VU) in England and endangered in Wales (Woods & Coppins 2012).

In Macaronesian sites, *S. canariensis* grows in laurisilva forests, an ecosystem highly threatened by global change (Fernández-Palacios *et al.* 2011; Perry 2011; Ritter *et al.* 2019; Castilla-Beltrán *et al.* 2021). Our study area (Fig. 2) includes

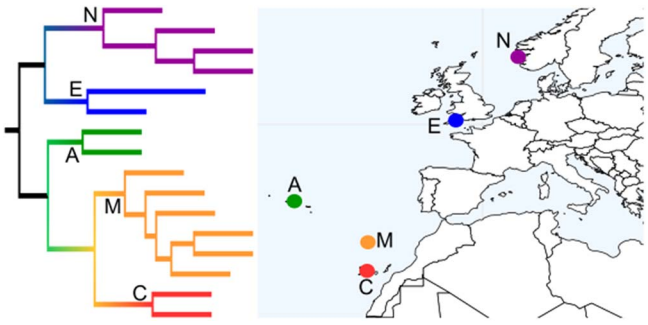

Figure 2. Tree model for the lichen-forming fungus *Sticta canariensis*. Midpoint-rooted neighbour-joining population tree from PHYLIP based on chord distance between populations, calculated with 1000 bootstrap replicates. A = Azores, E = England, N = Norway, M = Madeira, C = Canary Islands. In colour online.

populations from across the species range: south-western England, the south-west coast of Norway, and the Macaronesian archipelagos of Azores, Madeira, and Canary Islands. In this work, our aims were twofold: 1) to develop a novel set of microsatellite loci for *S. canariensis*; 2) to test their polymorphism in natural populations and determine their usefulness for further studies.

Materials and Methods

Development of microsatellite markers

At the onset of this study, microsatellites were available for several species of *Lobaria*. However, Walser *et al.* (2003) showed that markers developed for one lichen species often do not work for other species within the same genus. New microsatellite markers were therefore developed for the *Sticta canariensis* mycobiont (fungal partner). Markers were derived from genomic data of *Sticta canariensis*, the generation of which is described in Resl *et al.* (2022). We used MSATCOMMANDER v. 1.0.8 (Faircloth 2008) to locate a total of 147 microsatellite markers with flanking primer sequences (Supplementary Material Table S1, available online). Of these, we chose 27 with a motif size of 2–3 bp and copy number ≥ 8 (Vieira *et al.* 2016). Each genomic contig sequence that returned a candidate microsatellite was first verified as fungal using BLAST (www.ncbi.nlm.nih.gov/blast), optimized for highly similar sequences (megaBLAST) and excluding uncultured environmental sequences. Primers were then designed using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/), for a melting temperature (Tm) of 54–56 °C. The primers were checked using the OligoAnalyzer Tool (Integrated DNA Technologies; eu.idtdna.com/pages/tools/oligoanalyzer) for hairpin structure that had Tm > 10 °C lower than the primer Tm, and ≤ 4 complementary bases in self- and hetero-dimers; primers above these thresholds were excluded (Table 1).

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Table 1. Primers for 25 microsatellite markers developed for the lichen-forming fungus *Sticta canariensis*.

Locus ID		Primer sequence (5'→3')	Allele size range (bp)	T_a (°C)*	Repeat motif**	GenBank Accession No.
Scan3	F:	ACCTATAATACCGAATCTGTTCA	136–142	55.46	$(ATC)_{14}$	MW821801
	R:	TAAGGTTCCAAAGTTGGTGG				
Scan8	F:	TACTCCGTACCAAATTGCAG	281–321	55.48	$(TG)_{15}$	MW821802
	R:	AGTTTTGTGCAGAACTCCT				
Scan9	F:	CCGCAATCTCGATCATTTC	229–285	55.12	$(AG)_{10}$	MW821803
	R:	TATTAGTGTCTTGGTGCCTC				
Scan14	F:	TTGGGCTGCTTACATAATCG	155–180	55.91	$(TTC)_{17}$	MW821804
	R:	CTCTTGCTCCAATCACATCT				
Scan22	F:	TATACTCAGCAAAGAGCACG	140–170	55.31	$(ATC)_{8}$	MW821805
	R:	CAGGGGATAGGGAGTTTTTC				
Scan29	F:	GGAAATGACGATGAGAGGAA	287–354	54.87	$(TTC)_{31}$	MW821806
	R:	TGAAATTGTGAGGGTTGGAT				
Scan56	F:	GTACAAACGATGACAAGGAG	83–89	54.82	$(AGA)_{8}$	MW821807
	R:	GTTTCCAGTTTCAGTCTGTC				
Scan65	F:	GTAACCACAAGCCACATTC	326–352	55.03	$(GAA)_{9}$	MW821808
	R:	TTCTCTACATTTGCCCTCTC				
Scan102	F:	GGTGGTATCGTGGTTCAATA	196–200	54.87	$(CT)_{11}$	MW821809
	R:	CGGTTTTTATCTTGGATGGC				
Scan110	F:	TCTCTTCCCTCAATCTCTGT	376–390	54.86	$(TG)_{23}$ $(AG)_{11}$	MW821810
	R:	CATCAAACCATTTTTCCCGT				
Scan128	F:	AATCAGTGTGAGGAGGAGAT	100–108	55.23	$(GA)_{8}$	MW821811
	R:	GCTCGTCCATCGAATATCAA				
Scan145	F:	GATGTGGGGAGAATGATATAGA	98–106	54.27	$(TC)_{10}$	MW821812
	R:	GTGAGGTAAGGTGTTTGATTT				
Scan234	F:	GGAATAACAAATACTGCCTACA	246–278	53.90	$(AC)_{18}$	MW821813
	R:	AACTCCTTTTAAGTCCCTCG				
Scan255	F:	ATATACTGTACTGTCGAGGC	90–110	53.81	$(CT)_{8}$	MW821814
	R:	CCCCCTTGCATACCGA				
Scan308	F:	CGATCAAGTGTCATTTCGG	415–425	54.26	$(TG)_{9}$	MW821815
	R:	AAAACATGAAAGGCAAGGTG				
Scan471	F:	TACCTTTACGTGATTGCTGT	305–456	54.71	$(ACT)_{38}$	MW821817
	R:	GTACTCTGTACCTGTTGACC				
Scan492	F:	GAAATGGGTTGTGACAGCTA	192–213	55.66	$(CT)_{15}$	MW821818
	R:	TTGTACTGTTTGTTCCGTCA				
Scan503	F:	ACAAGTCTGAGGCGATTTG	385-583	55.54	$(ATTATCATC)_{6}$ $(ATC)_{35}$ $(ATC)_{6}$	MW821819
	R:	GGGAAAAACAATACGCATGAT				
Scan606	F:	TCAATGAAAGTTTTGCCTGC	239-267	55.35	$(CTT)_{22}$	MW821820
	R:	TTTGGTGCAGATCAAGTCAA				
Scan616	F:	CTAGCTCCGCTCTTCGT	251-258	55.58	$(CA)_{13}$	MW821821
	R:	GGCAGTCATCTTTTTCACTAC				
Scan680	F:	TTGGTGCAGTTCATGGAG	125-154	54.80	$(AAG)_{10}$	MW821822
	R:	ATCCAACAACAATGGAGTCT				
Scan1125	F:	TGATCCATCCTCAGTTCATC	367-382	54.98	$(AG)_{12}$	MW821823

(*Continued*)

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Table 1. (*Continued*)

Locus ID		Primer sequence (5'→3')	Allele size range (bp)	T_a (°C)*	Repeat motif**	GenBank Accession No.
	R:	GCATCAACTCAGTCATACTC				
Scan1321	F:	CCCTAATTCTCCCATTCCATC	384-503	55.48	$(ATC)_{27}$ $(TTC)_{10}$	MW821824
	R:	ATCAAAGAGACTGACGATTACT				
Scan1857	F:	GAAAGTCGGAAACACATGAG	257-292	54.66	$(AG)_{11}$ $(TG)_{12}$ $(GA)_{16}$	MW821825
	R:	ATCCTCAAAGCACTCTTCC				
Scan3179	F:	CCTCTCCATAGCACATCAAC	74-89	55.62	$(AC)_8$	MW821826
	R:	TTACTTTTGCGTTCCCAATC				

*T_a refers to the annealing temperature of a given primer pair.
**Repeat motif is reported as: $(motif)_x$ with x as the number of repeats for that motif.

DNA extraction and genotyping of microsatellites

This study included 65 samples, 32 Macaronesian (eight from the Azores, 20 from Madeira and four from the Canary Islands), 11 from England and 22 from Spissøy Island, Norway. The number of chloromorphs (ch) and cyanomorphs (cy) used from each location is reported in Supplementary Material Table S2 (available online). Thallus fragments *c.* 1 × 1 cm were manually cleaned of foreign debris, lyophilized overnight, and pulverized with a TissueLyser II mill (Retsch) at a frequency of 30.0 s^{-1} for 3 min. DNA extraction was performed with the NucleoSpin Plant II Mini Kit (Macherey-Nagel) according to the manufacturer's protocol.

PCRs were conducted on all samples with 25 mycobiont-specific primers (consisting of 14 di- and 11 trinucleotide sequences) (Table 1). The protocol was adapted from the M13 PCR protocol (Schuelke 2000) utilizing 6-FAM, VIC, PET and NED fluorescent dyes bound to M13(-21) primers (Blacket *et al.* 2012). PCRs were conducted in 25 µl final reaction volumes using the Type-IT Microsatellite PCR Kit (Qiagen) with 10 ng genomic DNA, 5 µl primer mixture (unique mixture for each locus containing: 0.2 µM fluorescence-labelled M13(-21) primer, 0.05 µM unlabelled forward primer including an M13(-21) tail at the 5′-end, 0.2 µM reverse primer, in 1× TE buffer), 12.5 µl Type-IT Master Mix, and ultrapure H_2O to bring to volume. All PCRs were performed under the following conditions: 95 °C for 5 min; followed by 30 cycles of 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 45 s; then 8 cycles of 95 °C for 30 s, 53 °C for 45 s, 72 °C for 45 s; with a final extension at 72 °C for 30 min. Fragment sizes were determined with a 3730 DNA Analyzer (Applied Biosystems). Microsatellite genotyping was performed with the software Geneious v. 7.1 (Kearse *et al.* 2012).

Statistical analyses

We used GenAlEx v. 6.5 (Peakall & Smouse 2006, 2012) to calculate allele frequencies and genetic diversity indices, including the total number of alleles per locus (A_L), the number of unique alleles per population for each locus (A_U), the effective number of alleles (N_e), and Nei's unbiased gene diversity (H_E) (Nei 1978). Population pairwise fixation indices (F_{ST}) were calculated in R Statistical Software (v. 4.2.2; R Core Team 2022 using the *hierfstat* package v. 0.5.11 (Goudet & Jombart 2022).

The 'seqboot' module of PHYLIP v. 3.698 (Felsenstein 2004) was used to generate 1000 bootstrapped microsatellite allele frequency datasets. The genetic relationships between the collection sites were evaluated using chord distance (D_C) (Cavalli-Sforza & Edwards 1967), as implemented in the 'gendist' module which is an appropriate distance for microsatellites. Neighbour-joining trees were constructed for each replicate with the 'neighbour' module. A majority-rule consensus tree was generated using the 'consense' module of PHYLIP. The consensus tree was midpoint rooted with FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

We analyzed 25 loci from 65 individuals of *Sticta canariensis*. All 25 mycobiont-specific primer pairs reliably amplified fragments, with less than 8% missing amplicons per locus, and 3% or less missing at each location (Supplementary Material Table S2, available online). We found a total of 175 alleles, with the total number of alleles per locus ranging from 2 (Sca1125) to 22 (Sca503) (Table 2). The highest percentage of polymorphic loci was found in the Azores (96%). Madeira had the highest number of total alleles (102), the highest number of unique genotypes (18 out of 20 individuals), and the highest number of private alleles (58). England had the lowest total allele count (29), and the fewest private alleles (1). The majority of private alleles came from only four loci (Sca1321: 16, Sca503: 14, Sca471: 15 and Sca1857: 10). Nei's unbiased gene diversity (H_E) ranged from 0.000 to 0.189 in locus Scan308, and from 0.000 to 1.000 in Scan1321. (Table 2). F_{ST} values were consistently high, ranging from 0.216 to 0.743 (Supplementary Material Table S3, available online).

A midpoint-rooted population tree produced with PHYLIP supports genetic patterns that resemble geographical distributions (Fig. 2). A southern group includes populations from the Canary Islands and Madeira, with populations from the Azores comprising a sister group. A strongly divergent northern group comprises the closely related populations from England and Norway.

Discussion

This study is the first to report and test microsatellite markers for the lichen-forming fungus *Sticta canariensis*. The set of 25 microsatellite loci that we developed had varying levels of polymorphism, with four highly polymorphic loci and several with moderate polymorphism. Even though individually some of these microsatellite loci exhibited low variability, when used concurrently they allowed us to reliably characterize the genetic relationships between individuals and populations of *S. canariensis* across this

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Table 2. Microsatellite diversity of the mycobiont *Sticta canariensis*, based on the analysis of 25 loci from 65 individuals.

	Total ($n = 65$)		Norway ($n = 22$)			England ($n = 11$)			Azores ($n = 8$)			Madeira ($n = 20$)			Canary Islands ($n = 4$)		
Locus	Ns	A_T	A_L	A_P	H_E	A_L	A_P	H_E	A_L	A_P	H_E	A_L	A_P	H_E	A_L	A_P	H_E
Sca3	60	3	1	0	0.00	1	0	0.00	2	0	0.43	3	0	0.511	2	0	0.67
Sca8	62	5	1	0	0.00	1	0	0.00	4	3	0.75	1	0	0.000	1	1	0.00
Sca9	64	4	1	0	0.00	1	0	0.00	4	3	0.64	1	0	0.000	1	0	0.00
Sca14	62	8	2	0	0.42	1	0	0.00	3	2	0.46	5	3	0.737	2	1	0.50
Sca22	64	4	1	0	0.00	1	0	0.00	2	1	0.54	2	0	0.189	3	1	0.83
Sca29	62	9	1	0	0.00	1	0	0.00	3	2	0.61	6	4	0.705	3	0	0.83
Sca56	65	4	1	0	0.00	1	0	0.00	2	1	0.54	3	1	0.468	1	0	0.00
Sca65	62	5	1	0	0.00	1	0	0.00	3	2	0.68	2	0	0.189	2	0	0.50
Sca102	63	3	1	0	0.00	1	0	0.00	2	1	0.25	2	1	0.100	1	0	0.00
Sca110	65	5	2	1	0.09	1	0	0.00	2	1	0.54	3	1	0.563	1	0	0.00
Sca128	64	3	1	0	0.00	1	0	0.00	3	2	0.61	1	0	0.000	1	0	0.00
Sca145	64	3	1	0	0.00	1	0	0.00	2	1	0.25	1	0	0.000	2	1	0.50
Sca234	61	12	1	0	0.00	2	0	0.55	3	0	0.46	11	8	0.900	1	0	0.00
Sca255	63	4	1	0	0.00	2	0	0.55	4	1	0.79	1	0	0.000	1	0	0.00
Sca308	60	3	2	1	0.09	1	0	0.00	1	0	0.00	2	1	0.189	1	0	0.00
Sca471	64	16	1	0	0.00	1	1	0.00	6	5	0.93	7	7	0.874	2	2	0.50
Sca492	64	4	1	0	0.00	1	0	0.00	2	1	0.54	3	2	0.279	1	0	0.00
Sca503	64	22	4	0	0.52	2	0	0.55	7	3	0.96	12	9	0.947	4	2	1.00
Sca606	65	9	1	0	0.00	1	0	0.00	6	3	0.93	4	1	0.363	2	1	0.50
Sca616	65	6	1	0	0.00	1	0	0.00	3	1	0.68	5	3	0.779	1	0	0.00
Sca680	65	4	1	0	0.00	1	0	0.00	2	1	0.54	2	1	0.100	2	1	0.50
Sca1125	64	2	1	0	0.00	1	0	0.00	2	0	0.54	1	0	0.000	1	0	0.00
Sca1321	62	21	1	1	0.00	2	0	0.55	6	3	0.93	12	9	0.926	4	3	1.00
Sca1857	63	13	1	0	0.00	1	0	0.00	4	2	0.75	10	7	0.921	3	1	0.83
Sca3179	65	3	1	0	0.00	1	0	0.00	3	1	0.68	2	0	0.505	1	0	0.00
Mean		7	1.24		0.04	1.2		0.09	3.2		0.60	4.1		0.41	1.8		0.33

Ns = number of successful amplifications; A_T = total number of alleles per locus across all locations; A_L = number of alleles per locus; A_P = total number of private alleles per locus; H_E = unbiased diversity by population; n = number of samples per location.

species' range. The only exception would be Sca1125 which had the lowest polymorphism (2), making it the least useful for analysis.

Madeira had the highest number of unique genotypes, private alleles and total alleles. This could partly be an effect of the large sample size and more diverse sampling locations, although most individuals were unique at several loci, while other locations showed less polymorphism. Additionally, we found the island populations were genetically distinct from populations in England and Norway, suggesting limited migration between these distant locations. Similarly, in their study on Macaronesian *Lobaria*, Werth *et al.* (2021) found high divergence between populations, high polymorphism and private alleles in populations from the Azores and Madeira, and a genetic distinction between island and mainland populations.

These novel microsatellite markers will provide a valuable tool for population genetic studies and for conservation assessments of *Sticta canariensis*. This is the first study to investigate the population genetic structure of *S. canariensis* and it provides an important starting point for understanding this rare species, which we will develop through more extensive sampling in Macaronesia and elsewhere.

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Competing Interests. The authors declare none.

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