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1 **Title:** Hybrid plants preserve unique genetic variation in the St Helena endemic trees
2 *Commidendrum rotundifolium* (Roxb.) DC and *C. spurium* (G.Forst.) DC.

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15 **Abstract**

16 The island of St Helena in the South Atlantic Ocean has a rich endemic flora, with 10 endemic
17 genera and 45 recognised endemic species. However, populations of most endemic species have
18 undergone dramatic reductions or extinction due to over-exploitation, habitat destruction and
19 competition from invasive species. Consequently, endemic species are likely to have lost
20 genetic variation, in some cases to extreme degrees. Here, the entire extant wild populations
21 and all planted trees in seed orchards, of two critically endangered species in the endemic genus
22 *Commidendrum* (Asteraceae), *C. rotundifolium* and *C. spurium*, were sampled to assess levels
23 of genetic variation and inbreeding. Six new microsatellite loci were developed from next-
24 generation sequence data, and a total of 190 samples were genotyped. Some seed orchard trees
25 contained alleles from both wild *C. rotundifolium* and *C. spurium* indicating they could be
26 hybrids and that some backcrossing may have occurred. Some of these trees were more similar
27 to *C. rotundifolium* than *C. spurium* both genetically and morphologically. Importantly, allelic
28 variation was detected in the putative hybrids that was not present in wild material. *C.*
29 *rotundifolium* is represented by just two individuals one wild and one planted and *C. spurium*
30 by seven, therefore the seed orchard trees comprise an important part of the total remaining
31 genetic diversity in the genus *Commidendrum*.

32 **Keywords:** allelic variation, genetic conservation, rarity, breeding programme, endemic plants

33 **Introduction**

34 Islands make a disproportionate contribution to global biodiversity as they house distinct
35 evolutionary lineages of endemic species, and many are biodiversity hotspots (Myers et al.
36 2000; Emerson & Kolm 2005). Island floras are widely threatened by invasive species,
37 exploitation, habitat degradation and climate change, the results of which can cause severe
38 reductions in population sizes (Cronk 1986; Glen et al. 2013; Courchamp et al. 2014). Small
39 population sizes and fragmentation can reduce genetic diversity, and disrupt gene flow and
40 inbreeding, with consequential declines in fitness (Ellstrand & Elam 1993). Another risk, for
41 small plant populations in particular, is hybridisation either through exposure to larger
42 populations of closely related species (e.g. *Hyacinthoides* spp. in the UK, see Kohn et al.
43 2009) or where previously geographically-separated close relatives are brought together (e.g.
44 *Trochetiopsis* on St Helena, see Cronk 1995). In combination, these threats highlight that
45 island biodiversity is in urgent need of assessment and conservation before genetic variation is
46 lost forever.

47 Hybridization among plants is an important evolutionary mechanism with the origin of 40–
48 80% of angiosperms estimated to involve either hybridisation or changes in ploidy (Stebbins
49 1950; Stace 1975; Rieseberg et al. 1993; Rhymer & Simberloff 1996). However,
50 hybridization is a conservation risk for rare and/or endangered species, potentially threatening
51 their genetic integrity (Levin et al. 1996). Gene flow between related species can compromise
52 fitness by the wastage of reproductive effort (Levin, Francisco-Ortega et al. 1996). It can also
53 be a threat where it occurs between differently-adapted populations of a single species by
54 disrupting co-adapted gene complexes (Rhymer & Simberloff 1996). Of particular concern is
55 when hybrids display greater fitness than either or both of the parental species (hybrid vigour

56 or heterosis) causing competition with the parental species (Rhymer & Simberloff 1996;
57 Emms & Arnold 1997). Hybridization is more likely where there are limited options for out-
58 breeding (Rhymer & Simberloff 1996; Kothera et al. 2007) or where isolation barriers
59 between two previously isolated species are broken (Ellstrand & Schierenbeck 2000). On the
60 other hand, where species have become critically endangered to the extent that only a few
61 individuals remain, hybridization may be the only means to preserve alleles that would be lost
62 to extinction (Fant et al. 2010), especially where outbreeding is obligate due to mechanisms
63 for self-incompatibility.

64 On St Helena, a small island (122 km²) in the South Atlantic (15° 58'S and 5°43'W, Suppl.
65 Figure S1a) several endemic species are at risk. The endemic genus *Commidendrum* DC.
66 (Asteraceae), the 'gumwoods', contains four very closely related species (Eastwood et al.
67 2004), all severely threatened in the main by introduced species. Hybridisation has also been
68 found among *Commidendrum* species (Eastwood 2003), but was not thought to be
69 widespread. *Commidendrum rotundifolium* (Roxb.) DC. was classified by IUCN as Extinct in
70 the Wild until recently as it was rediscovered at the top of a cliff edge (Suppl. Figure S1b) but
71 remains Critically Endangered and is likely to again be classified as Extinct in the Wild when
72 this individual dies unless other individuals are discovered. *Commidendrum spurium* (G.
73 Forst.) DC. is Critically Endangered with the largest population currently comprising just
74 seven individuals. The other two species, *C. rugosum* (Dryand) DC. and *C. robustum* DC., are
75 slightly more widespread and have larger populations (approximately 35,000 and 680
76 individuals respectively). The latter species is almost exclusively confined to a single site but
77 has been the focus of a successful community woodland restoration project (Figure S1b). The
78 extremely small sizes of these populations, allied with the self-incompatibility of the species
79 (Eastwood 2003), places substantial barriers to establishment of self-sustaining populations.

80 Until very recently all extant *C. rotundifolium* were the progeny of one individual tree which
81 has subsequently died. At least nine trees were established from this individual and grown in a
82 seed orchard (at Pounceys, Suppl. Figure S1b). In 1998, seedlings were raised from the
83 Pounceys seed orchard and planted in a second seed orchard at Scotland (Suppl. Figure S1b).
84 In 2002, more seedlings were raised and planted at a third seed orchard at Barren Ground
85 (Suppl. Figure S1b). All but one of the original nine progeny at Pounceys have since died,
86 leaving this individual and the seed orchard stock as the entire surviving *C. rotundifolium*
87 population at the time. However, as the seed orchard trees at Scotland and Barren Ground
88 matured, morphological ambiguity suggested that these may be of hybrid origin. Several *C.*
89 *spurium* trees grew adjacent to the original planting site (Pounceys), and are likely to be the
90 co-parental species. To inform decisions for the recovery and re-introduction of *C.*
91 *rotundifolium* and *C. spurium*, it was necessary to establish the hybrid status and levels of
92 extant genetic diversity in the seed orchards for both species. In this study, we specifically
93 aimed to:

- 94 1. establish the possible hybrid status of seed orchard trees, and
- 95 2. identify any pure *C. rotundifolium* or *C. spurium* plants for subsequent conservation
96 breeding.

97 **Methods**

98 *Collection of samples*

99 With the exception of samples of *C. spurium* taken from the living collection at the Royal
100 Botanic Gardens Edinburgh, all samples were collected on St Helena from wild and seed
101 orchard populations (Figure 1). A total of 191 individuals were collected including all four
102 *Commidendrum* species (*C. spurium*, *C. rotundifolium*, *C. rugosum* and *C. robustum*) and the
103 putative hybrid samples from the seed orchards at Scotland and Barren Ground and a few trees
104 planted at the George Benjamin Arboretum (GBA). Leaf samples were collected into polythene
105 bags containing silica gel, between 01/06/2010 and 15/07/2010. *C. rotundifolium* came from
106 the single planted individual at Pounceys and a wild plant near Botley's and four seedlings
107 planted at Drummond Point. Wild *C. spurium* was collected from the seven individuals at
108 Mount Vessey and included fresh material of *C. spurium* was donated by the Royal Botanic
109 Gardens Edinburgh (RBGE Accession number 20000247E; collected 05/10/2012) from which
110 initial sequences were generated. Additional material of *C. robustum* (Peak Dale, Thompsons
111 Wood, Deep Valley and Millenium Forest) and *C. rugosum* (Man and Horse, Horse Point and
112 Blue Point) was also included for comparison.

113 *Laboratory Methods*

114 DNA was extracted from leaf tissue using DNeasy 96 and Mini plant kits (Qiagen), following
115 manufacturer's instructions.

116 To identify microsatellite loci, over 48 million bases of genomic DNA sequence were obtained
117 from *C. spurium* by 454 sequencing using a GS FLX (GATC Biotech). The sequence was
118 searched for 3, 4, 5 and 6 base pair repeat sequences using msatcommander (Rozen & Skaletsky

119 2000; Faircloth 2008) and primers were designed for 48 potential marker loci. In all cases an
120 M13 sequence tag was added to the 5' end of the forward primer. Potential markers were used
121 in polymerase chain reaction (PCR) amplification in 8 individuals from the sample set and those
122 showing consistent amplification and potential for diagnostic purposes were amplified in a
123 further subset of 28 individuals. Five trinucleotide and one tetranucleotide microsatellite loci
124 were chosen and the whole set of 191 samples were genotyped.

125 All microsatellites were amplified using 10 µl PCR reactions, each comprising 1µl of genomic
126 DNA (diluted from original elution to 1:10), 1.5mM MgCl₂, 1 X PCR Buffer, 200 µM each
127 dNTP, 0.2 µM each primer, 0.2 µM IRD fluorescent labelled M13 primer (700 or 800), 20%
128 v/v BSA and 1 U Taq DNA polymerase. Reactions were run on a Hybaid MBS thermocycler
129 using the following protocol for all loci: 5 min at 95 °C, then 10 cycles of 30 sec at 94 °C, 1
130 min at 57 °C, 30 sec at 72 °C, followed by 22 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 30 sec
131 at 72 °C, followed by 10 min at 72 °C. PCR products were then separated on an 8% denaturing
132 polyacrylamide gel (25 cm), and visualised using a LI-COR 4200 IR2 automated genotyper.
133 PCR products were run out alongside a standard and fragment sizes were scored by eye.

134 *Data Analyses*

135 All summary statistics (number of different alleles; number of effective alleles; observed
136 heterozygosity; expected heterozygosity; unbiased expected heterozygosity; fixation index)
137 were calculated using GenAlex (version 6.501) (Peakall & Smouse 2006; Peakall & Smouse
138 2012). A Principal Coordinate Analysis (PCoA) was also computed based on the pairwise
139 genetic distance matrix to examine relatedness among samples. Pairwise genetic distance was
140 estimated among all pairs of samples using the squared distance method for codominant
141 genotypes as implemented in GenAlEx.

142 **Results and Discussion**

143 Across all species, very little variation was evident in marker screening, and only 6 loci had any
144 variation, probably due to the extremely small population sizes and inbred nature of the extant
145 trees. Although several loci were monomorphic within species, loci were retained on the basis
146 that they showed polymorphism either within or among species. It was not possible to determine
147 whether monomorphism at a locus was due to null alleles or homozygosity, but as we had
148 completely sampled all plants of the extant populations, these markers were nevertheless useful
149 for species resolution, and to indicate gene pool variation (comparing species with putative
150 hybrids).

151 Overall, levels of genetic variation within and among species were very low, with mean
152 numbers of alleles per locus, $N_a = 1 - 2.5$ (Table 1), especially for *C. rotundifolium* ($N_a = 1$).
153 Seed orchard plants had slightly higher levels of variation ($N_a = 2.5$) than wild populations,
154 except for *C. rugosum* where sample size was small. One allele was present in some of the
155 hybrids that was not found in any of the extant parent plants (Locus 6, allele 162, Supplementary
156 Table S1). Most samples shared the majority of alleles with *C. spurium*, but a few samples had
157 more alleles in common with *C. rotundifolium* (one sample shared 75%, Supplementary Table
158 S2, and displayed leaf morphology closer to *C. rotundifolium*). The first 3 axes of the PCoA
159 explained over 80% of the variation in the data (Figure 2). All of the seed orchard samples from
160 Barren Ground, Scotland and GBA had alleles found in both *C. rotundifolium* and *C. spurium*
161 supporting their putative hybrid origin (Figure 2). The distribution of hybrid samples on axes 1
162 & 2, range from being close to a putative parents or somewhat intermediate between the two,
163 suggesting both first generation hybrids and hybrid-parent backcrossed progeny may be present
164 (Figure 2).

165 The data indicated a very low level of genetic diversity in these threatened species as expected
166 from the extremely small extant population sizes. They also support the suspected hybrid origin
167 of the seed orchard plants, as had been suggested by previous studies (Eastwood 2003). Level
168 of heterozygosity in the putative hybrid samples were higher than expected ($H_o = 0.77$, $H_e =$
169 0.51 , mean $F = -0.52$, Table 1), possibly indicative of combination of gene pools as would occur
170 in hybridisation. None of the seed orchard plants were pure *C. rotundifolium* or *C. spurium* but
171 alleles were discovered in some of the hybrids that were not present in any of the wild or wild
172 derived individuals. These alleles may be derived from now-extinct *C. rotundifolium* or *C.*
173 *spurium* parent populations. The hybrid plants may therefore represent a repository of genetic
174 variation, which merits careful conservation on St Helena given the extremely limited genetic
175 variation present in both *C. rotundifolium* and *C. spurium* in the wild. This potential repository
176 is significant due to the self-incompatibility system in both species, which results in limited
177 seed production from mating between closely-related individuals.

178 *Conservation Implications*

179 The genus *Commidendrum* is endemic to St Helena and is a unique part of global plant diversity.
180 All species in the genus currently face extinction, being threatened by invasive species,
181 exploitation, habitat degradation and climate change. A further difficulty is effective
182 propagation due to self-incompatibility mechanisms. For species such as these, hybrid plants
183 may represent a valuable source of variation that would otherwise be lost via extinction (Fant
184 et al. 2010). In *C. rotundifolium* and *C. spurium*, reproductive success is dependent on mate
185 availability for cross-pollination success and the limited genetic diversity in extant populations
186 of *C. rotundifolium* and *C. spurium* will undoubtedly impede the recovery programme. Our
187 results show that seed orchard trees contain variation not found in the wider population; these

188 trees should therefore be considered a resource for a controlled breeding programme or genetic
189 rescue (Whiteley et al. 2015). To support this work, as well as continuing to develop the record
190 of their unique genetic variation through wider genomic sequencing, additional studies of inter-
191 fertility and propagation are urgently required for the *Commidendrum* species. However, any
192 moves to implement genetic rescue or hybrid breeding should take careful account of the ethical
193 questions that arise when dealing with highly threatened species. We recommend that the
194 natural populations are maintained 'as is' but that other mixed putative hybrid populations
195 should be established. In such threatened populations we suggest that the conservation of
196 *Commidendrum* should focus on all genetic diversity and this is as much of a priority as
197 conserving taxonomic species.

198 **Acknowledgments**

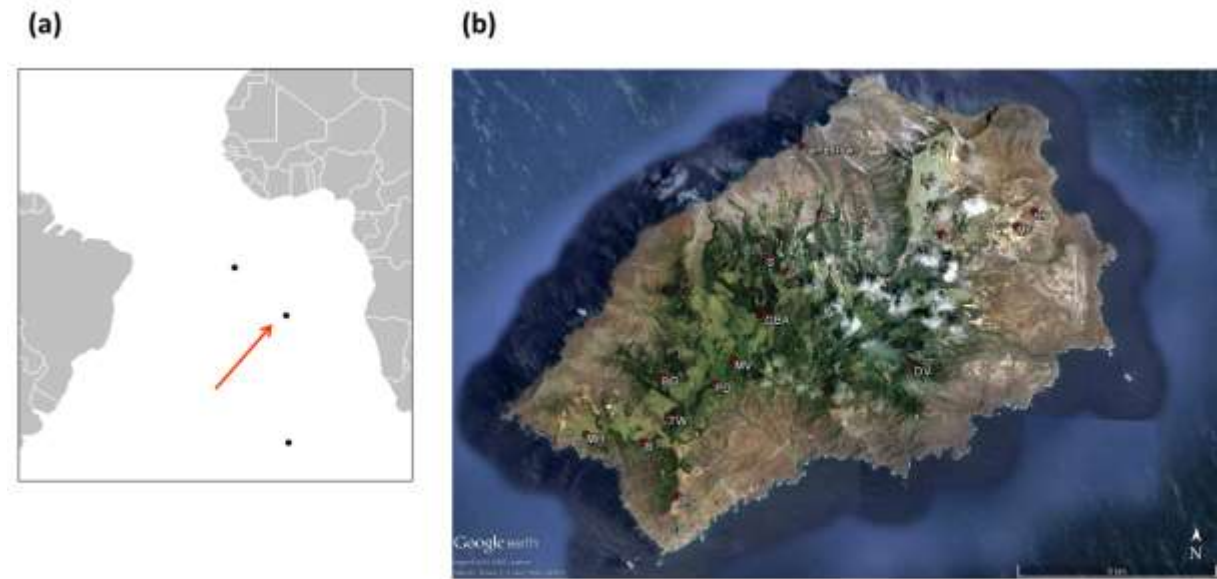
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206 **References**

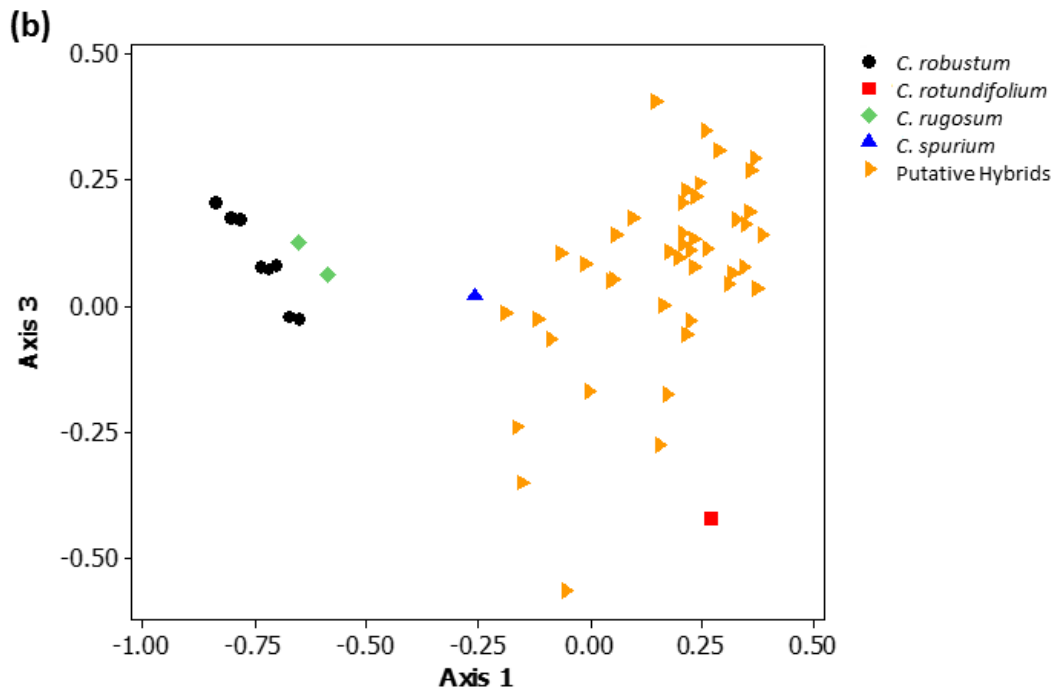
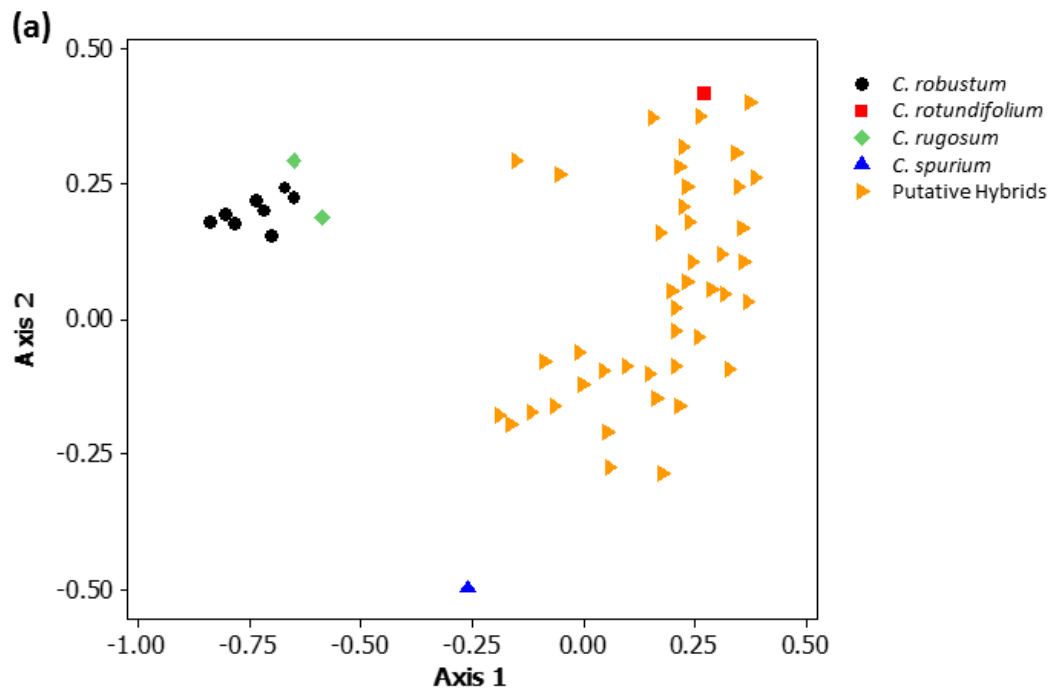
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259



260
 261 Figure 1: (a) Map showing general locality of St Helena in the south Atlantic Ocean with
 262 Ascension Island to the north west and Tristan da Cunha to the south. (b) Location of sites on
 263 St Helena mentioned in the main manuscript: BG - Barren Ground, BP - Blue Point, B -
 264 Botley's, DV - Deep Valley, DP - Drummond Point, GBA - George Benjamin Arboretum
 265 (Casons), HP - Horse Point, LF - Longwood Farm (Picolo), MH - Man and Horse, MF -
 266 Millennium Forest, MV - Mount Vessey, PD - Peak Dale, P – Pounceys, S – Scotland, and
 267 TW - Thompsons Wood.



268 Figure
 269 2: Axes 1 and 2 (a) and axes 1 and 3 (b) from a principal co-ordinates analysis based on genetic
 270 distance estimated using 6 microsatellite loci. The percentage of variation explained by these
 271 axes was: axis 1 - 47.26 %, axis 2 - 19.26 %, and axis 3 - 15.04 %, cumulative variation – 81.56
 272 %.

273 Table 1: Summary genetic diversity statistics for the four *Commidendrum* species and
274 putative hybrids. Number of genotypes; N_a - No. of Different Alleles; N_e - No. of Effective
275 Alleles; H_o - Observed Heterozygosity; H_e - Expected Heterozygosity; ${}_uH_e$ - Unbiased
276 Expected Heterozygosity; F - Fixation Index.

Samples	No. genotypes	Locus	N	Na	Ne	Ho	He	uHe	F
Seed Orchard	18	6	151	3.00	2.64	0.77	0.62	0.62	-0.25
		11	151	3.00	1.99	0.90	0.50	0.50	-0.81
		19	151	2.00	1.98	0.79	0.49	0.50	-0.59
		36	151	2.00	2.00	0.79	0.50	0.50	-0.59
		42	151	3.00	1.98	0.87	0.49	0.50	-0.77
		43	151	2.00	1.85	0.50	0.46	0.46	-0.10
		Mean	2.50	2.07	0.77	0.51	0.51	-0.52	
		SE	0.22	0.11	0.06	0.02	0.02	0.12	
<i>C. robustum</i>	4	6	0	0.00	0.00	0.00	0.00	0.00	
		11	17	2.00	1.99	0.94	0.50	0.51	-0.89
		19	18	1.00	1.00	0.00	0.00	0.00	#N/A
		36	19	2.00	1.87	0.32	0.47	0.48	0.32
		42	19	2.00	1.82	0.68	0.45	0.46	-0.52
		43	19	1.00	1.00	0.00	0.00	0.00	#N/A
		Mean	1.33	1.28	0.32	0.24	0.24	-0.36	
		SE	0.33	0.31	0.17	0.11	0.11	0.25	
<i>C. rotundifolium</i>	1	6	6	1.00	1.00	0.00	0.00	0.00	#N/A
		11	6	1.00	1.00	0.00	0.00	0.00	#N/A
		19	6	1.00	1.00	0.00	0.00	0.00	#N/A
		36	6	1.00	1.00	0.00	0.00	0.00	#N/A
		42	6	1.00	1.00	0.00	0.00	0.00	#N/A
		43	6	1.00	1.00	0.00	0.00	0.00	#N/A
		Mean	1.00	1.00	0.00	0.00	0.00		
		SE	0.00	0.00	0.00	0.00	0.00	0.00	
<i>C. rugosum</i>	6	6	0	0.00	0.00	0.00	0.00	0.00	
		11	7	4.00	2.51	0.57	0.60	0.65	0.05
		19	4	2.00	1.60	0.50	0.38	0.43	-0.33
		36	4	2.00	1.60	0.00	0.38	0.43	1.00
		42	7	3.00	2.65	0.86	0.62	0.67	-0.38
		43	5	1.00	1.00	0.00	0.00	0.00	#N/A
		Mean	2.00	1.56	0.32	0.33	0.36	0.09	
		SE	0.58	0.40	0.15	0.11	0.12	0.26	
<i>C. spurium</i>	1	6	8	1.00	1.00	0.00	0.00	0.00	#N/A
		11	8	2.00	2.00	1.00	0.50	0.53	-1.00
		19	8	1.00	1.00	0.00	0.00	0.00	#N/A
		36	8	1.00	1.00	0.00	0.00	0.00	#N/A
		42	8	2.00	2.00	1.00	0.50	0.53	-1.00
		43	8	1.00	1.00	0.00	0.00	0.00	#N/A
		Mean	1.33	1.33	0.33	0.17	0.18	-1.00	
		SE	0.21	0.21	0.21	0.11	0.11	0.00	
Across all loci and species			Mean	1.63	1.45	0.35	0.25	0.26	-0.39
			SE	0.17	0.12	0.07	0.05	0.05	0.10

