Development of 13 Microsatellite Markers in the Endangered Sinai Primrose
(*Primula boveana*, Primulaceae)

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Source: Applications in Plant Sciences, 1(6) 2013.
Published By: Botanical Society of America
DOI: [http://dx.doi.org/10.3732/apps.1200515](http://dx.doi.org/10.3732/apps.1200515)

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DEVELOPMENT OF 13 MICROSATellite MARKERS IN THE ENDANGERED SINAI PRIMROSE (PRIMULA BOVEANA, PRIMULACEAE)1

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• Premise of the study: We developed microsatellite markers for the endangered plant Primula boveana, the Sinai primrose, and assessed the cross-transferability of these markers to six related taxa.
• Methods and Results: DNA sequences containing microsatellites were isolated from a microsatellite-enriched library. We obtained successful amplification of 13 microsatellite primer pairs, seven of which were polymorphic in P. boveana. Eleven of these primers successfully cross-amplified to related taxa.
• Conclusions: The markers reported herein will be useful to characterize the genetic diversity of the endangered P. boveana and to evaluate its mating system, and have the potential to be useful for similar studies in close relatives.

Key words: cross-amplification; Dionysia; microsatellites; Primula boveana; Sinai; sect. Sphondylia.

The Sinai primrose, Primula boveana Decne. ex Duby (Primulaceae), has been reported as one of the rarest and most endangered plant species worldwide (Richards, 2003). It is endemic to Mount St. Catherine, in the Sinai mountains in southern Egypt, where it has been located in only five clearly delimited localities at least one kilometer from each other, all of them consisting of fewer than 10 to a few hundred adult plants. This species, restricted to wadis (i.e., valleys of intermittent streams) fed by meltwater near the top of Mount St. Catherine, is severely threatened by both natural and human factors. The most important natural threats are the fragmentation inherent to its habitat and the aridity of the area, with very scarce precipitation year round. Human impacts, especially water collection for human consumption, sheep and goat grazing, and traditional plant collection for medicinal uses, further intensify the natural threats of aridification and fragmentation, thus pushing P. boveana to the brink of extinction.

Primula boveana belongs to sect. Sphondylia (Duby) Rupr., which, together with its sister group, the genus Dionysia Fenzl, forms a well-supported clade within Primula L. (Mast et al., 2001, 2006). All the species included in sect. Sphondylia, as well as some Dionysia species, are rare, narrow endemics distributed in wet refugia in arid areas from northeastern Africa to Southwest Asia. Because of the rarity of these species, genetic diversity and mating system studies are needed to warrant the conservation of these taxa. Here, we report 13 microsatellite loci that will be used to characterize the genetic diversity and mating system of P. boveana, and test their cross-amplification with three other Primula species belonging to sect. Sphondylia and with three Dionysia species.

METHODS AND RESULTS

DNA isolated from our specimen AS35 of P. boveana from the population in Ain Shennarah (see below) was used by Genetic Marker Services (Brighton, United Kingdom; http://www.geneticmarkerservices.com) to develop a microsatellite-enriched library and to design and test microsatellite primer pairs. Enrichment involved incubating adapter-ligated, size-restricted DNA with filter-bonded synthetic repeat motifs, (AG)17, (AC)17, (AAC)10, (CCG)10, (CTG)10, and (AAT)10. Thirty-nine positive library colonies were selected for sequencing, from which 22 microsatellites were designed and tested for amplification. The primer pairs were designed using the software Primer3 version 3.0 (Rozen and Skaltsky, 2000), with the criterion of amplifying products in the range of 100–250 bp to minimize later overlap ambiguities during multiplexing genotyping projects. We tested each primer pair for amplification and polymorphism in eight individuals of P. boveana that represented four of the five populations in Mount St. Catherine: Ain Shennarah (28°31′N, 33°57′E; N = 2), wadi Shaq Mousa (28°31′N, 33°57′E; N = 2), wadi Gebal (28°33′N, 33°52′E; N = 2), and Kafl El-Ghoulia (28°32′N, 33°56′E; N = 2). The 13 primer pairs that resulted in amplification products in P. boveana (Table 1) were further tested for cross-amplification in one individual each of several closely related Primula and Dionysia species (Table 2). Representative voucher specimens for every taxon are deposited in herbariums E (Royal Botanic Garden Edinburgh), SCU (Suez Canal University), and Z (University of Zurich; Appendix 1).

Prior to DNA extraction, ~20 mg of dry leaf tissue per individual was ground with stainless steel beads using an MM 3000 shaker (Retsch GmbH, Han, Germany). Total genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hombrechtikon, Switzerland) following the manufacturer’s guidelines. Amplification of microsatellite loci was performed following the

1 Manuscript received 27 September 2012; revision accepted 16 November 2012.
2 The authors thank M. Meloni for her valuable advice on protocols to screen for microsatellite polymorphisms and S. Hussein for his help in sampling Primula boveana in the field. This study was supported by the Swiss National Science Foundation grant IZK0Z3_139418 to H.M. and E.C. and by the University of Zurich.
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doi:10.3732/apps.1200515
population.

equilibrium (one allele significantly departed from the expectations of Hardy–Weinberg
tions studied, all of the observed genotype frequencies of loci with more than
0 to 0.038 and from 0.032 to 0.144, respectively (Table 1). In the three popula-
tations (Ain Shennarah, wadi Shaq Mousa, and wadi Gebal) for a total of 60 indi-
viduals. The number of alleles observed for the seven polymorphic loci ranged
from two to four, and the observed and expected heterozygosities ranged from
0.074 to 0.144.

Smouse, 2006) on 20 individuals randomly sampled from each of three popula-
tions (Arif et al., 2010).

single-reaction, nested PCR method of Schuelke (2000), a cost-efficient
method best suited for projects with a small to moderate number of samples
(Blacket et al., 2012). PCRs were performed in a final volume of 25 μL con-
taining 2.5 μL of 10× reaction buffer, 1 μL of MgCl2 (50 mM), 0.5 μL of a
mix of all four dNTPs (10 mM), 0.2 μL of the forward primer incorporating
the M13-tail (10 ng/μL of the universal M13 primer (10 μM), 0.5 μL of the reverse primer (10 μM),
0.5 μL of the universal M13 primer (10 μM; Schuelke, 2000) labeled with
a fluorophore (FAM, NED, VIC, or PET), 0.1 μL of Taq DNA polymerase
(Bioline GmbH, Luckenwalde, Germany; 50 U/μL), 1.0 μL of bovine serum albumin (BSA; 20 mg/mL), 1.0 μL of ng/μL of genomic DNA, and sterilized
water up to the final volume. All PCRs were carried out in singleplexes using a
T1 Thermocycler (Biometra GmbH, Göttingen, Germany) under the follow-
ing conditions: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for
30 s, 55°C for 45 s, and 72°C for 1 min; eight cycles of 94°C for 30 s, 53°C for
45 s, and 72°C for 1 min; and a final extension step of 72°C for 5 min. The
resulting fluorescently labeled PCR products were run in multiplexes on an
ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California,
USA) using GeneScan 500 LIZ Size Standard (Applied Biosystems) as a size
standard and scored using GeneMapper 4.1 (Applied Biosystems), following
the recommendations given by Arif et al. (2010).

Seven of the 13 microsatellite primers amplified polymorphic products in
Primula boveana (Table 1). Genetic diversity and deviations from Hardy–
Weinberg equilibrium were estimated using GenAlEx version 6.4 (Peakall and
Smouse, 2006) on 20 individuals randomly sampled from each of three popula-
tions (Ain Shennarah, wadi Shaq Mousa, and wadi Gebal) for a total of 60 indi-
viduals. The number of alleles observed for the seven polymorphic loci ranged
from two to four, and the observed and expected heterozygosities ranged from
0 to 0.038 and from 0.032 to 0.144, respectively (Table 1). In the three popula-
tions studied, all of the observed genotype frequencies of loci with more than
one allele significantly departed from the expectations of Hardy–Weinberg
equilibrium (P < 0.001), with the exception of locus Prim61 in the wadi Gebal
population.

Eleven primer pairs amplified PCR products in at least one of the three
other Primula and three Dionysia species tested (Table 2). Accordingly with the
expectations of higher cross-transferability of microsatellites to the taxa more
closely related to the focal species (e.g., Primmer et al., 1996), the suc-
cess of cross-amplification was higher in the other Primula species of sect.

Sphondylia (11 out of 13 primers resulted in amplification) than in Dionysia
(eight out of 13 primers resulted in amplification).

CONCLUSIONS

The set of seven polymorphic loci out of the 13 microsatell-
ites reported here is adequate to further investigate the mating
system and population genetic structure of P. boveana. More
specifically, measurements of genetic diversity and estimations
of selfing and outcrossing rates will be useful to understand the
evolutionary responses of the mating system of P. boveana to
the factors threatening its persistence in the wild. Furthermore,
the six microsatellites reported here as monomorphic may still
provide useful genetic information if they are polymorphic in
other populations of P. boveana. Additionally, the successful
cross-amplification of 11 microsatellite loci to other species of
Primula sect. Sphondylia and of genus Dionysia open up the
possibility, provided that they amplify polymorphic products,
of studying the genetic variation of other endangered taxa in
this group of plants.

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A. H. A. FARIHAN, AND A. H. BAHIRL. 2010. Interpretation of electrophoretograms of seven microsatellite loci to
etermine the genetic diversity of the Arabian Oryx. Genetics and Molecular Research 9:
259–265.


TABLE 1. Characterization of 13 microsatellite loci isolated from Primula boveana.

<table>
<thead>
<tr>
<th>Locus</th>
<th>GenBank accession no.</th>
<th>Repeat motif</th>
<th>Size range (bp)</th>
<th>Primer sequences (5′–3′)</th>
<th>A</th>
<th>Hs</th>
<th>He</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prim45a</td>
<td>JX154138</td>
<td>(CT)n</td>
<td>200</td>
<td>F: CAGAGTCACAGTCTTTGAGCTTT &lt;— —— R: CACACACACACAGAGACCA</td>
<td>3</td>
<td>0</td>
<td>0.14</td>
</tr>
<tr>
<td>Prim45b</td>
<td>JX154138</td>
<td>(TG)12</td>
<td>197–203</td>
<td>F: GCCACTAGTAACCTCCTAAGG &lt;— —— R: GCCCAAAATCTGTATTTGTC</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Prim48</td>
<td>JX154139</td>
<td>(TCA)n</td>
<td>161</td>
<td>F: TCCCAATCTGCTGTATTGTC</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Prim49b</td>
<td>JX154155</td>
<td>(GT)12</td>
<td>186–188</td>
<td>F: GTGTGTTGTTGTTGTTGTTA &lt;— —— R: AGGTGAATCCAAAAATGGCAAA</td>
<td>2</td>
<td>0</td>
<td>0.32</td>
</tr>
<tr>
<td>Prim53</td>
<td>JX154159</td>
<td>(GA)11</td>
<td>218</td>
<td>F: CACACAGGAGCGCGGACTTA &lt;— —— R: AGGCGGGAAGAAAAATGGAA &lt;— ——</td>
<td>2</td>
<td>0</td>
<td>0.74</td>
</tr>
<tr>
<td>Prim54</td>
<td>JX154160</td>
<td>(GA)16</td>
<td>166–168</td>
<td>F: CGCTTGGAAACATCTCTTC &lt;— —— R: AGGCGGGAAGAAAAATGGAA &lt;— ——</td>
<td>2</td>
<td>0</td>
<td>0.74</td>
</tr>
<tr>
<td>Prim58</td>
<td>JX154142</td>
<td>(AC)n(AA)(AC)n</td>
<td>104</td>
<td>F: CACACATCTCTCTCCCCTTC &lt;— —— R: AAACCGCAAAACCTCTCTG &lt;— ——</td>
<td>3</td>
<td>0.031</td>
<td>0.127</td>
</tr>
<tr>
<td>Prim59</td>
<td>JX154143</td>
<td>(AG)10</td>
<td>200–216</td>
<td>F: GCCACATGACCATCTGTCT &lt;— —— R: GCAAGAAGACACGTTTACCA &lt;— ——</td>
<td>4</td>
<td>0</td>
<td>0.036</td>
</tr>
<tr>
<td>Prim61b</td>
<td>JX154167</td>
<td>(TG)12</td>
<td>206–228</td>
<td>F: GTGTGTTGTTGTTGTTGTTA &lt;— —— R: AAACCCGCTAAGATACGAG &lt;— ——</td>
<td>3</td>
<td>0.031</td>
<td>0.127</td>
</tr>
<tr>
<td>Prim62b</td>
<td>JX154168</td>
<td>(AC)n</td>
<td>128</td>
<td>F: GCGTGACGACATTACCAAAA &lt;— —— R: AGGAGGACACATCCTCCCT &lt;— ——</td>
<td>3</td>
<td>0</td>
<td>0.32</td>
</tr>
<tr>
<td>Prim65</td>
<td>JX154149</td>
<td>(GA)11</td>
<td>198</td>
<td>F: GCGTGACGACATTACCAAAA &lt;— —— R: AGGAGGACACATCCTCCCT &lt;— ——</td>
<td>3</td>
<td>0</td>
<td>0.32</td>
</tr>
<tr>
<td>Prim66</td>
<td>JX154148</td>
<td>(GA)12</td>
<td>251–254</td>
<td>F: CCCATATCCATCTCTCCTC &lt;— —— R: GACATCACATGCTCAG &lt;— ——</td>
<td>2</td>
<td>0</td>
<td>0.144</td>
</tr>
<tr>
<td>Prim67</td>
<td>JX154150</td>
<td>(AC)n</td>
<td>146–148</td>
<td>F: TCCCTTCCCTCTACCTCTCC &lt;— —— R: TGGGCTTACACGTGAA &lt;— ——</td>
<td>2</td>
<td>0.038</td>
<td>0.138</td>
</tr>
</tbody>
</table>

Note: — = monomorphic locus; A = number of alleles per locus; Hs = expected heterozygosity; He = observed heterozygosity.
TABLE 2. Cross-amplification of Primula boveana microsatellites to three other Primula species from sect. Sphondylia and to three Dionysia species.a

<table>
<thead>
<tr>
<th>Locus</th>
<th>P. edelbergii</th>
<th>P. floribunda</th>
<th>P. simensis</th>
<th>D. gaubae</th>
<th>D. hedgei</th>
<th>D. tapetodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prim45a</td>
<td>231</td>
<td>233</td>
<td>223</td>
<td>131</td>
<td>231</td>
<td>130</td>
</tr>
<tr>
<td>Prim45b</td>
<td>233</td>
<td>—</td>
<td>225</td>
<td>133</td>
<td>233</td>
<td>132</td>
</tr>
<tr>
<td>Prim48</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Prim49b</td>
<td>+</td>
<td>—</td>
<td>178</td>
<td>+</td>
<td>116</td>
<td>—</td>
</tr>
<tr>
<td>Prim53</td>
<td>219</td>
<td>—</td>
<td>237</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Prim54</td>
<td>153</td>
<td>—</td>
<td>169</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Prim58</td>
<td>+</td>
<td>+</td>
<td>122</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Prim59</td>
<td>+</td>
<td>—</td>
<td>218</td>
<td>187</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Prim61b</td>
<td>197</td>
<td>+</td>
<td>195</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Prim62b</td>
<td>122</td>
<td>—</td>
<td>141</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Prim64</td>
<td>265</td>
<td>258</td>
<td>249</td>
<td>+</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Prim65</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Prim66</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Note: + = amplification of not readily interpretable products requiring further optimization; — = no amplification.
aAll cross-amplifications were tested on a single individual per species. Numbers represent allele size (in base pairs).


APPENDIX 1. Information on voucher specimens for taxa included in this study. Vouchers are deposited in herbaria E (Royal Botanic Gardens Edinburgh), Z (University of Zurich), and SCU (Suez Canal University).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Voucher specimen (Herbarium)</th>
<th>Geographic origin (Geographical coordinates)</th>
<th>Distribution range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primula boveana Decne. ex Duby</td>
<td>S. A. Gamal El-din 340 (SCU)</td>
<td>University of Suez Canal, Ismailia, Egypt; ex Mount St. Catherine, Egypt (28°31′N, 33°57′E)</td>
<td>Egypt</td>
</tr>
<tr>
<td>P. edelbergii O. Schwarz</td>
<td>A. R. Mast 715 (Z)</td>
<td>University of Newcastle upon Tyne, Newcastle, England; ex Göteborg Botanic Garden</td>
<td>Afghanistan</td>
</tr>
<tr>
<td>P. floribunda Wall.</td>
<td>A. R. Mast 714 (Z)</td>
<td>University of Newcastle upon Tyne, Newcastle, England; ex Royal Botanic Gardens Edinburgh, Edinburgh, Scotland</td>
<td>Afghanistan, Pakistan, India, Nepal</td>
</tr>
<tr>
<td>P. simensis Hochst.</td>
<td>A. R. Mast 712 (Z)</td>
<td>University of Newcastle upon Tyne, Newcastle, England; source of plant uncertain</td>
<td>Ethiopia, Somalia</td>
</tr>
<tr>
<td>Dionysia gaubae Bornm.</td>
<td>F. Ghahremani-nejad 135 (Z)</td>
<td>Lorestan, Iran (33°23′N, 47°58′E)</td>
<td>Iran</td>
</tr>
<tr>
<td>D. hedgei Wendelbo</td>
<td>D. S. Feller 34113 (Z)</td>
<td>Mazar-I Sharif, Afghanistan (36°34′N, 67°05′E)</td>
<td>Afghanistan</td>
</tr>
<tr>
<td>D. tapetodes Bunge</td>
<td>CULTE 15012 (E)</td>
<td>Royal Botanic Gardens Edinburgh, Edinburgh, Scotland; ex Baghlan, Afghanistan: 18 miles E of Banu, S of the village of Pul-i-Sar</td>
<td>Afghanistan, Iran</td>
</tr>
</tbody>
</table>