MICROSATELLITE MARKERS FOR THE STUDY OF MIXED MATING IN *HIBISCUS APONEURUS* AND *H. FLAVIFOLIUS* FROM KENYA

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ABSTRACT

In a mixed mating system, plants use both self- and cross-pollination to produce seeds. Observations of pollinator behavior from populations of two Kenyan *Hibiscus* species suggest that *H. aponeurus* uses self-pollination at higher rates than does the co-occurring *H. flavifolius*. To begin testing this hypothesis, we sought to develop a suite of variable genetic loci to use in measuring levels of selfing and outcrossing in natural populations. We tested five microsatellite primer pairs from each of four *Hibiscus* species for amplification in *H. aponeurus* and *H. flavifolius.* Eight loci were subsequently tested for genetic variation, but were found to be monomorphic in both species. Gene sequencing suggested that evolutionary changes within these loci could account for the lack of inter-individual variation.

BACKGROUND

Hibiscus aponeurus and *H. flavifolius are* morphologically and ecologically similar species that co-occur in woody savannahs of Kenya. A field study of pollinator visitation indicated that the red-flowered *H. aponeurus* was visited only about half as often as the white-flowered *H. flavifolius* (Foutch et al. 2008). Controlled pollination experiments also showed that *H. aponeurus* more often used self-fertilization to produce seeds than did *H. flavifolius*. In both species, seeds can be produced by a process called delayed autonomous self-pollination, in which styles with unpollinated stigmas curve backwards towards the anthers throughout the day. If the stigmas are pollinated during the curving process, the styles will stop curving, but if stigmas are not pollinated, styles will continue curving, eventually causing the stigmas to touch the pollen on the anthers and effect self-pollination. This mechanism assures that a plant will produce at least some seeds, even when pollinator visits are few. Thus, the more a plant is visited by pollinators, the less it will self-pollinate.

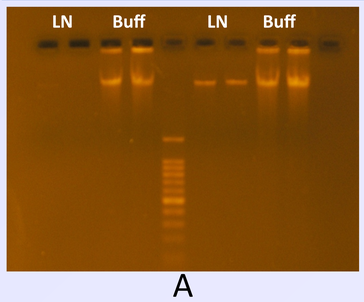
QUESTION & HYPOTHESES

What are the levels of selfing in natural populations of *H. aponeurus* and *H. flavifolius*?

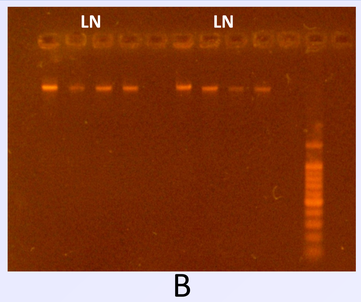
- ✧ Because the flowers of both species have been shown to use autonomous self-pollination in the field when pollinators don't visit, the populations should have a mating system that includes a mix of seeds produced by selfing and outcrossing.
- ✧ Because *H. aponeurus* is visited less often by pollinators, it will more often use delayed selfing to produce seeds and will therefore show a higher selfing rate than *H. flavifolius.*

Objective 1:
Extract clean DNA from *H. aponeurus* and *H. flavifolius*

Figure 1. Results of DNA extractions (Qiagen DNAeasy plant mini kit) from 8 seedlings each of *H. aponeurus* (A) and *H. flavifolius* (B). LN = seedlings were ground in liquid N_2 prior to adding tissue to lysis buffer; Buff = seedlings were ground directly in lysis buffer without aid of liquid N_2. In *H. aponeurus* (A), the LN method resulted in better quality DNA, while Buff method resulted in a greater quantity of DNA. During PCR, DNA extracted with both methods resulted in similar amplification of microsatellites.



A



B

Objective 2:
Choose and test the amplification of microsatellite loci from four other *Hibiscus* species.

Table 1. Number of loci (out of 5) that successfully amplified in *H. aponeurus* and *H. flavifolius.* We tested 20 microsatellite loci from four other *Hibiscus* species. A total of 11 loci successfully amplified in *H. aponeurus, and* 8 amplified in *H. flavifolius*. Only those loci that amplified with strong, single bands were considered for subsequent genotyping and sequencing.

Species for which primers were developed	*H. aponeurus*	*H. flavifolius*	Comments
H. tiliaceus Takayama et al. (2006)	5/5	2/5	Lots of non-specific amp.
H. aridicola Zhang et al. (2001)	4/5	4/5	Strong, single bands with good length
H. rosa-sinensis Bruna et al (2009)	2/5	2/5	Strong, single bands
H. glaber Ohtani et al (2008)	0/5	0/5	No amp. at any temperature

Figure 2. Variation in amplification success at different annealing temperatures in *H. aponeurus.* Microsatellites amplified better at 55°C than at 60°C. Primers from *H. aridicola* (HA primers) generally amplified well at the expected size, whereas primers from *H. tiliaceus* (Ht primers) showed a lot of non-specific amplification.

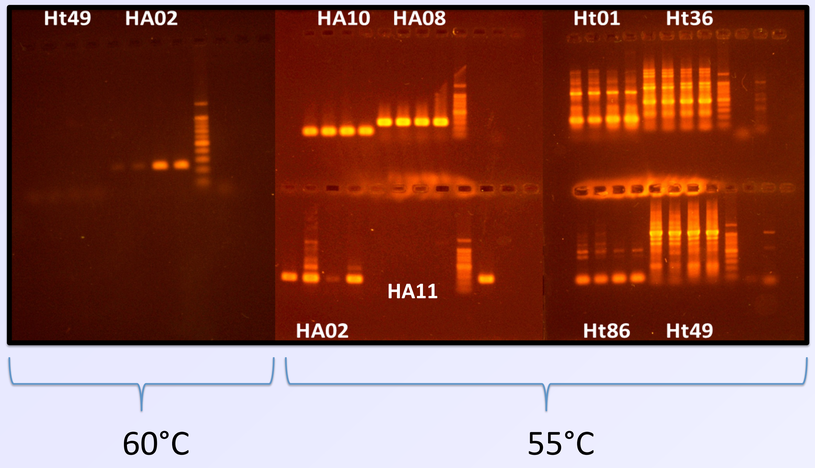
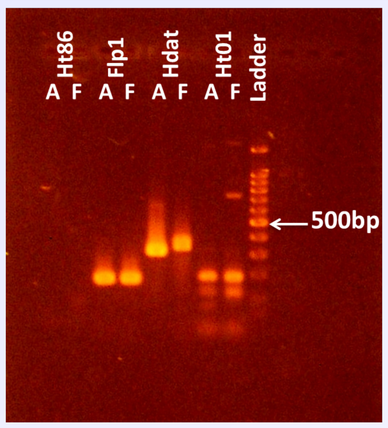
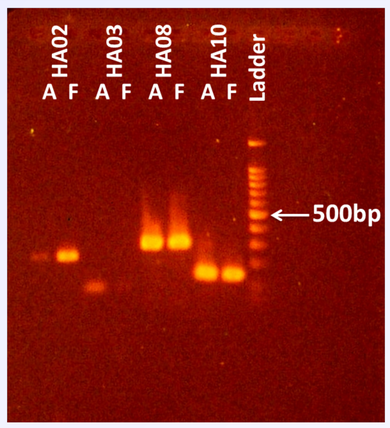


Figure 3. Amplification differences between *H. aponeurus* and *H. flavifolius* were evident. Amplification within the same locus in the two species differed in quantity and quality. There was also a difference in the amount of nonspecific amplification between the two species in the Ht01 microsatellite locus.





Objective 3:
Test microsatellite loci for levels of variation

Table 2. We chose 7 loci to test for levels of variability: 4 from *H. aridicola*, 1 from *H. tiliaceus*, and 2 from *H. rosa-sinensis.* Forward primers were fluorescently tagged, and amplification products were genotyped using an ABI 3130 genetic analyzer. We tested 12 individuals per locus per species and found no variation at any locus. At each locus, all individuals were homozygous for a single allele.

Locus	Repeat motif	Expected size (bp)	Primer sequences (5'-3')
HA-02	AG	410-445	F: CTGAATGCCAGAATGACT R: CAGGCGAAAGAGGAAGAT
HA-03	CT	141-154	F: ATCATTATCATCTTCGTTTC R: AAGGGACCAAAGTCTCAA
HA-08	CTTCT	230-242	F: CACTTCCACGAAGCTCTTAC R: GGAGATAAACAGAAAAGGGTA
HA-10	GT	197-204	F: CCCAAACCTCTATCATCT R: ATATCCCTTAGTTCTGCT
Ht-86	GA	94-99	F: GGACTTTTCTGTTTCTTTTCTCGTAG R: ATGCTTTCAGTTCCTTGACAAATG
H-DAT2	TTA	400-480	F: TGTCAAGCTGTCAAGGGTGA R: CCGATCCGTGTTTTTCAAGT
H-MAFLP1	CT	159-169	F: AGCCTGTCACCAACAAA R: GAGAGCTTACGAAGCGGAGA

Figure 4. Nucleotide sequence of H-dat2 microsatellite locus from *H. aponeurus* and *H. flavifolius* (primer sequences in **green**; variable sites in **red**). The locus length is ~100-150 bp shorter than expected, and there is no evidence of the TTA repeat motif (cf. Table 2). These results indicate that the locus has undergone extensive evolutionary change since the *H. flavifolius/H. aponeurus* lineage split from the *H. rosa-sinensis* lineage within the *Hibiscus* genus. They also help explain our finding of only a single allele within our populations. Similar results would be expected if we sequenced the 7 other loci that were also monomorphic.

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H.fl.  TGTCAAGCCT GGCGAAGGGT GAATTTCTTA TTTCTTATTA TTAATTTATT GATTTCTTAT  60
H.ap.  TGTCAAGCCT GGCGAAGGGT GAATTTCTTA TTTCTTATTA TTGATTTATT GATTTCTTAT  60

H.fl.  TATTGAGTTT ATATTATATA TAATTTATAT TAGATGTTAG AATATTAATT CTATTATATA 120
H.ap.  TATTGAGTTT ATATTATATA TAATATATAT TAGATGTTAG AATATTAATT CTATTATATA 120

H.fl.  TTTATATATT CTAATATATA TTCTAATTTA TTTATATATT TTATTTAGAA TAATATAATT 180
H.ap.  TTTATATATT CTAATATATA TTCTAACTTA TTT------- ---------- --ATATAATT 161

H.fl.  TTATTTAGAA TATATTCTAT TTAAATTAAT ATTTAATATT ATTATTTTTT ATATTCTTTT 240
H.ap.  TTATTTAGAA TATATTCTAT TTAAATTAAT ATTTAATATT ATTATTTTTT ATATTCTTTT 221

H.fl.  TATATCATTT ATTTTCATTT ATTTCATTTG CAT                               253
H.ap.  TATATCATTT ATTTTCATTT ATTTCATTTG CATTTCGATT CAAAAAAAAA GGAAGGAATT 281

H.fl.
H.ap.  ATAAACTTGA AAAACACGGA TCGGA                                       306
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LITERATURE CITED

Bruna S, Portis E, Braglia L, De Benedetti L, Comino C, Acquadro A, Mercuri A (2009) Isolation and characterization of microsatellite markers from *Hibiscus rosa-sinensis* (Malvaceae) and cross-species amplifications. *Conservation Genetics*, **10**, 771-774.

Foutch BR, Ruiz-Guajardo JC, Otero-Arnaiz A, Stone G, Schnabel A (2008) Field and greenhouse experiments into the pollination biology of *Hibiscus aponeurus* and *H. flavifolius* from Kenya. Annual Meeting of the Ecological Society of America, Milwaukee, Wisconsin.

Ohtani M, Tani N, Yoshimaru H (2008) Development and characterization of microsatellite markers for *Hibiscus glaber* Matsum. Ex Nakai, and endemic tree species of the oceanic Bonin Islands, Japan. *Molecular Ecology Resources*, **8**, 1316-1318.

Takayama K, Kajita T, Murata J, Tateishi Y (2006) Isolation and characterization of microsatellites in the sea hibiscus (Hibiscus tiliaceus L., Malvaceae) and related hibiscus species. *Molecular Ecology Notes*, **6**, 721-723.

Zhang L, Sun W, Wang Z, Guan K, Yang J (2011) Isolation and Characterization of Microsatellite Loci for *Hibiscus aridicola* (Malvaceae), and Endangered Plant Endemic to the Dry-Hot Valleys of Jinsha River in Southwest China. *International Journal of Molecular Sciences*, **12**, 5698-5704.

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