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DEVELOPMENT AND CHARACTERIZATION OF 27 MICROSATELITE MARKERS FOR THE MANGROVE FERN, ACROSTICHUM AUREUM (PTERIDACEAE)

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• Premise of the study: Twenty-seven nuclear microsatellite markers were developed for the mangrove fern, Acrostichum aureum (Pteridaceae), to investigate the genetic structure and demographic history of the only pantropical mangrove plant.

• Methods and Results: Fifty-six A. aureum individuals from three populations were sampled and genotyped to characterize the 27 loci. The number of alleles and expected heterozygosity ranged from one to 15 and 0.000 to 0.893, respectively. Across the 26 polymorphic loci, the Malaysian population showed much higher levels of polymorphism compared to the other two populations in Guam and Brazil. Cross-amplification tests in the other two species from the genus determined that seven and six loci were amplifiable in A. danaeifolium and A. speciosum, respectively.

• Conclusions: The 26 polymorphic microsatellite markers will be useful for future studies investigating the genetic structure and demographic history of A. aureum, which has the widest distributional range of all mangrove plants.

Key words: Acrostichum aureum; mangrove fern; microsatellite; pantropical distribution; Pteridaceae; pyrosequencing.

Mangroves are intertidal ecosystems that have a pantropical distribution. The distributional range of species inhabiting these ecosystems is typically restricted to either the Indo-West Pacific (IWP) region or the Atlantic-East Pacific (AEP) region (Tomlinson, 1986). How this pattern of distribution formed is one of the main biogeographic questions in mangrove research. Phylogenetic studies have detected significant levels of divergence in several tree species across the IWP and AEP (Rhizophora L. in Duke et al., 2002 and Takayama et al., 2013; and Hibiscus L. in Takayama et al., 2008). However, the divergence history, at a global scale, of many other mangrove plants remains to be clarified. Acrostichum aureum L. (common name “mangrove fern”; Pteridaceae) is of particular interest because this species is the only mangrove plant that is distributed pantropically (i.e., in both the IWP and AEP regions). This species also differs from other mangrove plants in that it has wind-dispersed spores, while most other mangrove plants have sea-dispersed seeds, fruits, or propagules. This different dispersal system might have enabled this species to achieve its relatively wide distribution compared to other mangrove plants. To address this question, it is important to perform population genetic studies to analyze the genetic structure and demographic history of the species using highly polymorphic microsatellite markers. Therefore, we developed novel microsatellite markers for A. aureum using next-generation sequencing. We tested the markers on samples from across the pantropical distribution of the species to check
Acrostichum aureum microsatellites

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Table 1. Characteristics of 27 microsatellite markers developed for *Acrostichum aureum*.\(^a,b\)

| Locus  | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | Fluorescent dye | Multiplex | DDBJ accession no. |
|--------|--------------------------|--------------|------------------------|-----------------|----------|-------------------|
| AA07   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (GA)\(^a\)_17 | 194–246 | FAM | Set 1 | LC065390 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AG)\(^b\)_16 | 120–150 | VIC | Set 4 | LC065391 |
| AA08   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^b\)_15 | 144–170 | FAM | Set 1 | LC065392 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AC)\(^b\)_14 | 179–259 | VIC | Set 4 | LC065393 |
| AA11   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_15 | 129–159 | NED | Set 8 | LC065394 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AC)\(^a\)_14 | 123–159 | VIC | Set 5 | LC065395 |
| AA14   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_14 | 169–177 | PET | Set 10 | LC065396 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AT)\(^a\)_13 | 104–124 | FAM | Set 2 | LC065401 |
| AA15   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_14 | 110–134 | VIC | Set 6 | LC065399 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AC)\(^a\)_13 | 114–134 | NED | Set 9 | LC065398 |
| AA16   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_14 | 169–182 | VIC | Set 6 | LC065399 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AT)\(^a\)_13 | 140–152 | NED | Set 7 | LC065400 |
| AA17   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_14 | 84–128 | VIC | Set 6 | LC065403 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AC)\(^a\)_14 | 104–124 | PET | Set 2 | LC065405 |
| AA19   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_14 | 103–135 | FAM | Set 3 | LC065404 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AT)\(^a\)_13 | 160–172 | FAM | Set 2 | LC065406 |
| AA24   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_14 | 118–256 | VIC | Set 6 | LC065403 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AC)\(^a\)_14 | 103–135 | FAM | Set 3 | LC065404 |
| AA27   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_14 | 196–214 | VIC | Set 6 | LC065399 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AT)\(^a\)_13 | 188–216 | FAM | Set 1 | LC065407 |
| AA33   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_14 | 352–376 | FAM | Set 1 | LC065408 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AT)\(^a\)_13 | 352–376 | FAM | Set 1 | LC065408 |
| AA35   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_14 | 173–199 | VIC | Set 5 | LC065409 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AC)\(^a\)_13 | 203–225 | NED | Set 9 | LC065410 |
| AA37   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_14 | 169–199 | VIC | Set 8 | LC065412 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AC)\(^a\)_13 | 145–165 | FAM | Set 12 | LC065413 |
| AA38   | F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC | (AG)\(^a\)_14 | 173–199 | VIC | Set 5 | LC065409 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AC)\(^a\)_13 | 145–165 | FAM | Set 12 | LC065413 |
| AA43   | F: GGTTTTCCCAGTCACGACGTTGGATGGGTTGACCTTCTTCGTC | (AG)\(^a\)_14 | 313–315 | VIC | Set 4 | LC065414 |
|       | R: GTTGGTGTTGAGATGGGTTGTTA | (AT)\(^a\)_13 | 176–224 | FAM | Set 3 | LC065415 |
| AA48   | F: GGTTTTCCCAGTCACGACGTTGGATGGGTTGACCTTCTTCGTC | (AG)\(^a\)_14 | 114–136 | PET | Set 10 | LC065416 |

Note: DDBJ = DNA Data Bank of Japan.

\(^a\)All values are based on 56 samples representing Sabah (Malaysia), Piti (Guam), and Pará (Brazil) populations (Appendix 1).

\(^b\)Annealing temperature for all loci was 57°C.

their levels of polymorphism and to determine their usefulness as markers for future studies.

METHODS AND RESULTS

One sample of *A. aureum* was collected from Sabah (Malaysia) (Appendix 1), and total DNA extracted using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). We then performed shotgun sequencing, using one-third of a run on a Roche 454 Genome Sequencer Junior (Roche Applied Science, Penzberg, Germany). The GS Junior Titanium Sequencing Kit (Roche Applied Science) and Multiplex Identifier (MID) adaptors (see Margulies et al., 2005) were used following the manufacturer’s protocol. The run generated a total of 81,415 reads with an average length of 490 bp.

The program QDD version 2.1 (Meglécz et al., 2010) was used to identify di- to hexanucleotide motif microsatellites with at least five repeats. Sequence similarity and establishment contigs were detected following the procedure in Takayama et al.
Table 2. Genetic variation of the 27 newly developed microsatellite markers in three Acrostichum aureum populations. 

| Locus | Sabah, Malaysia (N = 24) | Piti, Guam (N = 16) | Pará, Brazil (N = 16) |
|-------|------------------------|-------------------|---------------------|
|       | A | H\textsubscript{o} | H\textsubscript{e} | F\textsubscript{IS} | A | H\textsubscript{o} | H\textsubscript{e} | F\textsubscript{IS} | A | H\textsubscript{o} | H\textsubscript{e} | F\textsubscript{IS} |
| AA07  | 12 | 0.875 | 0.893 | 0.052 | 1 | 0.000 | 0.000 | NA | 1 | 0.000 | 0.000 | NA |
| AA08  | 10 | 0.870 | 0.883 | 0.037 | 1 | 0.000 | 0.000 | NA | 2 | 0.133 | 0.391 | 0.678 |
| AA09  | 9  | 0.563 | 0.758 | 0.288 | — | — | — | NA | 1 | 0.000 | 0.000 | NA |
| AA10  | 13 | 0.647 | 0.870 | 0.285 | 2 | 1.000 | 0.500 | −1.000* | 2 | 1.000 | 0.500 | −1.000* |
| AA11  | 6  | 0.542 | 0.682 | 0.226 | — | — | — | NA | — | — | — |
| AA12  | 8  | 0.625 | 0.752 | 0.200 | 1 | 0.000 | 0.000 | NA | 0 | 0.000 | 0.000 | NA |
| AA14  | 4  | 0.375 | 0.541 | 0.326 | 1 | 0.000 | 0.000 | NA | 1 | 0.000 | 0.000 | NA |
| AA15  | 6  | 0.583 | 0.523 | −0.095 | 2 | 0.000 | 0.305 | 1 | 2 | 0.000 | 0.219 | 1.000 |
| AA16  | 8  | 0.333 | 0.782 | 0.590* | — | — | — | NA | — | — | — |
| AA17  | 10 | 0.824 | 0.804 | 0.007 | 1 | 0.000 | 0.000 | NA | 1 | 0.000 | 0.000 | NA |
| AA23  | 5  | 0.762 | 0.667 | −0.119 | 1 | 0.000 | 0.000 | NA | 1 | 0.000 | 0.000 | NA |
| AA24  | 1  | 0.000 | 0.000 | NA | 1 | 0.000 | 0.000 | NA | — | — | — |
| AA27  | 8  | 0.773 | 0.744 | −0.016 | 1 | 0.000 | 0.000 | NA | 2 | 0.063 | 0.661 | 0.000 |
| AA28  | 15 | 0.722 | 0.773 | 0.094 | 1 | 0.000 | 0.000 | NA | 1 | 0.000 | 0.000 | NA |
| AA29  | 9  | 0.765 | 0.827 | 0.105 | 1 | 0.000 | 0.000 | NA | 1 | 0.000 | 0.000 | NA |
| AA30  | 6  | 0.583 | 0.681 | 0.164 | 1 | 0.000 | 0.000 | NA | 1 | 0.000 | 0.000 | NA |
| AA33  | 3  | 0.375 | 0.490 | 0.255 | 1 | 0.000 | 0.000 | NA | — | — | — |
| AA34  | 8  | 0.714 | 0.659 | −0.060 | 1 | 0.000 | 0.000 | NA | 3 | 0.188 | 0.174 | −0.047 |
| AA35  | 10 | 0.875 | 0.855 | −0.002 | 1 | 0.000 | 0.000 | NA | 2 | 0.188 | 0.170 | −0.071 |
| AA37  | 9  | 0.688 | 0.732 | 0.093 | 2 | 0.000 | 0.305 | 1.000* | 2 | 0.063 | 0.661 | 0.000 |
| AA38  | 9  | 0.682 | 0.830 | 0.201 | 1 | 0.000 | 0.000 | NA | 1 | 0.000 | 0.000 | NA |
| AA40  | 10 | 0.833 | 0.852 | 0.044 | 1 | 0.000 | 0.000 | NA | 2 | 0.067 | 0.664 | 0.000 |
| AA41  | 9  | 0.792 | 0.813 | 0.048 | 3 | 0.250 | 0.584 | 0.593 | 1 | 0.000 | 0.000 | NA |
| AA42  | 8  | 0.857 | 0.862 | 0.030 | 3 | 0.250 | 0.498 | 0.522 | 1 | 0.000 | 0.000 | NA |
| AA43  | 2  | 0.063 | 0.170 | 0.651 | — | — | — | 0.611 | — | — | — |
| AA46  | 13 | 0.941 | 0.796 | −0.153 | 1 | 0.000 | 0.000 | NA | — | — | — |
| AA48  | 9  | 0.583 | 0.745 | 0.237 | 1 | 0.000 | 0.000 | NA | 1 | 0.000 | 0.000 | NA |

Note: — = not amplified; A = number of detected alleles; F\textsubscript{IS} = fixation index; H\textsubscript{o} = expected heterozygosity; N = number of individuals genotyped; NA = not applicable.

*Voucher and locality information are provided in Appendix 1.

*Significant deviation from Hardy–Weinberg equilibrium (P < 0.05).

The amplified products were loaded into an ABI3500 automatic sequencer (Applied Biosystems, Waltham, Massachusetts, USA) with GeneScan 600 LIZ Size Standard (Applied Biosystems), and their sizes and genotypes were determined using GeneMarker (Holland and Parson, 2011). Excluded heterozygosity (H\textsubscript{o}) and fixation index (F\textsubscript{IS}) were calculated to evaluate genetic diversity of the three populations using FSTAT version 2.9.3.2 (Goudet, 2001; hereafter, FSTAT). The significance of deviations of F\textsubscript{IS} from zero, as evidenced by deviation from Hardy–Weinberg equilibrium, and genotypic disequilibrium for all locus pairs, were tested by randomization using FSTAT. The obtained P values (with a 0.05 significance threshold) were adjusted based on a sequential Bonferroni correction. The presence of null alleles and their bias on genetic diversity among the three populations (F\textsubscript{IS}) (Weir and Cockerman, 1984) were evaluated using FreeNA (Chapuis and Estoup, 2007). In the Sabah population, the number of alleles detected and H\textsubscript{o} ranged from one to 15 and 0.000 to 0.893, respectively, and 26 of the 27 loci were polymorphic (Table 2). A significant deviation in F\textsubscript{IS} was found in only one locus (AA16). Although null alleles were detected and their frequencies estimated at each locus (Table 4), the F\textsubscript{IS} value after the null allele correction was 0.619, the same as the original value without correction (= 0.619), suggesting that biases, due to null alleles, in genetic structure analysis would be limited. Although 19 of the 27 loci were amplified in samples from the other two populations, most were fixed for different alleles among populations. Seven and six loci were amplified in A. danaeifolium and A. speciosum, respectively (Table 3).

CONCLUSIONS

The 26 polymorphic microsatellite markers developed in this study will be useful to evaluate the genetic structure and to infer the past demographic history of A. aureum to study how this mangrove fern achieved the widest distributional range of all mangrove plants. Cross-species amplification also suggested that some markers could be used to evaluate genetic diversity in other species in the same genus.

http://www.bioone.org/loi/apps
Table 3. Fragment sizes detected in cross-amplification tests of the 27 newly developed Acrostichum aureum microsatellite markers in two closely related species.\textsuperscript{a}

| Locus | A. danaeifolium | A. speciosum |
|-------|-----------------|--------------|
|       | Pará, Brazil (N = 4) | Colima, Mexico (N = 4) | Sungei Buloh, Singapore (N = 4) |
| AA07  | 134–2154         | 134–2158      | 154–2158 |
| AA08  | 207–2217         | 207–2217      | 144     |
| AA09  | 344–2348         | 344–2348      |         |
| AA10  | 273             | 273           |         |
| AA11  | 213             | 213           |         |
| AA12  | 316             | 316           |         |
| AA13  | 273             | 273           |         |
| AA14  | 207–219         | 207–2217      |         |
| AA15  | 175             | 175           |         |
| AA16  | 344–2348         | 344–2348      |         |
| AA17  | 207–219         | 207–2217      |         |
| AA18  | 207–219         | 207–2217      |         |
| AA19  | 344–2348         | 344–2348      |         |
| AA20  | 207–219         | 207–2217      |         |
| AA21  | 344–2348         | 344–2348      |         |
| AA22  | 207–219         | 207–2217      |         |
| AA23  | 344–2348         | 344–2348      |         |
| AA24  | 207–219         | 207–2217      |         |
| AA25  | 344–2348         | 344–2348      |         |
| AA26  | 207–219         | 207–2217      |         |
| AA27  | 207–219         | 207–2217      |         |
| AA28  | 207–219         | 207–2217      |         |
| AA29  | 344–2348         | 344–2348      |         |
| AA30  | 344–2348         | 344–2348      |         |
| AA31  | 207–219         | 207–2217      |         |

Note: — = not amplified.

\textsuperscript{a}Voucher and locality information are provided in Appendix 1.

Table 4. Null allele frequencies at each locus estimated by FreeNA software in three Acrostichum aureum populations.\textsuperscript{a}

| Locus | Sabah, Malaysia (N = 24) | Piti, Guam (N = 16) | Pará, Brazil (N = 16) |
|-------|--------------------------|---------------------|-----------------------|
| AA07  | 0.000                    | 0.001               | 0.001                 |
| AA08  | 0.000                    | 0.001               | 0.200                 |
| AA09  | 0.122                    | NA                  | 0.001                 |
| AA10  | 0.114                    | 0.000               | 0.000                 |
| AA11  | 0.111                    | NA                  | NA                    |
| AA12  | 0.086                    | 0.001               | 0.001                 |
| AA13  | 0.111                    | 0.001               | 0.001                 |
| AA15  | 0.000                    | 0.263               | 0.224                 |
| AA16  | 0.247                    | NA                  | NA                    |
| AA17  | 0.000                    | 0.001               | 0.001                 |
| AA18  | 0.000                    | 0.001               | 0.001                 |
| AA19  | 0.000                    | 0.001               | 0.001                 |
| AA20  | 0.000                    | 0.001               | 0.001                 |
| AA21  | 0.000                    | 0.001               | 0.001                 |
| AA22  | 0.000                    | 0.001               | 0.001                 |
| AA23  | 0.000                    | 0.001               | 0.001                 |
| AA24  | 0.000                    | 0.001               | 0.001                 |
| AA25  | 0.000                    | 0.001               | 0.001                 |
| AA26  | 0.000                    | 0.001               | 0.001                 |
| AA27  | 0.000                    | 0.001               | 0.001                 |
| AA28  | 0.000                    | 0.001               | 0.001                 |
| AA29  | 0.053                    | 0.001               | 0.001                 |
| AA30  | 0.048                    | 0.001               | 0.001                 |
| AA31  | 0.090                    | 0.001               | NA                    |
| AA32  | 0.000                    | 0.001               | 0.000                 |
| AA33  | 0.000                    | 0.001               | 0.000                 |
| AA34  | 0.000                    | 0.001               | 0.000                 |
| AA35  | 0.000                    | 0.001               | 0.000                 |
| AA36  | 0.065                    | 0.263               | 0.000                 |
| AA37  | 0.054                    | 0.001               | 0.001                 |
| AA38  | 0.000                    | 0.001               | 0.000                 |
| AA39  | 0.000                    | 0.211               | 0.001                 |
| AA40  | 0.000                    | 0.175               | 0.001                 |
| AA41  | 0.138                    | NA                  | NA                    |
| AA42  | 0.000                    | 0.001               | 0.001                 |
| AA43  | 0.000                    | 0.001               | 0.001                 |
| AA44  | 0.000                    | 0.001               | 0.001                 |
| AA45  | 0.000                    | 0.001               | 0.001                 |
| AA46  | 0.000                    | 0.001               | 0.001                 |
| AA47  | 0.000                    | 0.001               | 0.001                 |
| AA48  | 0.098                    | 0.001               | 0.001                 |

Note: NA = not applicable.

\textsuperscript{a}Voucher and locality information are provided in Appendix 1.

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### APPENDIX 1. Voucher information for *Acrostichum* species used in this study.

| Species                   | Voucher specimen accession no.* | Collection locality         | Geographic coordinates          | N  |
|---------------------------|---------------------------------|----------------------------|---------------------------------|----|
| *A. aureum* L.            | TK 11072403 (348–371) (URO)     | Klias, Sabah, Malaysia      | 5.426454°, 115.559861°          | 24 |
| *A. aureum*               | TK 13122001 (1–16) (URO)        | Piti, Guam                  | 13.440381°, 144.678365°         | 16 |
| *A. danaefolium* Langsd. & Fisch. | GMM 14112102 (207–222) (UEC)  | Perimirim, Pará, Brazil    | −0.973032°, −46.591348°         | 16 |
| *A. danaefolium* Langsd. & Fisch. | GMM 14112201 (231–234) (UEC)  | Capanema, Pará, Brazil     | −1.299979°, −47.099699°         | 4  |
| *A. speciosum* Willd.     | TK 14071804 (50–53) (URO)       | Ciudad de Armería, Colima, Mexico | 18.912410°, −104.036289°      | 4  |
| *A. speciosum* Willd.     | TK 091112003 (61–63) (URO)      | Sungei Buloh, Singapore    | 1.449007°, 103.730684°          | 4  |

*Note:* N = number of individuals sampled.

*Collectors and herbaria: GMM = Gustavo Maruyama Mori; TK = Tadashi Kajita; UEC = Universidade Estadual de Campinas herbarium; URO = Herbarium, Faculty of Education, University of the Ryukyus.*