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Author(s): Takashi Yamamoto, Yoshiaki Tsuda, Gustavo Maruyama Mori, Mariana Vargas Cruz, Yoshimi Shinmura, Alison K. S. Wee, Koji Takayama, Takeshi Asakawa, Takeru Yamakawa, Monica Suleiman, Juan Núñez-Farfán, Edward L. Webb, Yasuyuki Watano, and Tadashi Kajita
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Primer Note

Development and characterization of 27 microsatellite markers for the mangrove fern, *Acrostichum aureum* (Pteridaceae)[1]

Takashi Yamamoto[2,3,12], Yoshiaki Tsuda[4,12], Gustavo Maruyama Mori[3,5], Mariana Vargas Cruz[5], Yoshimi Shinmura[6], Alison K. S. Wee[7], Koji Takayama[8], Takeshi Asakawa[6], Takeru Yamakawa[6], Monica Suleiman[9], Juan Núñez-Farfán[10], Edward L. Webb[11], Yasuyuki Watano[6], and Tadashi Kajita[2,3,13]

[2]United Graduate School of Agricultural Science, Kagoshima University, 1-21-24 Korimoto, Kagoshima 980-0065, Japan; [3]Iriomote Station, Tropical Biosphere Research Center, University of the Ryukyus, 870 Uehara, Taketomi-cho, Yaeyama-gun, Okinawa 907-1541, Japan; [4]Sugadaira Montane Research Center, University of Tsukuba, 1278-294 Sugadairakogen, Ueda, Nagano 386-2204, Japan; [5]Laboratório de Análise Genética e Molecular, Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, Brazil; [6]Department of Biology, Graduate School of Science, Chiba University, 1-33 Yayoi, Inage, Chiba, Chiba 263-8522, Japan; [7]College of Forestry, Guangxi University, Nanning, Guangxi, China; [8]Museum of Natural and Environmental History, Shizuoka, 5762 Oya, Suruga-ku, Shizuoka-shi, Shizuoka 422-8017, Japan; [9]Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Malaysia; [10]Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Apartado Postal 70-275, 04510 México D.F., México; and [11]Department of Biological Sciences, National University of Singapore, Singapore, Singapore

- *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 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

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[12]These authors contributed equally to this work.

[13]Author for correspondence: kajita@mail.ryudai.jp

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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: GGTTTTCCCAGTCACGACAATGGGCTACTCAAATGGG	$(GA)_{17}$	194–246	FAM	Set 1	LC065390
	R: GTTTGTGTTCCTTGTATGTCGATCAAT					
AA08	F: GGTTTTCCCAGTCACGACGAAGAGGTGGGACAAGCAAG	$(AG)_{16}$	120–150	VIC	Set 4	LC065391
	R: GTTTGTGGTTGAGAGTGGGTTGA					
AA09	F: GGTTTTCCCAGTCACGACGTGCGATGGCTACTTCTCCT	$(AG)_{15}$	144–170	FAM	Set 1	LC065392
	R: GTTTCCCTTTCTCCACACTCC					
AA10	F: GGTTTTCCCAGTCACGACAGCCTTGCAACCTGCTCTAC	$(AC)_{15}$	197–259	VIC	Set 4	LC065393
	R: GTTTCCATCATGGCCAGCTTTACT					
AA11	F: GGTTTTCCCAGTCACGACCCGTAGGCTCTGATACCAA	$(AC)_{15}$	129–159	NED	Set 8	LC065394
	R: GTTTCTCCCATGTCAAACACTCCA					
AA12	F: GGTTTTCCCAGTCACGACGCCAGCCTAGACACCTCTTG	$(TG)_{15}$	123–159	VIC	Set 5	LC065395
	R: GTTTGCATGCATAAGAAGACCAACC					
AA14	F: GGTTTTCCCAGTCACGACAGGTCAAGCACAAGCTCAA	$(AG)_{14}$	169–177	PET	Set 10	LC065396
	R: GTTTACACCTGCACACTGGTGAGT					
AA15	F: GGTTTTCCCAGTCACGACAGTTCTTGTCTTGGGTGAGCA	$(TG)_{14}$	269–281	PET	Set 10	LC065397
	R: GTTTGGAGTAAGCTTGGTGCATATC					
AA16	F: GGTTTTCCCAGTCACGACGGTGCAAGGAGATGCCATAG	$(GA)_{14}$	114–134	NED	Set 9	LC065398
	R: GTTTAGTCAGGGTCGTTCAAGCTG					
AA17	F: GGTTTTCCCAGTCACGACGGGTGTGAGGGATTTGAGAA	$(AG)_{14}$	118–182	VIC	Set 6	LC065399
	R: GTTTATCGTTGGAGATGATGGAGG					
AA23	F: GGTTTTCCCAGTCACGACGAGAGGAGAGAAGCAAATAGGG	$(GA)_{12}$	285–293	NED	Set 7	LC065400
	R: GTTTGGAGTCTTGGTAGACGGG					
AA24	F: GGTTTTCCCAGTCACGTTGAGCCAATGAAATGCT	$(TG)_{11}$	267–269	FAM	Set 2	LC065401
	R: GTTTAGGAAGAGAAAGCGAGGGAG					
AA27	F: GGTTTTCCCAGTCACGACGTTGTCCTCTACTTGAGCTCCC	$(CA)_{15}$	140–152	NED	Set 7	LC065402
	R: GTTTCACACAAGAGAGCATGTTTGTA					
AA28	F: GGTTTTCCCAGTCACGACGTCTCCTGAAGGGAGTGGTGA	$(GA)_{15}$	84–128	VIC	Set 6	LC065403
	R: GTTTGAGTTCCACACCATGCCAG					
AA29	F: GGTTTTCCCAGTCACGACGAAAGATGCAAAGAAAGGGAGA	$(AC)_{15}$	103–135	FAM	Set 3	LC065404
	R: GTTTGAAGATGAGAAGTGTGGTCG					
AA30	F: GGTTTTCCCAGTCACGACGTCTTCAAGTGTCTTGGGTTTGA	$(AC)_{14}$	104–124	FAM	Set 2	LC065405
	R: GTTTATTCATGAGGAGCATGACCTA					
AA33	F: GGTTTTCCCAGTCACGACGCGCACCTTGTCCAAGTAAGC	$(AT)_{13}$	160–172	FAM	Set 2	LC065406
	R: GTTTGGAATAGGTAATGGAGTAGACTTGA					
AA34	F: GGTTTTCCCAGTCACGACGTCTTCAATCCTCTCTATAAACTAGCG	$(CA)_{13}$	188–216	PET	Set 12	LC065407
	R: GTTTCTCACAAGGGAGGCTATCCA					
AA35	F: GGTTTTCCCAGTCACGACGATGAAGCCAAGATCCCAAA	$(GA)_{13}$	352–376	FAM	Set 1	LC065408
	R: GTTTGCCACCACACCTTCTCTGAT					
AA37	F: GGTTTTCCCAGTCACGACGTTCCGATCCTTGTTGGTAGC	$(AG)_{13}$	173–219	VIC	Set 5	LC065409
	R: GTTTAAGTGGACGGCGTAATCAAG					
AA38	F: GGTTTTCCCAGTCACGACGCAATGGCGAATAGCGAAGC	$(TG)_{13}$	205–223	NED	Set 9	LC065410
	R: GTTTGTCACCCAAGACTCCCTCT					
AA40	F: GGTTTTCCCAGTCACGACGTTGCAGGTTAGAGCTCCCAT	$(TC)_{13}$	145–163	PET	Set 11	LC065411
	R: GTTTAGTGTCCACCAACCATCCA					
AA41	F: GGTTTTCCCAGTCACGACGTTGATGCAAATCAACCCTTT	$(CT)_{13}$	167–199	NED	Set 8	LC065412
	R: GTTTCATGATCCTTACCTTGCCC					
AA42	F: GGTTTTCCCAGTCACGACGAAGGATTGATGCAACCAAGG	$(AC)_{13}$	145–165	PET	Set 12	LC065413
	R: GTTTCCAATGTGAGCCATCAAGG					
AA43	F: GGTTTTCCCAGTCACGACGTTGGATGGACCTTCTTCGTC	$(CA)_{13}$	313–315	VIC	Set 4	LC065414
	R: GTTTGATGCTCTGATCCCTCCTT					
AA46	F: GGTTTTCCCAGTCACGACGGGAGTGTGACAAGGTGTAAGA	$(CT)_{12}$	176–224	FAM	Set 3	LC065415
	R: GTTTGACCGAGGCCAAGAATAAGG					
AA48	F: GGTTTTCCCAGTCACGACGTTCTACACGTGGTGGGAGGT	$(AG)_{12}$	114–136	PET	Set 10	LC065416
	R: GTTTCAAGGCTTCATATGAGGTGAG					

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.[a]

	Sabah, Malaysia (N = 24)				Piti, Guam (N = 16)				Pará, Brazil (N = 16)			
Locus	A	H_o	H_e	F_{IS}	A	H_o	H_e	F_{IS}	A	H_o	H_e	F_{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	—	—	—	—	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	—	—	—	—	—	—	—	—
AA12	8	0.625	0.752	0.200	1	0.000	0.000	NA	1	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*	—	—	—	—	—	—	—	—
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	1	0.000	0.000	NA
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	5	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	0.061	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	0.064	0.000
AA41	9	0.792	0.813	0.048	3	0.250	0.584	0.593	1	0.000	0.000	NA
AA42	10	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	—	—	—	—	—	—	—	—
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_{IS} = fixation index; H_e = expected heterozygosity; N = number of individuals genotyped; NA = not applicable.

[a]Voucher and locality information are provided in Appendix 1.

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

(2011). A total of 1452 perfect microsatellite sequences were obtained and 48 primer pairs designed using the following criteria: (1) PCR product size of 80–300 bp; (2) flanking region containing at least five repetitions of any di- to hexanucleotide motifs; and (3) primers with length 18–27 bp, annealing temperature 57–63°C, and GC content 20–80%. Forty-eight primer pairs with at least 12 repeats of various fragment sizes appropriate for multiplex PCR were selected. The 5′-tailed primer method (Schuelke, 2000) was used to label and visualize the PCR amplicons of the selected primers. The 19-bp U19 sequence (GGTTTTCCCAGTCACGACG) was added to the 5′-tail of forward primers, and the GTTT PIG-tail was added to the 5′ end of the reverse primer. This PIG-tail facilitates the addition of adenosine by *Taq* polymerase, thereby reducing stuttering (Brownstein et al., 1996). PCR amplification tests of each primer pair were performed in individual PCR reactions using two individuals, collected from Sabah (Malaysia) and Pará (Brazil), using the standard protocol of QIAGEN Type-it Microsatellite PCR Kit (QIAGEN), with a final volume of 5.0 µL and 1.0 µM of each primer. The PCR thermal conditions were as follows: initial denaturation at 95°C for 5 min; 30–32 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 90 s, extension at 72°C for 30 s; and final extension at 60°C for 30 min. The PCR products were electrophoretically separated on 1.5% agarose gels stained with ethidium bromide. Thirty loci exhibited clear PCR amplification. Twenty-four individuals sampled from Sabah (Malaysia) were used to assess the quality of amplification and polymorphism of these 30 loci. Loci were amplified using QIAGEN Type-it Microsatellite PCR Kits (QIAGEN) in three tubes, each with 5.0-µL mixtures containing 0.5 µL of 1–10 ng of genomic DNA, 2.5 µL of multiplex PCR master mix buffer, 1.2 µL of primer mix (with the concentration of each primer pair adjusted from 1.0 µM), and 0.8 µL of U19 fluorescent dye–labeled primer (6-FAM, VIC, NED, or PET; 1.0 µM). We used the same PCR protocol as described above. Twenty-seven of the 30 loci showed clear fragment patterns using one singleplex and 11 multiplex PCR sets (two to three primer pairs per multiplex; Table 1). Samples from two more populations (16 individuals each from Piti [Guam] and Pará [Brazil]; Appendix 1) were then included to check the genetic diversity of these loci. Cross-species amplification of these loci was also assessed by testing in the other two species in the genus *Acrostichum* L.: four individuals of *A. danaeifolium* Langsd. & Fisch. collected in Pará (Brazil) and Colima (Mexico), and four individuals of *A. speciosum* Willd. from Sungei Buloh (Singapore) (Appendix 1).

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). Expected heterozygosity (H_e) and fixation index (F_{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_{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_{ST}) (Weir and Cockerham, 1984) were evaluated using FreeNA (Chapuis and Estoup, 2007). In the Sabah population, the number of alleles detected and H_e 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_{IS} was found in only one locus (AA16). Although null alleles were detected and their frequencies estimated at each locus (Table 4), the F_{ST} 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.

TABLE 3. Fragment sizes detected in cross-amplification tests of the 27 newly developed *Acrostichum aureum* microsatellite markers in two closely related species.[a]

	A. danaeifolium		*A. speciosum*
Locus	Pará, Brazil (*N* = 4)	Colima, Mexico (*N* = 4)	Sungei Buloh, Singapore (*N* = 4)
AA07	—	—	188
AA08	—	—	—
AA09	134–2154	134–2158	154–2158
AA10	—	—	—
AA11	—	—	—
AA12	—	—	120–2132
AA14	—	—	—
AA15	—	—	—
AA16	—	—	—
AA17	—	—	—
AA23	—	—	—
AA24	—	—	—
AA27	—	—	144
AA28	—	—	—
AA29	—	—	110–2112
AA30	—	—	—
AA33	—	—	—
AA34	213	—	—
AA35	316	—	—
AA37	—	—	—
AA38	273	—	—
AA40	—	—	—
AA41	207–219	207–2217	—
AA42	175	—	—
AA43	344–2348	344–2348	—
AA46	—	—	217–2221
AA48	—	—	—

Note: — = not amplified.
[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.[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
AA14	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
AA23	0.000	0.001	0.001
AA24	0.001	0.001	NA
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
AA33	0.090	0.001	NA
AA34	0.000	0.001	0.000
AA35	0.000	0.001	0.000
AA37	0.065	0.263	0.000
AA38	0.054	0.001	0.001
AA40	0.000	0.001	0.000
AA41	0.000	0.211	0.001
AA42	0.000	0.175	0.001
AA43	0.138	NA	NA
AA46	0.000	0.001	NA
AA48	0.098	0.001	0.001

Note: NA = not applicable.
[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.[a]	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. aureum	GMM 14112102 (207–222) (UEC)	Perimirim, Pará, Brazil	−0.973032°, −46.591348°	16
A. danaeifolium Langsd. & Fisch.	GMM 14112201 (231–234) (UEC)	Capanema, Pará, Brazil	−1.299979°, −47.099699°	4
A. danaeifolium	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.

[a]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.