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Development of microsatellite markers for the annual andromonoecious herb *Commelina communis* f. *ciliata* (Commelinaceae)

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Commelina communis f. *ciliata* (Commelinaceae), a newly distinguished taxon, is an annual andromonoecious herb exhibiting a mixed mating system, the details of which remain unclear. We developed microsatellite markers for use in exploring the evolution of andromonoecy and mixed mating in the species. Fifteen microsatellite loci were developed using next-generation sequencing. The primer sets were used to evaluate 65 *C. communis* f. *ciliata* individuals from three populations in Japan; we found 1–13 alleles per locus and the expected heterozygosity ranged from 0.00 to 0.76. The markers are potentially useful to examine intra- and interspecies genetic structure and the mixed mating strategy of *Commelina* species via paternity analysis.

Key words: andromonoecy, *Commelina*, next-generation sequencing, paternity analysis

INTRODUCTION

Commelina L. is cosmopolitan and is the largest genus (comprising about 170 species) within the family Commelinaceae (Faden, 1998). *Commelina communis* is an annual herb native to temperate and subtropical regions of eastern and northern Southeast Asia, often growing around rice fields and on roadsides (Satake et al., 1982; Ushimaru et al., 2014). The species has been introduced to non-native regions including Europe and North America. *Commelina communis* exhibits morphological and chromosomal diversity; several varieties and forms have been described (Fujishima, 2003). *Commelina communis* f. *ciliata* Pennell is distinguished from *C. communis* by the presence of bract hair and the number of chromosomes. *Commelina communis* f. *ciliata* and *C. communis* usually have 2n = 44 or 46 and 2n = 86 or 88, respectively (Fujishima, 2003, 2010, 2017). The two taxa often grow sympatrically but do not hybridize (Fujishima, 2010; Katsuhara and Ushimaru, 2019). We also found significant differences in floral morphologies between the two taxa (Fig. 1; K. R. Katsuhara and A. Ushimaru, unpublished data). These recent findings suggest that *C. communis* f. *ciliata* should be a new taxon, although sufficient taxonomic studies have not yet been made.

Like *C. communis*, *C. communis* f. *ciliata* is andromonoecious and has a relatively high pollen:ovule ratio of ca. 1,300–1,700 for perfect flowers, comparable to ca. 1,000–1,700 in *C. communis* (Katsuhara and Ushimaru, 2019). Flowers of *C. communis* f. *ciliata* are frequently visited by syrphid flies and bees, as are the flowers of *C. communis* (Ushimaru et al., 2007). *Commelina communis* f. *ciliata* can reproduce via both bud pollination and autonomously delayed self-pollination, suggesting that this taxon exhibits a mixed mating system, the details of which remain unclear. Accordingly, studies of

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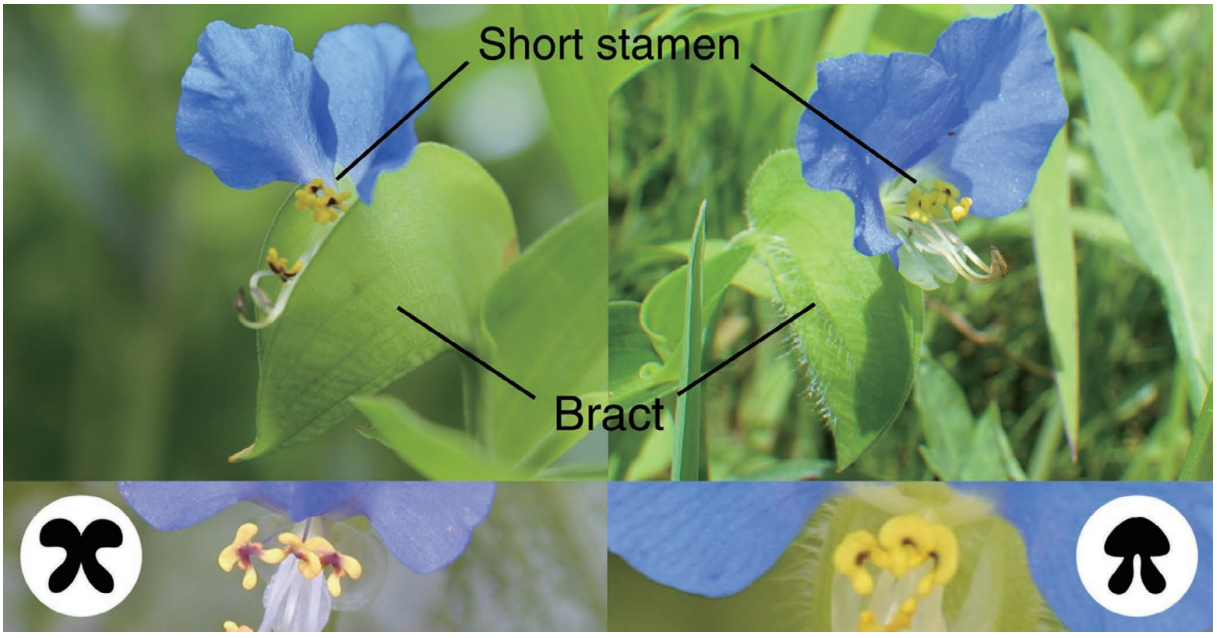

Fig. 1. Flower (upper) and short stamen (lower) morphologies of *Commelina communis* (left) and *C. communis* f. *ciliata* (right). *Commelina communis* f. *ciliata* is distinguished from *C. communis* by the presence of bract hair and the shape of short stamens; the stamen shape is illustrated as a silhouette in the circle for each taxon. These pictures are modified from Katsuhara (2017, https://doi.org/10.6084/m9.figshare.5732574.v1).

the mating systems (e.g., estimates of selfing rates and pollen transfer distances via paternity analysis using microsatellite markers) are necessary to clarify the relative contributions of pollinator-mediated outcrossings to reproductive success and to explore the evolution of andromonoecy. Microsatellite markers have already been developed for the closely related *C. communis* (Li et al., 2015), which is considered to be tetraploid (Fujishima, 2010). In a preliminary experiment, we found that only four of the twelve markers developed by Li et al. were applicable for analyses of *C. communis* f. *ciliata*: one more marker was amplified in more than half of the samples (Supplementary Table S1). Additionally, these previously developed markers may not be suitable for analyzing a large number of samples because they have different annealing temperatures (Li et al., 2015). Thus, markers specifically developed for *C. communis* f. *ciliata* are valuable to reveal the mating system of the genus because it is diploid and more suitable to genetic analyses. In addition, work on the population genetics of *C. communis* f. *ciliata* and related varieties and forms may afford new insights into the relationships between genetic and morphological diversity and taxonomy of the *C. communis* complex. Moreover, we are currently studying pollinator-mediated competition between *C. communis* and *C. communis* f. *ciliata* in natural conditions (Katsuhara and Ushimaru, 2019), and therefore genetic analyses using both the existing *C. communis* markers and our new *C. communis* f. *ciliata* markers will reveal the pollen flow dynamics of each taxon under pollinator-mediated competition. Thus, we developed 15 nuclear microsatellite markers for *C. communis* f. *ciliata* and explored their applicability to the study of related taxa.

MATERIALS AND METHODS

Genomic DNA was extracted from a fresh leaf sample of *C. communis* f. *ciliata* collected in Hyogo Prefecture, Japan, using a DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). A DNA fragment library was constructed and amplified using an Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific, Waltham, MA, USA) and an Ion PGM Hi-Q OT2 Kit, respectively. This was sequenced employing an Ion PGM Hi-Q Sequencing Kit and an Ion 318 Chip v2 BC (both from Thermo Fisher Scientific); 351,799 reads of mean size 237 bp were obtained (DNA Data Bank of Japan Sequence Read Archive accession no.: DRA006993). We screened these reads using PRIMER3 software (Rozen and Skaletsky, 2000) embedded in MSATCOMMANDER version 0.8.2 (Faircloth, 2008) to identify microsatellite loci containing seven or more dinucleotide repeats and eight or more trinucleotide repeats. Based on 128 putatively identified microsatellite loci, we designed a total of 44 primer pairs with 37–71% GC content, annealing temperatures of 57–61 °C, and fragment sizes of 150–317 bp. These primers were used for DNA amplification of four *C. communis* f. *ciliata* individuals. We labeled all forward primers of M13 with one of three fluorescent tag sequences (Boutin-Ganache et al., 2001). All PCR ampli-

fications were performed in a final volume of 6 μl, including approximately 16 ng template DNA, 3 μl 2× Multiplex PCR Master Mix (Qiagen), and primers as follows: 0.05 μM forward primer, 0.1 μM reverse primer and 0.05 μM M13 (fluorescently labeled) primer. One M13 primer (NED) and primer pairs tagged by NED were added to levels five-fold greater than indicated above to adjust the fluorescence level. The PCR thermal profile was as follows: initial denaturation at 95 °C for 15 min; 35 cycles of 94 °C for 30 s, 57 °C for 1.5 min and 72 °C for 1 min; and a final extension at 60 °C for 30 min. The PCR products were loaded onto an autosequencer (GenomeLab GeXP, Beckman Coulter, Indianapolis, IN, USA) and their sizes calculated using Fragment Analysis ver. 8.0 (Beckman Coulter). Fifteen of the 44 primer pairs yielded amplification products from all four individuals and the fragment lengths were scored uniquely (Table 1). Applicability of these 15 primers was tested by examining three populations of *C. communis* f. *ciliata* in Nagano (presumably 2n = 48, Fujishima, 2010), Kyoto and Hyogo Prefectures (presumably 2n = 46, Fujishima, 2010), and three related species, *C. communis*, *C. benghalensis* and *Murdannia keisak* in Hyogo (population localities and specimen data are listed in Supplementary Table S2).

To examine genetic structure in three populations of *C. communis* f. *ciliata*, we performed Bayesian-based

Table 1. Characteristics of microsatellite loci of *Commelina communis* f. *ciliata* based on all 65 samples

Locus	Primer sequences (5′-3′)	Repeat motif	Fluorescent label[a]	Size range (bp)	GenBank Accession No.
Ccfc01	F:CACGACGTTGTAAAACGAC	$(CGA)_8$	6-FAM	156–182	LC339837
	R:CGCATTACCAATTGAGTTTGACC				
Ccfc02	F:TGTGGAATTGTGAGCGG	$(CGA)_7$	VIC	229–235	LC339838
	R:CTACGTGCCTGCACATTACC				
Ccfc05	F:TGTGGAATTGTGAGCGG	$(ATT)_8$	VIC	171–178	LC339839
	R:GAGCAATGGAAGTTGGAGCC				
Ccfc08	F:TGTGGAATTGTGAGCGG	$(AT)_8$	VIC	177–183	LC339840
	R:TGTACATAGATGACTTGAGCATTGG				
Ccfc09	F:CTATAGGGCACGCGTGGT	$(AT)_8$	NED	275–296	LC339841
	R:TGTCGGGATAAGGGTTCGAG				
Ccfc19	F:CACGACGTTGTAAAACGAC	$(GAA)_7$	6-FAM	224–250	LC339842
	R:TAGGTCCACTTCACCTGGC				
Ccfc25	F:CACGACGTTGTAAAACGAC	$(GT)_9$	6-FAM	163–180	LC339843
	R:GCAAGCCCATTATATTGTTTCTCC				
Ccfc28	F:CACGACGTTGTAAAACGAC	$(AT)_9$	6-FAM	236–258	LC339844
	R:GGTCTTTGTTGGTGACCCTTG				
Ccfc29	F:TGTGGAATTGTGAGCGG	$(ACT)_7$	VIC	222–235	LC339845
	R:GGGATTAATATGGTTGGTCCTTTCC				
Ccfc31	F:CACGACGTTGTAAAACGAC	$(TA)_8$	6-FAM	248–264	LC339846
	R:ACACTACTGTAAGCCTCGCC				
Ccfc32	F:TGTGGAATTGTGAGCGG	$(TA)_8$	VIC	287–307	LC339847
	R:GCCTCTGCCTTCAGCCC				
Ccfc37	F:CACGACGTTGTAAAACGAC	$(AG)_8$	6-FAM	259–278	LC339848
	R:CTGGAGGTCCCTTCTAGCC				
Ccfc38	F:TGTGGAATTGTGAGCGG	$(AT)_9$	VIC	261	LC339849
	R:GATCGACTTGGCGCTTTGG				
Ccfc39	F:CTATAGGGCACGCGTGGT	$(AT)_8$	NED	170–184	LC339850
	R:CAACTCTGATACTGGTGAGCG				
Ccfc44	F:TGTGGAATTGTGAGCGG	$(AT)_{10}$	VIC	323	LC339851
	R:ACCTACTGGTTTCATAACAGGTC				

[a] Sequences of the fluorescently labeled primers: 6-FAM = 5′–CACGACGTTGTAAAACGAC–3′, NED = 5′–CTATAGGGCACGCGTGGT–3′, VIC = 5′–TGTGGAATTGTGAGCGG–3′.

clustering using STRUCTURE ver. 2.3.2 with no prior information on population origins (Pritchard et al., 2000). Ten independent runs each for K = 1 to 10 were performed with a burn-in period of 100,000 steps followed by 100,000 Markov chain Monte Carlo iterations. We determined the appropriate number of clusters (K) based on ΔK (Evanno et al., 2005), calculated by the program STRUCTURE HARVESTER (Earl and vonHoldt, 2012). To infer the genetic differentiation among *C. communis* and *C. communis* f. *ciliata* populations, we conducted principal component analysis (PCA) using genotypic distances between each individual based on presence/absence of a focal allele (R Development Core Team, 2010, http://www.R-project.org/).

RESULTS AND DISCUSSION

In the three populations of *C. communis* f. *ciliata*, the range and mean number of alleles per locus were 1 to 13 and 5.11, respectively (the means were 5.13, 4.53 and 5.67 for the Nagano, Kyoto and Hyogo populations, respectively). The mean observed and expected heterozygosities (H_O and H_E values, respectively) over loci and populations as calculated by GenAlEx version 6.4 (Peakall and Smouse, 2006) were 0.32 and 0.33, respectively. The H_O and H_E values for each population are shown in Table 2. The inbreeding coefficients (F_{IS} values) were −0.04, −0.06 and 0.16 in the Nagano, Kyoto and Hyogo populations, respectively. We used FSTAT version 2.9.3 (Goudet, 1995) to explore deviation from Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium (LD) between loci. Significant deviations from HWE were observed in three, three and six loci of the Nagano, Kyoto and Hyogo populations, respectively (Table 2), whereas we found no significant LD. These significant deviations from HWE could be the consequences of autonomous selfing in the species (Katsuhara and Ushimaru, 2019). The combined first- and second-parent non-exclusion probabilities for the 15 markers were 0.0320 and 0.0015, respectively, calculated using Cervus 3.0 software (Marshall et al., 1998; Kalinowski et al., 2007). Analyses of related taxa revealed that 13, 7 and 2 loci were amplified and that 10, 4 and 0 loci were polymorphic in *C. communis*, *C. benghalensis* and *M. keisak* (Table 3).

Our STRUCTURE HARVESTER analysis indicated that ΔK was at a maximum when K = 2 (Supplementary Fig. S1 in supplemental information). Results from STRUCTURE suggested that one genetic cluster was represented by the Nagano population and the other consisted of the Kyoto and Hyogo populations (Fig. 2). According to the PCA result, individuals in the Nagano population formed a group that was independent from those in the Kyoto and Hyogo populations of *C. communis* f. *ciliata* (Fig. 3). The genetic differentiation between the Nagano and the other two populations may

Table 2. Primer screening for 15 microsatellite loci in three *Commelina communis* f. *ciliata* populations

Locus	Total A	Nagano (N = 22)				Kyoto (N = 20)				Hyogo (N = 23)			
		A^a	H_O	H_E	F_{IS}	A^a	H_O	H_E	F_{IS}	A^a	H_O	H_E	F_{IS}
Ccfc01	21	**12**	0.41	0.61	0.33	9	0.65	0.56	−0.16	10	0.57	0.50	−0.12
Ccfc02	5	2	0.23	0.20	−0.13	**2**	0.95	0.50	−0.90	**5**	0.13	0.16	0.21
Ccfc05	3	1	0.00	0.00	—	1	0.00	0.00	—	**3**	0.13	0.49	0.74
Ccfc08	4	**2**	0.73	0.46	−0.57	3	0.65	0.45	−0.43	**2**	0.96	0.50	−0.92
Ccfc09	10	6	0.23	0.21	−0.07	**3**	0.10	0.18	0.46	**5**	0.22	0.58	0.62
Ccfc19	19	10	0.45	0.40	−0.15	11	0.65	0.53	−0.22	13	0.61	0.56	−0.08
Ccfc25	15	6	0.36	0.32	−0.13	9	0.60	0.69	0.14	7	0.35	0.61	0.43
Ccfc28	14	8	0.45	0.39	−0.15	8	0.60	0.70	0.14	8	0.48	0.76	0.37
Ccfc29	5	1	0.00	0.00	—	1	0.00	0.00	—	**5**	0.13	0.49	0.73
Ccfc31	11	**8**	0.27	0.57	0.52	**3**	0.15	0.37	0.60	6	0.22	0.28	0.21
Ccfc32	4	2	0.09	0.09	−0.05	2	0.05	0.05	−0.03	3	0.22	0.20	−0.10
Ccfc37	16	11	0.73	0.58	−0.26	11	0.65	0.53	−0.22	12	0.70	0.61	−0.14
Ccfc38	1	1	0.00	0.00	—	1	0.00	0.00	—	1	0.00	0.00	—
Ccfc39	7	6	0.36	0.46	0.21	3	0.10	0.10	−0.04	**4**	0.17	0.20	0.13
Ccfc44	1	1	0.00	0.00	—	1	0.00	0.00	—	1	0.00	0.00	—
Mean	9.07	5.13	0.29	0.29	−0.04	4.53	0.34	0.31	−0.06	5.67	0.33	0.40	0.16

A = number of alleles; F_{IS} = fixation index; H_E = expected heterozygosity; H_O = observed heterozygosity; N = sample size.

[a] Numbers in boldface show deviations from Hardy–Weinberg equilibrium.

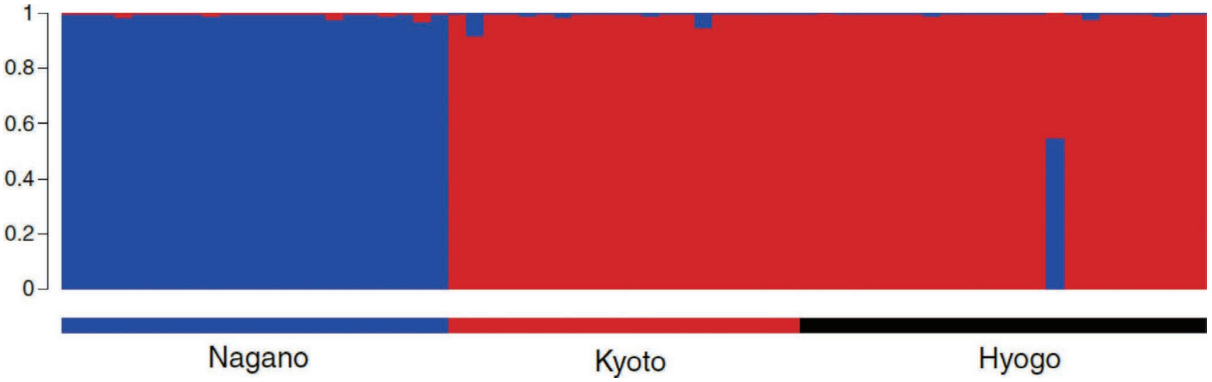

Fig. 2. Bar plots for $K = 2$ of STRUCTURE analysis results. y- and x-axis labels indicate the probability of a sample being assigned to each cluster represented by two different colors and samples grouped by three populations, respectively. K value was determined from ΔK values based on structure estimation ranging from 1 to 10 (Supplementary Fig. S1).

Table 3. Ranges of amplicon sizes in cross-amplification testing of three related species for 15 microsatellite loci

Locus	*C. communis* ($N = 8$)	*C. benghalensis* ($N = 6$)	*M. keisak* ($N = 6$)
Ccfc01	156–172	155–161	NG
Ccfc02	223–231	NG	NG
Ccfc05	171	176	NG
Ccfc08	177	171–181	NG
Ccfc09	292–296	NG	NG
Ccfc19	232–246	233–240	240
Ccfc25	168–179	NG	162
Ccfc28	234–247	NG	NG
Ccfc29	222–230	245	NG
Ccfc31	245–256	NG	NG
Ccfc32	285–293	317	NG
Ccfc37	NG	NG	NG
Ccfc38	261	NG	NG
Ccfc39	176–184	172–192	NG
Ccfc44	NG	NG	NG

NG = no signal or nonspecific amplification was detected in PCR amplification.

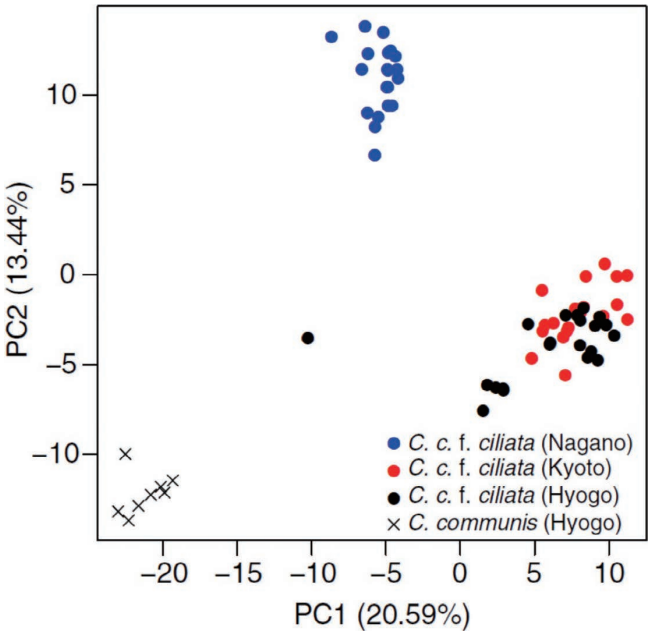

Fig. 3. Principal component analysis plot of individuals based on genotypic distances. Blue, red and black indicate the populations of Nagano, Kyoto and Hyogo, respectively. Axis PC1 explains 20.59% of the variance and axis PC2 explains 13.44% of the variance.

reflect geographic distribution (distance between populations: Nagano vs. Hyogo, ca. 350 km; Nagano vs. Kyoto, ca. 300 km; Hyogo vs. Kyoto, ca. 60 km) or differences in chromosome number (Fujishima, 2010). The PCA result also revealed a high degree of genetic differentiation between *C. communis* and *C. communis* f. *ciliata* populations in Hyogo, although they were geographically very narrowly distributed (Fig. 3).

We characterized 15 microsatellite loci of *C. communis* f. *ciliata* that are useful in terms of paternity analysis and for revealing mixed mating strategies. Although we added 13 new microsatellite loci for the closely related *C. communis*, only seven out of 15 loci could be applied to *C. benghalensis*. The results indicate that species-specific markers are necessary for each *Commelina* species. Our work will contribute to understanding genetic structure and the evolution of andromonoecy in *Commelina* species.

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