Annals of Botany **121**: 37–46, 2018
doi:10.1093/aob/mcx122, available online at www.academic.oup.com/aob

ANNALS OF BOTANY
Founded 1887

Relative contributions of neutral and non-neutral processes to clinal variation in calyx lobe length in the series *Sakawanum* (*Asarum*: Aristolochiaceae)

Daiki Takahashi[1,*], Tsutomu Teramine[2], Shota Sakaguchi[1] and Hiroaki Setoguchi[1]

[1]*Graduate School of Human and Environmental Studies, Kyoto University, Yoshida-Nihonmatsu, Sakyo-ku, Kyoto 606-8501, Japan and* [2]*Kochi Gakuen College, Asahi-Tenjin, Kochi 292-26, Japan*
**For correspondence: takahashi.daiki.35u@st.kyoto-u.ac.jp*

Received: 3 July 2017 Returned for revision: 7 July 2017 Editorial decision: 25 July 2017 Accepted: 8 September 2017

• **Background and Aims** Clines, the gradual variation in measurable traits along a geographical axis, play a major role in evolution and can contribute to our understanding of the relative roles of selective and neutral process in trait variation. Using genetic and morphological analyses, the relative contributions of neutral and non-neutral processes were explored to infer the evolutionary history of species of the series *Sakawanum* (genus *Asarum*), which shows significant clinal variation in calyx lobe length.
• **Methods** A total of 27 populations covering the natural geographical distribution of the series *Sakawanum* were sampled. Six nuclear microsatellite markers were used to investigate genetic structure and genetic diversity. The lengths of calyx lobes of multiple populations were measured to quantify their geographical and taxonomic differentiation. To detect the potential impact of selective pressure, morphological differentiation was compared with genetic differentiation (Q_{CT}–F_{ST} comparison).
• **Key Results** Average calyx lobe length of *A. minamitanianum* was 124.11 mm, while that of *A. costatum* was 13.80 mm. Though gradually changing along the geographical axis within series, calyx lobe lengths were significantly differentiated among the taxa. Genetic differentiation between taxa was low (F_{ST} = 0.099), but a significant geographical structure along the morphological cline was detected. Except for one taxon pair, pairwise Q_{CT} values were significantly higher than the neutral genetic measures of F_{ST} and G'_{ST}.
• **Conclusions** Divergent selection may have driven the calyx lobe length variation in series *Sakawanum* taxa, although the underlying mechanism is still not clear. The low genetic differentiation indicates recent divergence and/or gene flows between geographically close taxa. These neutral processes would also affect the clinal variation in calyx lobe lengths. Overall, this study implies the roles of population history and divergent selection in shaping the current cline of a flower trait in the series *Sakawanum*.

Key words: Cline, Q_{CT}–F_{ST} comparison, microsatellite marker, genetic structure, *Asarum*, Aristolochiaceae

INTRODUCTION

It has been established that evolutionary changes occur by selective and neutral effects (Gould and Lewontin, 1979). Investigating the relative roles of selective and neutral processes in shaping phenotypic traits has been a central issue in the understanding of the evolution of local adaptation and phenotypic diversity (Bridle and Vines, 2007).

Studying species and/or populations showing clinal variation can provide important insights in this request (Antoniazza *et al.*, 2010). Clinal variation, the gradual variation in measurable traits along a geographical axis, is generally considered to be the result of selection linked to micro-environmental heterogeneity (Huxley, 1938, 1939; Haldane, 1948; Endler, 1973; Salomon, 2002; Takahashi, 2015; Etterson *et al.*, 2016). Many examples of clinal variation of phenotypic traits linked to gradient environments across altitudes, latitudes and longitudes have been reported (Watt *et al.*, 2010; Hut *et al.*, 2013; Takahashi, 2015). Moreover, several neutral demographic processes can also generate clines (Endler, 1973; Zink and Remsen, 1986; Vasemagi, 2006). For example, spatial range expansion can form clines within a species along a geographical axis (Currat, 2012). Random genetic drift among populations with spatially limited gene exchange (isolation by distance) and genetic admixture of previously isolated populations (secondary contact) have also been shown to create clinal variations among populations and/or species (Endler, 1973; Slatkin, 1973; Barton and Hewitt, 1985; Campitelli and Stinchcombe, 2013). Thus, species showing interspecific and/or intraspecific clinal variations can offer important insights into the relative roles of selective and neutral processes (Haldane, 1948; Endler, 1973; Woods *et al.*, 2012; Laaksonen *et al.*, 2015; Takahashi, 2015).

The series *Sakawanum* in the genus *Asarum* (Aristolochiaceae) shows a typical cline in flower morphology among taxa, including three species and two varieties (*A. minamitanianum*, *A. sakawanum* var. *stellatum*, *A. sakawanum* var. *sakawanum* and *A. costatum*). Species of the series *Sakawanum* are diploid ($2n$ = 24) and have a synapomorphic character, with longitudinal ridges on the adaxial surface of the calyx tube (Sugawara, 2006); molecular phylogenetic analysis has also shown that these species are a monophyletic group (Takahashi and Setoguchi, 2017). Within the series, the length of the calyx lobes increases gradually among the taxa inhabiting regions from East to West in southern Japan (Akasawa and Shinma, 1984; Akasawa, 1985)

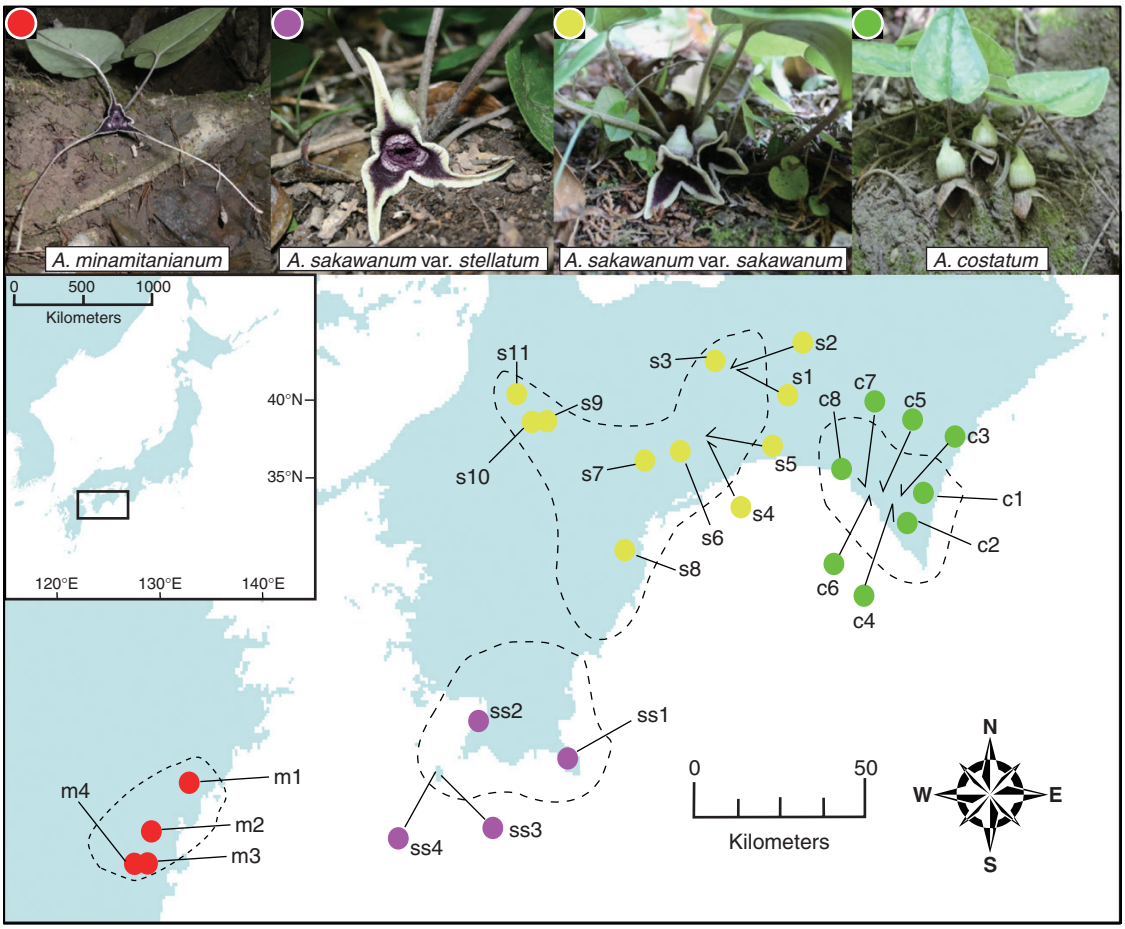

FIG. 1. Photographs of *A. minamitanianum*, *A. sakawanum* var. *stellatum*, *A. sakawanum* var. *sakawanum* and *A. costatum*, and a map with sampled populations of the series *Sakawanum* in this study. Green circles, *A. costatum*; yellow circles, *A. sakawanum* var. *sakawanum*; purple circles, *A. sakawanum* var. *stellatum*; red circles, *A. minamitanianum*. The distributions of each taxon are shown by broken lines.

(Fig. 1). The calyx lobe length of the western-most species, *A. minamitanianum*, is 50–150 mm, followed by that of the central *A. sakawanum* (20–40 mm) and then by *A. costatum* (8–20 mm) at the Eastern-most extent of the range (Akasawa and Shinma, 1984; Akasawa, 1985; Sugawara, 2006). The calyx lobes lengths are regarded as only one of the taxonomically significant characteristics that can separate all the species and varieties in the series *Sakawanum*, while the form of the calyx tube of *A. costatum* is tubular and that of the other taxa is depressed globose (Akasawa and Shinma, 1984; Sugawara, 2006).

It is known that the floral display (including flower size) is one of the most important aspects of attracting pollinators (Willmer, 2011). Many studies have shown that floral size is associated with efficiency of pollinator selection (Harder and Johnson, 2009). On the other hand, floral size is also influenced by other biotic and/or abiotic factors (e.g. cost of reproduction, defence against other organisms, and microenvironments), and the interactions of several pressures can lead to trade-offs in phenotypes (Gadgil and Bossert, 1970; Strauss and Irwin, 2004; Strauss and Whittall, 2006). Flowers of *Asarum* are pollinated by mushroom-visiting flies and/or ground-prowling insects (Lu, 1982; Sugawara, 1988; Mesler and Lu, 1993; Kaiser, 2006; Sugawara, 2006). Overall, we can assume that in the series *Sakawanum* the calyx lobes may play an important role in pollinator visitation, and that selective forces are involved in their morphological differentiation.

To investigate whether selection was involved in the evolution of the length of calyx lobes in the series, we adopted a Q_{ST}–F_{ST} comparison approach. Q_{ST} is an index measuring hierarchical differentiation of a quantitative trait (Spitze, 1993; Whitlock, 2008) and F_{ST} (and related statistics) measures the extent of neutral genetic differentiation. The results of a Q_{ST}–F_{ST} comparison can be interpreted as follows: when $Q_{ST} > F_{ST}$, divergent selection would operate on the trait, and drift migration would not explain the trait variation; when $Q_{ST} < F_{ST}$, stabilizing selection may affect the trait, whereas if $Q_{ST} = F_{ST}$ there is no evidence of geographical variation in natural selection (Latta, 1998; Merila and Crnokrak, 2001; Whitlock, 2008).

Additionally, to infer the evolutionary history of a species group, it is important to define evolutionary units (Rieseberg *et al.*, 2006). However, in the series *Sakawanum*, whether each taxon is morphologically differentiated is still unclear, and no extensive study on the morphometry or genetic structure of the series has been performed. Investigating the evolution of a species with

clinal variation may involve taxonomic difficulties. Extensive morphological measurements and statistical analyses can contribute to the detection of evolutionary units (Sokal and Sneath, 1963; Rieseberg *et al.*, 2006). Genetic approaches using neutral molecular markers are also effective in uncovering evolutionary units (Craft *et al.*, 2002; Selkoe and Toonen, 2006; Caddah *et al.*, 2012), but these may not be consistent with morphological variations influenced by non-neutral evolutionary forces.

The specific aims of this study were to (1) clarify whether the evolution of calyx lobe length in the series *Sakawanum* can be explained by neutral processes using the Q_{ST}–F_{ST} approach, (2) delineate the genetic structure of the series by molecular analyses using nuclear microsatellite (simple sequence repeat [SSR]) markers, and (3) test whether the taxonomic entity is significant in terms of calyx lobe length or whether a continuous cline is seen across the geographical range. Such comprehensive analyses of the series *Sakawanum* are an important step towards understanding the complex evolution of the morphological cline.

MATERIALS AND METHODS

Plant materials

For molecular analyses, we collected leaf materials from 322 individuals from 16 populations of the series *Sakawanum* (*A. minamitanianum*, 83 individuals from four populations; *A. sakawanum* var. *stellatum*, 62 individuals from three populations; *A. sakawanum* var. *sakawanum*, 89 individuals from five populations; and *A. costatum*, 88 individuals from four populations), with always >16 individuals sampled from each population. We also collected 433 flowers to investigate morphological variation. In total, 94 flowers of *A. minamitanianum* (four populations), 80 flowers of *A. sakawanum* var. *stellatum* (five populations), 126 flowers of *A. sakawanum* var. *sakawanum* (nine populations) and 133 flowers of *A. costatum* (six populations) were included in these morphological measurements. In populations where both morphological measurements and genotyping were conducted, the measured individuals were also used for genotyping. For each individual, one flower was selected randomly, and measurements were taken. Leaf samplings and morphological measurements were conducted during May 2012, 2014 and 2015, when the flowers of each taxon were fully open and the calyx lobes had been extended. Details of sample locations and the numbers of samples are shown in Fig. 1 and Table 1.

DNA extraction and genotyping

Leaf samples were dried in silica gel and subsequently pulverized to a fine powder (~20 mg) with a TissueLyser (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After removal of the polysaccharides with HEPES buffer (pH 8.0; Setoguchi and Ohba, 1995), total DNA was extracted using the cetyltrimethylammonium bromide method (Doyle and Doyle, 1987). Extracted DNA was dissolved in 150 µL of Tris-ethylenediamine tetraacetic acid buffer and used for polymerase chain reaction (PCR) analysis. The genotypes of 322 individuals were determined using six SSR markers, five of which were developed for this study, and one (Af-20) of which was adopted from Matsuda and Setoguchi (2012). A compound SSR primer $[(AC)_6(AG)_5]$ was labelled with the fluorochrome 6-carboxyfluorescein (Applied Biosystems, Foster City, CA, USA). The primers used are listed in Supplementary Data Table S1. PCR was performed in a 6-µL singleplex reaction volume containing 40–60 ng of DNA, 2.5 µL of Multiplex PCR Master Mix (Qiagen) and 0.5 µM of each primer. PCR amplification for all primer pairs started with 15 min at 94 °C for initial denaturation, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 1.5 min, extension at 72 °C for 1 min and a final extension for 10 min at 72 °C. Amplified products were loaded onto an ABI 3130 autosequencer (Applied Biosystems) using the GeneScan ROX-350 size standard (Applied Biosystems), POP7 polymer (Applied Biosystems) and a 36-cm capillary array; fragment size was determined using GeneMapper software (Applied Biosystems). About 5 % of all samples were amplified and genotyped at least twice to confirm reproducibility.

Genetic analysis

For each SSR marker, each population and each taxon, we calculated allelic richness, private allelic richness, observed heterozygosity (H_O), expected heterozygosity (H_E) and the fixation index ($F_{IS} = 1 - H_O/H_E$) using GENALEX 6.5.2 (Peakall and Smouse, 2012). Deviation from Hardy–Weinberg equilibrium was assessed using GENEPOP 1.2 (Raymond and Rousset, 1995). To check for the presence of null alleles, a maximum likelihood estimate of the frequency of null alleles was calculated for each locus and population using FreeNA (Chapuis and Estoup, 2007). The coefficients of genetic differentiation within each population and each taxon were estimated in terms of F_{ST} (Weir and Cockerham, 1984) using the diveRsity package (Keenan *et al.*, 2013) in R 2.6.2 (R Development Core Team, 2008). The pattern of spatial genetic structure described as the isolation-by-distance model (Wright, 1943) was evaluated using a Mantel test with 999 random permutations in the matrix of pairwise population differentiation ($F_{ST}/1 - F_{ST}$) and the matrix of the geographical distance using GenAlEx. To investigate relationships among populations, we constructed a phylogenetic tree by the neighbour-joining method, based on Nei's D_a distance (Nei *et al.*, 1983) using Populations 1.3.20 (Langella, 2002). To further assess the population structure, we used a model-based Bayesian clustering approach implemented in InStruct (Gao *et al.*, 2007). InStruct clusters individuals into subpopulations in cases where partial self-fertilization or inbreeding occurs, and can estimate inbreeding coefficients simultaneously at the population level without assuming Hardy–Weinberg equilibrium within a locus. As *Asarum* species are self-compatible (Wildman, 1950; Lu, 1982), we used InStruct to infer clusters of similar genotypes. To quantify the amount of variation in the likelihood for each cluster number (K), we performed a series of 15 independent runs for each value of K, ranging from 1 to 15, with a burn-in of 100 000, followed by 500 000 generations after burn-in. The log posterior probability of the data $L(D)$ and ΔK statistics (Evanno *et al.*, 2005) were used to determine an appropriate number for K. Graphical representations of population assignments from InStruct were produced using the program Distruct ver. 1.1 (Rosenberg, 2004).

TABLE 1. *Sample information for molecular and morphological analysis*

Taxon name	Population name	No. of flower samples for morphological analysis	No. of leaf materials for genetic analysis	Latitude	Longitude	Mean value of calyx lobe length (mm), mean ± s.d.	Three months* mean temperature (°C)	Three months* precipitation (mm)
A. minamitanianum	m1	10	22	N32°27′	E131°34′	124.78 ± 36.47	13.7	633
	m2	22	19	N32°26′	E131°34′	120.01 ± 30.52	13.9	634
	m3	40	19	N32°33′	E131°36′	137.69 ± 28.36	14.4	603
	m4	22	23	N32°40′	E131°44′	118.75 ± 31.74	13.7	561
A. sakawanum var. *stellatum*	ss1	10	22	N32°45′	E132°32′	27.30 ± 5.18	14.2	558
	ss2	17	–	N32°44′	E132°32′	35.56 ± 7.32	14.2	558
	ss3	18	21	N32°51′	E132°43′	25.74 ± 6.42	14.7	540
	ss4	24	19	N32°44′	E133°00′	25.60 ± 3.27	13.6	681
A. sakawanum var. *sakawanum*	s1	20	–	N33°41′	E132°52′	22.65 ± 4.04	10.8	522
	s2	16	–	N33°37′	E132°55′	17.97 ± 4.30	11.8	519
	s3	11	–	N33°37′	E132°58′	20.38 ± 4.50	11.6	535
	s4	16	19	N33°16′	E133°13′	19.33 ± 3.99	14.4	677
	s5	11	17	N33°31′	E133°17′	21.48 ± 2.44	14.3	646
	s6	–	19	N33°32′	E133°24′	–	14.9	670
	s7	10	–	N33°34′	E133°29′	22.83 ± 2.89	13.7	652
	s8	13	17	N33°34′	E133°30′	17.98 ± 3.29	14.3	669
	s9	19	–	N33°46′	E133°33′	21.17 ± 3.69	12.4	554
	s10	10	–	N33°45′	E133°35′	18.14 ± 4.99	12.9	560
	s11	–	17	N33°46′	E133°37′	–	13.1	555
A. costatum	c1	14	24	N33°28′	E133°56′	14.71 ± 1.82	14.5	595
	c2	26	–	N33°26	E134°02′	12.78 ± 2.26	14.4	605
	c3	–	22	N33°22′	E134°03′	–	14.8	614
	c4	39	–	N33°25′	E134°03′	14.87 ± 2.84	13.0	629
	c5	13	–	N33°22′	E134°05′	14.98 ± 3.13	14.1	624
	c6	16	–	N33°25′	E134°06′	11.84 ± 1.97	13.6	622
	c7	–	19	N33°15′	E134°10′	–	15.4	632
	c8	25	23	N33°22′	E134°12′	13.35 ± 3.06	15.3	615

*Flowering season of series *Sakawanum* species (March, April and May).

Morphological measurements and statistical analyses

Calyx lobe length was defined as the average distance from the orifice ring to three apices of calyx lobes (explained in Supplementary Data Fig. S1) and measured using digital callipers (at a resolution of 0.01 mm). To test morphological differentiation between taxa, a linear mixed-effects model (LME model) was used with population as the random factor. The LME model was used here because the data set included multiple individuals per population and multiple populations per taxon, and such nested data structures can be accommodated readily as mixed effects. To investigate whether calyx lobe length differed between all pairs of taxa, Tukey's multiple comparison test was performed subsequently. LME models using the maximum likelihood method were fitted using the lme4 package (Bates *et al.*, 2015) and Tukey's multiple comparison test was applied using the multcomp package (Hothorn *et al.*, 2008) in R.

Q_{CT}–F_{ST} *analysis*

To confirm the environmental uniformity of the distribution range within the series *Sakawanum*, we first conducted a statistical analysis of environmental differentiation. We used mean temperature and summed precipitation in the quarter of the flowering season in each population (i.e. March to May) as environmental variables. Each value was extracted from the WorldClim data set (Hijmans *et al.*, 2005) and is shown in Table 1. The significance of differences in environmental indices among taxa was tested using one-way ANOVA. The degree of pairwise taxon differentiation of calyx lobe lengths in wild populations was estimated using the modified Q_{ST} parameter. The Q_{ST} parameter is used to estimate the divergence of populations at a single spatial level (Spitze, 1993; Whitlock, 2008; Xu *et al.*, 2010). In this study we focused primarily on divergence between taxa, not populations, and we used the Q_{CT} parameter where populations were nested within taxa (Whitlock, 2008). Q_{CT} values were calculated as follows:

$$Q_{CT} = V_b/(V_b + V_p + 2V_w)$$

where V_b is the between-taxon variance component, V_p is the between-population (within taxon) variance component and V_w is the within-taxon variance component. The variance components were calculated from hierarchical LME models for each taxon pair. Q_{CT} values and their confidence intervals (CIs) were calculated using 200 hierarchical bootstrap samplings and the ape package (Paradis *et al.*, 2004) in R. As a comparison with Q_{CT}, both the F_{ST} value and G'_{ST} values, which are G_{ST} values standardized by expected heterozygosity, total gene diversity and within-gene diversity, were used. This is because F_{ST} values can often underestimate differentiation, while G'_{ST} values can overestimate genetic differentiation, particularly when using markers with a high mutation rate (Hedrick, 2005). Pairwise F_{ST} and G'_{ST} values with 95 % CIs were calculated using the diveRsity package (Keenan *et al.*, 2013) in R. We used χ^2 tests to investigate whether each Q_{CT} value was significantly different from the F_{ST} and G'_{ST} values.

RESULTS

Molecular diversity and genetic structure

The genetic diversity of each population (H_E) was high, ranging from 0.675 to 0.823, while the observed heterozygosity (H_O) was relatively low, ranging from 0.212 to 0.442 (Table 2). Consequently, inbreeding coefficients (F_{IS}) ranged from 0.390 to 0.661. Genetic diversity was high in each taxon [H_E = 0.869 (*A. minamitanianum*) to 0.754 (*A. costatum*)]. Hardy–Weinberg equilibrium exact tests indicated that no population or locus was in equilibrium (Supplementary Data Table S1, Table 2). Each locus showed the presence of null alleles and the estimated frequency per locus per population ranged from 0.042 to 0.384, depending on the population (Supplementary Data Table S2). The mean null allele frequency over all populations and loci was 0.186. Pairwise F_{ST} values between populations were generally low, ranging from 0.007 to 0.156 (Supplementary Data Table S3). Genetic differentiation was highest between *A. minamitanianum* and *A. costatum* (F_{ST} = 0.096) and lowest between *A. sakawanum* var. *sakawanum* and *A. sakawanum* var. *stellatum* (F_{ST} = 0.029) (Table 3); global F_{ST} was also low (F_{ST} = 0.099). Significant isolation by distance was detected among populations ($P < 0.0001$, $R^2 = 0.234$, Fig. 2). In the neighbour-joining phylogenetic tree, *A. costatum* and *A. minamitanianum* consisted of monophyletic clades, while *A. sakawanum* var. *sakawanum* and *A. sakawanum* var. *stellatum* were paraphyletic (Supplementary Data Fig. S2). Furthermore, *A. sakawanum* (including var. *stellatum* and var. *sakawanum*) was not monophyletic. The bootstrap value of each clade was low (<60).

In the clustering analysis $L(D)$ increased progressively up to K = 10, at which point it started to plateau, and variance across runs was very low (Fig. 3A). ΔK peaked at K = 2, slightly higher than at K = 3 (Fig. 3B). Thus, clustering with two or three clusters was considered to most accurately reflect the genetic structure represented in the data. At K = 2, individuals of *A. minamitanianum* and *A. costatum* were assigned to different clusters (green and red in Fig. 3C; the inbreeding coefficients were 0.582 and 0.527, respectively), while individuals of *A. sakawanum* var. *sakawanum* and var. *stellatum* showed no distinct structure, with most individuals being intermediate between the two clusters. At K = 3, each cluster corresponded well with the three morphospecies (*A. minamitanianum*, *A. sakawanum* and *A. costatum*; the inbreeding coefficients were 0.571, 0.561 and 0.519, respectively), although there was a sign of admixture of the clusters among the morphospecies. However, at K = 4 two varieties of *A. sakawanum* (*A. sakawanum* var. *sakawanum* and *A. sakawanum* var. *stellatum*) did not consist of single clusters.

TABLE 3. *Pairwise* F_{ST} *between taxa*

	A. minamitanianum	*A. sakawanum* var. *stellatum*	*A. sakawanum* var. *sakawanum*
A. sakawanum var. *stellatum*	0.058		
A. sakawanum var. *sakawanum*	0.055	0.029	
A. costatum	0.096	0.046	0.070

Morphological differentiation

The average calyx lobe lengths were 125.87 mm (s.d. = 31.12) in *A. minamitanianum*, 27.56 mm (s.d. = 7.09) in *A. sakawanum* var. *stellatum*, 20.15 mm (s.d. = 4.35) in

TABLE 2. *Estimation of genetic diversity in the series Sakawanum*

Taxon name	Population	AR	PA	H_O	H_E	F_{IS}
A. minamitanianum	m1	8.833	0.667	0.365	0.814	0.549*
	m2	7.333	0.333	0.255	0.787	0.661*
	m3	8.167	0.333	0.347	0.801	0.566*
	m4	9.000	0.667	0.406	0.823	0.506*
	all	16.000	3.000	0.346	0.869	0.599*
A. sakawanum var. *stellatum*	ss1	8.500	0.500	0.442	0.792	0.426*
	ss2	9.883	0.667	0.390	0.815	0.508*
	ss3	8.000	0.333	0.355	0.761	0.525*
	all	15.000	1.833	0.396	0.828	0.505*
A. sakawanum var. *sakawanum*	s4	8.000	0.000	0.212	0.794	0.740*
	s5	6.667	0.167	0.269	0.738	0.621*
	s6	8.833	0.000	0.411	0.818	0.464*
	s8	6.667	0.333	0.424	0.778	0.459*
	s11	6.667	0.333	0.257	0.730	0.650*
	all	14.500	1.000	0.319	0.849	0.621*
A. costatum	c1	7.833	0.167	0.413	0.733	0.441*
	c3	7.333	0.500	0.417	0.681	0.390*
	c7	6.833	0.000	0.392	0.675	0.427*
	c8	7.500	0.000	0.383	0.717	0.447*
	all	12.167	0.833	0.403	0.754	0.461*

AR, allelic richness; PA, private allelic richness.

*Significant deviation from Hardy-Weinberg equilibrium ($P < 0.01$).

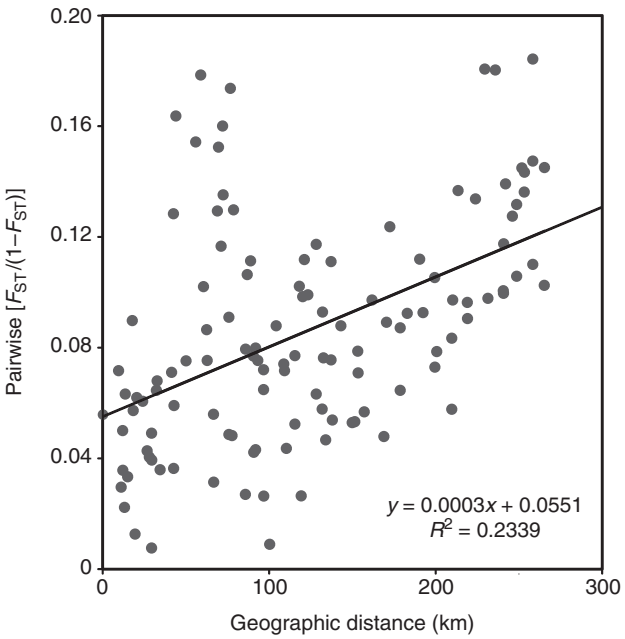

FIG. 2. Correlation between geographical distance (km) and pairwise genetic distance [F_{ST}/(1 − F_{ST})] across populations of the series *Sakawanum*.

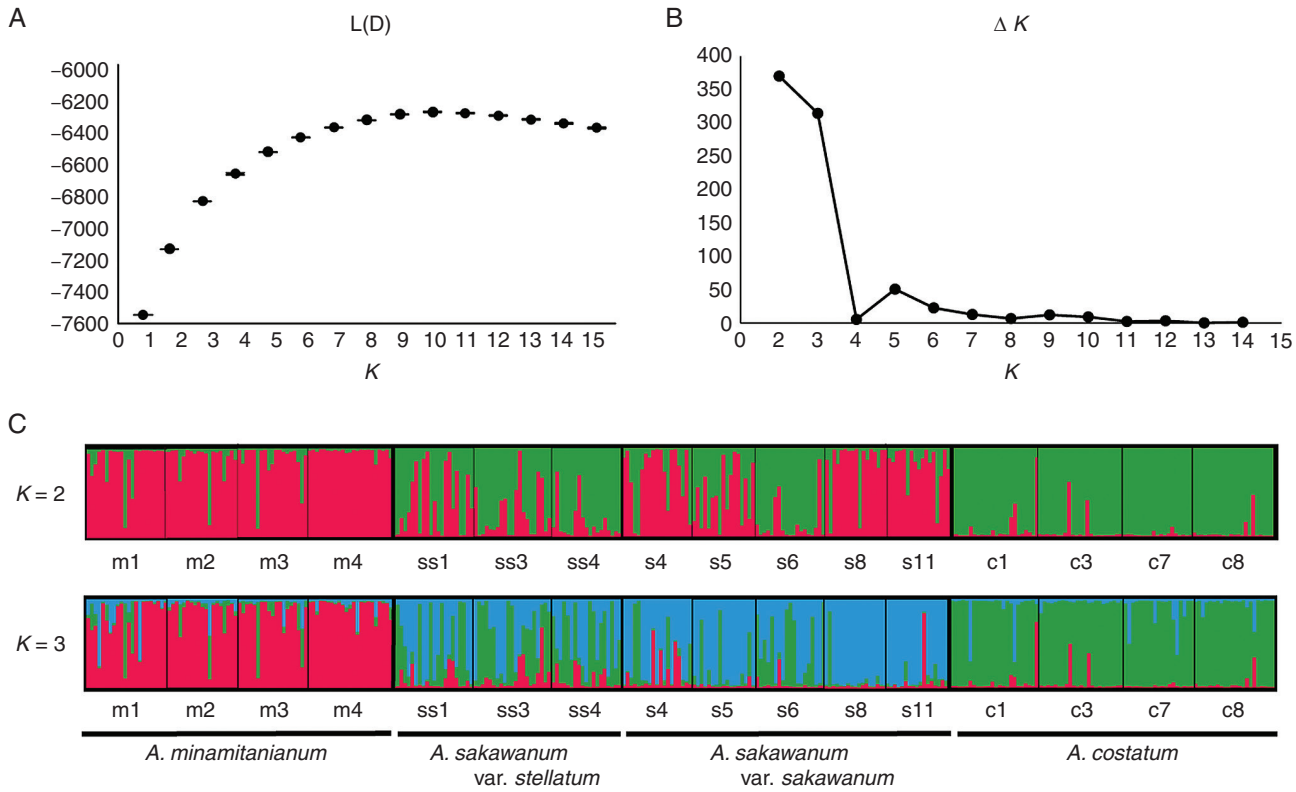

FIG. 3. Clustering results for 16 populations of the series *Sakawanum* using InStruct. (A) $L(D)$ values for each K. Error bars represent the standard deviation of each mean. (B) ΔK values for subsequent Ks. (C) Graphical results of the most strongly supported K values ($K = 2$ and 3). Individuals are represented as thin vertical lines partitioned into segments, corresponding to the clusters indicated by the different colours. Population names are shown under the bar.

A. sakawanum var. *sakawanum* and 13.80 mm (s.d. = 2.86) in *A. costatum* (Fig. 4A, Table 1). The LME analysis showed that taxa, a fixed term, exerted a significant effect on calyx lobe length ($P < 0.01$), and subsequent Tukey's multiple comparisons supported that each taxon has a different length ($P < 0.05$; Fig. 4B, Supplementary Data Table S4).

Q_{CT}–F_{ST} *comparison*

In the ANOVA analysis, no significant differentiation was detected for either environmental index among the taxa ($P > 0.05$; Supplementary Data Table S5). Pairwise Q_{CT}, F_{ST} and G'_{ST} values with 95 % bootstrap CIs are shown in Fig. 5 and Supplementary Data Table S6. In all pairs, Q_{CT} values were significantly higher than F_{ST} values ($P < 0.05$). Except for one G'_{ST} value between *A. sakawanum* var. *sakawanum* and *A. costatum*, Q_{CT} values were also significantly higher than G'_{ST} values ($P < 0.05$).

DISCUSSION

Evolutionary hypothesis for calyx lobe length variation

Based on the interspecific differentiation of calyx lobe lengths and neutral genetic differentiation, we showed that strong divergent selection would have affected calyx lobe lengths in series *Sakawanum* taxa. Our results should be interpreted with caution, however, because Q_{CT}–F_{ST} (G'_{ST}) comparisons should ideally be conducted using plants cultivated under controlled greenhouse conditions to eliminate any effect of the environment on traits. In our case, the measurement of a floral characteristic was performed using samples collected from natural populations, and we thus accept that our Q_{CT} estimate may be an overestimate due to environmental variance. Nevertheless, the major climatic variables for these populations only fluctuate across small ranges (Table 1), and they showed no significant differentiation among taxa (Supplementary Data Table S5). As these species are commonly cultivated ornamental plants in Japan, the length and shape of the calyx lobes are not known to vary greatly depending on cultivation conditions (Kishi and Irizawa, 2008). Thus, we expected that although there is plasticity in series *Sakawanum* taxa caused by environmental differences, the effect would be small.

In the genus *Asarum*, no species has calyx lobes as long as those of *A. minamitanianum*. Assuming that the ancestral state of calyx lobe length in the series *Sakawanum* would have been lower than that of *A. minamitanianum*, it is plausible that a morphological change to longer calyx lobes would have occurred within the series *Sakawanum*. Some plants have significantly extended sepals, petals or appendages [e.g. *Dracula lafleurii*, *Corybas iridescens* (Orchidaceae); *Tolmiea megarrhina* (Saxifragaceae); Fuller, 1994; Goldblatt *et al.*, 2004; Endara *et al.*, 2010; Policha *et al.*, 2016]. Considering that divergent selection is detected in calyx lobe lengths in the series *Sakawanum*, these extended flower organs could have

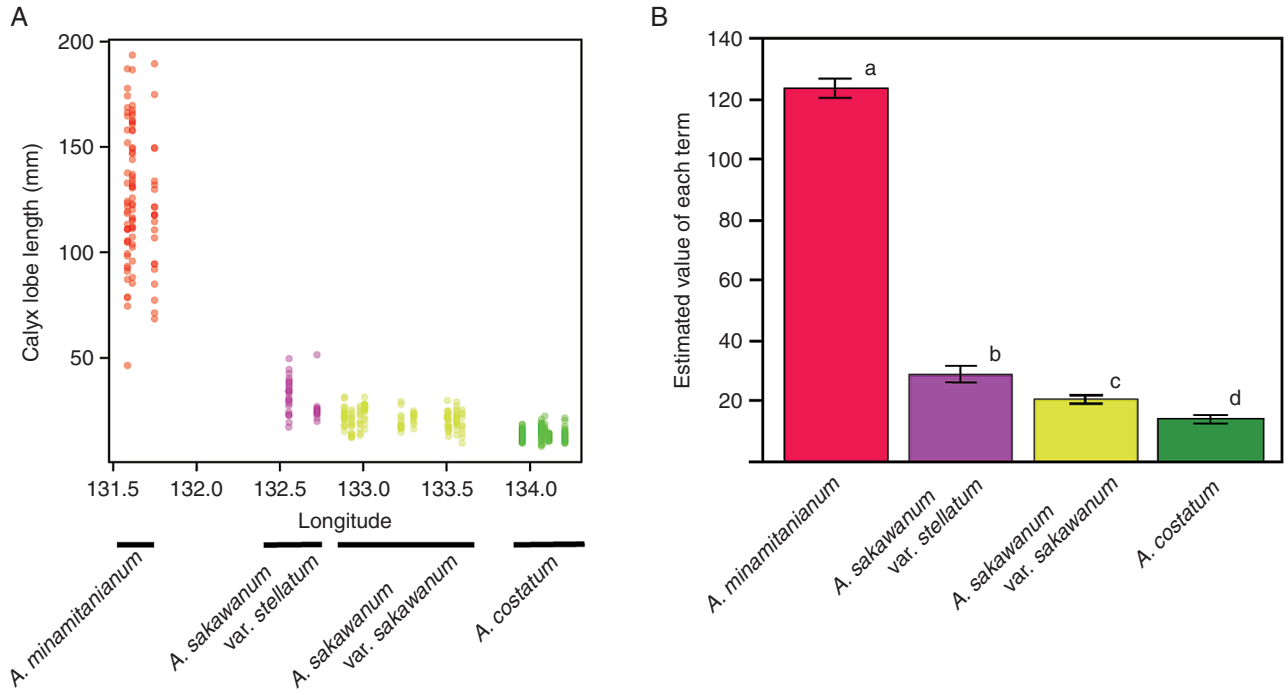

FIG. 4. (A) Plot of calyx lobe length of individuals against longitude. (B) Bar plots of lmer-estimate value (± s.e.) of each fixed effect term for calyx lobe length in the linear mixed effects model. Colours indicate taxa (see Fig. 1) and different lower-case letters indicate a significant difference ($P < 0.05$), based on Tukey's multiple comparison tests.

important roles (e.g. visual attraction, landing foothold for pollinators, and emitting olfactory attractants), as suggested for other flowers with elongated organs (Policha *et al.*, 2016; Katsuhara *et al.*, 2017). We could hypothesize that the variation of calyx lobes in the series *Sakawanum* is related to pollinator attraction, although other biotic and abiotic factors may also have affected calyx lobe length. Based on the current data, we cannot conclude that pollinators are responsible for the variation of calyx lobe length in series *Sakawanum*. Pollination research and identification of chemicals included in floral scents for the series *Sakawanum* would be important in further investigation of the role and evolution of calyx lobe length in the series *Sakawanum* and, in addition, manipulation experiments (e.g. cutting the calyx lobes or using artificial flowers) could be performed to confirm this.

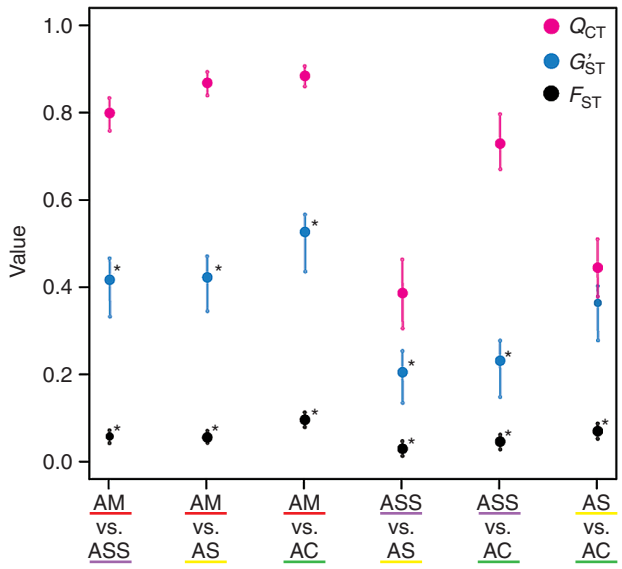

FIG. 5. Pairwise Q_{CT}, G'_{ST} and F_{ST} values for each taxon pair. Bars show the 95 % CI. Abbreviations indicate taxon names (AM, *A. minamitanianum*; ASS, *A. sakawanum* var. *stellatum*; AS, *A. sakawanum* var. *sakawanum*; AC, *A. costatum*). *$P < 0.05$ compared with Q_{CT} value (χ^2 test).

Population genetic variation in Asarum *populations*

An interesting characteristic of the genetic variation in *Asarum* populations was the remarkably high genetic diversity (species mean, $H_E = 0.825$) and the high inbreeding coefficients (species mean, $F_{IS} = 0.547$). The high F_{IS} within populations may be attributable to the presence of null alleles, inbreeding and a small effective population size. The presence of null alleles was shown in each locus (mean value for loci = 0.186). Null alleles are relatively common in genomic microsatellite markers and are considered to be one reason for high F_{IS} values (Dakin and Avise, 2004). Although automatic self-pollination does not occur in series *Sakawanum* species, *Asarum* species have self-compatibility. This could be another reason for high F_{IS} values in the series *Sakawanum*. In *Asarum* species, pollinators, including fungus gnats and ground-prowling insects, were observed visiting flowers at low frequencies (Wildman, 1950; Lu, 1982; Sugawara, 1988; Mesler and Lu, 1993). Seeds of *Asarum* species are dispersed by ants (Gorb and Gorb, 1995), and the dispersal ability of *Asarum* seeds has been estimated as

10–50 cm per year (Maekawa, 1953; Hiura, 1978). These inefficient pollinators and the low seed dispersal ability can result in the formation of reproductively isolated units within a population (Hamrick *et al.*, 1993; Gevaert *et al.*, 2013; Sant'Anna *et al.*, 2013). Substructuring of populations causes a reduction in heterozygosity in a population because of treating multiple subpopulations as a single large population (known as the Wahlund effect; Wahlund, 1928; Dharmarajan *et al.*, 2013). Accordingly, the deficiency in heterozygotes in the series *Sakawanum* in our study may have been shaped by a combination of the presence of null alleles, inbreeding and/or the Wahlund effect. Further research on the mating system and seed dispersal in *Asarum* species is needed to determine the precise mechanism by which high genetic variation is maintained, despite the deficiency in heterozygotes.

Implications for evolutionary history and the establishment of clinal variation

Our morphological and genetic analyses showed that all species (*A. costatum*, *A. sakawanum* and *A. minamitanianum*) in the series *Sakawanum* had differentiated significantly from one another. However, genetic differentiation levels were generally low (global F_{ST} = 0.099). A sign of admixture of the clusters among the morphospecies was detected by the clustering analysis, though each cluster corresponded with the morphospecies. There are two possible explanations for this low genetic divergence and genetic structure: recent species divergence and gene flow (Petit and Excoffier, 2009). Recent species divergence shows low genetic differentiation due to shared ancestral polymorphism and alleles not fixing within a population and/or a species (Pamilo and Nei, 1988; Linder and Rieseberg, 2004; Muir and Schlotterer, 2005; Krak *et al.*, 2013; Pillon *et al.*, 2013). In Japan, *Asarum* species seem to have diversified allopatrically in the Quaternary due to geographical fragmentation caused by climatic changes (Takahashi and Setoguchi, 2017; Matsuda *et al.* 2017). *Asarum* species take >7 years from sowing to first flowering and can live >20 years in wild populations (Kume, 1989, 1993). The recent origin and long lifetimes of Japanese *Asarum* species (Maekawa, 1933) suggest that each taxon in the series *Sakawanum* could have been formed in a recent timeframe, which would have resulted in low genetic differentiation, as estimated in this study.

Considering the isolation by distance within the series, interspecific gene flow is also plausible. It is known that clinal variations can involve neutral processes (Endler, 1973; Slatkin, 1973; Vasemagi, 2006; Antoniazza *et al.*, 2010; Campitelli and Stinchcombe, 2013), and the distribution range and morphology of *A. sakawanum* are intermediate between the other two species. We can therefore speculate that interspecific gene flow would have contributed to forming the clinal variation. However, based on the genetic data currently available, it is difficult to draw a definitive conclusion on whether recent species diversification or gene flow between taxa occurred in this series. In future studies, we need to test alternative population models that consider ancient gene flow, a recent population split and population admixture using more informative data sets (e.g. single-nucleotide polymorphisms) subjected to statistical population analyses (e.g. Bayesian inference, approximately Bayesian competition; Beaumont *et al.*, 2002; Hey, 2010).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: morphological characteristics measured in flowers of the series *Sakawanum* [front view (a) and cross-section view (b)]. The broken lines indicate measured length of the calyx lobe. Abbreviations: *cl*, calyx lobe; *or*, orifice ring; *dp*, degeneration petal. Figure S2: neighbour-joining population phylogenetic tree of the series *Sakawanum*. Table S1: characteristics of the six SSR markers analysed in this study. Table S2: frequencies of null alleles per population per loci estimated by freeNA. Table S3: pairwise F_{ST} values among populations. Table S4: results of Tukey's multiple comparison test for estimated values of each taxon from LME model. Table S5: results of ANOVA for two environmental indices. Table S6: Q_{ST}, F_{ST} and G'_{ST} values with 95 % CI between each taxon pair.

ACKNOWLEDGEMENTS

We thank M. Tanaka for help in sampling, taxonomic determination, Y. Umetsu and T. Tanada for providing research assistance and M. Yamasaki for valuable comments on the statistical analyses. This work was supported by Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science (24247013 and 26304013) to H.S., the Environmental Research and Technology Development Fund of the Ministry of the Environment (4-1403), and SICORP Program of the Japan Science and Technology Agency ('Spatial-temporal dimensions and underlying mechanisms of lineage diversification and patterns of genetic variation of keystone plant taxa in warm-temperate forests of Sino-Japanese Floristic Region') (grant 4-1403).

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