

## Inbreeding coefficients in six species of *Ainsliaea* and two species of *Pertya* (Asteraceae)

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**Abstract.** As a basis for comparative studies of mating systems, we estimated genetic diversities and inbreeding coefficients for six perennial species of *Ainsliaea* and two shrubby species of *Pertya* (Asteraceae). These related species are known to vary from frequent cleistogamy to xenogamy by bumblebee pollination, and we examined allozyme variation in these species to describe mating system variation in further detail. Significant heterozygote deficit was found in five species, while no heterozygote deficit was found in three species. A cleistogamous species showed no genetic polymorphism. Multilocus average of inbreeding coefficients among seven species ranged from  $-0.12$  to  $0.78$ . These findings indicated that related species can vary from predominant selfers to predominant outcrossers under perennial life cycles.

**Key words:** Inbreeding coefficient, allozyme, mating system, cleistogamy, *Ainsliaea*, *Pertya*, Asteraceae

### Introduction

One of the most remarkable characteristics of flowering plants is the high variability in mating systems, from complete outcrossing to complete selfing (Barrett and Harder 1996, Vogler and Kalisz 2001); at least one-third of flowering

plant species is known to be predominantly selfing (Allard 1975), and some other species are mixed mating (Darwin 1876, Uphof 1938, Fryxell 1957, Vogler and Kalisz 2001). Thus, flowering plants have been extremely important for comparative studies of mating systems (Lewontin 1974, Jain 1976, Ritland and Ritland 1989, Charlesworth et al. 1994, Johnston and Schoen 1996). Recent studies based on marker-based estimation of selfing rates (Schemske and Lande 1985, Barrett and Eckert 1990, Ritland and Gender 1985, Vogler and Kalisz 2001) or inbreeding coefficient (Ritland and Ritland 1989, Sweigart et al. 1999) reconfirmed classic observations that degrees of selfing vary often greatly within a genus and even within a species. However, only a few efforts have been made to thoroughly describe intrageneric and/or intra-specific variation of the selfing rate because estimating selfing rates for more than a few populations is labor intensive (Takebayashi and Morrell 2001). Thus, more comparative studies are needed to test macroevolutionary hypotheses of mating system evolution in flowering plants.

The genus *Ainsliaea* (Pertyeae: Asteraceae) and its relatives provide a suitable system for comparative studies of plant mating systems.

*Ainsliaea* is a small genus having ca. 30 perennial species in East Asia. Watanabe et al. (1992) reported that *A. apiculata* is cleistogamous while *A. faurieana* is chasmogamous. On the contrary, some other species of *Ainsliaea* have larger flowers than these two species and they are suggested to be more outcrossing. *Ainsliaea* is closely related to *Pertya* of 16 species (Bremer 1994) in which some shrubby species seem to be predominantly outcrossing: They have larger and more conspicuous heads, which consist of larger number of florets than those of *Ainsliaea*. The purpose of this paper is to demonstrate that seven species of *Ainsliaea* and two species of *Pertya* do show a wide range of variation in the level of selfing by estimating inbreeding coefficients of representative populations.

The inbreeding coefficient is one of the most important parameters to describe the mating systems in flowering plants. The inbreeding coefficient is correlated with population mean selfing rate that probably covaries with degree of inbreeding depression (Lande and Schemske 1985, Lande et al. 1994) and various floral traits (Cruden 1977, Ritland and Ritland 1989, Brunet 1992). Inbreeding coefficients may be more suitable for comparative studies of plant mating systems than selfing rates because of the following properties. First, because selfing rate often varies with non-genetic factors such as pollinator availability, fluctuates among years. On the other hand, the inbreeding coefficient represents the average level of inbreeding during recent 2-3 generations, because successive selfing accumulates homozygotes within a population. On the contrary, multilocus outcrossing rate shows the proportion of outcrossing only in a reproductive season. Therefore, inbreeding coefficient for an adult population can be a better measure of the average level of selfing in a population (Dole and Ritland 1993). Thirdly, estimation of inbreeding coefficient is less labor intensive than estimating selfing rate.

Considering these advantages, we compared inbreeding coefficients among eight species of *Ainsliaea* and *Pertya*. We discriminated

mixed-mating and outcrossing species by testing deviations from Hardy-Weinberg equilibrium. In addition, we compared some measures of genetic diversity among species. Specific questions addressed in this paper are as follows.

- (1) How do nine species of *Ainsliaea* and *Pertya* vary in inbreeding coefficient and genetic diversity?
- (2) In which species is significant deficit of heterozygosity observed?
- (3) Are cleistogamous species more selfing than chasmogamous species?

## Materials and methods

**Species.** The nine species we examined are listed in Table 1 with localities and sample size. Seven species of *Ainsliaea* are perennial herbs of at most 1.2m height, inhabiting understories or margins of temperate deciduous or warm-temperate evergreen forests except for *A. faurieana*, which is a rheophyte. *A. apiculata* (Watanabe et al. 1992) and *A. fragrans* (our observation) have both cleistogamous and chasmogamous flowers; the remaining five species have only chasmogamous flowers (Table 1). Two species of *Pertya* are small shrubs less than 1m tall, preferring steep slopes or ridgelines in temperate deciduous forests. While all species of *Ainsliaea* have three florets per head, two species of *Pertya* have more than 10 florets per head and appear to be more attractive for pollinators. There are no detailed observations of pollination biology for these species. According to our preliminary observations, flowers of *A. cordifolia* and *Pertya* spp. are mainly visited by bumblebees and those of other species are mainly visited by small opportunistic bees and syrphids.

**The estimation of the genetic diversity and inbreeding coefficients.** Polyacrylamide gel electrophoresis was performed to identify genetically variable loci following the procedures of Murakami et al. (1999). Fresh leaves were ground in extraction buffer, 0.1mM Tris-HCl, 1mM EDTA (4Na), 10mM KCl, 10mM MgCl<sub>2</sub>, 0.4% 2-mercaptoethanol and 10% polyvinyl-pyrrolidone with pH adjusted to 7.5. We screened eight isozyme system and six of these gave interpretable banding patterns; alcohol dehydrogenase (ADH: EC1.1.1.1), leucine aminopeptidase

**Table 1.** Locality of populations used for estimating of inbreeding coefficients. CH represents chasmogamous flowers, CL represents cleistogamous flowers

Species	Reproductive characters			Number of individuals
	Types of flowers	Number of florets per a head	Locality	
<i>A. acerifolia</i>	CH	3	Mt. Inuga-take, Fukuoka Pref.	50
<i>A. apiculata</i>	CH and CL	3	Mt. Houman, Fukuoka Pref.	22
<i>A. cordifolia</i>	CH	3	Mori-town, Shizuoka Pref.	50
<i>A. dissecta</i>	CH	3	Mt. Myoujin, Aichi Pref.	50
<i>A. faurieana</i>	CH	3	Yaku-Island, Kagoshima Pref.	50
<i>A. fragrans</i>	CH and CL	3	Miyazaki-city, Aya-chou, and Kunitomi-town, Miyazaki Pref.	50
<i>A. macroclinioides</i>	CH	3	Kuro-Island, Kagoshima Pref.	30
<i>P. glabrescens</i>	CH	Approximately 13	Shinshiro-city, Aichi Pref.	53
<i>P. scandens</i>	CH	Approximately 15	Shinshiro-city, Aichi Pref.	47

(LAP: EC 3.4.11.1), glutamate oxaloacetate transaminase (GOT: EC 2.6.1.1), phosphoglucosomerase (PGI: EC 5.3.1.9), phosphoglucosomutase (PGM: EC 5.4.2.2), and triosephosphate isomerase (TPI: EC 5.3.1.1). Staining recipes followed Murakami et al. (1999).

We could not determine genotypes at two loci, *6pg* and *pgm*, because of distortion of Mendelian segregation, perhaps due to gene duplications. These loci were not used for estimating inbreeding coefficients. Since we found no polymorphism in the population of *A. apiculata* we studied, we reanalyzed the published data of Watanabe et al. (1992) in which polymorphism at LAP is described.

To measure genetic diversity, we calculated the proportion of polymorphic loci, mean number of alleles per locus, and expected heterozygosity. Expected heterozygosity is estimated using the formula of  $H_s$  in gene diversity statistics (Nei 1987).

Single locus inbreeding coefficients were estimated as  $F_{is}$  of Weir and Cockerham (Reynolds et al. 1983) using GENEPOP version 3.3 (Raymond and Rousset 1995). Multilocus average of inbreeding coefficients  $F$ , were estimated by using the 7.25 equation of Nei (1987):

$$F = \frac{\sum w_k x_k (1 - x_k) F_{is}}{\sum w_k x_k (1 - x_k)}$$

where  $w_k$  represents the number of individuals at a locus  $k$ , and  $x_k$  is allele frequency at locus  $k$ . We calculated selfing rate by using inbreeding equilibrium equation  $s = 2f / (1 + f)$ , although this equation

is exact only when inbreeding depression is negligible.

Deviations from Hardy-Weinberg (H-W) equilibrium were tested under the alternative hypotheses of heterozygote excess and heterozygote deficiency (Rousset and Raymond 1995). The local tests for each locus in the population and global tests across loci were performed using GENEPOP version 3.3. Therefore, we examined 21 of 26 polymorphic loci by these tests. The global test assumed the independence among loci, however, we could not perform the test for independence of the loci before the global test, so the result of global test might be overestimated.

## Results

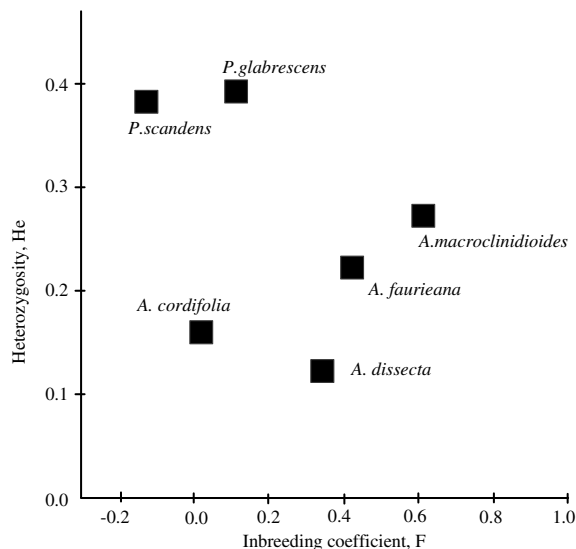
**Genetic diversity.** In Table 2, the three measures of genetic diversity are compared among nine species examined. One cleistogamous species, *A. fragrans*, had no polymorphic locus. In cleistogamous *A. apiculata* and seven chasmogamous species, the number of polymorphic loci ranged from one to seven, mean number of alleles per locus ranged from 2.0 to 3.0, and expected heterozygosity ranged from 0.11 to 0.38. Two species of *Pertya* showed higher heterozygosity than four species of *Ainsliaea*.

**Inbreeding coefficients and selfing rates.** Table 3 shows single-locus and multilocus average of inbreeding coefficients. Single-locus

**Table 2.** Estimates of genetic diversity parameters.  $He$  = expected heterozygosity

Species	Number of polymorphic loci	Mean number of allele per locus	$He$
<i>A. macroclinidioides</i>	3	2.0	0.29
<i>A. faurieana</i>	4	2.0	0.26
<i>A. cordifolia</i>	7	2.3	0.16
<i>A. dissecta</i>	6	2.3	0.11
<i>P. glabrescens</i>	–	2.0	0.39
<i>P. scandens</i>	–	3.0	0.38
<i>A. acerifolia</i>	–	–	–
<i>A. fragrans</i>	0	–	–
<i>A. apiculata</i>	0	–	–

inbreeding coefficients ranged from -0.13 to 1.0, and multilocus average of inbreeding coefficients ranged from -0.12 to 0.78. Among all species, selfing rates ranged from -0.27 to 0.88. *A. apiculata* and *A. faurieana* showed higher values of inbreeding coefficient (0.78 and 0.62, respectively; corresponding selfing rates 0.88 and 0.77), *A. dissecta* and *A. macroclinidioides* have intermediate values (0.35, and 0.43, respectively; selfing rate 0.52 and 0.60) and other four species had inbreeding coefficients lower than 0.18 (selfing rates higher than 0.28). Figure 1 shows the relationships between inbreeding coefficient and the gene diversity ( $He$ ). The highest diversity values were seen in the

**Fig. 1.** The relationships between the inbreeding coefficient and the gene diversity ( $He$ )

most outcrossing species (*P. glabrescens* and *P. scandens*), but another outcrossing species (*A. cordifolia*) showed low diversity.

**The deviation from Hardy-Weinberg equilibrium.** No heterozygosity excess was found (Table 3), while significant heterozygote deficit was found in *A. apiculata*, *A. cordifolia*, *A. dissecta*, *A. faurieana* and *A. macroclinidioides*. No heterozygote deficit was found in *A. acerifolia* and two species of *Pertya* (Table 3).

## Discussion

**Interspecific variation of mating system in *Ainsliaea* and *Pertya*.** Nine species of *Ainsliaea* and *Pertya* we examined varied from predominant outcrossers to predominant selfers. In *A. acerifolia* and two species of *Pertya*, the distribution of genotype frequencies did not deviate from Hardy-Weinberg equilibrium, supporting the conclusion that these species are predominantly outcrossing. On the other hand, a cleistogamous species *A. fragrans* showed no polymorphism in eight loci we examined and is suggested to be predominantly selfing. The other four species showed significant deficiencies of observed heterozygosity at several loci, indicating that these are mixed-mating. Among them, inbreeding coefficients varied from 0.02 in *A. cordifolia* to 0.78 in *A. apiculata*. *A. cordifolia* has relatively large flowers often visited by bumblebees while *A. apiculata* is mostly cleistogamous and a few small chasmogamous flowers per spike are scarcely visited by small opportunistic



**Table 4.** Summary of published studies on interspecific and/or intraspecific variation of outcrossing rate or inbreeding coefficient. \*, Species in which outcrossing rates varied between populations. †, Outcrossing rates varied between closely related species. ‡, Number of loci used for estimating outcrossing rate are not described in detail. §, Only two selfing species out of five species are investigated. ||, Only three outcrossing species out of five species were polymorphic and inbreeding coefficients of these species were estimated

Genus/Species	Number of populations	Number of loci	Outcrossing rate	Inbreeding coefficient	coeff- Life-history	References
<i>Amsinckia</i> *†	8	1	0.25 – 0.999	0.032 – 1.00	Annual	Johnson and Schoen (1996)
<i>Aquilegia</i> *†	11	2	0.00 – 0.83	0.002 – 0.60	Short-lived perennial	Routley et al. (1999), Brunet and Eckert (1998)
<i>Clarkia</i> *†	14	1 – 4	0.084 – 0.984	–0.0842 – 0.94	Annual	Holtsford and Ellstrand (1989), Vasek and Harding (1976)
<i>Gilia achilleifolia</i> †	9	1	0.15 – 1.06	[–]	Annual or short-lived perennial	Schoen (1982)
<i>Eichhornia paniculata</i> *	32	19 – 27	0.00 – 0.96	[–]		Barrett and Husband (1990)
<i>Linanthus</i> †	5	1 – 2	0.00 – 0.30	0.048 – 0.944	Annual	Goodwillie (2000)
<i>Lupinus</i> †	5	1 – 3 ‡	0.05 – 0.84	[–]		Karoly (1994), (1992)
<i>Leavenworthia</i> *†	15	5	0.03 – 0.33§	0.092 – 0.792	Annual	Lyons and Antonovics (1991), Charlesworth et al. (1994), Charlesworth and Yang (1998)
<i>Mimulus</i> †	9	> = 8 ‡	[–]	0.18 – 0.73	Perennial	Sweigart et al. (1999), Ritland and Ritland (1989)
<i>Silene</i> †	36	5 – 6	–0.28 – 0.89	[–]	Long-lived or short-lived perennial	Van Rossum et al. (1997), Kephart et al. (1999), Dudash and Fenster (2001)
<i>Turnera ulmifolia</i> *	10	< = 6 ‡	0.00 – 0.69	[–]	Small shrub	Belousoff and Shore (1995)

bees. These observations agree well with the different inbreeding coefficients between *A. cordiflora* and *A. apiculata*. *A. dissecta*, *A. macroclinidioides*, and *A. faurieana* had intermediate level of inbreeding coefficients (0.35, 0.43, and 0.62).

Significant deficiency of observed heterozygosity can result not only from selfing but also from population structure. However, population structure would not influence strongly in *Ainsliaea* spp. we examined. All sampling areas for these species are within an area of 10 to 20 m<sup>2</sup>. Because the *Ainsliaea* spp. we examined have small achenes with a well-developed pappus, seed dispersal distance in these species easily extends over sampling areas. In addition, all of these species are insect-pollinated, and flower visitors such as bumble bees, small opportunistic bees and syrphids were observed to move around sampling areas.

**Comparison with previous studies.** Large variation of outcrossing rate and/or inbreeding coefficient between and within species have been reported in 11 genera of nine families (Table 4); *Amsinckia* (containing heterostylous populations; Johnston and Schoen 1996), *Aquilegia* (Brunet and Eckert 1998, Routley et al. 1999), *Clarkia* (Vasek and Harding 1976, Holtsford and Ellstrand 1989), *Eichhornia* (including tristylous and monomorphic populations: Barrett and Husband 1990), *Gilia* (Schoen 1982), *Leavenworthia* (Lyons and Antonovics 1991, Charlesworth et al. 1994, Charlesworth and Yang 1998), *Linanthus* (Goodwillie 2000), *Lupinus* (Karoly 1992, 1994), *Mimulus* (Ritland and Ritland 1989; Sweigart et al. 1999), *Silene* (Van Rossum et al. 1997, Kephart et al. 1999, Dudash and Fenster 2001) and *Turnera* (Belaoussoff and Shore 1995).

In these studies, more efforts have been made to describe wide variation of mating system among interfertile populations within a species (see studies of *Aquilegia*, *Clarkia*, *Eichhornia*, *Gilia*, *Leavenworthia* and *Turnera*). In particular, the evolution of homostylous selfing lineages from heterostylous outcrossing

ancestors has been intensively studied in *Amsinckia*, *Eichhornia* and *Turnera*. On the other hand, our knowledge about mating system variation between related species still remains limited. In the best studied case of *Leavenworthia*, outcrossing rates were estimated only in three species. Thus, our study for eight related species has provided the largest data set of interspecific variation in plant mating systems and a better opportunity for further comparative studies.

Our study is also unique in that nine species of *Ainsliaea* are herbaceous perennials and two species of *Pertya* are shrubs. Most previous studies have been made on annuals or short-lived perennials (Table 4). Theoretically, it is expected that annuals will be more self-fertile than perennials (Lloyd 1992, Morgan et al. 1997), and variation of mating systems observed in *Leavenworthia* and other annual genera may be associated with annual habit. On the other hand, our study has provided an example that related species can vary from predominant selfers to predominant outcrossers even under perennial life cycles. In addition, this study is the first report of mating systems concerning taxa having varying frequency of cleistogamy. Further comparative studies using established phylogenetic relationship in *Ainsliaea* will enable us to test macroevolutionary hypotheses of breeding system evolution in flowering plants.

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