Int. J. Plant Sci. 167(3):495–502. 2006.

1058-5893/2006/16703-0010$15.00

REPRODUCTIVE BIOLOGY OF TWO SYMPATRIC SPECIES OF *POLYALTHIA* (ANNONACEAE) IN SRI LANKA. II. BREEDING SYSTEMS AND POPULATION GENETIC STRUCTURE

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The breeding systems of two sympatric species, *Polyalthia coffeoides* and *Polyalthia korinti* (Annonaceae), are assessed using a range of approaches, including controlled pollination experiments and analysis of intersimple sequence repeat markers within and between populations. Natural (open) pollination resulted in similar levels of fruit formation as artificial cross-pollination, suggesting that pollinator availability is not a limiting factor in reproduction. Both species possess facultatively xenogamous breeding systems, with 33%–36% fruit formation in artificially crossed experiments and 17%–19% fruit formation following geitonogamy. Reduced fruit set following geitonogamy suggests partial self-incompatibility; this is supported by index of self-incompatibility values of ca. 0.5 in both species. Analysis of population genetic structure supports the hypothesis of a mixed but largely xenogamous mating system. Genetic diversity within populations was estimated to be ca. 80% and 66% of total genetic diversity for *P. coffeoides* and *P. korinti*, respectively. The levels of gene flow between populations were moderate ($Nm = 2.033$ for *P. coffeoides* and 0.970 for *P. korinti*), and genetic identity (I) values between populations within species were high. This possibly reflects the fragmentation of a previously more extensive population, correlated with the historical deforestation associated with crop cultivation and irrigation in Sri Lanka.

Keywords: Annonaceae, breeding system, ISSR, pollination, *Polyalthia*.

Introduction

The Annonaceae exhibit a diversity of highly specialized pollination systems, although the majority of species are beetle-pollinated, with distinct small- and large-beetle pollination systems (e.g., Gottsberger 1999; Silberbauer-Gottsberger et al. 2003). In an accompanying article (Ratnayake et al. 2006), we present data to show that *Polyalthia coffeoides* and *Polyalthia korinti* are pollinated by an unidentified species of *Endaeus* weevil (Coleoptera: Curculionidae), with *Carpophilus plagiatipennis* (Coleoptera: Nitidulidae) as a secondary pollinator of *P. coffeoides*. Our study of the floral phenology and pollination ecology furthermore strongly suggests that both *Polyalthia* species are likely to possess a primarily xenogamous breeding system.

As recently noted by Zhang et al. (2005), however, it is important to confirm assumptions regarding pollination systems by investigating the breeding system operating within populations. Such research is rare, and the only previous studies to include population genetic analysis of Annonaceae species are studies on cultivated and wild populations of the American pawpaw *Asimina triloba* (Huang et al. 1997, 1998, 2000, 2003; Pomper et al. 2003). One of the main aims of our study is therefore to investigate whether the cantharophilous pollination system observed in *P. coffeoides* and *P. korinti* is mirrored by evidence of predominant outcrossing, assessed using controlled pollination experiments and intersimple sequence repeat (ISSR) markers.

Molecular phylogenetic data using combined *rbc*L and *trn*L-F sequences have shown that *Polyalthia* is polyphyletic (Mols et al. 2004). Supplementary data (Y. C. F. Su, unpublished data, based on *rbc*L and *trn*L-F sequences) suggest that *P. coffeoides* and *P. korinti* are not congeneric: *Polyalthia coffeoides* is most closely related to *Enicosanthum* and species in *Polyalthia* sect. *Monoon*, whereas *P. korinti* is associated with *Polyalthia cerasoides*, *Polyalthia pendula*, and *Polyalthia stuhlmannii*.

Material and Methods

Study Sites

Field experiments on the reproductive biology of both *Polyalthia coffeoides* and *Polyalthia korinti* were conducted at the Menikdena archaeological forest reserve (7°28′–56′N, 80°35′–45′E; fig. 1) in Sri Lanka, where the two species co-occur and are codominant. Details on the topography, climate, and ecology of the site are given by Ratnayake et al. (2006). The Menikdena forest is legally protected and is relatively well sheltered from human disturbances.

Leaf samples were collected from a total of six sites for ISSR analysis (fig. 1). Four populations of each species were used, namely, Menikdena, Omaragolla, Ritigala, and Walankatuwa (*P. coffeoides*) and Medirigiriya, Menikdena, Pannampitiya,

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Manuscript received August 2005; revised manuscript received January 2006.

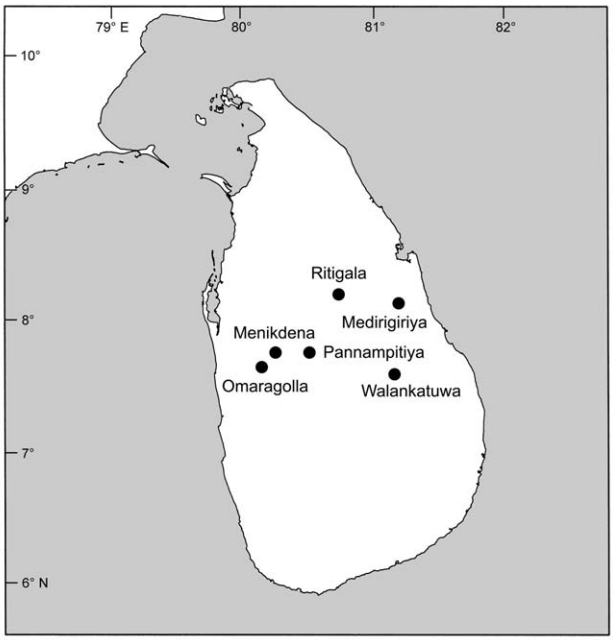

Fig. 1 Location of study sites in Sri Lanka.

and Ritigala (*P. korinti*). Voucher specimens have been deposited in HKU and PDA herbaria, namely, *R. M. C. S. Ratnayake 1/03* (*P. coffeoides*) and *R. M. C. S. Ratnayake 2/03* (*P. korinti*).

Pollen/Ovule Ratios

The ratio of the number of pollen grains and ovules produced by a flower can be used as an approximate indicator of breeding system as follows (Cruden 1977): 2.7–5.4 suggests cleistogamy, 18.1–39.0 suggests obligate autogamy, 39.1–396.0 suggests facultative autogamy, 244.7–2588 suggests facultative xenogamy, and 2108–195,525 suggests obligate xenogamy. The numbers of pollen grains and ovules were counted using standard techniques (Dafni 1992), based on 11 flowers from five individuals of each species.

Controlled Pollination Experiments

In order to determine whether viable pollen was available for controlled pollination experiments, pollen germination rates were assessed *in vitro* using artificial sucrose solutions. Sucrose solutions of various concentrations (0%, 5%, 10%, 20%, and 25%) were prepared and mixed with 50% (w/v) H_3PO_3 and 50% $Ca(NO)_3$(w/v) (Dafni 1992). Mature pollen was collected from five flowers from each of five individuals immediately after anther dehiscence and mixed with 10 μL of each sucrose solution on glass cavity slides. The slides were maintained in closed petri dishes with damp filter paper at ambient temperatures for 24 h. One hundred pollen grains were visually assessed to determine the proportion of pollen grains germinating, using six replicates of each treatment.

Controlled pollination experiments were conducted at the beginning of the peak flowering season. The flowers were covered with chiffon cloth bags to exclude pollinators (Dafni 1992) before the onset of stigmatic receptivity. Pale green cloth was used as camouflage to protect the bagged flowers from attack by animals, monkeys in particular. The field experiments involved 107–131 flowers (varying between treatments) from seven individuals of *P. coffeoides* and 152–220 flowers from eight individuals of *P. korinti*.

A total of four different controlled pollination treatments were conducted (adapted from Dafni 1992) to determine breeding system: treatment 1, control, flowers not bagged and left to freely pollinate; treatment 2, test for geitonogamy, flowers bagged and artificially pollinated with pollen from another flower of the same individual that had previously been bagged; treatment 3, test for xenogamy, flowers bagged and artificially pollinated with pollen from flowers of a different individual (10–100 m from recipient); treatment 4, test for autogamy, flowers bagged but not artificially pollinated. Standard pollination treatments requiring emasculation of the flowers were not possible because the flowers inevitably withered and abscised before the onset of stigmatic receptivity. The strong protogyny shown by flowers of both species (Ratnayake et al. 2006) precludes intraflower self-pollination (autogamy), and consequently, pollination treatment 4 can also be regarded as a test for agamospermy. Since autogamous self-pollination is not possible, all references to "self-pollination" in the present research refer to geitonogamous pollination (between different flowers of the same individual). In the absence of clearly discernible external morphological changes associated with postpollination events, it was not possible to record with certainty the extent of pollination success until 2 wk after petal abscission.

The number of monocarps (components of the fruit derived from individual carpels) and entire fruits that developed following each treatment were assessed at 2-wk intervals until fruit maturity (after ca. 20 wk). The overall time between pollination and fruit maturity was recorded for each species. The percentage fruit set in relation to the number of flowers pollinated in each treatment was also calculated. Some treated flowers were destroyed by monkeys and were consequently excluded from the analyses. The statistical significance of comparisons between different pollination treatments and between different individuals was assessed by ANOVA on rank after testing for equal variance and discriminated using the Tukey test for unequal n (Spjotvoll/Stoline test). For *P. korinti*, comparisons of the number of developing monocarps between different treatments after 2, 8, and 20 wk required $\log_{10}$ transformation to satisfy statistical assumptions of normal distribution. All statistical analyses were undertaken using Minitab software (Minitab 2001).

Index of Self-Incompatibility

A quantitative evaluation of possible self-incompatibility was achieved using the index of self-incompatibility (ISI), developed by Zapata and Arroyo (1978). ISI was calculated by dividing the number of fruits resulting from self-pollination by the number of fruits derived by cross-pollination. Resultant ISI values reflect the following possibilities: $0 =$ completely self-incompatible, $0-0.2 =$ mostly self-incompatible, $0.2-1 =$ partially self-incompatible, and $>1 =$ self-compatible (Zapata and Arroyo 1978).

ISSR Markers

A total of 97 accessions of *P. coffeoides* were studied from four populations (20–30 samples from each population), whereas a total of 47 samples of *P. korinti* were studied from four populations (10–14 samples per population).

Total DNA was extracted from silica-gel-dried leaves in the presence of liquid N_2, using an extraction procedure developed by Singh et al. (1999). A total of 100 primers, 15–23 nucleotides in length, were screened (USB ISSR Primer, Oligonucleotide Set 100/9, Biotechnology Laboratory, University of British Columbia, Vancouver), and 11 primers were used in single-primer PCR amplifications, namely, 807 [$(AG)_8$-T], 809 [$(AG)_8$-G], 810 [$(GA)_8$-T], 813 [$(CT)_8$-T], 815 [$(CA)_8$-G], 817 [$(CA)_8$-A], 822 [$(TC)_8$-A], 825 [$(AC)_8$-T], 829 [$(TG)_8$-C], 840 [$(GA)_8$-YT], and 850 [$(GT)_8$-YC].

After optimization, standard reaction conditions were as follows: reaction volumes of 25 μL, comprising 0.25 μM primer, 1× PCR buffer with 1.5 mM $MgCl_2$, 2 U Taq DNA polymerase (GE Healthcare, Piscataway, NJ), 0.1 mM dNTPs (Invitrogen, Carlsbad, CA), and ca. 25 ng DNA. PCR amplifications were performed in a PCT-100 programmable thermal cycler (MJ Research, Waltham, MA) with the following profile: one cycle at 94°C for 5 min; 40 cycles at 94°C for 45 s, 49°C for 45 s, and 72°C for 2 min; and one cycle at 72°C for 7 min. PCR products were loaded onto 1.8% agarose gels in 1× TAE buffer with ethidium bromide. The gels were visualized under UV and recorded using a UVP gel documentation system (UVP, Upland, CA). Fragment sizes were estimated based on a 100-bp DNA ladder (Invitrogen, Carlsbad, CA) and their sizes were used to assign loci for each primer; bands were scored as diallelic for each assigned locus (0 = absent; 1 = present).

The data matrix was analyzed using POPGENE (Yeh et al. 1997). Genetic parameters within populations were calculated (Nei 1987), including the percentage of polymorphic loci within populations (P_p), number of alleles per locus (A), effective number of alleles per locus (A_e), Nei's genetic diversity (H, where H_S = genetic diversity within populations and H_T = total genetic diversity in the pooled population), and estimated gene flow between populations (Nm). The genetic diversity values were multiplied by $2n/(2n-1)$, to correct for differences in sample size (Lowe et al. 2004). The number of alleles per locus (A) is large when the extent of polymorphism is high (Nei 1987) and can be used to measure genetic variability. Estimates of genetic diversity within populations were also measured using Shannon's information index (S) of polymorphic loci (Lowe et al. 2004), where S_S = genetic diversity within populations and S_T = total genetic diversity in the pooled population.

The relative magnitude of gene differentiation between the populations was assessed using Nei's (1987) coefficient of gene differentiation, G_{ST}. This does not rely on knowledge of genotype frequencies (Nei 1973) and can be estimated from allele frequencies in terms of the expected heterozygosities within and between populations: $G_{ST} = D_{ST}/H_T$, in which D_{ST} is the average genetic diversity between populations. The G_{ST} was also used to estimate the level of gene flow Nm, based on the relationship $G_{ST} = 1/(4Nm + 1)$, where G_{ST} is Nei's (1973) estimator of F_{ST} (Wright 1951).

The genetic identity (I) and Nei's (1978) unbiased genetic distance (D) between populations were also computed. UPGMA dendrograms were constructed using TFPGA, version 1.3 (Miller 1997).

Results

Pollen/Ovule Ratios

The pollen grains of both species are solitary when the anthers dehisce. In *Polyalthia coffeoides*, the mean numbers of

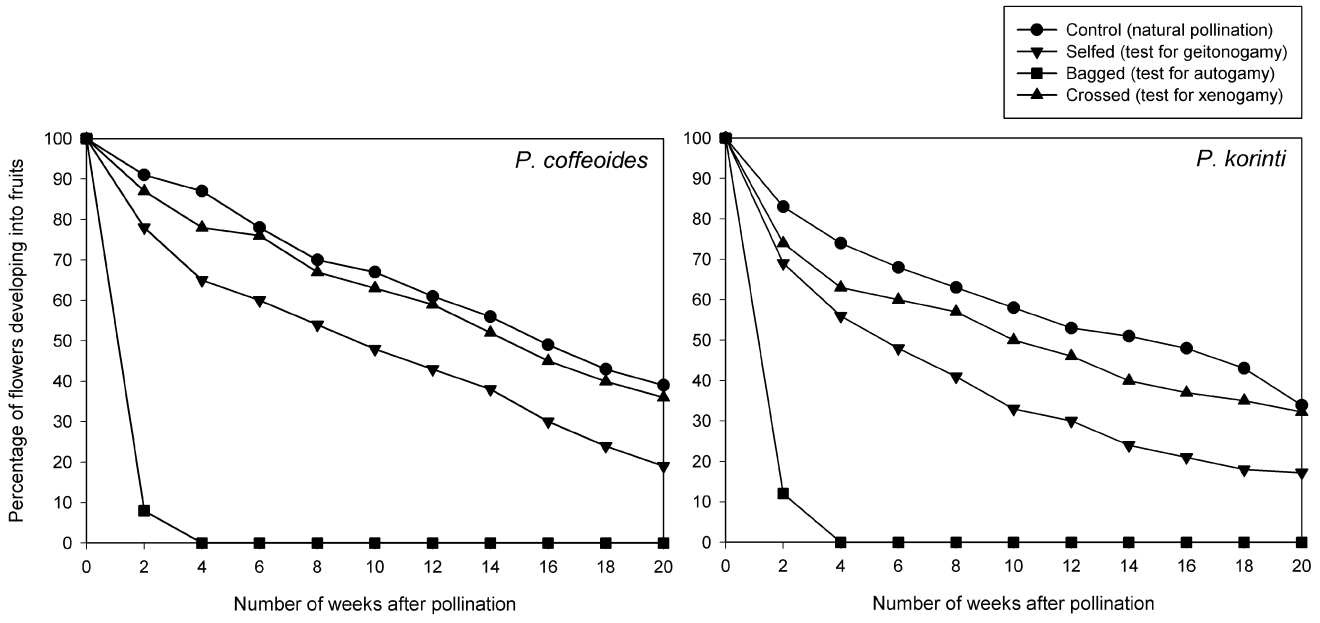

Fig. 2 Percentage fruit survival after different pollination treatments in *Polyalthia coffeoides* and *Polyalthia korinti*.

Table 1

Percentage Fruit Set in *Polyalthia coffeoides* Resulting from Controlled Pollination Experiments

Fruit set	Control	Crossed	Selfed	Bagged
No. individuals	7	7	7	7
No. flowers	107	110	112	131
Mean percentage fruit set (±SD)	39.2 ± 4.1[a]	35.6 ± 7.7[a]	18.7 ± 7.4[b]	0
Mean number of monocarps (±SD):				
After 2 wk	20.3 ± 5.4[a]	16.9 ± 1.9[a,b]	13.3 ± 2.6[b]	0
After 8 wk	13.1 ± 3.9[a]	9.5 ± 0.9[a]	5.0 ± 1.1[b]	0
After 20 wk	6.2 ± 1.6[a]	5.3 ± 1.0[a]	2.2 ± 0.8[b]	0

Note. Superscript letters summarize the results of a Tukey test for unequal n (Spjotvoll/Stoline test); treatments with the same letter do not differ significantly ($P < 0.05$).

pollen grains and ovules per flower (±SD) were 9701 ± 3864 and 14.3 ± 4.9, respectively, and corresponding data for *Polyalthia korinti* were 30,829 ± 4041 pollen grains and 14.6 ± 1.3 ovules per flower. The pollen/ovule ratios of *P. coffeoides* and *P. korinti* were therefore 722.6 ± 275.4 and 2296 ± 83, respectively. According to Cruden's (1977) scheme, both species are therefore likely to possess facultatively xenogamous breeding systems.

Controlled Pollination Experiments

The optimal sucrose concentrations for pollen germination for *P. coffeoides* and *P. korinti* were 15% and 10%, respectively. Under these conditions, *P. coffeoides* showed 19.5% (±3.2 SD) pollen viability, and *P. korinti* showed 13.0% (±3.4 SD). These results therefore confirmed the feasibility of undertaking controlled pollination experiments.

The overall time between pollination and fruit maturity was 125–138 d (mean = 134 d) for *P. coffeoides* and 86–115 d (mean = 101 d) for *P. korinti*. The 20-wk monitoring of postpollination events therefore covered the entire developmental range.

Abscission of the entire flower after 10–14 d was used as an indication of fertilization failure. Within the first 2 wk, ca. 90% of flowers that had been bagged without being artificially pollinated abscised (fig. 2), with 100% abscission after 4 wk. Significantly fewer fruits were aborted in the early stages of development in the other three treatments in both *Polyalthia* species. In *P. coffeoides*, the abortion rate for self-pollinated (geitonogamous), cross-pollinated, and control flowers (natural pollination) were 34.8%, 21.8%, and 13.1%, respectively, after 4 wk. Results for *P. korinti* were similar, with highest levels of fruit abortion after 4 wk in self-pollinated flowers (44.1%), followed by cross-pollinated flowers (36.8%) and control flowers (24.1%). Abscission of immature fruits was therefore greater in self-pollinated flowers than in either cross-pollinated or control flowers.

All fruits remaining on the trees after 20 wk were fully mature. Self-pollinated flowers showed significantly higher levels of fruit abortion than cross-pollinated and control flowers, with 18.7% and 17.2% fruits remaining in *P. coffeoides* and *P. korinti*, respectively (tables 1, 2). The results for cross-pollination and natural pollination were similar: in *P. coffeoides*, 35.6% and 39.2% of fruits remained, and in *P. korinti*, 32.2% and 33.9% remained.

Variation in the pollination success of the individual trees studied was not statistically significant (assessed using one-way ANOVA) in either species. In contrast, the differences between treatments were significant at $P < 0.05$: fruit production after geitonogamy was significantly lower than in either the open- or cross-pollination experiments for both *P. coffeoides* (table 1) and *P. korinti* (table 2) in terms of both overall percentage fruit set and the number of monocarps developing per fruit.

Index of Self-Incompatibility

The ISIs for *P. korinti* and *P. coffeoides* were 0.534 and 0.527, respectively, suggesting that both species are partially self-incompatible (Zapata and Arroyo 1978). The higher rate of fruit abortion in self-pollinated flowers (tables 1, 2; fig. 2) was also possibly indicative of an incomplete self-compatibility system. Even though the number of monocarps in the fruits resulting from cross- and self-pollination do not differ significantly 2 wk after pollination, significant differences become apparent 8 and 20 wk after pollination in both *Polyalthia* species (tables 1, 2).

ISSR Markers

One hundred and twenty loci were scored using 97 individuals of *P. coffeoides* from four populations, with 11 primers (table 3). Bands ranged in size between 250 and 2900 bp, and the number of amplified ISSR bands ranged from seven to 19 per primer (average = 10.9). A total of 82 loci were similarly scored using 47 individuals of *P. korinti* from four populations, with nine primers (table 3). Bands ranged from 250 to 2900 bp, and the number of amplified ISSR bands ranged from seven to 12 per primer (average = 9.1). The primers were selected to show maximum genetic variation within and between populations of the two species.

The parameters of genetic variability within and between populations of *P. coffeoides* and *P. korinti* are provided in tables 4 and 5, respectively. In general, both species showed similar patterns of variation, although there was evidence that populations of *P. coffeoides* were more genetically

Table 2

Percentage Fruit Set in *Polyalthia korinti* Resulting from Controlled Pollination Experiments

Fruit set	Control	Crossed	Selfed	Bagged
No. individuals	8	8	8	8
No. flowers	170	220	152	193
Mean percentage fruit set (±SD)	33.9 ± 12.5[a]	32.2 ± 14.3[a,b]	17.2 ± 9.2[b]	0
Mean number of monocarps (±SD):				
After 2 wks	13.4 ± 5.9[a]	10.9 ± 1.3[a]	8.6 ± 2.1[b]	0
After 8 wks	8.6 ± 4.7[a]	6.6 ± 0.9[a]	4.0 ± 1.4[b]	0
After 20 wks	5.7 ± 1.8[a]	3.4 ± 0.6[a]	1.6 ± 0.7[b]	0

Note. Superscript letters summarize the results of a Tukey test for unequal n (Spjotvoll/Stoline test); treatments with the same letter do not differ significantly ($P < 0.05$).

Table 3

Number of Loci per Primer and Their Range of Band Size in Intersimple Sequence Repeat Analysis of *Polyalthia coffeoides* and *Polyalthia korinti*

	P. coffeoides		*P. korinti*	
Primer	No. loci	Range of band size (bp)	No. loci	Range of band size (bp)
807	9	1900–400	11	2900–400
809	12	1990–450	...	...
810	19	2100–250	...	...
813	8	2500–550	6	2000–450
815	9	2000–450	10	1600–450
817	9	2800–500	12	1600–450
822	10	2800–500	8	2000–450
825	12	1900–350	7	1200–350
829	15	1900–500	7	1450–400
840	10	1600–300	12	1900–250
850	7	2900–1031	9	2500–450

variable: the population average of Nei's genetic diversity within populations (H_S), for example, was 0.242 ± 0.023 (SD) in *P. coffeoides* but only 0.185 ± 0.018 in *P. korinti*. For both species, comparisons between populations revealed that they were almost equally variable, with similar H values.

Most of the genetic variation in the two species was distributed within populations. In *P. coffeoides*, the within-population genetic diversity (H_S) was 0.242, representing ca. 80% of the total genetic variation ($H_T = 0.299$). Conversely, the average genetic diversity between populations (D_{ST}) was 0.059, and thus the G_{ST} value calculated for *P. coffeoides* was 0.197, indicating that the remaining ca. 20% of the total genetic variation was attributable to population differentiation. Results for *P. korinti* are similar, with ca. 66% within-population genetic variation ($H_S = 0.185$; $H_T = 0.281$) and ca. 34% between-population variation ($D_{ST} = 0.095$; $G_{ST} = 0.340$). The proportions of within- and between-population genetic variation were also estimated using Shannon's index (S). In *P. coffeoides*, the proportion of total diversity distributed within populations (S_S/S_T) was 77.3%, which was greater than that between populations ($[S_T - S_S]/S_T = 22.7\%$). Corresponding values for *P. korinti* were 63.2% and 36.8%, respectively.

In *P. coffeoides*, the genetic identity (I) values of individual populations ranged between 0.863 and 0.931, with an average of 0.904 (table 6). Data for *P. korinti* were similar, with I values ranging between 0.784 and 0.984, with an average of 0.853 (table 7). These values suggest that populations of each species are genetically very similar and are likely to indicate relatively high levels of gene flow.

Genetic distances (D) between populations were correspondingly low (tables 6, 7), in the range 0.072–0.147 for *P. coffeoides* (average = 0.102) and 0.016–0.259 for *P. korinti* (average = 0.163). The dendrogram based on the genetic distances between populations of *P. coffeoides* (fig. 3*A*) shows that the Menikdena population was most distant from the other three populations, although there is no obvious geographical or ecological explanation for this pattern. The dendrogram for *P. korinti* (fig. 3*B*) shows two branches, however, reflecting ecological differences: the Medirigiriya and Ritigala populations occur in the dry zone area, whereas the Menikdena and Pannampitiya populations occur in the intermediate climatic zone. The level of gene flow between populations (Nm) was estimated to be 2.033 and 0.970 in *P. coffeoides* and *P. korinti*, respectively, indicating that there is a moderate level of migration between populations of each species.

Discussion

Breeding System

The relatively high rates of fruit production recorded during the controlled pollination experiments after natural (open) pollination indicate that fruit set is unlikely to be limited by pollinator availability. The primary pollinator of both species has been shown to be an *Endaeus* weevil (Coleoptera: Curculionidae), which is locally abundant (Ratnayake et al. 2006).

In both species, the mean percentage fruit set after cross-pollination exceeded that following geitonogamous self-pollination (tables 1, 2; fig. 2), although the difference was not statistically significant for *P. korinti*. This suggests that xenogamy is likely to be more prevalent than geitonogamy in *P. coffeoides*. The significant decrease in the numbers of monocarps per fruit derived from self-pollination relative to cross-pollination may be due to a postfertilization

Table 4

Genetic Variability Within and Between Populations of *Polyalthia coffeoides*

	Omaragolla	Ritigala	Menikdena	Walankatuwa	Population average	Total pooled population
N	20	24	30	23	24.3	97
P_p (%)	71.7	57.5	73.3	72.5	68.8	98.3
$A \pm$ SD	1.717 ± 0.453	1.575 ± 0.496	1.733 ± 0.444	1.725 ± 0.448	1.688 ± 0.075	1.983 ± 0.129
$A_e \pm$ SD	1.389 ± 0.401	1.384 ± 0.422	1.444 ± 0.392	1.484 ± 0.418	1.425 ± 0.408	1.507 ± 0.343
$S \pm$ SD	0.333 ± 0.285	0.309 ± 0.306	0.374 ± 0.286	0.388 ± 0.298	0.351 ± 0.036	0.454 ± 0.216
$H \pm$ SD	0.227 ± 0.206	0.217 ± 0.218	0.257 ± 0.205	0.266 ± 0.215	0.242 ± 0.023	0.299 ± 0.166
D_{ST}	...	...	...	...	...	0.059
Nei's G_{ST}	...	...	...	...	...	0.197

Note. P_p = percentage of polymorphic loci within populations; A = number of alleles per locus; A_e = effective number of alleles per locus; S = Shannon's information index; H = Nei's genetic diversity; D_{ST} = average genetic diversity between populations; Nei's G_{ST} = Nei's coefficient of gene differentiation.

Table 5

Genetic Variability Within and Between Populations of *Polyalthia korinti*

	Medirigiriya	Ritigala	Menikdena	Pannampitiya	Population average	Total pooled population
N	10	10	14	13	11.8	47
P_p (%)	39.0	45.1	59.8	52.4	49.1	92.7
$A \pm$ SD	1.390 ± 0.491	1.451 ± 0.501	1.598 ± 0.493	1.524 ± 0.503	1.491 ± 0.090	1.926 ± 0.262
$A_e \pm$ SD	1.291 ± 0.398	1.327 ± 0.408	1.373 ± 0.406	1.323 ± 0.391	1.329 ± 0.034	1.46 ± 0.327
$S \pm$ SD	0.232 ± 0.304	0.264 ± 0.306	0.313 ± 0.295	0.274 ± 0.293	0.271 ± 0.033	0.429 ± 0.224
$H \pm$ SD	0.169 ± 0.213	0.191 ± 0.215	0.218 ± 0.209	0.193 ± 0.207	0.185 ± 0.018	0.281 ± 0.166
D_{ST}	...	...	...	...	...	0.095
Nei's G_{ST}	...	...	...	...	...	0.340

Note. Definitions as in table 4.

self-incompatibility mechanism, although embryo development in abscised monocarps was not examined during this study. There is little information available on monocarp abscission in relation to pollination in the Annonaceae, although there was apparently no significant difference between the monocarp abscission following self- and cross-pollination in the self-compatible species *Deeringothamnus rugelii* and *Deeringothamnus pulchellus* (Norman 2003).

The predominantly xenogamous breeding system suggested by the controlled pollination experiments with both *Polyalthia* species is further corroborated by the pollen/ovule ratios. Caution is required in the interpretation of these results, however, since pollen/ovule values are known to vary within and between populations and to be affected by various factors, including timing of flower formation in the flowering season (Cruden 2000 and references therein).

Fruit abortion was observed in all field experiments with both species, irrespective of pollination treatment (fig. 2). Although self-pollination is clearly a significant cause of fruit abortion, other possible causes include unfavorable weather patterns, animal consumption, and "over-fruiting" (i.e., immature fruit abscission when the carrying capacity of an individual tree is exceeded). The relatively low flowering and fruiting intensities of both species (Ratnayake et al. 2006) suggest that the latter explanation is less likely, however.

The ISI results suggest that both *P. coffeoides* and *P. korinti* are partially self-incompatible. There are very few reports of self-incompatibility in the Annonaceae, possibly because of widespread protogyny in the family that would limit the potential benefits of such a system. Partial self-incompatibility has been reported, however, in *Sapranthus palanga* (Bawa 1974), *Asimina parviflora* and *Asimina triloba* (Norman et al. 1992), *Uvaria elmeri* (Nagamitsu and Inoue 1997), and species in the *Polyalthia hypoleuca* complex (Rogstad 1994). *Polyalthia* is known to be polyphyletic (Mols et al. 2004), however, and not only are *P. coffeoides* and *P. korinti* unlikely to be congeners, neither are they congeneric with the *P. hypoleuca* complex (Y. C. F. Su, unpublished data).

The self-incompatibility mechanisms previously reported in the Annonaceae appear to be late-acting and either prezygotic (*Uvaria elmeri*; Nagamitsu and Inoue 1997) or postzygotic (*Asimina parviflora*; Norman et al. 1992). The initial development of fruits after geitonogamous self-pollination in *P. coffeoides* and *P. korinti* suggests that the self-incompatibility mechanism is postzygotic. Significantly, the stigmas in *P. coffeoides* and *P. korinti* are of the "wet" type (with copious stigmatic exudate at maturity). This feature is consistent with the gametophytic self-incompatibility mechanism (de Nettancourt 1977; Richards 1986), which is widespread in magnoliid taxa and is likely to have been the earliest self-incompatibility mechanism to have evolved in angiosperms (Richards 1986; Bernhardt and Thien 1987).

The putative self-incompatibility mechanism in *P. coffeoides* and *P. korinti* is presumably responsible only for fruit abscission within the first few weeks after pollination; although there is a continued gradual abscission of immature fruits between weeks 4 and 20 following geitonogamous self-pollination (fig. 2), the same pattern is also observed in artificially crossed treatments, suggesting that it is not due to self-incompatibility.

Population Genetic Structure

Long-lived woody perennial species typically show high levels of genetic diversity within populations ($P_p = 49.3$, $A = 1.76$, $A_e = 1.20$, $H = 0.148$; Hamrick et al. 1992) but comparatively low levels of genetic variation between populations ($G_{ST} = 0.084$; Hamrick et al. 1992). The results obtained here for *P. coffeoides* and *P. korinti* show a similar pattern (tables 4, 5), although both species show greater genetic diversity than is typical of most long-lived woody perennials; this may be attributable to the higher resolving power of ISSR markers compared to traditional genetic markers.

In an analysis of the population genetics of long-lived woody species, Hamrick et al. (1992) showed that outcrossing animal-pollinated species typically exhibit higher levels of within-population variation ($P_p = 47.6$, $A = 1.72$, $A_e = 1.22$,

Table 6

Nei's Unbiased Measure of Genetic Identity (*I*) and Genetic Distance (*D*) for *Polyalthia coffeoides*

Population	Omaragolla	Ritigala	Menikdena	Walankatuwa
Omaragolla	...	0.919	0.899	0.931
Ritigala	0.083	...	0.863	0.911
Menikdena	0.117	0.151	...	0.898
Walankatuwa	0.078	0.094	0.115	...

Note. Genetic identity above diagonal; genetic distance below diagonal.

Table 7

Nei's Unbiased Measure of Genetic Identity (*I*) and Genetic Distance (*D*) for *Polyalthia korinti*

Population	Medirigiriya	Ritigala	Menikdena	Pannampitiya
Medirigiriya	...	0.984	0.784	0.771
Ritigala	0.020	...	0.828	0.814
Menikdena	0.255	0.188	...	0.939
Pannampitiya	0.283	0.221	0.074	...

Note. Genetic identity above diagonal; genetic distance below diagonal.

$H = 0.163$) than mixed-mating system or wind-pollinated species. The results for *P. coffeoides* and *P. korinti* are similar (tables 4, 5) but again show higher levels of genetic diversity, with H values in the ranges 0.217–0.266 (*P. coffeoides*) and 0.169–0.218 (*P. korinti*). The values for *P. coffeoides* are similar to those reported for *Asimina triloba* ($H = 0.25$; Huang et al. 2000), however, which is predominantly fly-pollinated (Willson and Schemske 1980) but also visited by small beetles (Johnson and Willson, cited in Norman et al. 1992). Loveless and Hamrick (1984) have shown that the coefficient of gene differentiation between populations (G_{ST}) is generally 0.118 ± 0.036 ($\pm$SE) for outcrossing species and 0.243 ± 0.059 for species with mixed-mating systems. The G_{ST} values obtained for *P. coffeoides* and *P. korinti* are in agreement with corresponding values obtained for species with a mixed-mating system.

Genetic diversity within populations (H_S) accounted for ca. 80% and 66% of the total genetic diversity of *P. coffeoides* and *P. korinti*, respectively, suggesting a considerable level of gene flow between populations. The estimated levels of gene flow between populations are moderate, with Nm values of 2.033 for *P. coffeoides* and 0.970 for *P. korinti*. This level of gene flow is sufficient to counteract population differentiation through genetic drift (Hartl and Clark 1997). Slatkin (1985) has shown that if the number of migrants per generation is greater than four ($Nm > 4$), then gene flow will counteract genetic drift within populations, but if it is less than one ($Nm < 1$), then genetic drift can be the dominant factor influencing genetic structure.

The high genetic identity (I) values (tables 6, 7) also suggest that populations of each species are genetically very similar and may have exchanged genes in the recent past, possibly reflecting the fragmentation of a previously larger population. The dry and intermediate vegetation zones in Sri Lanka were extensively cultivated and irrigated between the fifth or sixth century BC and the thirteenth century AD (Ashton et al. 1997, p.12). This resulted in extensive fragmentation of the natural forests, and most of the existing forests are therefore of secondary origin. The sites selected for our study are essentially reproductively isolated from one another, without intervening forested areas other than small "home gardens" consisting of cultivated trees and scrubland resulting from "slash and burn" cultivation. Although there is no published information on the geographical movements of the pollinating *Endaeus* weevils, the relative isolation of the study sites suggests that it is unlikely that extensive intersite migration will occur, hence reducing the chances of long-distance pollen dispersal. *Polyalthia korinti* trees were observed to grow in and around a few of these home gardens, however, thereby providing possible "stepping stones" for pollinator movement.

Although interpopulation gene flow via pollen dispersal is probably low, the seeds of *P. korinti* are bird dispersed (as possibly are *P. coffeoides* seeds, although no such field observations have been made), and it is feasible that the seeds could be transported between the sites, which are ca. 30–40 km apart. At maturity, the monocarps of *P. korinti* are bright red and are therefore within the color vision range of birds. The pericarp is furthermore sweet and the seeds are small, with a tough testa, thereby enabling passage through the birds' guts without damage. Further studies are needed to elucidate whether the low level of population differentiation observed in *P. coffeoides* and *P. korinti* is due to contemporary gene flow (possibly through seed dispersal) or whether the presently fragmented populations were part of a historically large, panmictic population.

Conclusions

The data presented here on the breeding systems and population genetic structure of *P. coffeoides* and *P. korinti* can be interpreted in conjunction with the data on the floral phenology and pollination ecology presented in an accompanying article (Ratnayake et al. 2006). Both species are pollinated by the same species of *Endaeus* weevil, which is locally abundant and does not show host specificity. High levels of fruit production under natural conditions suggest that fertilization is not limited by pollinator availability. Both species furthermore show marked protogyny, with a reproductively inactive phase between the pistillate and staminate phases. This precludes intrafloral self-pollination, although geitonogamous self-pollination is nonetheless possible because flowers

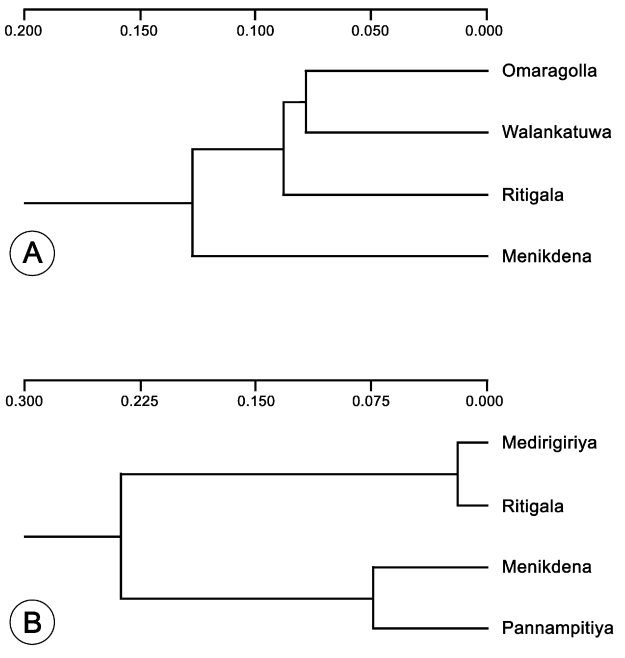

Fig. 3 UPGMA dendrograms of Nei's genetic distance between populations of *Polyalthia coffeoides* (*A*) and *Polyalthia korinti* (*B*).

in different sexual phases occur concurrently on the same individual tree. Controlled pollination experiments and genetic analysis of breeding systems confirm that both species show a mixed-mating system, although xenogamy is more prevalent than geitonogamy. It is also suggested that a partial self-incompatibility system (presumably gametophytically controlled) is operating in both species. Data on the population genetic structure of the two species indicate low levels of genetic variation between populations, with moderate levels of interpopulation gene flow, possibly reflecting the historical fragmentation of larger populations or contemporary gene flow through seed dispersal.

Acknowledgments

This research was funded by a grant to R.M.K. Saunders from the University of Hong Kong Committee on Research and Conference Grants. We are grateful to the Sri Lankan Archaeological Department for permission to conduct this research at Menikdena forest; the Sri Lankan Forestry Department for allowing the collection of leaf samples for ISSR analysis; Heidi Kong for assistance with the ISSR study; U. G. U. R. Bandara for assistance in the field; and Dr. R. T. Corlett, Dr. S. Dayanandan, Professor G. Gottsberger, Dr. L. Ramsden, and two anonymous reviewers for helpful discussions.

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