AN ABSTRACT OF THE THESIS OF

Sharon K. Krueger for the degree of Doctor of Philosophy in Crop Science presented on December 5, 1989.

Title: Allozyme Genetics and Mating Systems of *Cuphea laminuligera* and *Cuphea lutea*

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Cuphea laminuligera Koehne and *Cuphea lutea* Rose in Koehne are undomesticated species having potential as oilseed crops. Genetic markers and mating systems have not been described for these species. Our objectives were to survey these species for allozyme variation, analyze the segregation and linkage of polymorphic loci, estimate autofertility and outcrossing rates, and investigate the effect of plant density on outcrossing rate. We analyzed allozyme variation among 11 F_2 populations of *C. laminuligera* and one F_2 population of *C. lutea*. Both species were assayed for aconitase, diaphorase, esterase, fluorescent esterase, glutamine oxaloacetate transaminase, malate dehydrogenase, menadione reductase, 6-phosphogluconic dehydrogenase, phosphoglucose isomerase, and shikimate dehydrogenase activity. *Cuphea laminuligera* was also assayed for phosphoglucomutase activity. We observed 14 polymorphic loci and two monomorphic loci in *C. laminuligera*. Variation was observed within and between parental populations. Observed segregation ratios were generally not significantly different ($P > 0.05$) from expected ratios. We observed three polymorphic loci and at least

10 monomorphic loci in *C. lutea*. We found no within population variation in parental populations. Observed segregation ratios were not significantly different from expected ratios and linkage was not detected. Autofertility was estimated using plants isolated under insect-proof cages. The mean seed set per flower for *C. laminuligera* was 0.00 in 1986 and 1987. The mean seed set per flower of *C. lutea* was 4.75 and 4.64 in 1986 and 1987, respectively. Outcrossing rates (t) were estimated for four populations of *C. laminuligera* and three populations of *C. lutea* using allozyme phenotypes of open-pollinated individual plant families. Populations were grown at low (1.0 x 1.0 m) and high (0.04 x 0.3 m) density. Mating system parameters were estimated using the mixed mating model. Multilocus estimates of t ranged from 0.83 to 0.98 and 1.00 to 1.01 for low and high density populations of *C. laminuligera*, respectively. Multilocus estimates of t ranged from 0.17 to 0.26 and 0.36 to 0.54 for low and high density populations of *C. lutea*, respectively. *C. laminuligera* is strongly allogamous; however, we observed selfing rates as high as 17%. *C. lutea* is predominantly autogamous, but outcrossing rates occasionally exceeded 50%. Outcrossing rates were greatly affected by plant density.

Allozyme Genetics and Mating Systems of *Cuphea laminuligera* and *Cuphea lutea*

by

Sharon K. Krueger

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ALLOZYME GENETICS AND MATING SYSTEMS OF *CUPHEA LAMINULIGERA* AND *CUPHEA LUTEA*

INTRODUCTION

Medium-chain fatty acids (MCFAs), $C_{8:0}$ - $C_{14:0}$, have unique physical characteristics (stability to oxidation, and steep melting curves) which make them useful in the manufacture of surfactants and certain foods (Young 1983). Lauric oils are primarily used by the chemical industry for the manufacture of soaps and detergents (Young 1983, Thompson 1984, Arkcoll 1988). In addition, they are used as cooking fats and shortening and as replacements for dairy fat and cocoa-butter (Young 1983, Arkcoll 1988). Monolaurin, a derivative of lauric acid ($C_{12:0}$), has antibacterial and antifungal properties (Hierholzer and Kabara 1982). It solubilizes lipids and phospholipids in viral envelopes, thereby imparting virucidal activity. Capric acid ($C_{10:0}$) is used medicinally in the treatment of gallstones and lipid disorders (Bach and Babayan 1982). In addition, it is metabolized as quickly as glucose, but has more than twice the caloric density of protein and carbohydrate. Included in feeding solutions of hospitalized patients, capric acid together with other medium chain triglycerides is easily utilized as fuel and prevents protein (tissue) degradation (Babayan 1987).

Lauric seed oils are commercially derived exclusively from coconut and palm kernel oils (Young 1983, Thompson 1984). The United States annually imports about 455 million kilograms of coconut oil (Princen 1983, Young 1983). Tropical storms, drought, disease, and pests cause coconut oil yields to fluctuate (Young

1983, Arkcoll 1988). Because of this and the economic importance of MCFAs, a domestic source of these oils is desirable. *Cuphea* was identified as a possible domestic source of MCFA oilseeds in a USDA germplasm screening program (Earle et al. 1960).

Cuphea is a member of the Lythraceae. This genus is comprised of approximately 260 species. Many, including *Cuphea laminuligera* Koehne and *Cuphea lutea* Rose in Koehne, have seed oils rich in MCFAs (Graham et al. 1981, Wolf et al. 1983, Graham 1988, Graham 1989). Typical capric and lauric acid percentages of *C. laminuligera* and *C. lutea* are 15 and 60 and 30 and 40%, respectively (Graham et al. 1981, Graham 1988, Graham 1989).

C. laminuligera and *C. lutea* are herbaceous annuals and are fairly well adapted to temperate climates, but certain traits impede their domestication (Hirsinger and Knowles 1984, Knapp 1989). The most serious barrier to domestication is seed shattering. The placenta, with seeds attached, emerges through the capsule and floral tube, leaving maturing seeds exposed and free to dehisce. Other undesirable characteristics include indeterminate growth habit and seed dormancy (Hirsinger and Knowles 1984, Graham 1989, Knapp 1989).

The floral morphology of *C. laminuligera, C. lutea*, and several other *Cuphea* species has been described (Hirsinger and Knowles 1984, Graham 1988). Flowers of *C. lutea* and *C. laminuligera* have two large dorsal petals and four smaller ventral petals attached to a calyx tube which is eight and 11 mm in length, respectively (Hirsinger and Knowles 1984, Graham 1988). Both species are self-compatible and protandrous. In *C. lutea* anthers

are inserted in the floral tube at anthesis, whereas *C. laminuligera* anthers are exserted (Graham 1988). *C. laminuligera* and *C. lutea* have been classified as cross-pollinated and self-pollinated, respectively, based on the autofertility of greenhouse grown plants (Hirsinger and Knowles 1984); however, quantitative estimates of the mating systems of these species have not been made.

An understanding of the mating system of a species is a fundamental necessity. The mating system of a species has a significant bearing on the distribution of genetic variation of wild populations, strategies and methods used to collect, preserve, and maintain germplasm, and methods used to breed a species (Clegg 1980, Ritland and Jain 1981, Jain 1983, Ritland 1983).

Sophisticated statistical methods for estimating mating system parameters have been developed to exploit allozymes (Fyfe and Bailey 1951, Clegg 1980, Ritland and Jain 1981, Shaw et al 1981, Schoen and Clegg 1986). In the mixed mating model, the mating process of plants is comprised of both random-mating and self-fertilization (Clegg 1980; Ritland and Jain 1981). This model assumes pollen gene frequency distributions are constant and identical across maternal plants, outcrossing rates are independent of maternal genotype, there are no mutations or post-fertilization selection, and alleles at different loci segregate independently.

Ritland and Jain (1981) described maximum likelihood methods to estimate individual or multilocus mixed mating model parameters. Outcrossing rates (t), pollen allele frequencies (p), and maternal genotypic frequencies (m) are estimated using individual plant

progeny arrays. The variances of multilocus estimates of t and p are less than those of single locus estimates, and multilocus estimates are less sensitive to violations of model assumptions than single locus estimates. These properties can be exploited using allozymes.

Allozymes have several desirable properties as genetic markers (Tanksley 1983). Allozyme phenotypes can be determined at the whole plant, tissue and cellular level. Relatively large numbers of allozyme alleles are naturally occurring. There are usually no deleterious effects associated with allozyme loci, and epistatic and pleiotropic effects are usually absent. Additionally, most allozyme alleles are codominantly inherited.

We investigated several breeding and genetics problems in *C. laminuligera* and *C. lutea*, which we discuss in the following two papers. The first paper reports on the segregation of allozyme alleles and linkage among allozyme loci in *C. laminuligera* and *C. lutea*. This work was motivated by our need for codominant markers to use in cytogenetics, mating systems, and other genetics experiments. In addition, we wanted to have markers which could be assayed using seedlings; thus, we investigated several enzyme systems. In the second paper, we report our estimates of outcrossing rates of *C. laminuligera* and *C. lutea* using mixed mating model methods, and the effect of plant density on outcrossing rate.

Genetics of Allozyme Variation in *Cuphea laminuligera* and *Cuphea lutea*

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ABSTRACT

Cuphea laminuligera Koehne and *Cuphea lutea* Rose in Koehne are undomesticated species having potential as new oilseed crops. Genetic markers have not been described for these species. Our objectives were to survey these species for allozyme variation and analyze the segregation and linkage of polymorphic loci in several F_2 populations. We analyzed allozyme variation among 11 F_2 populations of *C. laminuligera* and one F_2 population of *C. lutea*. Both species were assayed for aconitase (ACO), diaphorase (DIA), esterase (EST), fluorescent esterase (FES), glutamate oxaloacetate transaminase (GOT), malate dehydrogenase (MDH), menadione reductase (MNR), 6-phosphogluconic dehydrogenase (PGD), phosphoglucose isomerase (PGI), and shikimate dehydrogenase (SKD) activity. *Cuphea laminuligera* was also assayed for phosphoglucomutase (PGM) activity. We observed 14 polymorphic loci and two monomorphic loci in *C. laminuligera*. Variation was observed within and between parental populations. Observed segregation ratios were generally not significantly different ($P > 0.05$) from expected ratios. We observed three polymorphic loci and at least 10 monomorphic loci in *C. lutea*. We found no within population variation in parental populations of *C. lutea*. Observed segregation ratios were not significantly different from expected ratios and linkage was not detected.

INTRODUCTION

Cuphea is a member of the Lythraceae and is comprised of approximately 260 species. Many of these species have seed oils rich in medium-chain fatty acids (MCFAs) (Graham et al. 1981, Wolf et al. 1983, Graham 1988, Graham 1989). Because of this and the economic importance of MCFAs (Princen 1983, Young 1983), certain species have potential as new annual oilseed crops. *C. laminuligera* Koehne and *C. lutea* Rose in Koehne are among them (Hirsinger and Knowles 1984).

The MCFAs of commercial importance are capric and lauric acid (Princen 1983, Young 1983, Thompson 1984). Typical capric and lauric acid percentages of *C. laminuligera* and *C. lutea* are 15 and 60 and 30 and 40%, respectively (Graham et al. 1981, Graham 1988, Graham 1989). These species are herbaceous annuals and are fairly well adapted to temperate climates, but certain traits impede their domestication, e.g., seed shattering (Hirsinger and Knowles 1984, Graham 1989, Knapp 1989). We investigated a broad range of breeding and genetics problems in these species. In this paper, we report on the segregation of allozyme alleles and linkage among allozyme loci in *C. laminuligera* and *C. lutea*. This work was motivated by our need for codominant markers to use in cytogenetics, mating systems, and other genetics experiments. In addition, we wanted to have markers which could be assayed using seedlings; thus, we investigated several enzyme systems.

MATERIALS AND METHODS

Seed was harvested from two *C. laminuligera* (LA26 and LA27) and two *C. lutea* (LU06 and LU07) populations grown in the field at Corvallis, Oregon in 1986. Populations of the same species were grown in separate locations and were reproductively isolated from other populations by a distance of at least 1.0 km. The four populations comprised the entire germplasm collection of these species.

We surveyed allozyme variation within these populations using the seed harvested in 1986. Extensive variation was observed within and between *C. laminuligera* populations. Because inbred lines were not available in this species, we randomly selected 11 individuals from LA26 and LA27 and self-pollinated them in the greenhouse. This gave us 11 F_2 populations segregating at various allozyme loci.

No within population variation was observed in *C. lutea*, but polymorphisms were observed between the two populations. An F_2 population (LU15) was derived from the F_1 between LU06 and LU07.

We assayed enzyme activities of cotyledon extracts using horizontal starch gel electrophoresis. Seed coats were removed from F_2 seeds and seeds were germinated at 26°C in a 16 h light : 8 h dark photoperiod. Cotyledons were removed from four to seven-day-old seedlings and were manually homogenized in a 24 well sample grinder (Diamond Research Products) in 35 μl of extraction buffer at 4°C (Wendel and Parks 1982, Knapp and Tagliani 1989). Samples were kept on ice during preparation and were absorbed on two 2 x 11

mm wicks. Wicks were immediately inserted into slots cut in the starch gels.

Starch gels were prepared using starch concentrations of 12.3% (Cardy et al. 1983). Slightly modified AC (Clayton and Tretiak 1972) and RWC (Ridgeway et al. 1970) gel and electrode buffer systems were used (Knapp and Tagliani 1989). The run conditions we used have been described (Knapp and Tagliani 1989).

The AC buffer system (electrode and gel buffer pH 6.1) was used to resolve aconitase (ACO, E.C.4.2.1.3), malate dehydrogenase (MDH, E.C.1.1.1.37), menadione reductase (MNR, E.C.1.6.99.2), 6-phosphogluconic dehydrogenase (PGD, E.C.1.1.1.44) shikimate dehydrogenase (SKD, E.C.1.1.1.25) bands in *C. laminuligera* and *C. lutea* and phosphoglucomutase (PGM, E.C.2.7.5.1) bands in *C. laminuligera*. The RWC buffer system (electrode buffer pH 8.1, gel buffer pH 8.4) was used to resolve diaphorase (DIA, E.C.1.6.4.3), esterase (EST, E.C.3.1.1.1), fluorescent esterase (FES, E.C.3.1.1.1), glutamate oxaloacetate transaminase (GOT = aspartate aminotransferase, E.C.2.6.1.1), and phosphoglucose isomerase (PGI, E.C.5.3.1.9) bands in *C. laminuligera* and *C. lutea*. The ACO, DIA, FES and GOT stains of Marty et al. (1984) were used except pyridoxal-5'-phosphate was not used in the GOT stain and one percent $MgCl_2$ (w/v) was added to the SKD stain. The EST, MDH, PGD, PGI and PGM stains of Cardy et al (1983) were used. The MNR stain of Cheliak and Pitel (1984) was used.

Allozyme loci for each species were sequentially numbered from anode (lowest) to cathode (highest). The most common allele was arbitrarily assigned the number 100. Other allozyme alleles were

identified by their protein band mobilities relative to band 100. Allele identities and migration distances were confirmed by electrophoresing all lines of a species on a single gel.

We performed tests of segregation and independent assortment using Williams' corrected G-statistics (Sokal and Rohlf 1981). Pooled and heterogeneity goodness of fit statistics were estimated when two or more lines were segregating for phenotypically identical alleles. G-statistics were used because they are additive, while χ^2-statistics are not (Sokal and Rohlf 1981). Linkage statistics were estimated when Mendelian segregation was observed at one or both loci, but not when aberrant segregation ratios were observed at both loci (Bailey 1961).

Linkage-1 (Suiter et al. 1983) was used to estimate recombination frequencies (r) using maximum likelihood methods. We used pooled segregation ratios to estimate recombination frequencies when data were available on more than one F_2 population. Linkage phases of parents were not known because F_2 lines were derived from randomly chosen open-pollinated individuals; however, the linkage phase was inferred by examining the frequencies of the four double homozygote phenotypic classes (Bailey 1961). Coupling phase linkage was inferred when the sum of 11/11 and 22/22 classes was greater than the sum of 11/22 and 22/11 classes; otherwise, repulsion phase was inferred.

We used multipoint linkage analysis (MAPMAKER) (Lander et al. 1987) to estimate maximum likelihood map distances for *C. laminuligera*. To construct the linkage map, we constructed a single F_2 population by pooling F_2 populations with at least 96

individuals. Data were corrected for linkage phase differences. A likelihood odds ratio (LOD score) of three was used to estimate map distances. A maximum recombination frequency of 0.35 was used. Map distances were estimated using the Haldane mapping function (map distance = -1/2ln(1 - 2r) where r is recombination frequency) (Bailey 1961, Lander et al. 1987).

RESULTS AND DISCUSSION

Enzyme Phenotypes

Cuphea laminuligera

Polymorphisms were associated with all four ACO loci (Figure 1). Three and four alleles were segregating at *Aco-1* and *Aco-2*, respectively. Segregation ratios were not significantly different ($P > 0.05$) from those expected for codominant alleles (Table 1). Pooled ratios were not significantly different ($P > 0.05$) from expected ratios and heterogeneity statistics were not significant ($P > 0.05$).

The bands observed on gel slices stained for DIA, EST and MNR were poorly resolved; consequently, segregation of these bands was not investigated. Six monomorphic bands were observed for MDH activity (Figure 1). Because there was no segregation in our populations the number of loci could not be determined. A single monomorphic locus was detected for PGM.

Two FES loci were detected. *Fes-1* was monomorphic. Two alleles were segregating at *Fes-2*. Heterozygotes for *Fes-2* were dimeric, forming a third hybrid band. Segregation ratios for *Fes-2* were consistent with ratios expected for codominant segregation (Table 1). Pooled and heterogeneity estimates were not significant (Table 1). A group of bands located between *Fes-1* and *Fes-2* (Figure 1) exhibited complex segregation patterns and were not scored.

A single polymorphic locus was detected for GOT (Figure 1). We detected two *Got-1* alleles. Segregation for *Got-1* (Table 1) was

Figure 1. Enzyme phenotypes observed in *Cuphea laminuligera* on starch gels assayed for various enzymes. Allele names (numbers) are electrophoretic mobilities relative to a common allele (100). A solid band indicates that one band was clearly resolved, a hollow band indicates numerous clear bands, and a narrow band indicates one or more poorly resolved band(s).

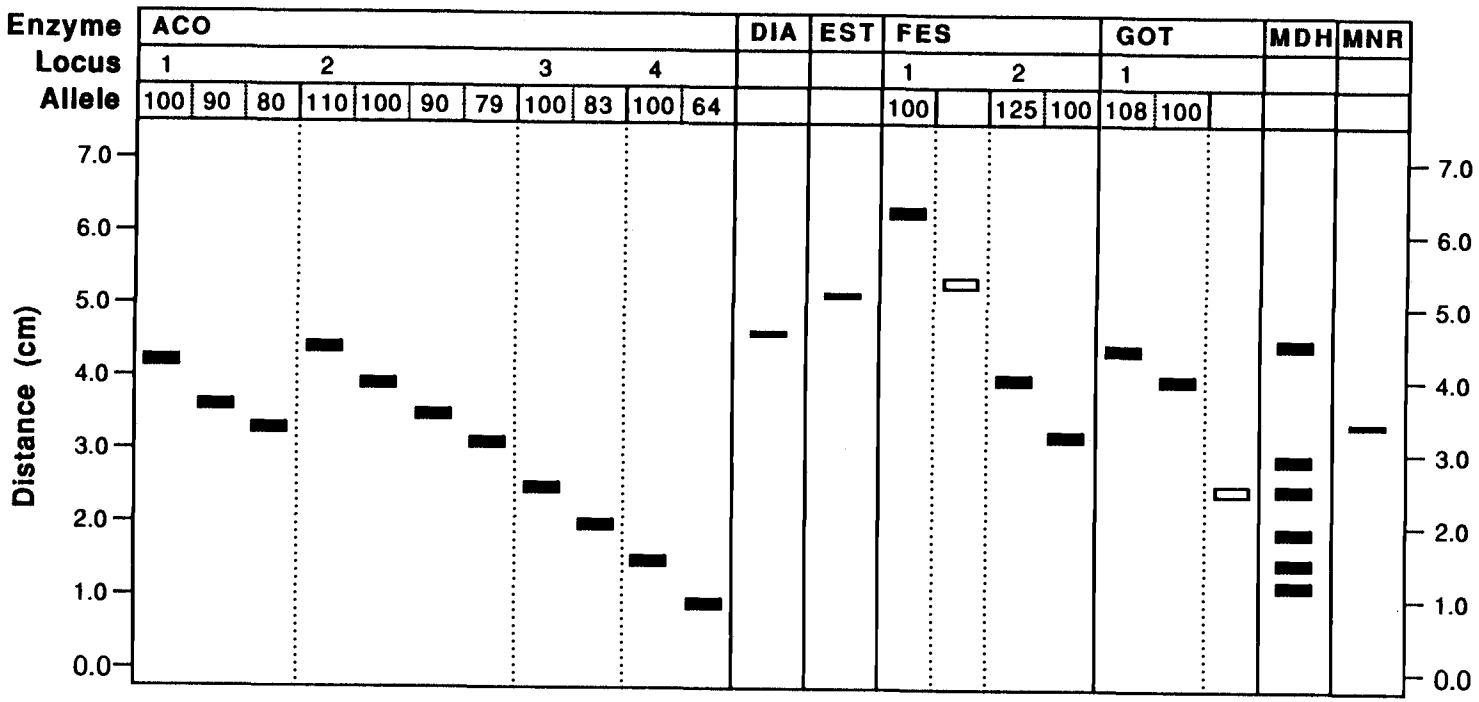
Enzyme
Locus
Allele
ACO
1
2
3
4
100
90
80
110
100
90
79
100
83
100
64
DIA
EST
FES
1
2
100
125
100
GOT
1
108
100
MDH
MNR
Distance (cm)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0

Figure 1.

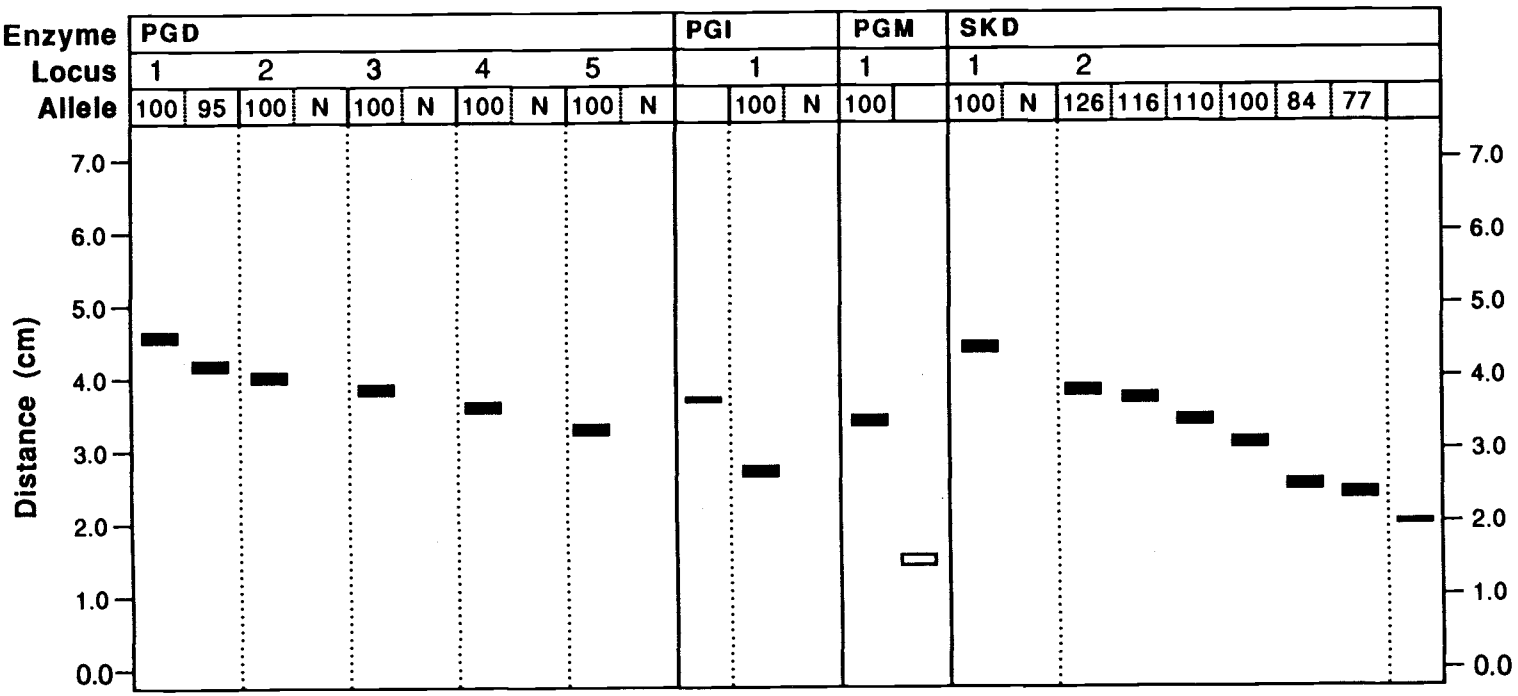

Figure 1. continued

Table 1. *Cuphea laminuligera* F_2 progeny allozyme allele segregation statistics.

Locus	Line	Alleles[a]	Ratios[b]	G[c]	P[d]
Aco-1	27-4	100, 90	24:47:26	0.26	0.88
	27-8	100, 90	29:48:29	0.94	0.62
	Pooled		53:95:55	1.10	0.58
	Heterogeneity			0.10	0.95
	26-4	100, 80	18:20:09	4.10	0.13
Aco-2	27-8	100, 90	26:51:27	0.06	0.97
	26-10	100, 90	51:98:57	0.82	0.66
	Pooled		77:149:84	0.77	0.68
	Heterogeneity			0.11	0.95
	27-3	100, 110	6:23:15	4.08	0.13
	27-7	100, 110	56:119:52	0.68	0.71
	26-5	100, 110	26:45:24	0.34	0.84
	Pooled		88:187:91	0.23	0.89
	Heterogeneity			4.88	0.30
	27-2	90, 110	13:14:09	2.52	0.28
	26-4	79, 90	14:19:13	1.44	0.49
	26-6	100, 79	13:25:07	2.38	0.30
Aco-3	26-5	100, 83	12:20:12	0.36	0.83
	26-10	100, 83	30:55:42	4.29	0.12
	Pooled		42:75:54	4.09	0.13
	Heterogeneity			0.57	0.75
Aco-4	27-2	100, 64	11:11:08	2.63	0.27
	27-3	100, 64	6:15:7	0.22	0.90
	27-4	100, 64	20:36:18	0.16	0.92
	Pooled		37:62:33	0.71	0.70
	Heterogeneity			2.30	0.68
Fes-2	27-3	100, 125	09:26:09	1.46	0.48
	27-4	100, 125	26:57:22	1.11	0.58
	27-7	100, 125	60:103:45	2.17	0.38
	Pooled		95:186:76	2.75	0.25
	Heterogeneity			1.99	0.74
Got-1	27-2	100, 108	10:17:11	0.47	0.79
	27-4	100, 108	25:48:32	1.63	0.44
	26-6	100, 108	09:23:15	1.54	0.46
	Pooled		44:88:58	2.96	0.23
	Heterogeneity			0.68	0.95

Table 1. continued

Locus	Line	Alleles[a]	Ratios[b]	G[c]	P[d]
Pgd-1	26-6	100, 95	13:22:14	0.55	0.76
	26-10	100, 95	23:76:37	5.18	0.07
	Pooled		36:98:51	3.25	0.20
	Heterogeneity			2.48	0.29
Pgd-2	27-2	100, N	30:08	0.33	0.57
	27-3	100, N	33:11	0.00	1.00
	27-4	100, N	89:16	5.94	0.01*
	26-6	100, N	34:14	0.43	0.51
	26-10	100, N	167:41	3.27	0.07
	Pooled		353:90	5.43	0.02*
	Heterogeneity			4.54	0.34
Pgd-3	27-1	100, N	78:17	2.75	0.10
	27-2	100, N	25:13	1.60	0.21
	27-3	100, N	35:9	0.51	0.48
	27-6	100, N	39:16	0.47	0.49
	27-7	100, N	173:45	2.30	0.13
	27-8	100, N	80:26	0.01	0.91
	26-6	100, N	24:25	15.22	<0.01*
	26-10	100, N	132:70	9.34	<0.01*
	Pooled		586:221	2.40	0.12
	Heterogeneity			29.81	<0.01*
Pgd-4	27-1	100, N	67:25	0.23	0.63
	27-4	100, N	91:14	8.71	<0.01*
	27-7	100, N	162:55	0.01	0.91
	27-8	100, N	80:26	0.01	0.91
	Pooled		400:120	1.04	0.31
	Heterogeneity			7.92	0.05*
Pgd-5	27-4	100, N	80:24	0.21	0.65
Pgi-1	27-1	100, N	39:14	0.06	0.81
	27-2	100, N	15:23	21.42	<0.01*
	27-6	100, N	24:07	0.10	0.75
	27-7	100, N	153:43	1.01	0.32
	27-8	100, N	62:22	0.06	0.80
	Pooled		293:109	0.94	0.33
	Heterogeneity			21.70	<0.01*
Skd-1	27-4	100, N	78:27	0.03	0.87

Table 1. continued

Locus	Line	Alleles[a]	Ratios[b]	G[c]	P[d]
Skd-2	27-1	116, 126	28:51:23	0.49	0.78
	27-6	116, 126	12:30:13	0.50	0.78
	Pooled		40:81:36	0.37	0.83
	Heterogeneity			0.62	0.73
	27-2	84, 126	25:12:1	32.85	<0.01*
	27-7	84, 126	66:103:61	2.71	0.26
	Pooled		91:115:62	10.95	<0.01*
	Heterogeneity			24.62	<0.01*
	27-3	84, 116	10:24:10	0.36	0.83
	27-4	100, 116	18:61:25	4.28	0.12
	27-8	77, 116	13:65:29	11.23	<0.01*
	26-4	110, 126	09:21:08	0.48	0.79
	26-5	100, 126	05:59:30	26.01	<0.01*
	26-10	100, 84	58:93:50	1.71	0.42

[a] N was used to denote a null allele.

[b] Ratio is the observed allozyme phenotype ratio.

[c] G is the G-statistic or log likelihood ratio.

[d] P is the p-value or percentage point of a χ^2-distribution.

consistent with the expected segregation of codominant alleles. Numerous additional bands were present (Figure 1), but the complexity of banding patterns prevented their analysis.

Five PGD loci were found (Figure 1). Two alleles were segregating for *Pgd-1* (Figure 1). Observed segregation ratios were not significantly different from expected ratios for codominant alleles (Table 1). *Pgd-2*, *Pgd-3*, *Pgd-4* and *Pgd-5* each had two alleles, one of which was a null allele (absence of enzyme activity). Ambiguous segregation data were obtained for these loci (Table 1). For *Pgd-2*, for example, probabilities associated with estimated G-statistics ranged from $P = 0.01$ for line 27-4 to $P = 1.00$ for line 27-3. The pooled estimate of G was 5.43 ($P = 0.02$), while the heterogeneity estimate was not significant ($P = 0.34$). The pooled goodness of fit for *Pgd-3* (Table 1) was not significant, but heterogeneity was significant. The same pattern was obtained for *Pgd-4*. The banding pattern observed for *Pgd-5* fit that expected for dominant alleles.

A single *Pgi-1* locus was identified (Figure 1). Segregation ratios for *Pgi-1* were not significantly different from expected ratios, except for those observed for line 27-2 ($P < 0.01$). The pooled ratio was not significant ($P = 0.33$), but there was significant heterogeneity ($P < 0.01$). We observed a variable, fast migrating group of bands. These bands were difficult to differentiate and were not analyzed (Figure 1).

Two SKD loci were identified (Figure 1). A 3:1 segregation pattern was observed ($P = 0.87$) for *Skd-1*; there was a null allele. We detected six *Skd-2* alleles (Figure 1). The segregation ratios

observed for most F_2 populations were not significantly different from those expected for codominant alleles (Table 1). Lines 27-2, 27-8 and 26-5 were exceptions (Table 1). There was significant segregation distortion in these lines ($P < 0.01$). Segregation distortion for SKD has also been observed in *C. lanceolata* (Knapp and Tagliani 1989). Poorly resolved bands segregating anodal to *Skd-1* and *Skd-2* were not scored (Figure 1).

Cuphea lutea

Three monomorphic bands were observed for ACO (Figure 2). These bands may be associated with one to three monomorphic loci. We detected two alleles segregating for *Aco-1* (Figure 2). The observed segregation ratio was not significantly different from the expected ratio for codominant alleles (Table 2).

The bands observed on gel slices stained for DIA, EST, FES GOT, MDH, MNR and PGI were monomorphic (Figure 2). The number of bands observed for a given enzyme ranged from one poorly resolved region (MNR) to four clear monomorphic bands (EST and PGI).

Two loci were identified for PGD (Figure 2). Heterozygotes for *Pgd-1* were dimeric. The alleles for *Pgd-1* showed Mendelian segregation (Table 2). *Pgd-2* was characterized by a single monomorphic band (Figure 2).

We identified a single polymorphic SKD locus (Figure 2). The segregation ratio observed for *Skd-1* was not significantly different ($P = 0.06$) from expected ratios for codominant alleles (Table 2). Four fast moving monomorphic bands were observed. The number of loci associated with these bands could not be determined.

Figure 2. Enzyme phenotypes observed in *Cuphea lutea* on starch gels assayed for various enzymes. Allele names (numbers) are electrophoretic mobilities relative to the common allele (100). A solid band indicates that one band was clearly resolved, and a narrow band indicates one or more poorly resolved band(s).

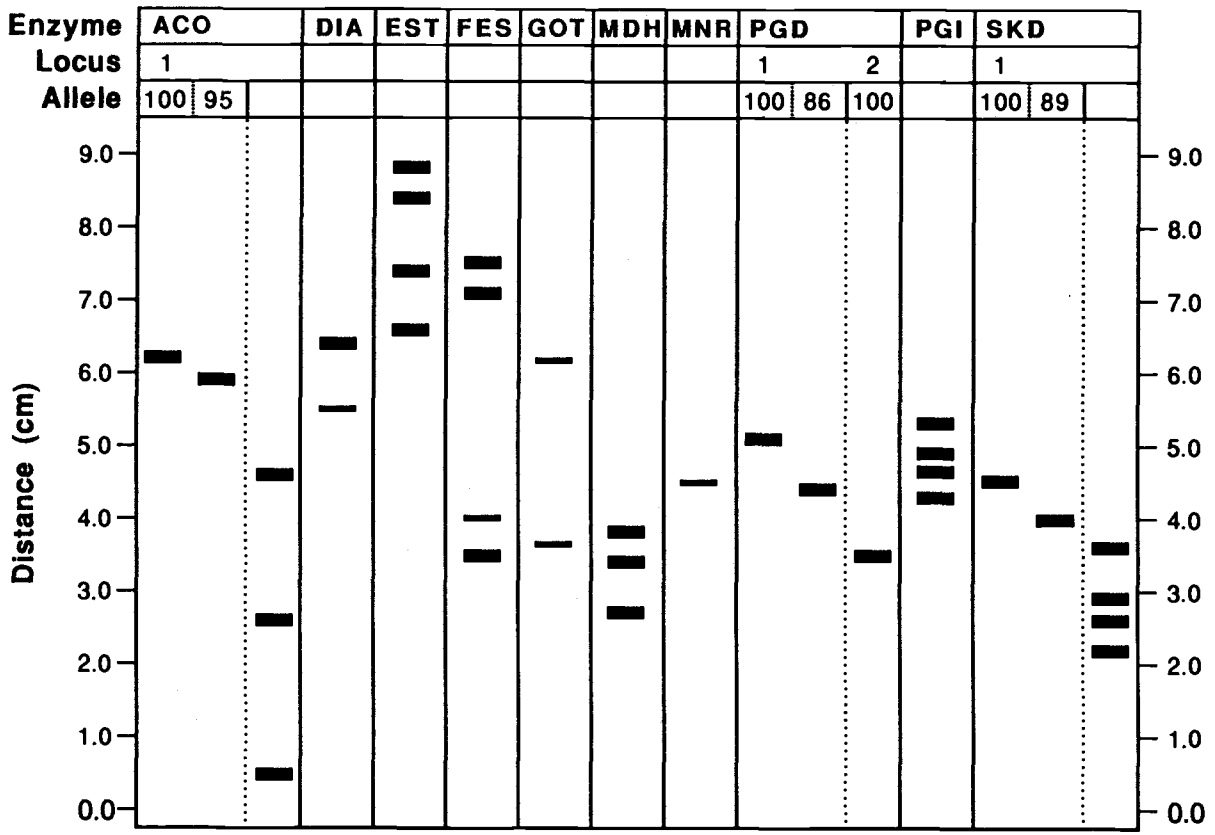
Enzyme
Locus
Allele
ACO
1
100
95
DIA
EST
FES
GOT
MDH
MNR
PGD
1
2
100
86
100
PGI
SKD
1
100
89
Distance (cm)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0

Figure 2.

Table 2. *Cuphea lutea* F_2 progeny allozyme allele segregation statistics.

Locus	Alleles	Ratios[a]	G[b]	P[c]
Aco-1	95, 100	78:147:81	0.53	0.77
Pgd-1	86, 100	79:156:71	0.54	0.76
Skd-1	89, 100	94:147:65	5.79	0.06

[a] Ratio is the observed allozyme phenotype ratio.

[b] G is the G-statistic or log likelihood ratio.

[c] P is the p-value or percentage point of a χ^2-distribution.

Linkage Analyses

Cuphea laminuligera

Fourteen loci were available to study linkage in *C. laminuligera*. Data were available to analyze linkage relationships among 69 of 91 pairs of loci (Table 3). There was significant segregation distortion at *Pgd-2* and *Pgd-4* in Line 27-4 and *Pgi-1* and *Skd-2* in line 27-2 (Table 1). Linkage statistics were not estimated using these data because distortion at both loci biases linkage estimates (Bailey 1961).

Large differences in P-values were observed in analyses of linkage between *Aco-2* and *Pgd-3*, *Aco-2* and *Skd-2*, *Aco-4* and *Got-1*, *Aco-4* and *Skd-2*, *Fes-2* and *Pgd-2*, *Fes-2* and *Pgd-4*, *Got-1* and *Pgd-2*, *Got-1* and *Skd-2*, *Pgd-2* and *Pgd-3*, *Pgd-3* and *Skd-2*, and *Pgi-1* and *Skd-2* (Table 4). P-values for *Aco-2* x *Pgd-3* were 0.72 and <0.01 for lines 27-2 and 27-7, respectively. The pooled estimate supports linkage but there was significant heterogeneity (Table 4). There were fewer than 50 individuals in F_2 populations where the data support independence for *Aco-2* x *Pgd-3* (Table 4). Differences among populations were observed for *Fes-2* x *Pgd-4* cosegregation (Table 4). Heterogeneity was significant and recombination frequency estimates ranged from 0.17 ± .04 to 0.46 ± .04 in lines 27-4 and 27-7, respectively.

P-values for *Pgd-3* x *Skd-2* were < 0.01 and 0.49 for lines 27-6 and 27-8, respectively (Table 4). The pooled G-statistic was not significant (P = 0.60), however there was significant heterogeneity (P = 0.03). Recombination frequencies ranged from 0.09 ± .04 to 0.45 ± .06.

Table 3. Allozyme locus pairs with data for linkage analyses in *Cuphea laminuligera* F_2 populations. Values in the body of the table indicate the number of F_2 populations cosegregating for the locus pair in question. A - indicates data were not available.

Locus	*Aco -1*	*Aco -2*	*Aco -3*	*Aco -4*	*Fes -2*	*Got -1*	*Pgd -1*	*Pgd -2*	*Pgd -3*	*Pgd -4*	*Pgd -5*	*Pgi -1*	*Skd -1*	*Skd -2*
Aco-1		2	-	1	1	1	-	1	1	2	1	1	1	3
Aco-2			2	2	2	2	2	4	6	2	-	3	-	7
Aco-3				-	-	-	1	1	1	-	-	-	-	2
Aco-4					2	2	-	3	2	1	1	1	1	3
Fes-2						1	-	2	2	2	1	1	1	3
Got-1							1	3	2	1	1	1	1	2
Pgd-1								2	2	-	-	-	-	1
Pgd-2									4	-	1	1	1	4
Pgd-3										3	-	5	-	7
Pgd-4											1	3	1	4
Pgd-5												-	1	1
Pgi-1													-	4
Skd-1														1
Skd-2														

Table 4. *Cuphea laminuligera* F_2 population allozyme allele linkage statistics.

Loci	Line	G[a]	P[b]	R[c] ± SE
Aco-1 x *Aco-2*	27-8	3.87	0.42	0.44 ± .05
	26-4	1.49	0.83	0.43 ± .07
	Pooled	3.90	0.42	0.44 ± .04
	Heterogeneity	1.46	0.83	
Aco-1 x *Aco-4*	27-4	5.50	0.24	0.44 ± .06
Aco-1 x *Fes-2*	27-4	3.23	0.52	0.44 ± .05
Aco-1 x *Got-1*	27-4	1.28	0.86	0.46 ± .05
Aco-1 x *Pgd-2*	27-4	1.10	0.58	0.46 ± .06
Aco-1 x *Pgd-3*	27-8	2.35	0.31	0.42 ± .06
Aco-1 x *Pgd-4*	27-4	3.26	0.20	0.48 ± .06
	27-8	0.47	0.79	0.49 ± .06
	Pooled	2.63	0.27	0.49 ± .04
	Heterogeneity	1.10	0.58	
Aco-1 x *Pgd-5*	27-4	1.52	0.47	0.47 ± .06
Aco-1 x *Pgi-1*	27-8	1.00	0.61	0.43 ± .07
Aco-1 x *Skd-1*	27-4	0.94	0.63	0.44 ± .06
Aco-1 x *Skd-2*	27-4	1.39	0.85	0.50 ± .05
	27-8	0.98	0.91	0.50 ± .05
	26-4	1.30	0.86	0.43 ± .08
	Pooled	0.41	0.98	0.49 ± .03
	Heterogeneity	3.26	0.92	
Aco-2 x *Aco-3*	26-5	2.36	0.67	0.47 ± .07
	26-10	6.82	0.15	0.49 ± .04
	Pooled	2.21	0.70	0.49 ± .04
	Heterogeneity	6.97	0.14	
Aco-2 x *Aco-4*	27-2	7.73	0.10	0.41 ± .09
	27-3	7.08	0.13	0.44 ± .09
	Pooled	2.73	0.60	0.43 ± .06
	Heterogeneity	12.08	0.02*	
Aco-2 x *Fes-2*	27-3	4.02	0.40	0.43 ± .07
	27-7	1.07	0.90	0.47 ± .03
	Pooled	1.83	0.77	0.47 ± .03
	Heterogeneity	3.27	0.51	

Table 4. continued.

Loci	Line	G[a]	P[b]	R[c] ± SE
Aco-2 x *Got-1*	27-2	2.00	0.74	0.48 ± .08
	26-6	5.50	0.24	0.40 ± .07
	Pooled	1.25	0.87	0.42 ± .05
	Heterogeneity	6.24	0.18	
Aco-2 x *Pgd-1*	26-6	1.94	0.75	0.44 ± .07
	26-10	6.05	0.20	0.47 ± .04
	Pooled	6.80	0.15	0.50 ± .04
	Heterogeneity	1.19	0.88	
Aco-2 x *Pgd-2*	27-2	2.68	0.26	0.44 ± .10
	27-3	0.28	0.87	0.47 ± .09
	26-6	0.01	0.99	0.48 ± .09
	26-10	0.34	0.84	0.49 ± .04
	Pooled	0.54	0.76	0.50 ± .03
	Heterogeneity	2.77	0.84	
Aco-2 x *Pgd-3*	27-2	0.66	0.72	0.46 ± .10
	27-3	3.10	0.21	0.41 ± .09
	27-7	18.27	<0.01*	0.35 ± .04
	27-8	6.29	0.04*	0.36 ± .06
	26-6	0.67	0.72	0.49 ± .09
	26-10	5.98	0.05*	0.47 ± .04
	Pooled	10.07	0.01*	0.43 ± .02
	Heterogeneity	24.91	0.01*	
Aco-2 x *Pgd-4*	27-7	16.75	<0.01*	0.47 ± .04
	27-8	35.28	<0.01*	0.17 ± .04
	Pooled	33.63	<0.01*	0.36 ± .03
	Heterogeneity	18.39	<0.01*	
Aco-2 x *Pgi-1*	27-2	0.18	0.92	0.45 ± .10
	27-7	1.77	0.41	0.44 ± .04
	27-8	1.70	0.43	0.49 ± .07
	Pooled	2.15	0.34	0.48 ± .03
	Heterogeneity	1.50	0.83	
Aco-2 x *Skd-2*	27-2	2.47	0.65	0.43 ± .08
	27-3	2.66	0.62	0.47 ± .07
	27-7	1.88	0.76	0.49 ± .03
	27-8	2.71	0.61	0.49 ± .05
	26-4	0.35	0.99	0.48 ± .08
	26-5	4.80	0.31	0.41 ± .05
	26-10	9.44	0.05*	0.44 ± .03
	Pooled	10.89	0.03*	0.46 ± .02
	Heterogeneity	13.42	0.96	

Table 4. continued.

Loci	Line	G[a]	P[b]	R[c] ± SE
Aco-3 x *Pgd-1*	26-10	5.97	0.20	0.49 ± .05
Aco-3 x *Pgd-2*	26-10	1.10	0.58	0.48 ± .05
Aco-3 x *Pgd-3*	26-10	1.64	0.44	0.45 ± .05
Aco-3 x *Skd-2*	26-5	7.46	0.11	0.48 ± .08
	26-10	2.14	0.71	0.49 ± .04
	Pooled	3.58	0.47	0.49 ± .04
	Heterogeneity	6.03	0.20	
Aco-4 x *Fes-2*	27-3	3.66	0.45	0.37 ± .09
	27-4	4.09	0.39	0.43 ± .06
	Pooled	4.10	0.39	0.41 ± .05
	Heterogeneity	3.65	0.46	
Aco-4 x *Got-1*	27-2	4.29	0.37	0.44 ± .09
	27-4	14.16	0.01*	0.37 ± .05
	Pooled	14.90	<0.01*	0.39 ± .05
	Heterogeneity	3.56	0.47	
Aco-4 x *Pgd-2*	27-2	26.56	<0.01*	0.04 ± .04
	27-3	10.36	0.01*	0.19 ± .08
	27-4	42.68	<0.01*	0.09 ± .03
	Pooled	72.88	<0.01*	0.10 ± .03
	Heterogeneity	6.72	0.15	
Aco-4 x *Pgd-3*	27-2	19.36	<0.01*	0.23 ± .09
	27-3	8.37	0.02*	0.20 ± .08
	Pooled	27.42	<0.01*	0.22 ± .06
	Heterogeneity	0.31	0.86	
Aco-4 x *Pgd-4*	27-4	13.43	<0.01*	0.25 ± .06
Aco-4 x *Pgd-5*	27-4	3.36	0.19	0.45 ± .07
Aco-4 x *Pgi-1*	27-2	14.87	<0.01*	0.34 ± .10
Aco-4 x *Skd-1*	27-4	4.62	0.10	0.41 ± .07
Aco-4 x *Skd-2*	27-2	0.85	0.93	0.50 ± .09
	27-3	8.04	0.09	0.38 ± .09
	27-4	9.92	0.04*	0.48 ± .06
	Pooled	6.38	0.17	0.46 ± .04
	Heterogeneity	12.44	0.13	

Table 4. continued.

Loci	Line	G[a]	P[b]	R[c] ± SE
Fes-2 x *Got-1*	27-4	6.64	0.16	0.48 ± .05
Fes-2 x *Pgd-2*	27-3	2.13	0.34	0.38 ± .09
	27-4	9.50	0.01*	0.34 ± .05
	Pooled	10.59	0.01*	0.35 ± .05
	Heterogeneity	1.03	0.60	
Fes-2 x *Pgd-3*	27-3	1.39	0.50	0.37 ± .09
	27-7	0.83	0.66	0.48 ± .04
	Pooled	0.54	0.76	0.50 ± .04
	Heterogeneity	1.68	0.43	
Fes-2 x *Pgd-4*	27-4	38.20	<0.01*	0.17 ± .04
	27-7	0.98	0.61	0.46 ± .04
	Pooled	13.60	<0.01*	0.38 ± .03
	Heterogeneity	25.66	<0.01*	
Fes-2 x *Pgd-5*	27-4	103.96	<0.01*	0.01 ± .01
Fes-2 x *Pgi-1*	27-7	1.52	0.47	0.47 ± .05
Fes-2 x *Skd-1*	27-4	1.75	0.42	0.42 ± .06
Fes-2 x *Skd-2*	27-3	3.54	0.47	0.35 ± .07
	27-4	1.86	0.76	0.44 ± .05
	27-7	3.53	0.47	0.48 ± .03
	Pooled	4.39	0.36	0.46 ± .03
	Heterogeneity	4.54	0.81	
Got-1 x *Pgd-1*	26-6	5.58	0.23	0.36 ± .07
Got-1 x *Pgd-2*	27-2	0.12	0.94	0.49 ± .10
	27-4	9.41	0.01*	0.33 ± .05
	26-6	2.94	0.23	0.42 ± .09
	Pooled	7.07	0.03*	0.40 ± .04
	Heterogeneity	5.41	0.25	
Got-1 x *Pgd-3*	27-2	4.13	0.13	0.36 ± .09
	26-6	1.11	0.57	0.49 ± .09
	Pooled	0.46	0.80	0.45 ± .07
	Heterogeneity	4.78	0.09	

Table 4. continued.

Loci	Line	G[a]	P[b]	R[c] ± SE
Got-1 x *Pgd-4*	27-4	0.19	0.91	0.46 ± .06
Got-1 x *Pgd-5*	27-4	0.82	0.66	0.49 ± .06
Got-1 x *Pgi-1*	27-2	0.84	0.66	0.49 ± .10
Got-1 x *Skd-1*	27-4	2.56	0.28	0.41 ± .06
Got-1 x *Skd-2*	27-2	4.45	0.35	0.42 ± .08
	27-4	9.11	0.06	0.39 ± .05
	Pooled	10.34	0.04*	0.40 ± .04
	Heterogeneity	3.21	0.52	
Pgd-1 x *Pgd-2*	26-6	37.49	<0.01*	0.06 ± .04
	26-10	46.90	<0.01*	0.17 ± .04
	Pooled	76.67	<0.01*	0.14 ± .03
	Heterogeneity	7.71	0.02*	
Pgd-1 x *Pgd-3*	26-6	56.42	<0.01*	0.46 ± .09
	26-10	100.54	<0.01*	0.30 ± .05
	Pooled	153.14	<0.01*	0.38 ± .04
	Heterogeneity	3.82	0.15	
Pgd-1 x *Skd-2*	26-10	1.89	0.76	0.45 ± .04
Pgd-2 x *Pgd-3*	27-2	1.08	0.29	0.40 ± .13
	27-3	0.05	0.83	0.47 ± .12
	26-6	5.84	0.02*	0.32 ± .13
	26-10	24.18	<0.01*	0.32 ± .06
	Pooled	25.32	<0.01*	0.35 ± .05
	Heterogeneity	5.83	0.12	
Pgd-2 x *Pgd-5*	27-4	0.04	0.84	0.47 ± .08
Pgd-2 x *Pgi-1*	27-2	9.39	<0.01*	0.36 ± .14
Pgd-2 x *Skd-1*	27-4	0.51	0.48	0.49 ± .06
Pgd-2 x *Skd-2*	27-2	2.58	0.28	0.46 ± .10
	27-3	2.42	0.30	0.46 ± .09
	27-4	2.74	0.25	0.49 ± .06
	26-10	1.76	0.41	0.45 ± .04
	Pooled	0.13	0.94	0.48 ± .03
	Heterogeneity	9.38	0.15	

Table 4. continued.

Loci	Line	G[a]	P[b]	R[c] ± SE
Pgd-3 x *Pgd-4*	27-1	12.16	<0.01*	0.00 ± -
	27-7	30.10	<0.01*	0.00 ± -
	27-8	17.21	<0.01*	0.00 ± -
	Pooled	59.61	<0.01*	0.00 ± -
	Heterogeneity	0.01	1.00	
Pgd-3 x *Pgi-1*	27-1	2.45	0.12	0.35 ± .12
	27-2	0.37	0.54	0.48 ± .12
	27-6	0.01	0.98	0.49 ± .14
	27-7	0.46	0.50	0.45 ± .06
	27-8	2.61	0.11	0.38 ± .09
	Pooled	3.45	0.06	0.43 ± .04
	Heterogeneity	2.44	0.66	
Pgd-3 x *Skd-2*	27-1	2.29	0.32	0.45 ± .06
	27-2	1.68	0.43	0.36 ± .09
	27-3	3.00	0.22	0.33 ± .08
	27-6	35.71	<0.01*	0.09 ± .04
	27-7	5.97	0.05*	0.41 ± .04
	27-8	1.42	0.49	0.43 ± .06
	26-10	1.75	0.42	0.45 ± .04
	Pooled	1.01	0.60	0.42 ± .02
	Heterogeneity	23.04	0.03*	
Pgd-4 x *Pgd-5*	27-4	38.32	<0.01*	0.14 ± .10
Pgd-4 x *Pgi-1*	27-1	0.63	0.43	0.41 ± .12
	27-7	0.07	0.79	0.49 ± .06
	27-8	1.68	0.20	0.40 ± .09
	Pooled	1.38	0.24	0.46 ± .04
	Heterogeneity	1.00	0.61	
Pgd-4 x *Skd-1*	27-4	0.80	0.37	0.45 ± .08
Pgd-4 x *Skd-2*	27-1	2.70	0.26	0.40 ± .06
	27-4	1.90	0.39	0.42 ± .06
	27-7	2.05	0.36	0.45 ± .04
	27-8	0.85	0.65	0.39 ± .06
	Pooled	1.53	0.47	0.49 ± .03
	Heterogeneity	5.97	0.43	

Table 4. continued.

Loci	Line	G[a]	P[b]	R[c] ± SE
Pgd-5 x *Skd-1*	27-4	2.05	0.15	0.41 ± .08
Pgd-5 x *Skd-2*	27-4	1.37	0.50	0.44 ± .06
Pgi-1 x *Skd-2*	27-1	14.25	<0.01*	0.47 ± .08
	27-6	0.64	0.73	0.46 ± .11
	27-7	1.88	0.39	0.49 ± .04
	27-8	3.14	0.21	0.40 ± .06
	Pooled	10.86	<0.01*	0.47 ± .03
	Heterogeneity	9.06	0.17	
Skd-1 x *Skd-2*	27-4	96.01	<0.01*	0.05 ± .02

[a] G is the G-statistic or log likelihood ratio.

[b] P is the p-value or percentage point of a χ^2-distribution.

[c] R and SE are recombination frequency and standard error.

P-values for *Aco-2* x *Skd-2* were 0.05 and 0.99 for lines 26-10 and 26-4, respectively. The pooled G-statistic was significant ($P = 0.03$); however, the heterogeneity estimate was not significant ($P = 0.96$). This pattern was also observed for *Aco-4* x *Got-1*, *Fes-2* x *Pgd-2*, *Got 1* x *Pgd-2*, *Pgd-2* x *Pgd-3*, and *Pgi-1* x *Skd-2* (Table 4). Evidence for linkage between *Fes-2* x *Pgd-2* and *Pgd-2* x *Pgd-3* was marginal ($r = 0.35 \pm .05$). The other recombination frequency estimates indicated weak or no linkage (Table 4).

There was variation in the cosegregation of *Aco-4* and *Skd-2* among F_2 populations. The G-statistics for the cosegregation of *Aco-4* and *Skd-2* ranged from 0.85 to 9.92. P-values for lines 27-2 and 27-4 were 0.93 and 0.04, respectively. The pooled G-statistic was not significant.

Linkage between *Aco-2* and *Pgd-4*, and *Pgd-1* and *Pgd-2* was detected ($P < 0.01$). There was significant heterogeneity (Table 4). This heterogeneity was reflected in variation among recombination frequency estimates.

Aco-4 and *Pgd-2*, *Aco-4* and *Pgd-4*, *Aco-4* and *Pgi-1*, *Fes-2* and *Pgd-5*, *Pgd-1* and *Pgd-3*, *Pgd-2* and *Pgi-1*, *Pgd-3* and *Pgd-4*, *Pgd-4* and *Pgd-5*, and *Skd-1* and *Skd-2* did not segregate independently (Table 4). Significant associations ($P \leq 0.02$) were observed in populations cosegregating for *Aco-4* and *Pgd-3* (Table 4). There were no significant heterogeneity statistics (Table 4). Independent assortment was observed between all other pairs of loci (Table 4).

In addition to two-point analysis we used multipoint analysis to detect linkage, since this should provide the most accurate

estimates of map distance. In two instances the results of two-point analysis were not in agreement with the results of multipoint analysis. Multipoint analysis of the data did not detect linkage between *Pgd-3* x *Pgd-4*. This contrasted to the results of two-point analyses techniques where the p-value was < 0.01 and the recombination was 0.0 ± - (Table 4).

Pgd-3 and *Pgd-4* were segregating for an enzyme producing allele (100) and a null allele (N) (Figure 1). Alleles from the two loci were found in all phenotypic combinations (100/100, N/100, 100/N), except double null (N/N). The enzymes produced by these loci may be interchangeable. If production of enzyme by *Pgd-3* or *Pgd-4* is required for survival, absence of the enzyme (N/N) may be lethal.

We tested this hypothesis by including the number of N/N (28) individuals expected. The observed (221:88:106:28) ratio was tested against the expected ratio (9:3:3:1). The recombination fraction was 0.45 ± .04 (P = 0.51). Thus, the hypothesis of a null lethal was strongly supported by our data. *Pgd-3* and *Pgd-4* are not linked.

Linkage was not detected in the multipoint analysis of *Fes-2* and *Pgd-5* either. The recombination frequency estimated by two-point analysis was 0.01 ± 0.01 (P < 0.01) (Table 4). The expected ratio was 3:6:3:1:2:1 (1:2:1 x 3:1), but the ratio we observed was 1:57:22:24:0:0. The linkage phase was unambiguously found to be repulsion. The observed ratios fit the results of two-point analysis.

Eight codominant and six dominant allozyme markers were found

in *C. laminuligera* (Figure 1). We constructed a linkage map of these loci (Figure 3) by pooling data from several F_2 populations (Table 3). Based on two-point and multipoint analysis, we concluded that nine loci could be assigned to three linkage groups (Figure 3). The remaining five loci were not linked. The assignment of linkage groups to specific chromosomes will not be possible until further genetic markers, or appropriate genetic stocks are identified.

C. laminuligera is a highly polymorphic species. There were three to nine segregating loci within the F_2 populations and an average of 2.31 alleles per locus (Figure 1). While the complex segregation patterns observed in *C. laminuligera* for some enzyme systems precluded their characterization in this study, their characterization should be possible by studying a large F_2 population under modified run conditions.

Cuphea lutea

Three loci were available to study linkage in *C. lutea*. Independent assortment was observed between all pairs of loci (Table 5). Many monomorphic bands were observed in *C. lutea* (Figure 2). Germplasm resources in this species are limited, but there was substantially less variation in *C. lutea* than in *C. laminuligera*. These results were expected. *C. laminuligera* (n = 10) and *C. lutea* (n = 14) have been classified as allogamous and autogamous, respectively (Hirsinger and Knowles 1984, Graham 1988). Allogamous species are expected to exhibit both between and within population genetic variation, while autogamous species have limited within population variation.

Figure 3. Allozyme linkage map for *Cuphea laminuligera*. Haldane map distances (cM) are given between loci.

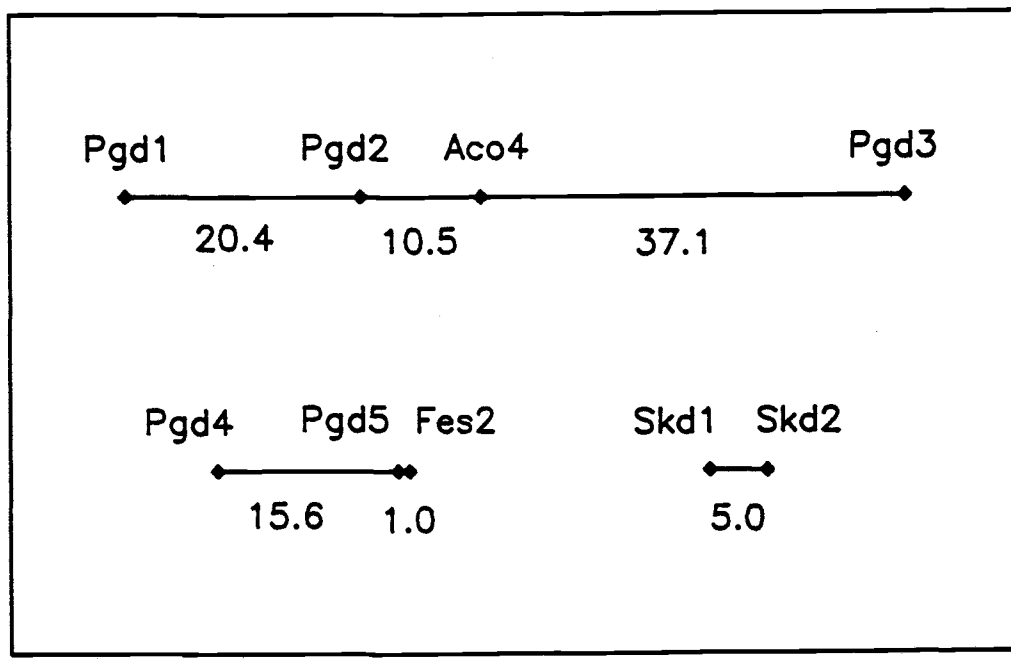
Pgd1
Pgd2
Aco4
Pgd3
20.4
10.5
37.1
Pgd4
Pgd5
Fes2
Skd1
Skd2
15.6
1.0
5.0

Figure 3.

Table 5. *Cuphea lutea* F_2 population allozyme allele linkage statistics.

Loci	G[a]	P[b]	R[c] ± SE
Aco-1 x *Pgd-1*	7.76	0.10	0.45 ± .03
Aco-1 x *Skd-1*	3.70	0.45	0.47 ± .03
Pgd-1 x *Skd-1*	2.56	0.63	0.48 ± .03

[a] G is the G-statistic or log likelihood ratio.

[b] P is the p-value or percentage point of a χ^2-distribution.

[c] R and SE are recombination frequency and standard error.

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Mating Systems of *Cuphea laminuligera* and *Cuphea lutea*

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ABSTRACT

Cuphea laminuligera Koehne and *Cuphea lutea* Rose in Koehne are new oilseed crop species characterized by seed oils rich in medium-chain fatty acids. The mating systems of these species have not been described. Our objective was to estimate autofertility and outcrossing rates in these species. In addition, we investigated the effect of plant density on outcrossing rate. Autofertility was estimated using plants isolated under insect-proof plant cages. The mean seed set per flower for *C. laminuligera* was 0.00 in 1986 and 1987. The mean seed set of *C. lutea* was 4.75 and 4.64 in 1986 and 1987, respectively. Outcrossing rates (t) were estimated for four populations of *C. laminuligera* and three populations of *C. lutea* using allozyme phenotypes of open-pollinated individual plant families. Populations were grown at low (1.0 x 1.0 m) and high (0.04 x 0.3 m) plant density. Pollen and ovule frequencies and single and multilocus outcrossing rates were estimated for each population using the mixed mating model. Multilocus estimates of t ranged from 0.83 to 0.98 and 1.00 to 1.01 for low and high density populations of *C. laminuligera*, respectively. Multilocus estimates of t ranged from 0.17 to 0.26 and 0.36 to 0.54 for low and high plant density populations of *C. lutea*, respectively. *C. laminuligera* is strongly allogamous; however, we observed selfing rates as high as 17%. *C. lutea* is predominantly autogamous, but outcrossing rates occasionally exceeded 50%. Outcrossing rate was affected by plant density. In *C. lutea*, the mean multilocus outcrossing rates for low and high plant densities were 0.24 and

0.45, respectively.

INTRODUCTION

Many species of *Cuphea*, a member of the genus Lythraceae, have seed oils rich in medium-chain fatty acids (MCFAs) (Graham et al. 1981, Wolf et al. 1983, Graham 1988, Graham 1989). MCFAs are commercially derived from coconut and palm kernel oil (Young 1983, Thompson 1984). A source of these oils from plants adapted to temperate climates would be desirable.

Cuphea laminuligera and *C. lutea* are herbaceous annuals and are fairly well adapted to temperate climates, but certain traits impede their domestication (Hirsinger and Knowles 1984, Knapp 1989). The most serious barrier to domestication is seed shattering. The placenta, with seeds attached, emerges through the capsule and floral tube, leaving maturing seeds exposed and free to dehisce (Graham 1989). Other undesirable characteristics include indeterminate growth habit and seed dormancy (Hirsinger and Knowles 1984, Graham 1989, Knapp 1989).

The floral morphology of *C. laminuligera*, *C. lutea* and several other *Cuphea* species has been described (Hirsinger and Knowles 1984, Graham 1988). Flowers of *C. laminuligera* and *C. lutea* have two large dorsal petals and four smaller ventral petals attached to a calyx tube which is 11 and 8 mm in length, respectively (Hirsinger and Knowles 1984, Graham 1988). Both species are self-compatible and protandrous, but anthers are exserted from the floral tube of *C. laminuligera* at anthesis while they are inserted in the floral tube in *C. lutea* (Graham 1988). *C. laminuligera* and *C. lutea* have been classified as cross-pollinated and self-

pollinated, respectively, based on the autofertility of greenhouse grown plants (Hirsinger and Knowles 1984); however, quantitative estimates of the mating systems of these species have not been made.

An understanding of the mating system of a species is a fundamental necessity. The mating system of a species has a significant bearing on the distribution of genetic variation of wild populations, strategies and methods used to collect, preserve, and maintain germplasm, and selection methods (Clegg 1980, Ritland and Jain 1981, Jain 1983, Ritland 1983).

Sophisticated statistical methods for estimating mating system parameters have been developed which utilize allozyme markers (Fyfe and Bailey 1951, Clegg 1980, Ritland and Jain 1981, Shaw et al 1981, Schoen and Clegg 1986). The mixed mating model recognizes that the mating process of plants is comprised of both random-mating and self-fertilization (Clegg 1980; Ritland and Jain 1981). This model assumes pollen gene frequency distributions are constant and identical across maternal plants, outcrossing rates are independent of maternal genotype, no mutations or post-fertilization selection, and alleles at different loci segregate independently (Ritland and Jain 1981, Brown et al. 1985).

Ritland and Jain (1981) described maximum likelihood methods to estimate individual or multilocus mixed mating model parameters. Outcrossing rates (t), pollen allele frequencies (p), and maternal genotypic frequencies (m) are estimated using individual plant progeny arrays. The variances of multilocus estimates of t and p are lower than those of single locus estimates, and multilocus

estimates are less sensitive to violations of model assumptions than single locus estimates. These properties of multilocus estimates can be exploited using allozyme markers.

Our objectives were to estimate the outcrossing rates of *C. laminuligera* and *C. lutea* using mixed mating model methods and to investigate the effect of plant density on outcrossing rate.

MATERIALS AND METHODS

Estimates of mating system parameters were made in populations of *C. laminuligera* and *C. lutea* derived from reproductively isolated open-pollinated seed increase plots of wild populations. Plots were separated by a distance of two km to insure reproductive isolation.

The *C. laminuligera* populations we used were LA86, LA87, LA11, and LA13. LA11 and LA13 were open-pollinated seed increases of wild populations. There are numerous polymorphic loci with multiple alleles in these populations (Krueger and Knapp in review). Because the mixed-mating model analysis program is capable of utilizing a maximum of three alleles per locus, we derived LA86 and LA87 from LA11 and LA13 by bulking three F_2 populations to maximize the number of polymorphic loci and to minimize the number of alleles segregating at a given locus.

The *C. lutea* populations we used were LU15, LU34, and LU36. LU15 was an F_2 population derived from the cross between parental populations LU06 and LU07. These parental populations are homozygous for different allozyme phenotypes at three loci (Krueger and Knapp in review). LU34 was an open-pollinated derivative of LU15. LU36 was derived by bulking equal amounts of seed of LU06 and LU07.

Two Corvallis, Oregon locations, separated by a distance of two km, were used for isolation in 1987 and 1988. We relied on wild bee populations as pollinators. *C. laminuligera* and *C. lutea* populations were grown in separate plots at the same location.

Populations of a given species were grown at separate locations.

Eight-week-old plants were transplanted to the field on May 29, 1987 using a 1 x 1 m spacing (low density). Sixty plants were planted in each plot. Individual plants were separately harvested. Harvest dates were August 21, 1987 and September 8, 1987. Not more than two seeds per flower were harvested, and several flowers were sampled, to eliminate the possibility that all seeds colected were the result of a single pollination event or a single pollinator trip.

In space-planted nurseries, bumble bees, the primary pollinators of experimental *Cuphea* plantings in western Oregon, tend to intensively work individual plants; thus, we hypothesized there may be a significant effect of plant density on outcrossing rate. To test this hypothesis, we used low density (1 x 1 m) and high density (0.04 x 0.30 m) planting rates. Plots were established by direct seeding on May 10, 1988. Seed was separately harvested from several flowers from each plant on September 20, 1988. We harvested 60 plants from each plot; however, poor stand at one location limited the number of plants available to 25, in one *C. lutea* population grown at low density.

Seeds of individual plant families were germinated on blotter paper at 26°C using twelve hours of fluorescent light. Four-to-seven-day-old cotyledons were electrophoretically assayed as previously described (Knapp and Tagliani 1989). Twenty individuals per family were assayed from populations grown in 1987. Fifteen individuals per family were assayed from populations grown in 1988. A slightly modified AC buffer system (Clayton and Tretiak 1972,

Knapp and Tagliani 1989) was used to resolve aconitase (ACO, E.C.4.2.1.3), fluorescent esterase (FES, E.C.3.1.1.1) and shikimate dehydrogenase (SKD, E.C.1.1.1.25) bands in *C. laminuligera* and ACO, 6-phosphogluconic dehydrogenase (PGD, E.C.1.1.1.44) and SKD bands in *C. lutea*. Standard ACO, FES, PGD and SKD stains were used (Cardy et al 1983, Marty et al 1984). The terminology and genetics of these allozymes have been described (Krueger and Knapp in review).

Single and multilocus outcrossing rates and gene frequencies were estimated using maximum likelihood methods based on the mixed mating model (Ritland and Jain 1981). Maternal genotypes were inferred. When more than three alleles were segregating at a locus the less frequent alleles were combined to form a synthetic allele.

Bias-corrected percentile confidence intervals were estimated for outcrossing rates using bootstrapping (Efron 1979, Liu and Knapp in review). One-thousand bootstrap replicates were used to estimate these intervals.

We examined our data for possible violations of mixed-mating model expectations by estimating pollen and ovule allele frequencies and expected genotype frequencies. Pollen and ovule allele frequency estimates were calculated using maximum likelihood methods based on the mixed-mating model. Expected genotype frequencies were calculated as the binomial or trinomial square of allele frequencies (Ritland and Jain 1981). Heterozygote deficiency or excess was calculated from observed and expected genotype frequencies as the observed number of hetgerozygotes minus the expected number divided by the expected number of heterozygotes.

In addition to outcrossing rates, we estimated autofertility. We direct seeded *C. laminuligera* and *C. lutea* at Corvallis, Oregon in 1986 and 1987 using a 0.10 m within row by 0.75 m between row plant spacing. A randomized complete blocks design with three replications was used. When plants began flowering, three plants from each row were randomly selected for caging. These plants were isolated under insect-proof single plant cages to exclude pollinators. Ten unopened flowers were tagged on each caged plant. The number of seeds set per flower was used to estimate autofertility.

RESULTS AND DISCUSSION

Several single locus estimates and multilocus estimates of t were made for each population. Single locus estimates of t ranged from 0.82 to 1.16 and 0.86 to 1.26 in populations of *C. laminuligera* grown at low density and at high density, respectively (Table 6). Multilocus estimates of t ranged from 0.83 to 0.98 and 1.00 to 1.01 for populations grown at low and at high plant density, respectively.

The effect of plant density on outcrossing rate in *C. laminuligera* was marginal. Outcrossing rates at low plant densities were lower than at high densities; however, the confidence interval estimates of t for the high and low density populations overlapped (Table 6).

Single locus estimates of t frequently exceeded one for *C. laminuligera* populations (Table 6). Estimates of t exceeding one do not necessarily imply that the mixed-mating model is invalid. Estimates greater than one may be caused by sampling effects, disassortative mating, or heterozygote selection (Brown et al. 1985).

Because single locus estimates of t for a given population sample the same mating events estimates should be identical; however, single locus estimates of t differed by as much as 35 % for a given population (Table 6). Confidence intervals for these estimates were large and did not necessarily overlap, another indication that the estimates were not uniform (Table 6). Variability could be due to selection acting differentially on loci

Table 6. Single and multilocus mixed-mating model outrcrossing rate estimates (t) and 90% bias-corrected percentile bootstrap confidence interval estimates (CI) for *C. laminuligera*.

Year	Location	Population	Density	Family	Locus	t	CI
1987	1	LA86	Low	60	Multilocus	0.83	0.80, 1.03
					Aco-2	0.87	0.77, 0.96
					Aco-3	0.90	0.83, 0.97
					Fes-2	0.97	0.88, 1.26
					Skd-2	0.85	0.75, 0.94
1987	2	LA87	Low	60	Multilocus	0.95	0.93, 1.03
					Aco-2	1.02	0.97, 1.09
					Aco-3	1.02	0.92, 1.13
					Fes-2	1.09	1.01, 1.19
					Skd-2	1.01	0.93, 1.08
1988	1	LA11	Low	60	Multilocus	0.91	0.87, 1.04
					Aco-1	0.82	0.68, 0.93
					Aco-2	1.06	0.94, 1.13
					Aco-3	0.87	0.75, 0.98
					Skd-2	1.01	0.91, 1.10
1988	1	LA11	High	60	Multilocus	1.01	0.99, 1.04
					Aco-1	1.02	0.93, 1.14
					Aco-2	1.16	1.06, 1.25
					Aco-3	0.86	0.76, 0.94
					Skd-2	1.08	1.00, 1.15
1988	2	LA13	Low	56	Multilocus	0.98	0.99, 1.05
					Aco-1	1.03	0.96, 1.20
					Aco-2	0.98	0.92, 1.05
					Aco-4	0.98	0.82, 1.11
					Fes-2	0.87	0.75, 0.97
					Skd-2	1.16	1.08, 1.28
1988	2	LA13	High	60	Multilocus	1.00	1.01, 1.08
					Aco-1	1.00	0.88, 1.09
					Aco-2	0.98	0.83, 1.09
					Aco-4	0.92	0.73, 1.05
					Fes-2	0.91	0.83, 1.06
					Skd-2	1.26	1.19, 1.30

linked to the allozyme marker locus (Brown et al. 1985); however, with the exception of *Skd-2* for which segregation distortion has been demonstrated (Krueger and Knapp in review), this is an unlikely explanation. Heterogeneity among single locus estimates is a common phenomenon and may be attributed to the unequal precision with which mating events can be detected with different marker loci, due to differing levels of polymorphism among loci and differing allele frequencies (Ritland and Jain 1981, Brown et al. 1985).

Multilocus estimates of t are an alternative to single locus estimates, and are the best approximation of actual outcrossing rates. The mixed-mating model assumes loci are unlinked, because using unlinked loci decreases the correlation among estimates, in effect increasing the probability of detecting an outcrossing event (Ritland and Jain 1981, Brown et al. 1985). In *C. laminuligera*, all of the loci used are unlinked (Krueger and Knapp in review).

We compared observed and expected numbers of genotype frequencies to detect violations of the assumptions of the mixed-mating model. Observed frequencies were generally not significantly different from expected frequencies (Table 7); our data did not violate model assumptions. Observed frequencies were significant for five of the 26 single locus estimates, but were generally limited to *Aco-2* and *Skd-2* (Table 7). There were no heterozygote deficiencies or excesses among the *C. laminuligera* populations that could explain the variation in genotype frequencies demonstrated by *Aco-2* and *Skd-2*. Observed numbers of heterozygous individuals were within 5% of expected values (data not shown).

Table 7. Observed and expected numbers of single-locus adult genotypes of *C. laminuligera*. Expected genotype frequencies were calculated as the binomial or trinomial square of allele frequencies.

Year	Location	Population	Density	Locus	Genotype 11	12	13	22	23	33	χ^2	P
1987	1	LA86	Low	*Aco-2*	46 51.4	218 217.3	84 72.8	350 339.5	209 235.7	63 55.3	6.71	0.15
				Aco-3	53 49.5	291 298.1		627 624.4			0.43	0.51
				Fes-2	645 640.9	295 295.2		30 35.0			0.74	0.39
				Skd-2	318 293.2	164 195.2	265 262.2	44 44.7	87 90.3	88 86.4	7.28	0.12
1987	2	LA87	Low	*Aco-2*	39 44.8	273 271.0	138 122.1	343 331.5	261 291.2	65 58.2	7.16	0.13
				Aco-3	35 44.8	389 366.2		697 710.1			3.80	0.05*
				Fes-2	577 578.0	487 477.6		56 65.0			1.43	0.23
				Skd-2	344 311.4	232 245.3	305 337.1	32 42.6	141 115.4	67 66.1	15.52	<0.01**
1988	1	LA11	Low	*Aco-1*				521 539.7	318 293.6	56 61.7	3.20	0.07
				Aco-2	151 160.4	256 268.8	174 181.0	126 100.3	139 143.4	50 42.1	9.63	0.05*
				Aco-3	69 65.3	293 308.9		493 480.9			1.33	0.25
				Skd-2	54 58.1	210 202.9	152 143.2	168 168.1	234 239.5	76 82.2	1.55	0.82
1988	1	LA11	High	*Aco-1*				566 575.4	294 280.6	28 32.0	1.29	0.26

Table 7. continued

Year	Location	Population	Density	Locus	Genotype 11	12	13	22	23	33	χ^2	P
				Aco-2	172 149.9	239 263.4	181 188.9	88 84.3	165 158.8	42 41.7	6.26	0.18
				Aco-3	85 84.2	317 323.8		466 460.0			0.23	0.63
				Skd-2	33 31.9	188 177.2	106 121.4	170 191.4	315 281.7	74 84.2	10.21	0.04*
1988	2	LA13	Low	*Aco-1*	13 16.5	226 214.5		511 519.0			1.48	0.22
				Aco-2	22 27.8	46 52.5	194 174.0	35 29.3	169 177.0	284 288.0	5.84	0.21
				Aco-4	9 8.2	157 155.2		584 587.2			0.12	0.73
				Fes-2	424 417.8	263 273.7		63 58.5			0.86	0.36
				Skd-2	87 89.1	209 208.2	145 154.3	67 80.1	192 167.8	49 48.7	6.30	0.18
1988	2	LA13	High	*Aco-1*	17 18.9	240 227.7		643 653.4			1.02	0.31
				Aco-2	64 62.0	80 92.6	229 253.5	41 36.0	179 191.5	306 265.2	11.93	0.02*
				Aco-4	26 27.5	243 235.3		618 624.3			0.40	0.53
				Fes-2	506 509.4	338 311.2		58 60.3			0.25	0.62
				Skd-2	86 91.6	189 176.0	280 275.7	26 30.5	200 202.9	116 120.3	2.23	0.69

Segregation distortion associated with *Skd-2* may be affecting estimates of t. Pollen and ovule allele frequencies were usually nearly equal except in LA87 where they differed by as much as 22% for *Skd-2* alleles (data not shown). The shift in allele frequency may be related to segregation distortion associated with *Skd-2*.

Three single locus estimates and a multilocus estimate of t were made for each *C. lutea* population. Single locus estimates of t ranged from 0.14 to 0.27 and 0.27 to 0.54 in populations grown at low and high plant density, respectively (Table 8). Multilocus estimates of t ranged from 0.17 to 0.26 and 0.36 to 0.54 for populations grown at low and high density, respectively.

In *C. lutea*, plant density greatly affected outcrossing rate (Table 8). A lower outcrossing rate was observed at low plant density than at high density and confidence intervals associated with low plant density generally did not overlap the confidence intervals associated with high plant density, indicating that plant density had a significant effect on outcrossing rates.

Single locus estimates of t for *C. lutea* were similar (Table 8). Single locus estimates of t did not differ by more than two to six percent, except in LU34 grown at high plant density. Single locus estimates of t differed by 16 % in this population.

We tested the *C. lutea* data for goodness of fit to model expectations. Observed and expected genotype frequencies were significantly different for LU36 grown at a low plant density (Table 9). This is probably due to the poor stand establishment in this population which limited the number of plants available to 25

Table 8. Single and multilocus mixed-mating model outrcrossing rate estimates (t) and 90% bias-corrected percentile bootstrap confidence interval estimates (CI) for *C. lutea*.

Year	Location	Population	Density	Family	Locus	t	CI
1987	1	LU15	Low	60	Multilocus	0.21	0.17, 0.28
					Aco-1	0.20	0.14, 0.27
					Pgd-1	0.20	0.16, 0.27
					Skd-1	0.22	0.15, 0.37
1987	2	LU15	Low	60	Multilocus	0.17	0.13, 0.21
					Aco-1	0.22	0.16, 0.29
					Pgd-1	0.20	0.13, 0.26
					Skd-1	0.14	0.10, 0.19
1988	1	LU34	Low	60	Multilocus	0.22	0.16, 0.27
					Aco-1	0.25	0.17, 0.32
					Pgd-1	0.21	0.15, 0.28
					Skd-1	0.20	0.13, 0.28
1988	1	LU34	High	60	Multilocus	0.36	0.30, 0.42
					Aco-1	0.36	0.29, 0.43
					Pgd-1	0.43	0.34, 0.51
					Skd-1	0.27	0.21, 0.41
1988	2	LU36	Low	25	Multilocus	0.26	0.20, 0.52
					Aco-1	0.27	0.23, 0.56
					Pgd-1	0.27	0.23, 0.55
					Skd-1	0.27	0.23, 0.55
1988	2	LU36	High	50	Multilocus	0.54	0.38, 0.60
					Aco-1	0.50	0.31, 0.58
					Pgd-1	0.53	0.39, 0.60
					Skd-1	0.54	0.29, 0.61

Table 9. Observed and expected numbers of single-locus genotypes of *C. lutea*. Expected genotype frequencies were calculated as the binomial or trinomial square of allele frequencies.

Year	Location	Population	Density	Locus	Genotype 11	Genotype 12	Genotype 22	χ^2	P
1987	1	LU15	Low	*Aco-1*	400 393.6	332 338.4	468 468.0	0.23	0.64
				Pgd-1	430 448.4	315 305.7	453 444.8	1.19	0.28
				Skd-1	517 501.2	363 369.3	319 327.3	0.82	0.37
1987	2	LU15	Low	*Aco-1*	426 430.8	372 382.8	402 386.4	0.99	0.32
				Pgd-1	452 440.0	354 366.0	394 391.2	0.56	0.46
				Skd-1	337 334.2	351 349.8	510 512.7	0.04	0.84
1988	1	LU34	Low	*Aco-1*	285 270.2	193 206.8	402 403.0	1.73	0.19
				Pgd-1	322 335.3	219 210.4	358 353.3	0.94	0.33
				Skd-1	299 285.9	249 252.6	351 360.5	0.90	0.34
1988	1	LU34	High	*Aco-1*	234 239.3	271 273.4	398 391.0	0.26	0.61
				Pgd-1	433 432.5	285 287.2	185 184.2	0.02	0.89
				Skd-1	284 279.9	273 264.6	345 358.5	0.84	0.36
1988	2	LU36	Low	*Aco-1*	171 187.5	91 73.7	110 110.9	5.52	0.02*
				Pgd-1	173 187.7	89 73.3	112 112.9	4.52	0.03*

Table 9. continued

Year	Location	Population	Density	Locus	Genotype 11	12	22	χ^2	P
				Skd-1	169 187.4	92 73.3	113 112.9	6.58	0.01**
1988	2	LU36	High	*Aco-1*	428 425.9	168 175.1	145 141.0	0.41	0.52
				Pgd-1	424 423.9	178 180.8	139 136.3	0.10	0.76
				Skd-1	421 420.9	182 185.2	138 134.9	0.13	0.72

(Table 8). Therefore, this is not a lack of fit to the mixed-mating model; otherwise, both the low and high plant density population should have shown a lack of fit, since the LU36 plots grown at high and low plant density were identical in every respect except plant density. Observed and expected genotype frequencies were not significantly different in any other population (Table 9).

In addition to estimating outcrossing rates, we estimated autofertility under field conditions. The mean seed set per flower for *C. laminuligera* was 0.00 $\pm$ 0.00 in 1986 and 1987 when pollinators were excluded. The mean seed set per flower for *C. lutea* was 4.75 $\pm$ 0.28 and 4.64 $\pm$ 0.42 in 1986 and 1987, respectively.

Autofertility estimates, outcrossing rate estimates, and floral morphology provide important information about the mating system of *C. laminuligera*. *C. laminuligera* has an absolute requirement for pollinators to affect pollination. This is generally limited to *Bombus sp.*. These species are able to access the nectaries at the base of the floral tube. *C. laminuligera* is self-compatible, protandrous, and anthers are exserted from the floral tube at anthesis (Graham 1988). Protandry and anther exsertion minimize self-fertilization; however, pollinator movement can affect self-fertilization at rates of up to 18%.

In contrast to *C. laminuligera*, *C. lutea* is autofertile, and prior to this study, was presumed to be highly self-pollinated. *C. lutea* is protandrous and self-compatible, but its anthers dehisce inside the floral tube (Graham 1988). While anther dehiscence inside the floral tube insures seed set in the absence of insect

pollinators, outcrossing rates from 14 to 54% can be achieved when pollinators are available. Bees of the genus *Bombus* and *Apis* have been observed visiting *C. lutea* flowers. This is a significant observation since *C. lutea* is one of the few if not only *Cuphea* species actively pollinated by honeybees.

Our estimates of mating system parameters have important ramifications for breeding, domesticating, and maintaining germplasm of the species. *C. laminuligera* is highly cross-pollinated and recurrent selection programs should be used to develop improved populations. Reproductive isolation is necessary for breeding this species, and insect pollinators must be provided to insure seed set.

Recurrent selection breeding methods can also be used to improve *C. lutea*, since cross-pollination occurs at significant levels. The integrity of lines can only be maintained by reproductive isolation. Self-pollination is insured by excluding pollinators using bags or cages.

Germplasm resources are limited in *C. laminuligera* and *C. lutea* and the breeding programs of both species are going to require additional germplasm. Germplasm collection in *C. laminuligera*, a highly polymorphic species, should emphasize the sampling of a large number individuals from a relatively limited number of collection sites. If natural populations of *C. lutea* are as highly differentiated as the populations in our program, and the majority of populations contain one or a few homozygous genotypes, then the optimum collection strategy is to collect seeds from a few individuals from many collection sites (Marshall and Brown 1975).

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