Mate recognition in *Cryptomyzus* aphids: copulation and insemination

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Abstract

The specificity of the mate recognition system in three species of the aphid genus *Cryptomyzus* (Homoptera: Aphididae: Aphidinea) that reproduce sexually on the same host plant, red currant, was studied. Mate recognition at the level of life cycle forms, sister species and more distantly related species was studied. A host race on black currant was used to determine the effect of the host plant on mate preference. The males do not have a specific courtship behaviour and matings readily occurred between different species. However, in choice tests the matings between the more distantly related species resulted only in intraspecific inseminations, and those between the sister species in a higher frequency of intraspecific, compared to interspecific, matings. In no-choice experiments mating between the more distantly related species occurred but did not result in insemination, and in sister species in a reduced insemination success compared to intraspecific matings. Thus, there is specific mate recognition in aphids, which prevents or reduces interspecific insemination but not interspecific mating. In the host races, host plant choice determined mating and insemination, and the life cycle forms did not show any difference in mate recognition.

Introduction

The ability of the sexes to recognize each other (Paterson, 1985) is important for the integrity of a species and its reproductive isolation from other closely related species (Mayr, 1963; Templeton, 1989). Paterson (1985) defined the specific mate recognition system as the chain of signals and responses of a male and female that lead to fertilization, thus all prezygotic processes, not only mating but also insemination, are included in specific mate recognition (see also Eberhard, 1985; Coyne & Orr, 1989, also used the rate of insemination as a measure of divergence). The degree of insemination, fertilization and successful development to maturity, and the viability and fertility of hybirds and their offspring determine whether gene flow and introgression will occur (Mayr, 1963; Templeton, 1989).

Studies on reproductive isolation in aphids have tended to concentrate on hybird inviability and differential habitat use, i.e. different host plants (Shaposhnikov, 1966; Müller, 1985; Guldemond, 1990a).

In olfactory tests the sex pheromones of the different species or biotypes have some specificity (Pettersson, 1971; Eisenbach & Mittler, 1987; Hardie *et al.*, 1990; Pickett *et al.*, 1992; Guldemond & Dixon, 1994). In mating experiments males discriminated between the species specific ratio of sex pheromone components (Hardie *et al.*, 1990), nevertheless interspecific matings are observed (Marsh, 1975; Steffan, 1990). These studies were carried out with species from different genera, and results obtained by studying closely related species might shed light on the role of specific mate recognition in speciation.

The aphids we studied, *Cryptomyzus galeopsidis* (Kalt.), *C. maudamanti* Guldemond and *C. ribis* (L.) (Homoptera: Aphididae: Aphidinea) all occur sympatrically on red currant, *Ribes rubrum*. The first two are sister species, because they share a common ancester: these species are each other's closest relatives; the latter is more distantly related to both sister species (Fig. 1, Guldemond, 1991a). They host-alternate, by migrating in spring from the winter host, red cur-
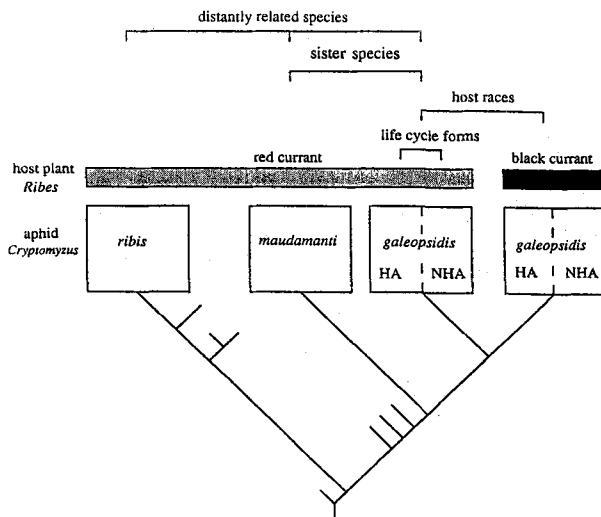
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Fig. 1. Phylogenetic relationships of the species and forms of *Cryptomyzus* and the host plants on which sexual reproduction takes place. (N)HA = (non) host-alternating life cycle form; branches on cladogram indicate other species of *Cryptomyzus* (after Guldemond & Eggers-Schumacher 1989 and Guldemond 1990c).

rant, to species specific summer hosts. On the summer hosts, which all belong to the Labiatae, they reproduce parthenogenetically. In autumn the males and the sexual females meet on the winter host where sexual reproduction takes place (for a more general account on the life cycle of aphids, see Dixon, 1985). Therefore, the fact they have different summer host plants is not important for the study of mating systems, because they only reproduce parthenogenetically on the summer hosts. Further, there is a non host-alternating life cycle form of *C. galeopsidis*, which remains on red currant throughout the year. This form interbreeds freely with the host-alternating form and differs in life cycle (Guldemond, 1991a). Further, a host race of this species on black currant, *R. nigrum*, is distinguished (Fig. 1), which differ in preference for and performance on the two currant species, and when interbred their hybirds show a reduced reproductive performance (Guldemond, 1991a & b).

The question to be addressed is: Do closely related species and life cycle forms of *Cryptomyzus*, which reproduce on the same host plant, red currant, have different specific mate recognition systems? We will focus on the copulatory behaviour, thus: are there factors effective at close range before or during copulation, that inhibit interspecific insemination? In order to answer these questions we have studied i) preferential insemination in choice experiments, and ii) mat-

ing behaviour, duration of copulation and insemination success in no-choice experiments. The inclusion of the host race on black currant enables us to determine the effect of the host plant on insemination success within a species.

Materials and methods

Aphid material and production of sexuals. All aphid species and forms were collected in the vicinity of Wageningen, The Netherlands. Each form or species was represented by one clone. Stock cultures reproduced parthenogenetically on their summer host plants at 15°C, L16:D8. *C. maudamanti* was cultured on yellow dead-nettle, *Lamium galeobdolon*, the non host-alternating *C. galeopsidis* on red currant, and the other species on hempnettle, *Galeopsis tetrahit* (Guldemond, 1991b). Sexuals were produced by transferring females from long to short day conditions (L12:D12) at 15°C. Their winged offspring were placed for 1–2 days on currant plants where these produced the sexual females. Sexual females of the non host-alternating form of *C. galeopsidis* were produced concurrently with the males, and were separated before they became adult. Only virgin sexual females were used in the experiments and are referred to in the text as females.

Experiments. The host-alternating form of the species *C. galeopsis* on red currant was chosen as the species with which to compare the others, because it is the only species which can be compared to a non host-alternating life cycle form, host race, sister species and distantly related species. The phylogenetic relationships of the red currant form of *C. galeopsidis* in relation to the other species and forms, and the host plants on which sexual reproduction occurs, are shown in Fig. 1.

i) In choice tests preferential insemination was measured in a growth cabinet at 15°C at L12:D12 on caged plants that had all their leaves but one removed. In the experiments 5–15 day old adult virgin females and young males were used. Equal numbers of females of two different forms or species (N = 5–10) were placed on the leaf and left for at least an hour to settle. Then a male was released onto the plant and removed after 24 h, when the females were immediately examined to determine whether they had been inseminated (see below). Choice tests with the two host races of *C. galeopsidis* were similarly performed in a cage, 30×34×38 cm, with dark walls and illuminated from

above, which contained two similar sized red and black currant plants. There were 15 females of the red currant form of *C. galeopsidis* on the red currant, and 15 females of the black currant form of this species on the black currant. A male was released from a glass tube placed between the two plants and removed 24 h later.

ii) In no-choice tests the duration of copulation was measured by observing pairs confined on their host plant at 20°C. Copulation started when the genitalia of male and female made firm and prolonged contact and ended when the male withdrew its aedeagus from the vulva. The mating behaviour of the male and female was observed through a 10× magnifying lens.

Insemination was assessed by dissecting each female and preparing a microscope slide of its spermatheca (pictures in Grassi, 1912; Blackman, 1987) in Grace's insect medium. The slides were examined at 100–400 times magnification, and the presence or absence of sperm noted. Insemination success refers to the first copulation of a male except for *C. galeopsidis* from red currant and *C. ribis*, for which the following copulations are also included. There was no difference in insemination success of males when assessed after one or several copulations (*C. galeopsidis*: all but one females inseminated; *C. ribis*: all females inseminated).

Results

Interspecific mating behaviour. No difference was observed in the behaviour of males approaching females of their own and other species. Mating occurred between all the species and forms in no-choice experiments. Males were even observed trying to copulate with the veins of a leaf bearing 'calling' females, i.e. females with their abdomen and hind tibia lifted, which indicates the release of sex pheromone (Pettersson, 1968, 1970; Marsh, 1975; Eisenbach & Mittler, 1980; Hardie et al., 1990; Steffan, 1990).

Interspecific matings between ♂ *C. galeopsidis* and ♀ *C. ribis* were terminated in 27% of the cases (N = 56) by the female vigorously lifting her abdomen several times and trying to push or even kick the male away with her hind legs. Also, females attempted to raise their body perpendicular to the leaf surface, or walk away. In conspecific matings of both species only in 2% of the cases (N = 50) the females showed similar behaviour, indicating a difference in the females' behaviour between inter- and intraspecific copulations ($\chi^2 = 6.43$, P<0.025, N = 106). Apparently, males are

not very selective, but females try, at least in some instances, to interrupt an interspecific mating.

Distantly related species. In choice tests between the two distantly related species, *C. galeopsidis* and *C. ribis*, males only inseminated conspecific females (Fig. 2A). However, in no-choice tests they copulated, but only in the combination ♂ *C. galeopsidis* × ♀ *C. ribis* were a few females inseminated, with the copulation time (7.7 min) rather similar to those in unsuccessful copulations (6.5 min; Table 1). The duration of copulation is shorter in matings between than within species (Table 1). Comparable results were obtained in no-choice experiments, when 5 or 10 males of *C. ribis* were kept with 10 females of the distantly related *C. maudamanti* and only 2% were inseminated (N = 58).

In one instance the sperm of *C. galeopsidis* was found in one of the accessory glands of a female of *C. ribis* rather than in her spermatheca, suggesting that the sperm could not be transferred or migrate to the spermatheca. However, virtually all the interspecific matings resulted in no insemination, although 96% (n = 24) of the matings between male *C. galeopsidis* and female *C. ribis* lasted as long as the shortest successful intraspecific mating of *C. galeopsidis*, and two as long as those of *C. ribis*. Apparently, the duration of copulation was not a factor in the failure to inseminate.

Copulations of *C. ribis* were longer (range 9.8 to 48 min) than in *C. galeopsidis* (range 3 to 13.5 min) (Table 1). Males can copulated again shortly after their first copulation with another (virgin) female: in *C. ribis* intraspecific second matings took place after 35 sec to 13.7 min (mean 3.2 min ± 0.7 s.e., N = 21). In *C. ribis* the second copulation lasted for shorter time than the first copulations (P<0.05, two-tailed Wilcoxon matched-pairs test, N = 21).

Sister species. Only males of *C. galeopsidis* inseminated more conspecific than heterospecific females in a choice test between the sister species *C. galeopsidis* and *C. maudamanti* (Fig. 2B). In some of the replicates more than half the females were inseminated, which could mask a difference. However, when these replicates are excluded from the analysis the results show the same trend. Males are able to inseminate a considerable number of females in 24 h, e.g. *C. galeopsidis* an average of 7.9 with a maximum of 14, and *C. maudamanti* 8.9 and maximally 19.

In a no-choice situation the likelihood of insemination was lower in matings between the sister species than in intraspecific matings (Table 2). The duration of
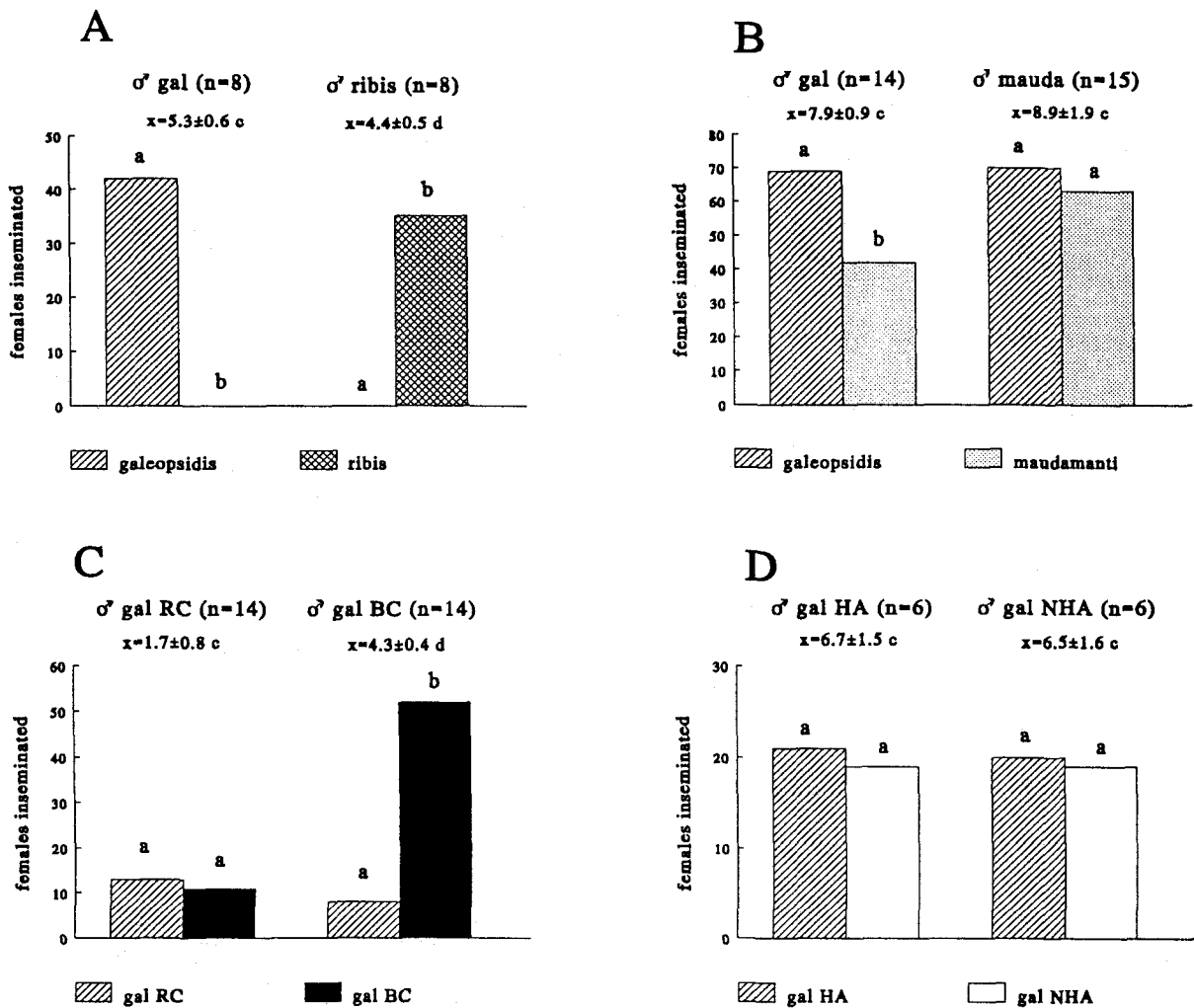
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Fig. 2. The number of inseminated females of the red currant form of *C. galeopsidis* and those of the A) more distantly related *C. ribis*; B) sister species *C. maudamanti*; C) black currant host race of *C. galeopsidis*; and D) non host-alternating (NHA) form of *C. galeopsidis* from red currant, by their own and other species'/forms males, with one male and 5–10 females (15 with host races) of each species/form per replica. a, b indicates differences at P<0.05, Wilcoxon matched-pairs test; x = mean number of inseminated females per male ± s.e., with c, d indicating differences at P<0.05, Mann-Whitney U-test.

Table 1. Duration of copulation (in min) ± s.e., and percentage inseminated in intra- and interspecific matings between the red currant form of *C. galeopsidis* and *C. ribis* in a no-choice situation. In brackets the sample size, differences tested with a one-way ANOVA

| | ♂ *C. galeopsidis* | | ♂ *C. ribis* | | |
	Copulation time (min)	% insemination	Copulation time (min)	% insemination	Cop time
♀ *C. galeopsidis*	8.9±0.6 (18)	100 (28)	2.9 ±0.5 (26)	0 (42)	P<0.000
♀ *C. ribis*	6.5±0.5 (24)	4 (48)	21.2 ±1.7 (27)	90 (19)	P<0.000
	P = 0.0055		P = 0.0000		

Table 2. Duration of copulation (in min, ± s.e.), when the females are inseminated and not inseminated, and percentage inseminated in intra- and interspecific matings between the red currant form of C. galeopsidis and C. maudamanti in a no-choice situation. In brackets the sample size, differences in insemination time tested with a one-way ANOVA, differences in % insemination with a χ^2-test with 1 df

		♂ C. galeopsidis		♂ C. maudamanti			
		Copulation time (min)	% inseminated	Copulation time (min)	% inseminated	Cop time	% ins
♀							
C. galeopsidis	Inseminated	8.9±0.6 (18)	100 (28)	9.4±1.1 (12)	52 (23)	P = 0.6990	P<0.001
	Not inseminated	— (0)		4.7±0.3 (11)			
♀							
C. maudamanti	Inseminated	10.2±0.5 (17)	60 (30)	21.4±2.9 (23)	88 (26)	P = 0.0024	P<0.05
	Not inseminated	3.3±0.5 (11)		3.4±0.4 (3)			
		P = 0.1176	P<0.01	P = 0.0069	P<0.01		

copulation was shorter in unsuccessful matings (range 1.7 to 7.4 min); successful matings were much longer in C. maudamanti than in the other combinations of matings (range 8.0 to 66.3 min) (Table 2).

Host races. Of the two host races of C. galeopsidis the males of the black currant form more frequently inseminated homogamous females in a choice test. The males of the red currant form, however, inseminated homo- and heterogamous females equally (Fig. 2C). It appeared that in each replicate insemination only took place on one of the two currants. This indicates that these males first choose a host plant and then mate. An additional experiment revealed that 73% of the black currant host race males preferred to settle on black currant in a host plant choice test in the absence of females ($\chi^2 = 50.58$, P<0.001, N = 248; test conditions the same as in the mate choice experiments). However, the red currant host race did not discriminate between the two currants (57% on red currant, $\chi^2 = 3.14$, NS, N = 156, Guldemond, 1990a). There is no difference between the incidence of insemination of the red and black currant races and the choice of the males for red and black currant in those experiments (black currant race: $\chi^2 = 0.006$, NS; red currant race: $\chi^2 = 0.041$, NS). Thus, the incidence of insemination (Fig. 2C) reflects the host plant preference of the males.

Duration of copulation and insemination success measured in a no-choice test was similar in 3 out of 4 combinations; the only exception were males of the black currant host race which inseminated fewer females of the red currant form than did homogamous males (Table 3).

Life cycle forms The males of the host-alternating and non host-alternating life cycle forms of C. galeopsidis from red currant inseminated each other's females equally in a choice test (Fig. 2D). In a no-choice test the success of insemination in the mating between the various forms did not differ ($\chi^2 = 4.58$, df = 3, P<0.20; the tests were performed with 10 females and 5 or 10 males on a small (ca 20 cm) red currant plant at 20°C; 29 to 60 females per combination were examined for insemination). The differences in mating success between the various species and forms of *Cryptomyzus* are summarized in Table 4.

Discussion

Different elements of the specific mate recognition system of *Cryptomyzus* can be distinguished: i) host plant preference, ii) pheromonal attraction at long range, iii) attraction at close range by means of visual and olfactory cues (courtship behaviour) leading to copulation, and iv) the act of copulation and insemination. The results show that at close range, males of *Cryptomyzus* are not very selective and mating occurred between the two relatively distantly related species, C. galeopsidis and C. ribis. Females sometimes try to avoid matings with heterospecific males which lends support to the idea of female choice (e.g. Eberhard, 1985). Species specific courtship behaviour has not been described for aphids and even intergeneric mating has been recorded in no-choice tests (Pettersson, 1971; Marsh, 1975; Steffan, 1990). However, males of the aphids *Acyrthosiphon pisum*, *Aphis fabae* and *Megoura viciae* (all belonging to the Aphidinea and occurring on the same host plant, pea) preferentially

Table 3. Duration of copulation (in min, ± s.e.), when the females are inseminated and not inseminated, and percentage inseminated in matings within and between the red and black currant host race forms of *C. galeopsidis* in a no-choice situation. In brackets the sample size, differences in insemination time tested with a one-way ANOVA, differences in % insemination with a χ^2-test with 1 df

		♂ Red currant form		♂ Black current form			
		Copulation time (min)	% inseminated	Copulation time (min)	% inseminated	Cop time	% ins
♀ Red currant	Inseminated	8.9±0.6 (18)	100 (28)	9.1±0.4 (18)	69 (26)	P=0.8509 NS	P<0.01
	Not inseminated	— (0)		3.9±0.6 (8)			
♀ Black currant	Inseminated	9.4±0.5 (26)	93 (28)	9.1±0.8 (20)	7 (26)	P=0.6974 NS	NS
	Not inseminated	4.2±1.1 (2)		4.1±0.5 (6)			
		P=0.5545	NS	P=0.9825	NS		

Table 4. Comparison of the specific mate recognition systems measured as the level of preferential insemination, duration of copulation and insemination success observed in matings between species, host races and life cycle forms of *Cryptomyzus*

Phylogenetic relationship	Preferential insemination	Duration of copulation	Insemination success
Distantly related	yes	shorter	none
Sister species	partial	shorter	reduced
Host races	host preference = mate preference	similar	similar
Life cycle forms	no	not measured	similar

copulate with parthenogenetic females treated with a sex pheromone mixture of the species to which the test male belongs. Mating attempts with females treated with pheromone mixtures of the other species have also been observed (Hardie *et al.*, 1990). Apparently, odour cues can play a role in specific mate recognition at close range in aphids.

The sister species and conspecific forms of *Cryptomyzus* have a similar morphology and colour and can only be distinguished by a morphometric analysis (Guldemond, 1991a). Thus, the sister species also represent sibling species. Therefore, visual recognition is not likely to play an important role, because only strong visual cues, such as a dark or light colour of a female and long or short appendages, seem to be an important means of identification for the male at close range (Pettersson, 1970).

Eberhard (1985) suggests that during copulation females can make a choice whether to accept a male or not. When *C. galeopsidis* and *C. maudamanti* mated with the distantly related *C. ribis*, either in a choice or a no-choice situation, no or very few inseminations resulted. This indicates that the possibility of gene flow

between these species is greatly reduced by a mechanism that comes into operation after mating but before fertilization. Similar extremely low insemination rates have only previously been reported for intergeneric crosses in aphids (Hardie *et al.*, 1990). The behaviour of the female in rejecting the male during copulation suggests that in the early phase of mating a signal is received, or not received, which leads to the termination of copulation.

Morphological differences in male genitalia between closely related insect species can be tremendous (e.g. Eberhard, 1985; Otte, 1989) and may be a cue for a female to accept or reject a mate during copulation (Eberhard, 1985). However, morphological differences in the shape and structure of the apical part of the aedeagus, which is inserted in the female and is membraneous and rather soft (Heie, 1980; Miyazaki, 1987), have not been reported in the Aphidinea (Polaszek, 1987). More detailed studies of male genitalia may reveal subtle differences in morphology or manoeuvrability (West-Eberhard, 1984), or the existence of substances in the sperm that are used by the female to select a male (Eberhard, 1985). Alternative-

ly, penetration of alien sperm might be prevented by (the lack of) a chemical cue in the females' genital system that directs the sperm into the spermatheca, which, for example, has been recorde in the weevil *Anthonomis grandis* (Grodner & Steffens, 1978). That crosses between two closely related *Dysaphis* species (Aphidinea) failed to produce fertile eggs has been attributed to the death of the sperm in the female tract (Shaposhnikov, 1966). It is unlikely that the reduction of insemination success has played a major role in the initial reduction of gene flow between closely related species, because although marked in matings between the more distantly related *Cryptomyzus* species it was only slightly present between the sister species.

There is evidence for a more frequent insemination of conspecific females in the sister species *C. galeopsidis* and *C. maudamanti*, at least by males of *C. galeopsidis*; also the insemination success in no-choice tests is reduced in these matings. Further, the sister species also differ in the timing of release and specificity of their sex pheromones, which act at a distance (Guldemond & Dixon, 1994). This indicates that the specific mate recognition system of the sister species of *Cryptomyzus* has diverged.

The experiments with the host races of *C. galeopsidis* show that i) in no-choice tests no or little difference was observed in insemination success between the host races and ii) it is the host preference of the male, and not its mate preference, which determines which females are inseminated. The sexual females of the black and red currant host races used in this study prefer to settle on their respective host plants, and, moreover, can only develop on black and red currant, respectively, (Guldemond, 1990a); this implies that the host plant preference of the males is rather crucial in determining the females of which host race they will encounter.

No preference or reduced level of insemination was found between the conspecific life cycle forms of *C. galeopsidis*, indicating they share the same mate recognition system, which is in agreement with earlier observations that they can freely interbreed (Guldemond, 1990b). However, in some years the sexuals of the non host-alternating form are produced earlier in the season than those of the host-alternating form (Guldemond & Tigges, 1991). The general pattern which emerges from this study is that of an increasing differentiation between mate recognition systems in *Cryptomyzus*, from life cycle forms (little differentiation), through host races, sister species to more distantly related species (Table 4).

What might be the origin of the divergence between the host races of *C. galeopsidis*? The concurrence of host preference and mating on the host plant in aphids is an important condition for rapid host race formation by a host shift and subsequent speciation (Bush & Diehl, 1982; Diehl & Bush, 1989). The importance of host preference is well illustrated by the host races of *Cryptomyzus*. Males of the black currant host race of *C. galeopsidis* show a distinct preference for this host and it is this that results in positive assortative mating. An earlier study (Guldemond, 1990a) revealed that all the black currant genotypes studied were well adapted to black currant, e.g., both females and males preferred and reproduced on this host. However, the genotypes obtained from red currant were more variable, ranging from well adapted to red currant to more intermediate in their host plant preference and reproductive performance, and these were probably hybirds between the two host races (Guldemond, 1990a). Because these intermediates reproduce less well on both currants, as do laboratory bred hybrids (Guldemond, 1990b), natural selection will favour the development of more selective genotypes. This could have resulted in the reduced gene flow between the host races, which has been demonstrated by differences in allozyme frequencies (Guldemond & Eggers-Schumacher, 1989). Thus, the scenario of speciation in the host races of *Cryptomyzus* is that of a change in host preference resulting in the colonization of an unused host plant. This is the start of ecological differentiation and independent regulation of the two populations on both host plants. In combination with hybrid inferiority between the new and ancestral population this might lead to reinforcement of differences in host plant preference between the host races.

Hybrids (F1, F2 and backcrosses) between the sister species of *Cryptomyzus* have reduced viability in terms of their reproductive performance (Guldemond, 1990b). The sister species produce their sexuals at the same time of the year (Guldemond et al., 1994) thus interspecific matings could occur in the field. Individuals with a more effective specific mate recognition would be at a selective advantage because they are more likely to chose a conspecific mate, and be more fecund (Mayr, 1963; Dobzhansky, 1970). Thus, selection is still acting on specific mate recognition in the sister species of *Cryptomyzus*.

Many host races of aphids are thought to have originated sympatrically, in the face of gene flow, and their hybrids have a reduced fitness (Mackenzie & Guldemond, 1994). In these circumstances any muta-

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tion which improves mate recognition will be favoured because it reduces hybridization and increases the fitness of the population. Simultaneously, this enhances the cohesion of the host races, which might eventually lead to (sympatric) speciation by reinforecement.

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