Temporal and host-related variation in frequencies of genes that enable *Phyllotreta nemorum* to utilize a novel host plant, *Barbarea vulgaris*

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Abstract

The flea beetle, *Phyllotreta nemorum* L. (Coleoptera: Chrysomelidae), is an intermediate specialist feeding on a small number of plants within the family Brassicaceae. The most commonly used host plant is *Sinapis arvensis* L., whereas the species is found more rarely on *Cardaria draba* (L.) Desv., *Barbarea vulgaris* R.Br., and cultivated radish (*Raphanus sativus* L.). The interaction between flea beetles and *Barbarea vulgaris* ssp. *arcuata* (Opiz.) Simkovics seems to offer a good opportunity for experimental studies of coevolution. The plant is polymorphic, as it contains one type (the P-type) that is susceptible to all flea beetle genotypes, and another type (the G-type) that is resistant to some genotypes. At the same time, the flea beetle is also polymorphic, as some genotypes can utilize the G-type whereas others cannot. The ability to utilize the G-type of *B. vulgaris* ssp. *arcuata* is controlled by major dominant genes (R-genes). The present investigation measured the frequencies of flea beetles with R-genes in populations living on different host plants in 2 years (1999 and 2003). Frequencies of beetles with R-genes were high in populations living on the G-type of *B. vulgaris* ssp. *arcuata* in both years. Frequencies of beetles with R-genes were lower in populations living on other host plants, and declining frequencies were observed in five out of six populations living on *S. arvensis*. Selection in favour of R-genes in populations living on *B. vulgaris* is the most likely mechanism to account for the observed differences in the relative abundance of R-genes in flea beetle populations utilizing different host plants. A geographic mosaic with differential levels of interactions between flea beetles and their host plants was demonstrated.

Introduction

Within phytophagous insect lineages there has been an evolutionary trend towards specialisation on a small number of host plants (Thompson, 1994; Schoonhoven et al., 1998). The processes leading to specialisation are not well understood, but adaptations to secondary plant compounds are likely to be involved. One possible scenario for evolutionary interactions between plants and insects is: (1) the plant evolves a novel type of secondary compound which confers resistance to a phytophagous insect, and (2) the phytophagous insect evolves a type of counter-adaptation to the secondary compound and these adapted insects are able to utilize the resistant plant and may eventually specialize to feed preferentially on this plant. This type of interaction is often referred to as a coevolutionary arms race between plants and insects. It is only one possible scenario for evolutionary interactions between plants and insects (Futuyma & Keese, 1992; Thompson, 1994, 1999), but it is a scenario that is suitable for experimental manipulation in cases where interacting species are polymorphic, e.g., plants are polymorphic with respect to resistance, and insects are polymorphic with respect to counter-adaptations to plant defences.

The interaction between *Barbarea vulgaris* R.Br. (Brassicaceae) and the flea beetle, *Phyllotreta nemorum* L. (Coleoptera: Chrysomelidae), offers a good opportunity for experimental studies of coevolutionary interactions (Nielsen,

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1997a,b; de Jong & Nielsen, 2002). The plant is polymorphic, as it contains one type (P-type) that is fully susceptible to flea beetles and another type (G-type) that is fully resistant to the most common flea beetle genotypes (Nielsen, 1997a; Agerbirk et al., 2001, 2003). The flea beetle is polymorphic, as some genotypes are susceptible to defences in the G-type, whereas others are resistant (de Jong & Nielsen, 1999). The ability to utilize the G-type is controlled by major, dominant genes (R-genes). The number of R-genes in the species is unknown, but segregation patterns suggest that autosomal as well as sex-linked loci are present (Nielsen, 1997b; de Jong et al., 2000). A single copy of an R-gene is sufficient to transform a susceptible genotype (rr) into a resistant genotype (Rr), which is able to utilize the G-type of *B. vulgaris*. R-genes influence larval survival and adult behaviour on the G-type of *B. vulgaris*. Larvae without R-genes (rr) do not develop on the G-type, whereas larvae with them (RR and Rr) do. Adult flea beetles without R-genes do not feed on the G-type, whereas beetles with them do (Nielsen, 1996, P.W. de Jong & J.K. Nielsen, unpubl.).

Phyllotreta nemorum is an intermediate specialist which feeds on a small number of crucifer species under field conditions in Denmark. The most important host plant is the weed *Sinapis arvensis* L., whereas the species is found more rarely on *Cardaria draba* (L.) Desv., *B. vulgaris,* and on cultivated radish (*Raphanus sativus* L.) (de Jong & Nielsen, 1999). Less than 20% of the known *B. vulgaris* populations (G- and P-type) are attacked by *P. nemorum* (J.K. Nielsen, unpubl.). Utilisation of *B. vulgaris* as a host plant therefore seems to be an expansion of the host plant range, at least at the local scale. The rarity of utilisation of *Barbarea* patches suggest that there are some constraints to the successful colonisation of this plant.

Colonisation of novel *Barbarea* patches may depend on the abundance of R-genes in flea beetle populations occurring within the region. Previous results have suggested that R-genes are found at high frequencies in flea beetle populations living on the G-type of *B. vulgaris*, but at lower frequencies in populations living on other host plants (de Jong & Nielsen, 1999). The method used by de Jong & Nielsen (1999) to detect the flea beetle genotypes consisted of crossing field-collected beetles with conspecifics from a susceptible laboratory line (rr), and investigating the progeny. This method is very laborious, and it is not possible to obtain estimates of genotype frequencies by the investigation of large samples from many populations using this method. A faster method based on adult behaviour can distinguish between beetles with R-genes (RR and Rr: feeding on the G-type of *B. vulgaris*) and beetles without R-genes (rr: do not feed on the G-type of *B. vulgaris*), but it cannot detect whether the resistant beetles are homozygous or heterozygous, and it cannot discriminate between autosomal and sex-linked loci. The latter method was used in the present investigation in order to obtain more exact information on the abundance of flea beetle genotypes carrying R-genes in populations living on the G-type of *B. vulgaris* (where R-genes are known to influence fitness), as well as on three alternative host plants where R-genes do not have any known effects on fitness. Populations living on the P-type were not included, as they were small.

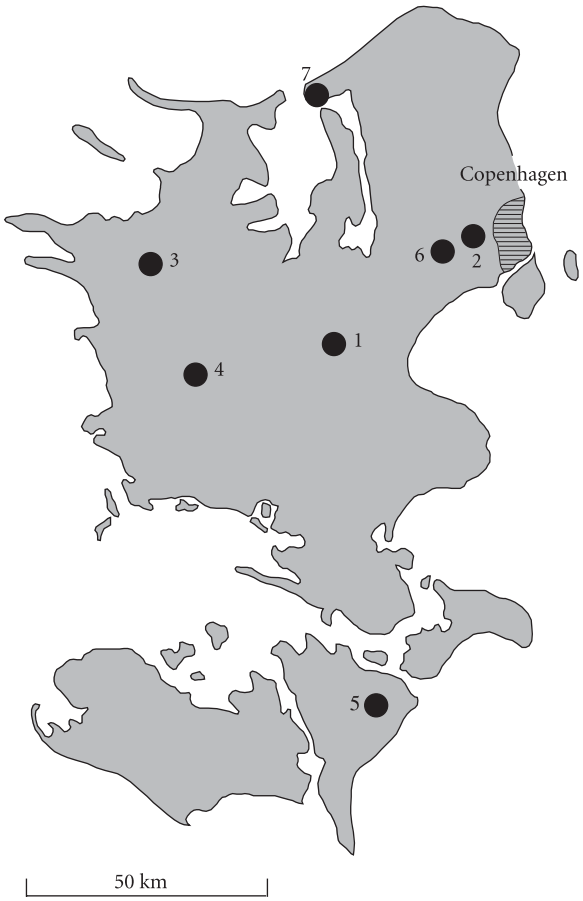

Figure 1 Map of localities in eastern Denmark where collections were made: (1) Kvaerkeby; (2) Ejby; (3) Sveboelle; (4) Suserup; (5) Maglebraende; (6) Taastrup; and (7) Lynaes.

Samples of flea beetle larvae were collected in eastern Denmark (Figure 1) on different host plants in 2 different years (1999 and 2003). The emerging adults were tested in a laboratory bioassay on the G-type of *B. vulgaris*, and a distinction was made between feeders (representing the genotypes RR and Rr) and non-feeders (representing the genotype rr). The study specifically addressed the following questions: (1) does the frequency of feeders depend on the host plant? and (2) is there a temporal variation (between years) in the frequencies of feeders in populations living on different host plants?

Materials and methods

Leaves with leaf-mining flea beetle larvae were collected from various localities (Figure 1) and host plants: (1) Kvaerkeby: G-type of *B. vulgaris* ssp. *arcuata* (two local sites) and *S. arvensis* (three local sites); (2) Ejby: G-type of *B. vulgaris* ssp. *arcuata* and *S. arvensis*; (3) Sveboelle: G-type of *B. vulgaris* ssp. *arcuata*; (4) Suserup: *S. arvensis*; (5) Maglebraende: *S. arvensis*; (6) Taastrup: *R. sativus*; and (7) Lynaes: *C. draba*. Each leaf sample represented plants growing within an area of at least 100 m^2. Full grown larvae pupated in a mixture of moist peat (Enhetsjord-K-jord) and vermiculite (5 : 1). Adult flea beetles were used in bioassays within 24 h of emergence, and before they had access to any food source.

Bioassays were performed as non-choice tests in 185 ml plastic containers containing a moist gypsum-charcoal bottom layer (Nielsen, 1978). The G-type of *B. vulgaris* ssp. *arcuata* was grown in a growth chamber at 20 ± 2 °C and under a L18:D6 photoperiod. Light was supplied by 400 W HPI/T-lamps that produced 160–200 µmol quanta m^{-2} s^{-1} at the level of the leaf surface. Two leaf discs (diameter = 14 mm) from young leaves of 4–8-week-old plants were introduced to each plastic container. Individual flea beetles had access to two leaf discs for 72 h at 24 ± 2 °C and a L18:D6 photoperiod. The amount of feeding after 72 h was judged visually, and a distinction was made between non-feeders (<15 mm^2 leaf area consumed) and feeders (>20 and usually more than 50 mm^2 leaf area consumed). More than 99% of the beetles could clearly be classified as non-feeders or feeders using this method. Statistical analyses were made with the likelihood-ratio test (G-test) using the GENMOD procedure in SAS (SAS Institute, 1993).

Results

There are significant differences in the frequencies of feeders (an estimate of the genotypes RR and Rr) on different host plants (Tables 1 and 2). There were no significant overall differences between years, but there was a highly significant interaction between host plant and year (Table 2). Frequencies of feeders were similar in females and males, and the data were combined in Table 1 and in all statistical analyses. The frequencies of feeders were higher in populations living on *B. vulgaris* than in populations living on three other host plants in both years ($P<0.0001$ for all pair-wise comparisons in both years). Frequencies of feeders were stable in populations living on *B. vulgaris*, *R. sativus*, and *C. draba*, but declining in five out of six populations living on *S. arvensis* (Table 1). Although frequencies of feeders on *S. arvensis* were declining during the study period, they were still abundant over a large geographical area, and the rarity of colonisation of novel *B. vulgaris* patches within this area cannot be explained by a local absence of R-genes.

Discussion

There are two large populations of *P. nemorum* which utilize *B. vulgaris* (in Ejby and Kvaerkeby) within the geographical region covered by the present survey. The population living on *B. vulgaris* in Sveboelle was not really well established, and only small numbers of *P. nemorum* were found on the plant in 2003 (Table 1) and again in 2004 (J.K. Nielsen, unpubl.). Nevertheless, frequencies of genotypes with R-genes were high in all populations living on *B. vulgaris*,

Table 1 Temporal variation in the frequencies of R-genes in *Phyllotreta nemorum* populations living on different host plants (n = number of beetles). P-values indicate the differences between 1999 and 2003

			Frequencies of beetles with R-genes						
			1999		2003			Distance (km) from	
Locality	Site	Host plant	%	n	%	n	P	Kvaerkeby Site 1	Ejby
Kvaerkeby	1	*Barbarea*	99.0	200	99.0	99	ns	0	43
Kvaerkeby	2	*Barbarea*	99.0	100	95.2	21	ns	1.2	43
Kvaerkeby	3	*Sinapis*	53.6	56	41.9	105	ns	1.4	43
Kvaerkeby	4	*Sinapis*	71.4	35	42.6	54	<0.01	2.6	42
Kvaerkeby	5	*Sinapis*	60.0	100	41.7	100	<0.01	3.5	42
Ejby	1	*Barbarea*	98.5	199	100	51	ns	43	0
Ejby	1	*Sinapis*	96.0	101	32.7	101	<0.0001	43	0
Sveboelle	–	*Barbarea*	98.9	87	100	7	ns	42	69
Suserup	–	*Sinapis*	43.0	100	23.9	92	<0.01	25	66
Maglebraende	–	*Sinapis*	48.0	100	21.7	23	<0.05	67	104
Taastrup	–	*Raphanus*	20.0	100	24.2	62	ns	33	9
Lynaes	–	*Cardaria*	15.5	97	21.4	28	ns	55	43

Table 2 The effect of host plant and year on the frequencies of beetles with R-genes. Outcome of analysis using the likelihood ratio test (G-test) on data from Table 1

Explanatory variable	G	d.f.	P
Host plant	747.4	3	<0.0001
Year	0.11	1	ns
Interaction	16.35	3	<0.001

whereas frequencies of R-genes were substantially lower in populations living on three other host plants. This pattern of distribution of R-genes could originate from two scenarios: (1) associations between flea beetles and host plants have been stable over long periods of time, and populations living on *B. vulgaris* and other plants have coexisted without any gene flow between them, or (2) flea beetle populations living on *B. vulgaris* and other plants within a region form one metapopulation connected by gene flow among subpopulations living on different host plants. Differences in the abundance of R-genes are caused by differential selection regimes and differential rates of emigration and immigration among populations living on different host plants. The latter scenario is supported by several independent observations: (1) the low differentiation in allozyme patterns among flea beetle populations living on different host plants within a region (i.e., Kvaerkeby and Ejby) suggests that these populations are connected by gene flow, and (2) mechanisms which could maintain separate flea beetle populations on *B. vulgaris* and other host plants in the field have not yet been documented. Reproductive barriers (prezygotic or postzygotic) have not been observed between populations living on different host plants (Nielsen, 1997b; de Jong & Nielsen, 1999; de Jong et al., 2000). Beetles with R-genes can feed on the G-type of *B. vulgaris*, but no preference for this plant has been documented (Nielsen, 1996). Finally, no fitness consequences of R-genes (either negative or positive) have been documented when beetles are feeding on plants outside the genus *Barbarea* in the laboratory (Nielsen, 1999; Agerbirk et al., 2003, P.W. de Jong & J.K. Nielsen, unpubl.). It is therefore most likely that models involving gene flow are more realistic than models assuming a long-lasting isolation of flea beetle populations on different host plants.

Gene flow among flea beetle populations living on different host plants has important evolutionary and ecological implications (Figure 2). Flea beetle genotypes containing R-genes are produced in high frequencies in populations living on *B. vulgaris*, and from there they may disperse to neighbouring populations living on other host plants, in most cases *S. arvensis*, which is the most abundant alternative host plant. The present study demonstrates that R-genes may be retained in these populations over at least a 4-year period. In the absence of detailed knowledge about emigration and immigration rates, it is not yet known whether there is selection against the R-genes in populations living on *S. arvensis*, although frequencies were declining in five out of six populations living on this plant. Until any fitness consequences of R-genes in flea beetles living on alternative host plants have been documented, it must be assumed that the frequencies of R-genes could increase as well as decrease in these populations (Figure 2). Whereas the R-allele is likely to spread from populations living on *B. vulgaris* to populations living on alternative host plants, the r-allele is likely to spread in the opposite direction, from alternative host plants to *B. vulgaris*. On the G-type of *B. vulgaris* there will be strong selection against the r-allele, as homozygous rr genotypes cannot survive on this plant. The successful colonisation of patches of *B. vulgaris* by immigrants from alternative host plants is therefore bound to have immediate evolutionary consequences leading to a rapid increase in the abundance of genotypes carrying R-genes. Rapid evolutionary adaptations to novel host plants have been documented in a few cases (Carroll et al., 1998; Singer et al., 1993; Thompson, 1998), but rapid changes in the frequencies of particular genes conferring adaptations to particular host plants have not previously been documented.

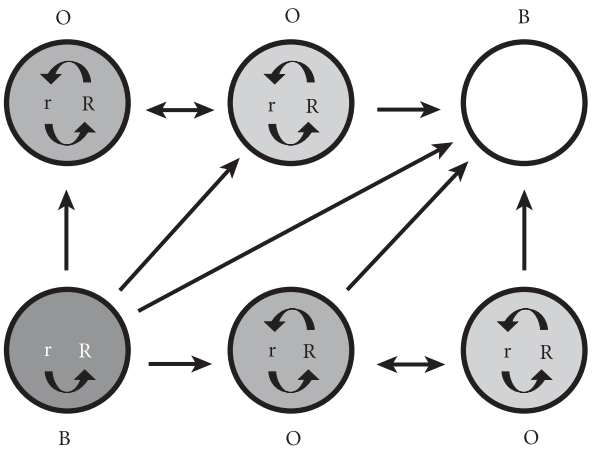

Figure 2 Geographic mosaic of interactions between the G-type of *Barbarea vulgaris* ssp. *arcuata* (B), and *Phyllotreta nemorum*, as influenced by bridging populations of flea beetles living on other host plants (O). High frequencies of R-genes are found in flea beetle populations in one *Barbarea* patch (bottom left) whereas another patch (top right) has not yet been colonized. Increasing frequencies of R-genes are indicated with progressive shades of grey, main directions of gene flow are indicated by straight arrows, and shifts in gene frequency due to selection or genetic drift are indicated by curved arrows.

Gene flow conferred by emigration and immigration among adjacent flea beetle populations living on different host plants has ecological as well as evolutionary implications for the interaction between flea beetles and their host plants. The present study demonstrates that flea beetle populations living on other host plants form a reservoir for the R-genes, which theoretically could initiate the colonisation of novel *Barbarea* patches. It also demonstrates – and confirms previous findings (de Jong & Nielsen, 1999) – that R-genes are found in many regions where *B. vulgaris* is not utilized as a host plant. The present investigation also confirms that there is substantial geographic variation in the interactions between *B. vulgaris* and flea beetles, as stated in the theory on the geographic mosaic of coevolution (Thompson, 1994). Flea beetle adaptation to *B. vulgaris* is controlled by sex-linked as well as autosomal genes in one population (Ejby), and solely by autosomal genes in another (Kvaekeby) (Nielsen, 1997b; de Jong et al., 2000). Most *Barbarea* patches are not utilized at all (J.K. Nielsen, unpubl.) and the rarity of successful colonisation events seems surprising since R-genes are widely distributed in Eastern Denmark.

Novel *B. vulgaris* patches could be colonized by flea beetles originating from other (distant) *Barbarea* populations or from neighbouring populations living on other host plants. It is unlikely that one of the populations from Ejby and Kvaerkeby has been the founder of the other, as they differ in genetic architecture. Furthermore, the allozyme patterns in the populations living on *B. vulgaris* in Ejby and Kvaerkeby are more similar to neighbouring populations living on other host plants than they are to each other (de Jong et al., 2001). There is as yet no evidence that the populations in Ejby and Kvaerkeby have founded any satellite populations in the neighbourhood (J.K. Nielsen, unpubl.). The geographical distance between *Barbarea* patches may therefore be a severe constraint to the colonisation of novel *Barbarea* patches from existing populations living on the same plant.

Successful colonisation of novel *Barbarea* patches by emigrants from populations living on other plants also seems to be a very rare event, even in areas where R-genes are abundant. Most beetles found on old host plants are heterozygous (Rr), whereas beetles living on *B. vulgaris* are homozygous (RR) (de Jong & Nielsen, 1999), and the change from heterozygosity to homozygosity may require some additional evolutionary changes, e.g., the interaction of R-genes with modifier genes (de Jong & Nielsen, 2000, 2002). Furthermore, different R-genes may have different effects. Our knowledge about R-genes originates from investigations of populations that use *B. vulgaris* as a natural host plant, and where major R-genes with large effects on the survival and behaviour on the G-type of *B. vulgaris* predominate (Nielsen, 1996, 1999; de Jong et al., 2000). However, previous surveys have demonstrated the presence of so-called minor genes in some populations living on other host plants (de Jong & Nielsen, 1999). These genes have minor effects on the survival of flea beetle larvae on the G-type of *B. vulgaris*, and their effect on flea beetle behaviour is unknown. It is possible that some of these minor genes have been detected in the present study, and that the abundance of the major R-genes has been over-estimated. A closer examination of these processes will require a better method for the detection of, and discrimination between, different R-genes.

The growth characteristics of *B. vulgaris* may impose particular barriers that are independent of the origin of flea beetle colonizers. It is remarkable that the P-type of *B. vulgaris* is rarely used as a natural host plant (J.K. Nielsen, unpubl.) although this plant does not contain any chemical defences against flea beetles (Nielsen, 1997; Agerbirk et al., 2003). Plant phenology may play a role, as rosette leaves from flowering *Barbarea* plants may wilt in June before the flea beetle larvae have finished feeding in the leaf mines. The P-type comes into flower approximately 2 weeks earlier than the G-type, but both types tend to lose the rosette leaves before the flea beetle larvae have left their mines. The flea beetle larvae then depend on the smaller cauline leaves or on any rosette leaves from plants that did not flower in the spring.

The present state of our knowledge about factors influencing the abundance of R-genes in a region is summarised in Figure 2. There is local variation in the interaction between *B. vulgaris* and flea beetles, as many plant patches are not utilized, whereas others are. There seems to be a strong selection in favour of R-genes in flea beetle populations living on the G-type of *B. vulgaris*, whereas it is uncertain whether selection influences the abundance of R-genes in populations living on old host plants. No estimates of gene flow have yet been made. Novel *Barbarea* patches may be colonised by: (1) beetles from adjacent populations living on other plants, and/or (2) from distant populations living on the G-type of *B. vulgaris*. The results demonstrate the importance of geographic variation in interactions between plants and insects (Thompson, 1994, 1999). Local interactions in one *Barbarea* patch may have far-reaching effects in other patches conferred by gene flow and the maintenance of pools of R-genes in bridging populations of flea beetles living on other host plants.

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