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Genetic and morphological differentiation in *Tephritis bardanae* (Diptera: Tephritidae): evidence for host-race formation

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wing length.

Abstract

The fruit fly *Tephritis bardanae* infests flower heads of two burdock hosts, *Arctium tomentosum* and *A. minus*. Observations suggest host-associated mating and behavioural differences at oviposition indicating host-race status. Previously, flies from each host plant were found to differ slightly in allozyme allele frequencies, but these differences could as well be explained by geographical separation of host plants. In the present study, we explicitly test whether genetic and morphological variance among *T. bardanae* are explained best by host-plant association or by geographical location, and if this pattern is stable over a 10-year period. Populations of *A. tomentosum* flies differed significantly from those of *A. minus* flies in (i) allozyme allele frequencies at the loci *Pep-A* and *Pgd*, (ii) mtDNA haplotype frequencies and (iii) wing size. In contrast, geographical location had no significant influence on the variance estimates. While it remains uncertain whether morphometric differentiation reflects genotypic variability or phenotypic plasticity, allozyme and mtDNA differentiation is genetically determined. This provides strong evidence for host-race formation in *T. bardanae*. However, the levels of differentiation are relatively low indicating that the system is in an early stage of divergence. This might be due to a lack of time (i.e. the host shift occurred recently) or due to relatively high gene flow preventing much differentiation at loci not experiencing selection.

Introduction

Host races can be defined as 'genetically differentiated, sympatric populations of parasites that use different hosts, and between which there is appreciable gene flow' (Dres & Mallet, 2002). They are somewhere between polymorphic populations and distinct species, and are considered a crucial intermediate step in sympatric speciation (Diehl & Bush, 1984). Phytophagous insects are prime candidates for host-race formation, as the close relationship to their host plants can often result in restricted gene flow via pleiotropy (Bush, 1969; Via, 2001). Genetically based differences in host preference (Craig *et al.*, 1993; Sezer & Butlin, 1998), allochronic isolation of host plants (Seitz & Komma, 1984; Itami *et al.*, 1998) or nongenetic induction of host fidelity by learning or conditioning (Prokopy *et al.*, 1982, 1986) can result in assortative mating and hence maintenance of host-related differentiation. The existence of sympatric host races is well documented for a variety of species (reviewed by Dres & Mallet, 2002) and completion of speciation in sympatry might occur frequently (Payne & Berlocher, 1995; Bush & Smith, 1998; Berlocher, 1999). However, for evaluating the likelihood and extent of sympatric speciation further case studies have to be conducted (Jiggins & Mallet, 2000; Schluter, 2001; Via, 2001). Particularly, the role of reproductive isolation for stability of host races has to be studied in more detail: what is the minimum of reproductive isolation for host race formation and which level of reproductive isolation is required for completion of sympatric speciation?

In the present paper, we establish a new model system for host-race formation, which might prove useful for

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answering the above questions. The tephritid fly *Tephritis bardanae* (Schrank, 1803) is a seed parasite of the plant genus *Arctium* (Asteraceae). *Tephritis bardanae* mate and oviposite exclusively on host plants. Mating takes place at the start of flower head development in May. Larvae feed on the achenes until pupating. Flies start to emerge in August and hibernate as adults. Previous studies investigating flies caught on *Arctium tomentosum* Mill. (woolly burdock) and *A. minus* Bernh. (common burdock) showed that *T. bardanae* flies differ in respects of behaviour relative to host plant, indicating a possible host-race status (Eber *et al*., 1991; Lehr, 1994). Given a choice between *A. tomentosum* and *A. minus*, females collected in the field preferred to mate and oviposite on the plant species from which they were collected (Lehr, 1994). As mating sites are often highly correlated with mate choice in tephritids (Zwölfer, 1974) assortative mating can arise as a by-product. This leads to restricted gene flow, which may result in host-race formation (Craig *et al*., 1993). The egg-laying behaviour differs between *T. bardanae* parasitizing *A. minus* and *A. tomentosum* (Eber *et al*., 1991). Eggs are laid in the flower heads of *A. minus*, whereas on *A. tomentosum* eggs are oviposited into the stem beneath the flower head. Although this might be a mere behavioural response to different flower head structures (*A. tomentosum* flower heads are covered with woolly filaments), a genetic basis is not unlikely. Eber *et al.* (1991) reported host-plant-related allozyme divergence but because their sampling was geographically biased they could not fully discriminate between host-plant and geographical separation as the agent of divergence.

The present study examines divergence of *T. bardanae* for host-race formation on *A. minus* and *A. tomentosum*. Both *Arctium* species occur syntopically in Middle and Eastern Europe (Hulten & Fries, 1986). We test explicitly whether host affiliation or geographical separation explains genetic and morphological variance among *A. minus* and *A. tomentosum* flies. We include flies sampled over a period of 10 years, from 1989–2000. We investigate whether host-plant and/or geographical associations are constant over time. Restricted gene flow is examined using nuclear (allozyme) and mitochondrial DNA genetic markers. In addition to genetic markers, we examine wing length as a quantitative trait of size. Affirmation of host-plant associations and stability over time should indicate host races.

Materials and methods

Sampling

Flower heads of *A. minus* and *A. tomentosum* were collected in the years 1989–90 and 1999–2000. At each locality, 100–200 flower heads were sampled from several plants. Flower heads were cut from branch tips and placed in darkened plastic bottles with a hole in the lid. The lid was covered with a glass bottle for trapping emerging flies. Adults were collected on a daily basis and stored at −80 °C. We refer to flies from *A. tomentosum* as tomentosum flies and those from *A. minus* as minus flies.

Populations of both plant species were sampled geographically to test for geographical separation vs. host-plant affiliation. Allowing for host-plant occurrence, host-plant populations were collected in equal numbers. All populations were located in Germany except for three in Denmark, one in Switzerland and one from England (Fig. 1). Samples from identical localities but from different years and/or host plants were treated as separate samples. For analysing the influence of geographical location on genetic and morphological variance, we grouped localities into two regions. Sites located in or south of the Central German Uplands were labelled 'south', while the remaining localities were labelled 'north'. The division between the Central German Uplands and the North German Basin may act as a dispersal barrier for insects (Sternberg, 1998). The north/south division corresponds to the study of Eber *et al.* (1991). We applied a similar sampling design but included populations of both hosts within each division to partition the effects of geography and host-plant on genetic and morphological divergence. Within each year 'north' and 'south' populations were separated in latitude by at least 200 km. The linear distance was at least 260 km.

We sampled 87 *A. tomentosum* sites (26 in 1989–90; 61 in 1999–2000) and 68 *A. minus* sites (23 in 1989–90; 45 in 1999–2000). *Tephritis bardanae* emerged from 72 *A. tomentosum* sites, but only from 23 of 68 *A. minus* sites. The emergence of minus flies was particularly low in the years 1999 and 2000, when only seven of the 45 *A. minus* sites provided *T. bardanae*. For analysis we included all seven minus populations from 1999–2000 as well as most minus populations from 1989–90 (13 populations). We limited the number of analysed tomentosum populations to 45. Populations were selected to analyse the influence of geographical region on differentiation.

Allozyme electrophoresis

Allozyme analysis was carried out using cellulose acetate electrophoresis (Hebert & Beaton, 1989). A total of 1854 flies belonging to 63 populations were analysed.

Flies collected in 1989–90 originally were analysed for allozyme variation at eight scorable enzyme systems (Lehr, 1994). Only three loci were polymorphic at the 95% level: isocitrate dehydrogenase (*Idh*, EC 1.1.1.42), phosphoglucomutase (*Pgm*, EC 5.4.2.2) and peptidase A (*Pep-A*, EC 3.4.11.11). Flies collected in 1999–2000 were screened anew. Loci previously found to be monomorphic revealed no additional polymorphisms and were omitted from further analysis. However, we found two new enzymes polymorphic at the 95% level, alcohol dehydrogenase (*Adh*, EC 1.1.1.1) and 6-phoshogluconate

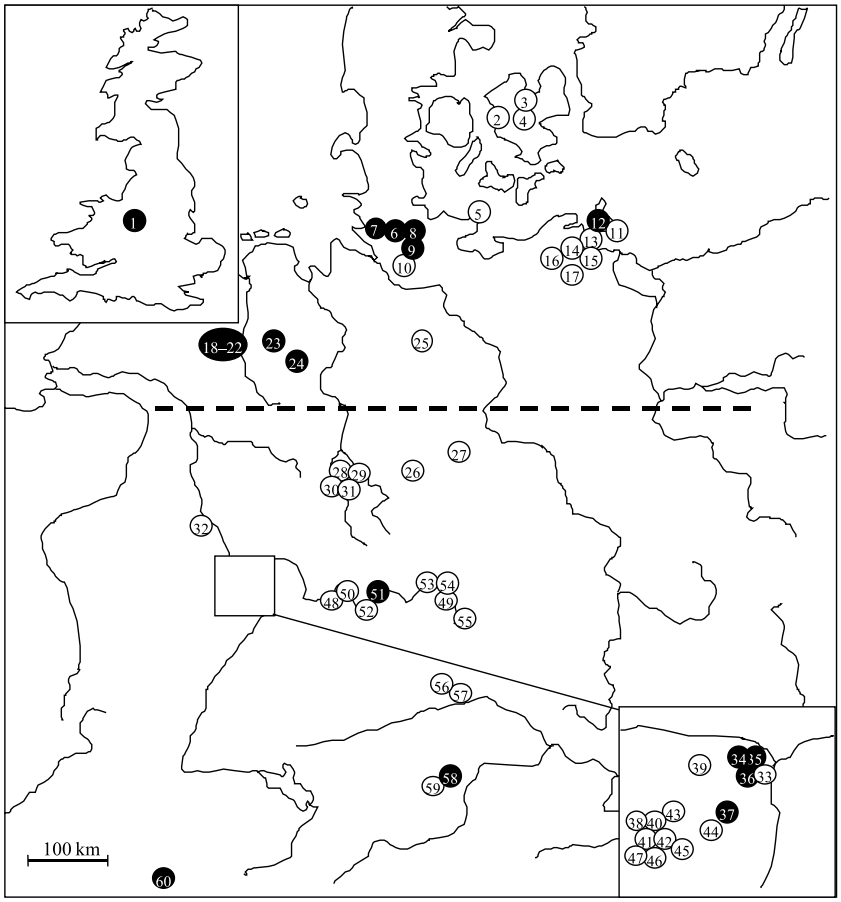

Fig. 1 Sampling sites of *Tephritis bardanae*. Black circles represent minus population and white circles represent tomentosum populations. The separation of regions is indicated by the dashed line. Location names are given in Appendix S1, web materials.

dehydrogenase (*6-Pgdh*, EC 1.1.1.44), which were included in genetic analyses of flies collected 1999–2000. All enzymes were run at 250 V for 30 min using the buffer systems Tris–Citrate pH = 8.2 (Richardson *et al.*, 1986) for *Idh* and *6-Pgdh*, and Tris–Glycine pH = 8.5 (Hebert & Beaton, 1989) for *Adh*, *Pgm* and *Pep-A*. The most common allele at each locus was given the arbitrary score 100. All other alleles were named relative to the 100 allele from the point of application. For comparison of Lehr's (1994) data and data from the present study, we used the three loci used in both studies. We reanalysed the *Pep-A* zymograms of the years 1989–90, because we could only reliably score three alleles in 1999–2000. These three alleles must be identical to those found by Eber *et al.* (1991). In contrast, Lehr (1994) found up to seven alleles because of higher electrophoretic resolution. For *Pep-A* data comparison we converted Lehr's data by pooling rare alleles with the common allele with the nearest relative migration score.

Sequencing of mtDNA

Mitochondrial DNA sequence variation was examined only for *T. bardanae* collected in 1999–2000. A total of 140 specimens, belonging to 26 *A. tomentosum* populations and seven *A. minus* populations, were included in the mtDNA sequence survey. DNA was extracted from tissue pellets remaining from allozyme electrophoresis (see above) using the Roche High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany). Subsequent polymerase chain reaction (PCR) was performed with Ready.to.Go™ beads (0.5 mL tubes; Amersham Pharmacia Biotech, Piscataway, NJ, USA) in 20 μL end volume, consisting of 1 μL DNA extraction, 1 μL forward and backward primer (10 pmol μL^{-1}) and 17 μL Rotisolv® water (Carl Roth GmbH and Co., Karlsruhe, Germany). We amplified a 830 bp fragment of the cytochrome oxidase subunits I and II (COI and COII) with the primers S2792 (5′-ATACCTCGACGTTAT TCAGA-3′) and A3661 (5′-CCACAAATTTCTGAACATT-GACCA-3′) (Brown *et al.*, 1996). The PCR reaction was started by denaturing at 94 °C for 1.5 min, followed by 35 cycles of: annealing 47 °C/30 s, extension 72 °C/1.5 min and denaturation 94 °C/30 s. After purification of the PCR product (Roche High Pure PCR purification kit) single-stranded PCR for sequence analysis was performed in an end volume of 20 μL, consisting of 5 μL purified DNA, 1 μL primer, 4 μL premix and 10 μL water. PCR

cycling procedure started with denaturation for 1 min at 96 °C, followed by 25 cycles of: annealing 45 °C/15 s, extension 72 °C/4 min and denaturation 96 °C/30 s. PCR products were sequenced in both directions with an ABI-377A (PE Applied Biosystems, Foster City, CA, USA) automatic sequencer. Sequences were aligned using SEQUENCE NAVIGATOR (PE Applied Biosystems). The alignment was subsequently checked manually.

Morphological measurements

Wing lengths of 929 adult *T. bardanae* (469 males, 460 females) flies were measured from 49 populations. For each population a maximum number of 24 flies (12 males and 12 females) were studied, if possible. Wings were dissected, mounted with tape on slides and digitized using *ImageTool 2.00* (Wilcox *et al.*, 1996). For every wing, 12 co-ordinates were defined and distances between all pairs were calculated as a pixel-value distance (1 mm corresponds to 121.72 pixel) (Fig. 2). Principle component analysis (PCA) showed that these pairwise distances were highly correlated: the first principle component explained 87.37% of the total variance and a single measurement between points A and B (Fig. 2) accounted for 99.3% of the first principle component. Based on this PCA we limited the morphological size analysis of wing length to the distance between A and B. Only right wings were included in analysis.

Data analysis

Allozyme variability estimates were calculated with G-Stat (Siegismund, 1993). Populations were examined for deviations from Hardy–Weinberg proportions and for linkage disequilibrium using exact tests with genetic data analysis (GDA) (Lewis & Zaykin, 2001). Genetic distances were calculated according to Reynolds *et al.* (1983) and Nei (1972), and phenograms for population relationships were computed with the UPGMA method (Fitch & Margoliash, 1967). Genetic distances and phenograms were calculated applying the software package PHYLIP (Felsenstein, 1993). Population differentiation was analysed with fixation indices using the method of Weir & Cockerham (1984). Due to only one population in 1990 and low number of minus populations in 1999–2000, populations from the consecutive sampling years 1989–90 and 1999–2000 were analysed together. We assume that dispersal cannot randomize genotypes within one generation across the total area of sampling. Hierarchical *F*-statistics were performed to examine the influence of host plant and/or geographical region on population differentiation. Here F_{ST} describes the variance among populations relative to the total level of sampling, F_{HT} is the variance among host plants relative to the total level of sampling (independent of region) and F_{RT} is the variance among regions relative to the total level of sampling (independent of host plant). Furthermore, we hierarchically tested for regional effects within host plants. Fixation indices were calculated using Arlequin 2.0 (Schneider *et al.*, 2000), with 95% confidence intervals obtained by a nonparametric permutation approach (Excoffier *et al.*, 1992). Further tests for geographical associations of populations (isolation by distance) were tested with Mantel tests (GENEPOP 3.2, Raymond & Rousset, 2000) by comparing pairwise F_{ST} estimates between populations (Slatkin, 1993).

A minimum spanning network of mtDNA haplotypes was constructed using TCS 1.12 (Clement *et al.*, 2000). Homogeneity of haplotype distributions among host plants and geographical regions were examined with chi-square tests. Arlequin 2.0 was used to calculate the mean number of pairwise differences between populations (Schneider *et al.*, 2000).

We applied *t*-tests and, if possible, analysis of variances (ANOVA) to quantify the influence of host plant, geographical region and year on wing length. Interaction terms could not be performed for all ANOVA analyses as populations of both host species were not present in each year and region (because of low emergence of minus flies).

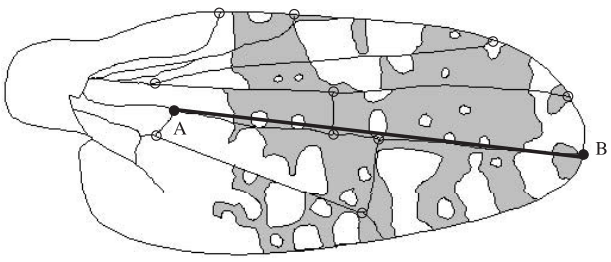

Fig. 2 Wing of *Tephritis bardanae*. The line between A and B was defined as wing length. Open circles show remaining measurement points.

Results

Allozyme variation

Expected heterozygosity (H_e) calculated for three loci (all years) and five loci (1999–2000) ranged from 0.226 to 0.736 (mean 0.372) and from 0.166 to 0.525 (mean 0.301), respectively. Minus populations had significantly higher expected heterozygosity than tomentosum populations at *Pep-A* (minus $H_e = 0.532$, tomentosum $H_e = 0.466$; Mann–Whityney *U*-test: $Z_{61} = -3.976$, $P < 0.001$). In contrast, there was no difference between geographical regions or years for *Pep-A*. Apart from *Pep-A* heterozygosity, no differences in genetic variability between host plants, geographical regions or years were detected. There were no diagnostic alleles; all alleles were present in both host-plant populations and regions. An allele frequency table is available on request.

After Bonferroni correction, no linkage disequilibrium between loci was observed. Three sites showed highly

Table 1 Hierarchical *F*-statistics over all loci and for single loci.

	All loci	*Idh*	*Pgm*	*Pep-A*	*Adh*	*Pgd*
All years						
F_{ST}	**0.057**	**0.040**	**0.037**	**0.107**		
F_{HT}	**0.023**	0.002	−0.002	**0.068**		
F_{RT}	−0.002	−0.002	−0.001	−0.002		
1989–90						
F_{ST}	**0.058**	**0.025**	**0.036**	**0.111**		
F_{HT}	**0.034**	−0.003	−0.002	**0.089**		
F_{RT}	0.001	−0.004	0.001	−0.002		
1999–2000						
F_{ST}	**0.047**	**0.062**	**0.047**	**0.081**	**0.056**	**0.076**
F_{HT}	**0.013**	0.013	0.010	**0.020**	−0.004	**0.036**
F_{RT}	−0.001	0.001	−0.003	0.003	−0.003	**0.012**

Idh, isocitrate dehydrogenase; *Pgm*, phosphoglucomutase; *Pep-A*, peptidase A; *Adh*, alcohol dehydrogenase; *Pgd*, phoshogluconate dehydrogenase.
Significant *F*-values are indicated in bold.

significant deviations from Hardy–Weinberg equilibrium: two populations from Rheinhessen had a homozygote excess at one locus (Lerchenberg at *Pep-A*, Jakobsberg at *IDH*), whereas the population Holzhausen consisted only of heterozygotes at *PGM* ($n = 24$).

Population differentiation over all samples was moderate but significant, $F_{ST} = 0.057$ (Table 1). Differentiation was within the range typical of within-species variation (Itami *et al.*, 1998). We applied a hierarchical analysis of variance to examine whether host-plant affiliation or geographical region contributes to population differentiation.

Differentiation among host-plants was significant over all years, $F_{HT} = 0.023$ (Table 1), indicating that population structure is at least partly caused by host plants. Single locus F_{HT} values revealed that only two loci contribute significantly to differentiation between host plants, namely *Pep-A* and *Pgd*. Allele frequencies at *Pgd* and *Pep-A* differed significantly due to host-plant affiliation (*Pgd-131*: $t_{32} = 3.384$, $P < 0.01$; *Pep-A-91*: $t_{61} = 5.269$, $P < 0.001$). For all populations, host affiliation explained 63.4% of total inter-population variance at *Pep-A* and 47.4% at *Pgd*, respectively. Noticeable is the difference in F_{HT} among years at *Pep-A*. The comparatively low value for 1999–2000 could partly result from outliers as only seven minus populations could be analysed for this period (because of low emergence). One of these populations is Wipfeld (51a), which seems to be a 'nontypical' minus population (see also Fig. 3, mtDNA and morphometric data). Exclusion of Wipfeld from analysis raises F_{HT} of *Pep-A* from 0.020 to 0.045 and of *Pgd* from 0.036 to 0.048 in 1999–2000.

In contrast to host-plant affiliation, no significant population differentiation due to geographical region (F_{RT}) was detected (Table 1). These findings hold over all loci and for all single locus estimates except for *Pgd* in 1999–2000. However, in 1999–2000 six of seven minus sites are located in the north, so the significant F_{RT} for *Pgd* might be caused by the heterogenous distribution of minus populations. To test this hypothesis, F_{RT}-values were calculated for each host plant separately. Again, no significant population differentiation due to geographical region was detected, i.e. the afore significant F_{RT} is host-plant rather than geographically related.

Isolation by distance tests (Mantel tests) provided further evidence for lack of geographical divergence. We observed no significant correlations between genetic and geographical distances within or among host-plant populations (all populations: 1953 contrasts, n.s.; among populations affiliated to the same host plant: tomentosum, 903 contrasts, n.s.; minus, 190 contrasts, n.s.) nor in any year (all n.s.).

Genetic distances between populations ranged from 0.001 to 0.393 (mean 0.077) (Reynolds *et al.*, 1983). Genetic distances between populations affiliated to the same host were significantly lower than between populations of the alternative host (Mann–Whitney *U*-test: $Z_{126} = -6.313$, $P < 0.001$). This pattern is visualized in the UPGMA phenogram of all 63 populations calculated over the three loci available for all years (*Pep-A*, *Idh*, *Pgm*): a host-related separation of populations is apparent, although not complete (Fig. 3). The clustering of populations due to host affiliation into two main branches is highly significant ($\chi^2_1 = 33.63$, $P < 0.001$). The distribution of 'north' and 'south' populations between the two main branches is heterogeneous too ($\chi^2_1 = 4.67$, $P < 0.05$). However, this latter significance is likely due to heterogeneous sampling of host plants: testing within tomentosum and minus populations, respectively, did not reveal geographically related clustering ($\chi^2_1 = 0.91$, n.s. and $\chi^2_1 = 2.54$, n.s., respectively).

The UPGMA phenograms constructed for single loci and all loci combinations revealed that *Pep-A* alone causes this host-related topology (*Pep-A* $\chi^2_1 = 22.58$, $P < 0.001$; all other loci combinations $\chi^2_1 < 1.53$, n.s.). Analysing the years 1999–2000 separately for all five polymorphic loci (including the loci *Adh* and *Pgd*) revealed that the locus *Pgd* caused host-related topology too ($\chi^2_1 = 17.49$, $P < 0.001$) (tree not shown). These results corroborate the hierarchical *F*-statistics.

mtDNA variation

We observed 18 haplotypes (H1–H18) among the 140 sequenced *T. bardanae* flies (GenBank accession numbers AY343502–AY343519). The two most common haplotypes H1 and H2 were separated by three synonymous substitutions (sites 8, 110 and 143). Another four haplotypes were found in more than two individuals, H3–H6. They differed only in the combination of the before mentioned three substitutions. The remaining 15 haplotypes were found only once. Mitochondrial diversity was significantly higher in tomentosum populations than in minus populations: mean number of haplotypes per

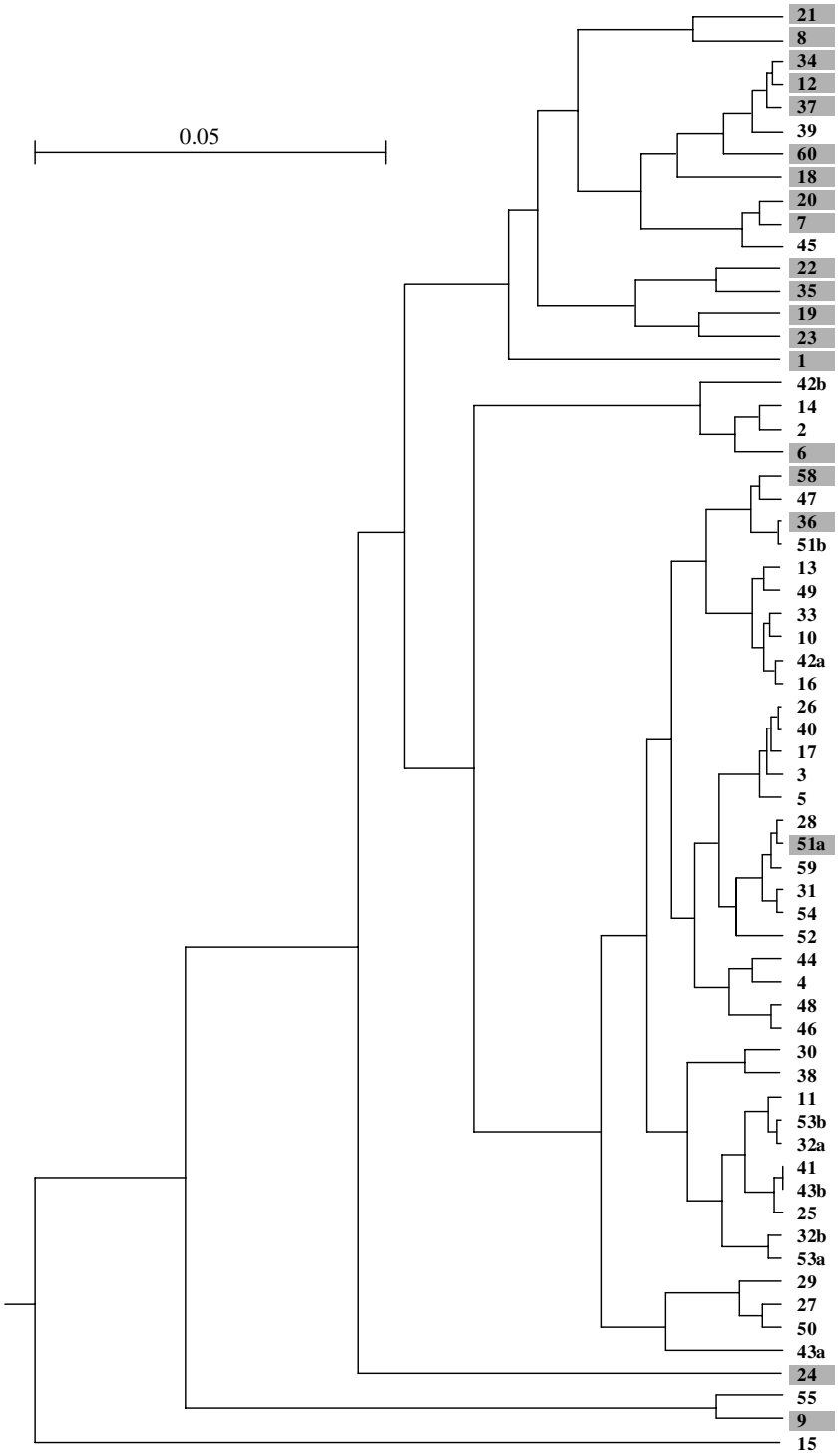

Fig. 3 UPGMA phenogram for all populations (*Idh*, *Pgm*, *Pep-A*). Minus populations are shaded.

sample, 2.52 vs. 1.57 (Mann–Whitney *U*-test: $Z_{26} = 1.990$, $P < 0.05$); nucleotide diversity (π) 0.0016 vs. 0.0002 ($Z_{26} = 2.759$, $P < 0.01$) (Appendix S1, available as web materials). However, due to heterogeneous sample sizes (107 tomentosum vs. 33 minus) and an uneven distribution of haplotype frequencies rare haplotypes will probably not be found in small samples (Archie, 1985). Hence, mtDNA diversity in minus may be underestimated. No differences were observed among geographical regions (independent of host plant).

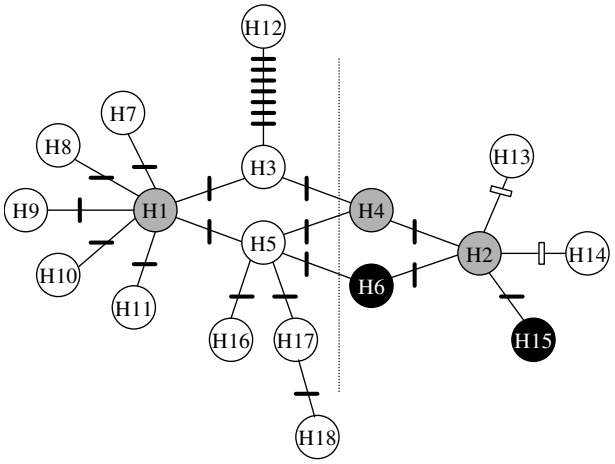

Fig. 4 Minimum spanning network showing the relationships among *Tephritis bardanae* mtDNA haplotypes. Each bar represents one substitution (black bar = transition; open bar = transversion). Black circles represent haplotypes found exclusively in minus populations, shaded haplotypes were found in minus and tomentosum populations. The dashed line separates haplotype cluster (H12 was not assigned to a cluster). H12 could equally well be connected to H10.

Genealogical relationships among haplotypes are shown in a minimum spanning network (Fig. 4). Most haplotypes observed in single individuals radiate from the two common haplotypes H1 and H2. Haplotypes H3–H6 occupy an intermediate position. All haplotypes could be assigned to either H1 (referred to as H1 cluster) or H2 (H2 cluster) except for H12. The latter differs by at least seven substitutions from all other haplotypes and was, therefore, not allocated to a cluster. All haplotypes except for H6 and H15 were found in tomentosum samples, whereas minus flies showed nearly exclusively haplotypes belonging to the H2 cluster. Only the minus population Wipfeld exhibited H1 individuals, again making Wipfeld particular among the minus sites.

The common haplotypes H1 and H2 and their haplotype clusters were distributed significantly heterogeneously among host plants, $P < 0.001$ (Table 2). In contrast, the haplotype distribution between north and south tomentosum populations was homogenous (we could not test for regional effects among minus populations because flies did not emerge from *A. minus* in the south). Thus, the significant haplotype–host plant distribution was not affected by geographical distributions, i.e. a higher H2 frequency in northern populations.

Table 2 Tests for homogeneity of haplotype distributions among host plants and among geographical regions for *Arctium tomentosum* flies showing the observed and expected (in parentheses) numbers of individuals.

	Host plants		Geographical regions	
	A. tomentosum	*A. minus*	North	South
H1 cluster	78 (61.91)	3 (19.09)	30 (32.07)	48 (45.93)
H2 cluster	29 (45.09)	30 (13.91)	14 (11.93)	15 (17.07)
χ^2 (df = 1)	42.11 ($P < 0.001$)		0.84 ($P = 0.36$)	

Level of significance does not change when haplotype distributions of H1 and H2 are tested separately (among host plants $P < 0.001$, among regions $P = 0.26$).

Morphometric variation

Due to sex-related differences, wings of males and females were analysed separately. Wing length of female *T. bardanae* was on average 6.7% greater than in males (mean 406.28 and 380.66, respectively; $t_{994} = 18.407$, $P < 0.001$). Size dimorphism was consistent for both host plants, regions and over all years; no two-way interactions could be observed (sex × host: $F_{1,925} = 1.005$, n.s.; sex × region: $F_{1,925} = 0.024$, n.s.; sex × year: $F_{1,921} = 0.591$, n.s.). The results below refer to females, but hold also for males. We could not pool data over years, because interaction terms were significant (year × region: $F_{3,452} = 3.657$, $P < 0.05$; year × host: $F_{3,452} = 3.761$, $P < 0.05$). As a consequence, two-way ANOVA (host × region) could not be performed (populations of both host species were not present in each year and region).

In each year the average wing length of minus flies was larger than for tomentosum flies (Fig. 5). This difference was significant in 1989 ($t_{129} = -4.920$, $P < 0.001$), 1999 ($t_{123} = -2.833$, $P < 0.01$) and 2000 ($t_{149} = -7.254$, $P < 0.001$), but not in 1990 ($t_{51} = -0.472$, n.s.). The nonsignificance of the latter year might be explained by a lack of statistical power as only 12 minus flies from one population could be measured.

There was significant wing size variation among years ($F_{487} = 9.900$, $P < 0.001$). Particularly the wing length of minus flies differed (Fig. 5). Small sample size (1990) and

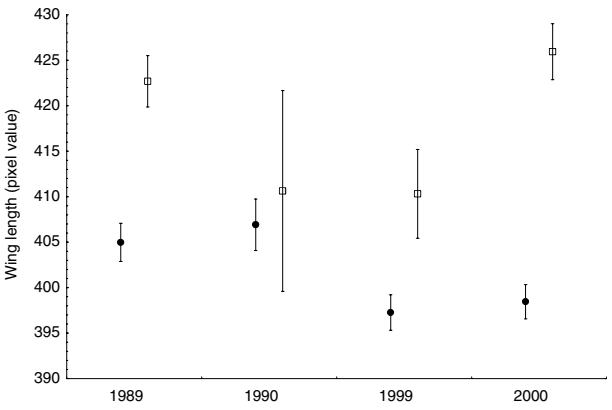

Fig. 5 Mean wing length (±standard error) for each year and host plant. Filled circles and open squares represent *Arctium tomentosum* and *Arctium minus* populations, respectively.

Table 3 Mean female wing length (pixel value) of *Tephritis bardanae* for each year and region (standard deviation in parentheses).

	Arctium to mentosum		*Arctium minus*	
	Wing length	*n*	Wing length	*n*
1989				
North	–	–	430.10 (18.12)	25
South	404.97 (16.38)	61	418.56 (25.38)	45
All	404.97 (16.38)	61	422.68 (23.58)	70
1990				
North	407.50 (19.83)	29	410.63 (38.23)	12
South	405.50 (13.82)	12	–	–
All	406.92 (18.13)	41	410.63 (38.23)	12
1999				
North	411.72 (15.60)	21	427.49 (14.58)	14
South	393.42 (18.75)	79	388.44 (14.60)	11
All	397.26 (19.55)	100	410.31 (24.40)	25
2000				
North	398.63 (21.30)	57	425.94 (18.46)	36
South	398.28 (19.34)	58	–	–
All	398.45 (20.25)	115	425.94 (18.46)	36
All years				
North	403.60 (20.50)	107	425.27 (22.07)	87
South	398.81 (18.57)	210	412.64 (26.45)	56
All	400.43 (19.35)	317	420.33 (24.58)	143

n, number of flies.

outlier populations (1999) might cause this. The latter year contains the population with the smallest wings of all minus sites, namely Wipfeld. This population is also a 'nontypical' minus population with respect to allozymes and mtDNA. Exclusion of Wipfeld increases the mean wing length of minus populations in 1999 from 410.30 to 427.49.

The influence of geographical region on wing size is less clear. Separate analyses for each year and host plant gave inconsistent results (Table 3). Tomentosum populations did not differ among regions in 1990 and 2000 ($t_{39} = -0.318$, n.s. and $t_{113} = -0.091$, n.s., respectively), but wing size was significantly larger in the north in 1999 ($t_{98} = -4.105$, $P < 0.001$). For the two years 1989 and 1999, where data are available for minus populations in the north, northern minus populations had larger wings than southern ones ($t_{68} = -2.004$, $P < 0.05$ and $t_{23} = -6.645$, $P < 0.001$, respectively). However, in 1999 minus in the south consist only of the population Wipfeld.

Discussion

Genetic variation

Allozyme frequencies and mtDNA haplotype distributions revealed significant associations to host plant but not to geographical location. Genetic differentiation between minus and tomentosum flies seemed to be solely a consequence of affiliation to specific hosts and this affiliation was repeated among samples collected 10 years apart.

The degree of differentiation among host races was much greater for mtDNA than for allozymes. The heterogenous distribution of mtDNA haplotypes suggests that gene flow is restricted. Assuming that the sexes have equal effective population sizes, mtDNA is expected to have fourfold smaller effective population size than allozymes (Birky *et al.*, 1983). Consequently, mtDNA should be more sensitive to the effects of genetic drift and lineage sorting. This may result in population differentiation in mtDNA markers, while allozyme markers do not reveal population structure (Piel & Nutt, 2000). As most base substitutions were silent, it seems unlikely that selection of mtDNA phenotypes has caused host-race differentiation, although it cannot be ruled out that mechanistically neutral mtDNA variants are linked to selected mtDNA mutations (Avise *et al.*, 1987).

Lehr (1994) could show host preferences for mating and oviposition sites in *T. bardanae* (defined as 'host fidelity' by Feder *et al.*, 1998), but did not examine whether sexes differ in their host fidelity. If host fidelity is high in females, *T. bardanae* would oviposite mostly in the host species of origin. Consequently, there would be nearly no gene flow between host races for the maternally inherited mitochondrial genomes. If the host fidelity of males is lower, they may partly mate on the alternate host. Therefore, migrating males would produce gene flow between host-races preventing differentiation of nuclear loci. Indeed, Itami *et al.* (1998) detected higher host fidelity in females than in males in *Eurosta solidaginis*. This was suggested to be caused by a sex-influenced gene (Craig *et al.*, 2001).

The underlying mechanism of allozyme differentiation might be different from that of mtDNA. Differentiation at two of five loci, *Pep-A* and *Pgd*, is likely not caused by genetic drift alone as all five loci should be affected to some extent. Reanalysis of Eber *et al.*'s (1991) allozyme data showed here that *Pep-A* and its 91 allele were the causes of host-related clustering [the locus/alleles causing clustering were not quantified (*Pgd* was not scored)]. The variance heterogeneity among loci implies that natural selection is acting to differentiate certain loci (Slatkin, 1987). Certain alleles of *Pep-A* and *Pgd* could increase (or decrease, respectively) fitness of flies, thereby being subjected to natural selection. Alternatively, the *Pep-A* and *Pgd* alleles themselves might be selectively neutral, but linked to genes under selection. Feder *et al.* (1993, 1997) found evidence that particular allozyme alleles are correlated with an early eclosion of *Rhagoletis pomonella* pupae, implying that selection on eclosion time changes allele frequencies of linked allozymes as a by-product. If *Pep-A* and *Pgd* are linked to fitness genes, the frequency of alleles defining host-races will differ somewhat randomly depending on the degree of linkage to the fitness genes.

Morphological variation

Tephritis bardanae of both sexes differed in wing length relative to host-plant affiliation where minus flies were larger than tomentosum flies. However, the degree to which geographical location influences wing length within either host-race remains uncertain. Unequal sampling of, and emergence from, the ephemeral host-plants prevented interaction tests of host and region on a yearly basis. Mean wing length differed in both host-races among years, but there was a tendency in both host races to have larger wings in the north. Latitudinal size increase is common in insects (e.g. Pfriem, 1983; Coyne & Beecham, 1987) and likely has a hereditary background (van't Land *et al.*, 1999; Loeschcke *et al.*, 1999, 2000). Temperature is discussed to be the pivotal factor, as it is associated with latitude and is known to influence adult size traits in many insects (Atkinson, 1994). Unfortunately, we have no microclimatic data for our sample sites to test whether size differences result from temperature effects. If microclimatic variation partly covers up a slight overall temperature gradient between north and south populations, this could explain ambiguous morphological results.

Geographical location could not explain differences in wing length *between* host races without latitudinal temperature variation. These size differences might either reflect phenotypic plasticity or genotypic variability, or both. Host plants provide habitat and food for *T. bardanae* larvae, thereby affecting larval development. Larval growth can be influenced by nutrition value (Stoyenoff *et al.*, 1994), amount of secondary metabolites (Roitberg & Isman, 1992) or time of larval development, which is host related because of the necessary synchronization of larval stages with particular flower head stages. Thus, host-related differentiation of morphological traits can be merely host plant induced without a genetic basis (Gillham & Claridge, 1994). In that case, the morphological data would not provide evidence for host-race formation in *T. bardanae*.

However, in flower head-attacking tephritids, it is assumed that body size is under strong selection because oviscape and ovipositor lengths relate to the dimensions of flower heads when these are suitable for oviposition (Zwölfer, 1987). Straw (1989) found that due to larvae requirements, *A. minus* flower heads had to be in a particular developmental stage for *T. bardanae* to oviposite and that *T. bardanae* of suboptimal size cannot exploit flower heads ideally. Therefore, there is likely strong selection on body size. Although selection might have stopped acting on this trait in tomentosum flies (due to oviposition into the stem), this argument still applies to minus flies. If indeed adaptive selection is acting on size in *T. bardanae*, the observed host-related differentiation must have a genetic basis. In that case, the morphological data have to be interpreted as evidence for the existence of host races.

It is notable in this context that the population Wipfeld, the 'nontypical' minus population, shows not only allozyme frequencies and mtDNA haplotypes characteristic for tomentosum flies, but also consists of the smallest flies among minus populations. We may assume that this minus site was colonized by flies of the tomentosum host race. Erroneous oviposition could result from competition for mating sites or, more likely, imperfect host fidelity caused by the absence of the 'correct' host (Craig *et al.*, 1993). The *A. minus* site in Wipfeld might have been an *A. tomentosum* site in former years (*A. minus* was not observed the following year). *Arctium* plants are ephemeral and die after flowering. Sites are furthermore often subjected to anthropogenic disturbance (e.g. mowing). If the analysed flies from Wipfeld belong to the tomentosum host race, this may be seen as a further hint for a genetic basis of wing length differences among host races. However, to which degree the morphological differences between host races are genetically determined, remains speculative until controlled rearing experiments have been carried out.

Implications for host-race formation in *T. bardanae*

Dres & Mallet (2002) propose four important criteria which have to be fulfilled in order to apply the term 'host race': (i) host association and fidelity, (ii) sympatry, (iii) genetic differentiation and (iv) some gene flow. Evidence for host association and fidelity is provided by Lehr (1994). The second criterion of sympatry is also clearly met in *T. bardanae*. While the cause of morphometric differentiation remains uncertain for the time being, significant and consistent host-related genetic differentiation could be shown for allozymes and mtDNA in this study, providing evidence for the third criterion. However, the level of differentiation is relatively low: there were no private alleles, only two allozyme loci showed host-related differentiation and the frequency differences of those two loci were small compared with other host-race systems (e.g. Itami *et al.*, 1998; Berlocher, 1999; Via, 1999; Emelianov *et al.*, 2001). This indicates that the *T. bardanae* system is in an early stage of divergence. The crucial question is whether the low level of differentiation is due to lack of time to diverge or due to high levels of gene flow. If the host shift occurred recently, there might have been too little time for genetic drift to act on all loci. In this case, *T. bardanae* would provide the opportunity to study the early stages in a speciation process. Alternatively, the system could be either young or old, but high levels of gene flow would prevent much differentiation of neutral loci anyway. The maintenance of genetic distinctness in spite of high levels of gene flow would indicate that host races can be stable in sympatry, although the level of reproductive isolation is low. However, to answer this question the degree of gene flow has to be estimated. As *Nm* is not a suitable gene flow estimator in speciation studies (Mallet, 2001; Dres &

Mallet, 2002), further studies analysing gene flow (e.g. via mark-recapture or observation of mating behaviour) have to be conducted to clarify *T. bardanae*'s position in the continuous process of speciation.

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Supplementary material

The following material is available at http://www.blackwellpublishing.com/products/journals/suppmat/JEB/JEB637/JEB637sm.htm

Appendix A1: Locality names, sample size and morphological/genetical variability measures.

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