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Genetic structure of *Cirsium palustre* (Asteraceae) and its role in host diversification of *Tephritis conura* (Diptera: Tephritidae)

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Whether or not a host plant is incorporated into a phytophagous insect's diet depends on both the insect's ability to colonize the novel host and the host plant's susceptibility to the insect. The latter, again, will be influenced by the genetic structure of the host plant. *Cirsium palustre* (marsh thistle) is heavily infested by the tephritid fly *Tephritis conura* in northern Britain, whereas infestation is not only absent in southern England, but also absent on the European continent where *T. conura* is common on other *Cirsium*. To understand why regional infestation patterns evolve, we studied how genetic structure and phylogeographic ancestry of *C. palustre* are related to the probability of infestation in Britain. The probability of infestation in Britain was related neither to phylogeographic lineage (two lineages were observed in Europe), nor to introgressive hybridization with the original host of *T. conura*, *C. heterophyllum*. However, infested British *C. palustre* were strongly genetically deprived. British *C. palustre* showed continuous loss of genetic variability towards the northern range edge. The loss was explained best by genetic drift during geographic range expansion, rather than by genetic drift in isolated patches or mating system change from predominately outcrossing to selfing. The phylogeographic pattern of *C. palustre* strongly suggests Scotland as the geographic origin of a recent host-plant expansion of *T. conura*. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, **95**, 221–232.

ADDITIONAL KEYWORDS: British Isles – *Cirsium heterophyllum* – co-evolution – host plant – host race – ITS – microsatellites – peripatric speciation – phylogeography – range edge – sympatric speciation.

INTRODUCTION

Utilization of novel host plants by phytophagous insects involves adaptations to host plants and, conversely, plant responses to the insect. These assertions are linked by what makes it possible for an insect to survive and evolve on host plants and what makes the plants susceptible to attack. The latter will be influenced by genetic variance among host plants (Fritz, 1995; Cronin & Abrahamson, 1999; Nuismer & Thompson, 2001; Fritz *et al.*, 2003), suggesting that geographic genetic divergence of host plants creates an innate liability for different infestation levels and host range hierarchies of the associated insects (Thompson, 2005). For example, it is well known that isolated and patchily distributed plants at range borders often experience higher frequencies of selfing (Stebbins, 1957; Barrett & Shore, 1987; Busch, 2005) and clonality (Eckert, 2002). Although isolation and selfing do not *per se* lead to new infestations, they will alter the genetic constitution of plants by increasing inbreeding, which, in some instances, may enhance susceptibility to infestation (Stephenson *et al.*, 2004; Hull-Sanders & Eubanks, 2005; Koslow & DeAngelis, 2006). Plants with clonal propagation in peripheral environments often retain outcrossing (Schaal & Leverich, 1996; Vallejo-Marin & O'Brien, 2007) but,

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by increasing clonally, intra-locality genetic variance may be reduced. Interestingly, herbivory may itself influence a plant's breeding system and increase inbreeding (Hayes, Winsor & Stephenson, 2004; Ivey & Carr, 2005). This may lead to feedback loops that change the probability of infestation (Carr & Eubanks, 2002).

The oligophagous tephritid fly *Tephritis conura* (Loew, 1844) is reported to parasitize nine to ten *Cirsium* (Asteraceae) species but the geographical infestation pattern differs significantly among potential hosts (Zwölfer, 1988; Romstöck-Völkl, 1997). For example, although *Cirsium oleraceum* and *Cirsium heterophyllum* are attacked over their entire distribution range and harbour host races of *T. conura*, *T. conura* in other incidences probably exploits *Cirsium* species on a temporary basis only. The marsh thistle *Cirsium palustre* (L.) Scop. provides an exceptional example of how infestation patterns differ consistently on a regional scale. *Cirsium palustre* is highly attacked by *T. conura* in Scotland where it occurs with the original host *C. heterophyllum* (Romstöck & Arnold, 1987). Genetic analyses of *T. conura* have confirmed that it in Scotland belongs to the *C. heterophyllum* host race (Komma, 1990; T. Diegisser, C. Tritsch, A. Seitz & J. Johannesen, unpublished data). By contrast, infestation is almost absent in southern England, where *C. heterophyllum* is missing, but also absent on the European continent in areas of sympatry with *C. heterophyllum* and *C. oleraceum* (H. Zwölfer, pers. comm.). The disjunct infestation pattern makes the *C. palustre–T. conura* interaction promising for studying host range expansion processes in plant–insect systems. Immediate questions include whether local infestation in Scotland is caused by *T. conura* adapted to *C. palustre* specific to Scotland, thereby hindering the spread further south, or by an only recent host range expansion where *T. conura* has not had enough time to colonize southern English *C. palustre* populations? Also, if infestation of *C. palustre* in Scotland is merely a consequence of co-occurrence with *C. heterophyllum* then why is it not infested in the European Alps where both species co-occur and *T. conura* is abundant on *C. heterophyllum*?

In the present study, we focused on whether infestation of *C. palustre* by *T. conura* in northern Britain is associated with the genetic structure and ancestry of *C. palustre*. To understand how local infestation patterns evolve, we compared infestation of western European *C. palustre* populations, and genetic parameters of infested and non-infested populations. We studied four genetic scenarios: (1) mating system modification; (2) genetic structure; (3) phylogeographic history; and (4) hybridization. Although common in Scotland, *C. palustre* occurs at a range border where the distribution and amount of genetic diversity may differ to more central distribution areas. *Cirsium palustre* is generally outcrossing (van Leeuwen, 1981) but it is also self-compatible (Mogford, 1974), and high levels of selfing have been reported when insect pollinators are scarce (Hagerup, 1951, cited in Mogford, 1974). We therefore hypothesized that the mating system should shift towards selfing, involving departures from Hardy-Weinberg proportions and linkage disequilibria in local populations. Alternatively, random mating may persist but the genetic structure may change, with consequences for genetic diversity. Genetic drift may reduce diversity in isolated patches with interrupted gene flow or due to repeated founder events during range expansion. The magnitude of between-population genetic variance in these two scenarios is difficult to predict because this depends on the mode of gene flow, time subsequent to population fission and effective population sizes (Slatkin, 1977), and such parameters are typically unknown in natural populations. However, if isolation without gene flow dominates, the probability that genetic variance is unrelated to geographic distance between patches is higher than when it is caused by inbreeding propagules in a moving front. The origin of infestation in Scotland could also lie in phylogeographic diversification of *C. palustre*. This scenario may involve the presence of two immigrant lineages in Britain (infested and non-infested) or *de novo* evolution of an infested lineage unique to Britain. Recent evidence suggests that parts of England or adjacent areas may have sheltered refugia during the last ice age (Sinclair, Morman & Ennos, 1998, 1999; Steward & Lister, 2001; Hänfling *et al.*, 2002). Indeed, Steinert (1992) found Scottish *C. palustre* populations to be diverged in allozyme frequencies from continental European populations. Finally, we considered the possibility that infestation of *C. palustre* was mediated by introgressive hybridization with *C. heterophyllum*. Introgression changes the genetic makeup of species, which may create a genetic 'bridge' between the hybridizing plants and mediate the transfer of insects from one parental plant to the other (Floate & Whitman, 1993). Although there is little evidence for insect host-shifts mediated by introgressive genomes (Pilson, 1999), *T. conura* may successfully infest *Cirsium* hybrids (Diegisser, Seitz & Johannesen, 2007). Hybridization with *C. heterophyllum* is likely rare and first generation hybrids are easily identified (Stöhr, 2006). However, signs of past hybridization might not be visible in morphological *C. palustre*. In the present, we restricted an introgression test to one syntopic locality in Scotland, but we further evaluated the likelihood of hybridization by comparing allele frequencies of Scottish *C. heterophyllum* populations

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using data provided by Dr A. Jump (Jump, Woodward & Burke, 2003).

MATERIAL AND METHODS

Host plant distribution and sampling

Cirsium palustre (L.) Scop. ($2n = 34$, Bureš *et al.*, 2004) is widely distributed in Europe, occurring from northwest Spain to western Russia. The Apennine Mountains in Italy and the northern Balkans mark the southern distribution limits; the northern limit is central Fennoscandinavia (Meusel & Jäger, 1992). It is found from sea level to 2000 m a.s.l. In Britain, the density of *C. palustre* generally increases towards the north (Romstöck & Arnold, 1987).

Cirsium palustre was sampled in Britain and western continental Europe between 26 June and 17 July in 2004 and 2005. All encountered plants were unambiguously identified as *C. palustre* (i.e. no intermediate forms indicative of first or early generation hybrids were observed). British populations were sampled approximately 1 week later than continental populations because plant phenology and the emergence of *T. conura* are delayed by approximately 1 week compared to continental Europe (Romstöck & Arnold, 1987). Inference of infestation levels was gained from 24 British and 19 continental European *C. palustre* populations (Table 1). Plants were sampled when *T. conura* had pupated; thus, flower heads could be transported back to the laboratory without increasing fly mortality. At each site, approximately 100 apical flower heads were collected from several plants and placed in darkened plastic bottles with a hole in the lid covered by a glass bottle. Emerging flies were attracted by the light and trapped in the bottle. Adult flies were collected on a daily basis. The assessment of infestation was only aimed at judging the presence or absence of infestation. Therefore, apical shoots, which are parasitized first, were preferably sampled. Plants from both regions were sampled in areas with and without *C. heterophyllum*. For genetic analyses, 17 *C. palustre* populations, comprising nine populations from Britain and eight from continental Western Europe, were included (Fig. 1, Table 1). Leaf material from individual plants was placed in paper folders sorted by population, and stored in airtight plastic bags filled with silica gel until DNA extraction.

DNA isolation, microsatellite, and ITS procedure

Dried leaf tissue (1–3 cm^2) was ground to a fine white powder in Eppendorf tubes immersed in liquid nitrogen. DNA was extracted from the powder with the 'DNeasy-Plant-Mini Kit' from Qiagen using the standard protocol. Ten individuals of *C. palustre* per population (except Er4, six individuals) were genotyped for seven loci isolated from *Cirsium acaule* (Jump *et al.*, 2002). Polymerase chain reaction (PCR) programs reported for *C. acaule* (Jump *et al.*, 2002) were fitted to *C. palustre*: *Caca01* (primer concentration: 10 pmol μl^{-1}, program 55); *Caca04* (3 pmol μl^{-1}, 65/55TD); *Caca05* (3 pmol μl^{-1}, 65/55TD); *Caca10* (4 pmol μl^{-1}, 65/55TD); *Caca16* (4 pmol μl^{-1}, 64); *Caca22* (4 pmol μl^{-1}, 64); and *Caca24* (10 pmol μl^{-1}, 60). Amplification was performed in PuReTaq Ready-to-Go 96-well PCR beads (GE Healthcare Bio-Sciences) with 1 µl of DNA and 1 µl of primer with an end volume of 25 µl (LiCrosolvwater). Each DNA amplification product was added to 0.4 µl of GeneScan-500 ROX-size standard. Twenty *C. heterophyllum* from the population Sc8 were genotyped for five of the above loci (*Caca01*; *Caca04*; *Caca05*; *Caca10*; *Caca16*). The PCR was performed with the GenAmp PCR System 9600 (Perkin-Elmer). Genotypes were analysed on an ABI 377 Sequencer with Gene Scan 2.1 (Perkin-Elmer) and scored with Genotyper 2.0 (Perkin-Elmer).

ITS sequences were amplified via PCR in an end volume of 25 µl, consisting of 1 µl of forward and backward primer (10 pmol μl^{-1}), 1 µl of genomic DNA and 22 µl of LiCrosolv water. PCR reactions were performed with PuReTaq Ready-to-Go beads (0.5 mL tubes). Primers ITS1 (Urbatsch, Baldwin & Donoghue, 2000) and ITS4 (White *et al.*, 1990) were used to amplify the ITS region (ITS-1, 5.8S RNA, ITS-2) using the PCR amplification protocol described by Kelch & Baldwin (2002). PCR products were sequenced using an ABI-377 automatic sequencer. Sequences were aligned and edited using the program Sequence Navigator (ABI).

Statistical analysis

The principle aim of this study was to characterize genetic diversity within and among *C. palustre* populations to test whether genetic parameters of *C. palustre* are related to the probability of infestation by *T. conura*. We partitioned the genetic analyses in to three analysis-sets that study: (1) genetic diversity; (2) within-population structure (breeding structure); and (3) among-population genetic structure, including the genetic ancestry of populations. Genetic variation within populations was quantified by analysing allelic richness (alleles per locus per population) and the unbiased expected heterozygosity with BIOSYS-2 (Swofford & Selander, 1989). Breeding structure was quantified by testing for Hardy–Weinberg (HW) and linkage disequilibrium (LD) (Genepop, version 3.4; Raymond & Rousset, 1995), and identical multilocus

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Table 1. Sample locations of *Cirsium palustre* in western Europe

	Locality abbreviation	Country	Locality	Coordinates		Altitude (m)	Date of collection	Emergence of *Tephritis conura*
				Latitude	Longitude			
1	**Er2**	Germany	Frauenstein	50°48′N	13°30′E	641	28.06.2004	2
2	**Er3**	Germany	Zöblitz	50°39′N	13°14′E	634	29.06.2004	0
3	**Er4**	Germany	Waldfrieden	50°39′N	13°04′E	436	29.06.2004	0
4	**Fi1**	Germany	Bischofsgrün	50°05′N	11°57′E	630	29.06.2004	8
5	**RP1**	Germany	Ober-Olmer Wald	49°56′N	08°11′E	151	06.07.2007	0
6	**RP2**	Germany	Erbeskopf	49°43′N	07°05′E	600	26.06.2004	0
7	**A1**	Austria	Gries	47°04′N	10°58′E	1359	07.07.2004	0
8	A2	Austria	Infang	46°56′N	11°00′E	1745	07.07.2004	0
9	A3	Austria	44bis	46°52′N	11°09′E	1597	08.07.2004	0
10	A4	Austria	Ischgl	46°59′N	10°16′E	1392	09.07.2004	0
11	A5	Austria	Kaunertal	47°01′N	10°44′E	1320	09.07.2004	1
12	A6	Austria	Silian111	46°42′N	12°34′E	1438	11.07.2004	0
13	**Pyr1**	France	Mounicou	42°42′N	01°27′E	–	14.07.2005	9
14	Pyr2	France	Lac de Soulcan	–	–	–	14.07.2005	1
15	Pyr3	France	Suc-et-Sentenac	42°47′N	01°27′E	–	14.07.2005	0
16	Pyr7	France	Le Mourtis	43°00′N	00°48′E	–	14.07.2005	0
17	MC1	France	Aubrac	44°36′N	02°58′E	–	15.07.2005	0
18	**MC2**	France	Aubrac	44°36′N	02°58′E	–	15.07.2005	0
19	**En3a**	England	Hertford	51°47′N	00°06′W	40	12.07.2005	0
20	**En3b**	England	Hertford	51°47′N	00°07′W	54	12.07.2005	0
21	En4	England	Wolvercote	51°47′N	01°17′W	58	12.07.2005	0
22	**En1**	England	Broadwas	52°11′N	02°26′W	151	12.07.2004	0
23	En5	England	Wye Forest	52°23′N	02°24′W	80	13.07.2005	0
24	En6	England	Coalport	52°36′N	02°27′W	109	14.07.2005	0
25	**En7**	England	Uttoxeter	52°54′N	01°51′W	75	14.07.2005	0
26	En8	England	Carsington Water	53°02′N	01°37′W	207	14.07.2005	0
27	En11	England	Buxton	53°16′N	01°56′W	436	15.07.2005	12
28	**En12**	England	Comberbach	53°17′N	02°31′W	45	15.07.2005	3
29	En13	England	Clough	53°47′N	01°53′W	322	15.07.2005	0
30	**En14**	England	Starbottom	54°09′N	02°04′W	224	16.07.2005	0
31	En15	England	Haile	54°27′N	03°29′W	64	17.07.2005	0
32	En2	England	Bowes	54°30′N	02°03′W	–	13.07.2004	13
33	En16	England	Moorsholm	54°30′N	00°57′W	215	17.07.2005	9
34	Sc1	Scotland	Littlemill	55°23′N	04°29′W	221	07.07.2004	8
35	**Sc2**	Scotland	Skares	55°27′N	04°19′W	234	07.07.2004	36
36	Sc3	Scotland	Lugar/Cabellow	55°30′N	04°08′W	228	07.07.2004	16
37	Sc4	Scotland	Clifton	56°27′N	04°42′W	315	08.07.2004	–
38	**Sc5**	Scotland	Tyndrum	56°31′N	04°42′W	265	08.07.2004	61
39	Sc6	Scotland	Loch Tay/Lawers	56°31′N	04°09′W	215	09.07.2004	42
40	Sc7	Scotland	Tullimet	56°39′N	03°38′W	176	10.07.2004	36
41	**Sc8**	Scotland	Inver/Trochry	56°33′N	03°37′W	182	11.07.2004	68
42	Sc9	Scotland	Amulree/Crieff	56°27′N	03°47′W	215	11.07.2004	166
43	**Wa1**	Wales	Pen y Gwryd	53°04′N	04°00′W	274	12.07.2004	98

–, no data. Locations in bold were analysed genetically.

genotypes as an estimate of clonality. The number of expected identical multilocus genotypes per population was calculated as sample size multiplied by the expected frequency of each genotype under random mating. Among-population differentiation was tested at different levels of organization. First, among-population differentiation, F_{ST}, was quantified for all populations and for British and continental populations separately. Second, hierarchical analyses of genetic variance were applied to test whether genetic

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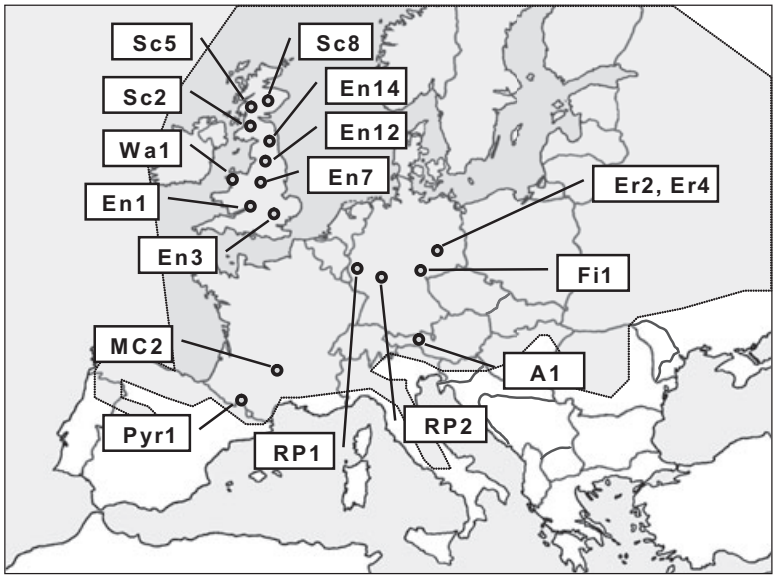

Figure 1. Sample locations of *Cirsium palustre* for population genetic analyses. Full locality names are presented in Table 1.

variance differed between: (1) British versus Continental European *C. palustre*; (2) parasitized versus nonparasitized *C. palustre* (total sample); and (3) parasitized versus nonparasitized *C. palustre* in Britain. The total among-population variance, F_{ST}, was partitioned into variance that can be assigned to region, F_{RT}, or parasite susceptibility, F_{PT}, and region/parasite-independent variance (F_{SR} or F_{SP}). Hierarchical analyses of genetic variance were performed with ARLEQUIN, version 3.11 (Excoffier, Laval & Schneider, 2005) using frequency based F_{ST} estimates (Weir & Cockerham, 1984), and ordered based R_{ST} estimates (Slatkin, 1995).

Phylogenetic ancestry of *C. palustre* populations was calculated assuming a pure drift model with maximum likelihood using the subroutine CONTML in PHYLIP (options: global rearrangements allowed, random addition of taxa, jumble = 100, random population as grouping taxon) (Felsenstein, 1993). Significant branching patterns were estimated by 95% confidence intervals (CIs) on branch lengths.

To analyse the effect of gene flow and genetic drift on population structure, isolation by distance (IBD) tests were performed within phylogenetic relevant clades identified above. IBD tests were performed on British and continental populations separately by comparing the observed spearman rank correlation coefficient between genetic distance ($F_{ST}/1 - F_{ST}$) and geographic distance (km) to 1000 random correlation coefficients (Mantel test in GENEPOP, version 3.4). Significance was estimated by one-tailed *P*-values.

The geographic change of within-population genetic variability was analysed by regressing mean allele number/locus/population and mean expected heterozygosity/population on latitude and longitude, respectively. The level of within-population genetic variability between regions was tested with Student's *t*-test. Analyses were done performed using JMP (SAS Institute, 1995).

Inference for introgression with *C. heterophyllum* was tested in the Scottish population Sc8. This syntopic population was chosen because both host plants were present in large numbers. Twenty *C. heterophyllum* plants were sampled throughout the location with a minimum distance of 2 m between plants. Admixture analysis was performed with STRUCTURE, version 2.1 (Pritchard, Stephens & Donnolly, 2000) using the admixture model and assuming $K = 2$ populations (burn-in = 10 000; iterations = 100 000).

RESULTS

INFESTATION BY *T. CONURA*

Table 1 presents infestation numbers of *T. conura* in 43 *C. palustre* populations in Western Europe. Infestation was high in Scotland, Wales and North England, and virtually absent in southern England and on the continent. In Britain, the geographic area of infestation overlaps with the range of *C. heterophyllum* (Perring & Walters, 1962). By contrast, only 11 individuals emerged in 13 German and Austrian *C. palustre* populations that occurred in *C. heterophyllum* habitat (Table 1) where *T. conura* is common (Diegisser, Johannesen & Seitz, 2006a; Diegisser, Seitz & Johannesen, 2006b). Thus, lack of infestation

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Table 2. Genetic differentiation among *Cirsium palustre* populations of all populations (Total level), between and within regions (Britain versus continental Europe) and *Tephritis conura* infestation (parasitized and nonparasitized)

	Total level		Between regions		Within regions		Between parasitized and nonparasitized	
Hierarchy	F_{ST}	R_{ST}	F_{RT}	R_{RT}	F_{SR}	R_{SR}	F_{PT}	R_{PT}
All populations	0.33	0.50	0.20	0.50	0.25	0.24	(0.03)	(0.12)
Continental Europe	0.28	0.24					(–0.05)	(–0.08)
Britain	0.20	0.25					(0.01)	0.15

All estimates were significantly different from zero except those in parenthesis (nonsignificant).

on the continent cannot be explained by missing *T. conura*.

Variability

The seven investigated microsatellite loci showed 67 alleles (6–17 alleles per locus). The nine British populations displayed seven private alleles; only four were found more than once and only three in more than one population. British populations had significantly less alleles per locus than continental populations (GB = 2.27, continent = 3.46, $t = -4.67$, d.f. = 15, $P < 0.001$) and lower expected heterozygosity per population (GB = 0.25, continent = 0.49, $t = -5.84$, d.f. = 15, $P < 0.001$). Genetic variability in Britain decreased significantly with latitude (allele number A: $t = -5.29$, d.f. = 8, $P = 0.001$, expected heterozygosity H_e: $t = -4.76$, d.f. = 8, $P = 0.002$) and was marginally significant with longitude (A: $t = 2.32$, d.f. = 8, $P = 0.053$, H_e: $t = 2.17$, d.f. = 8, $P = 0.063$) (Fig. 3). There was no effect of longitude and latitude on genetic variability among continental populations (all $P > 0.20$). Lack of significance on continental Europe was not biased by greater distances (compared to within Britain) because regression analyses for central Europe populations (excluding southern France) were also nonsignificant (all $P > 0.25$).

Two populations (En3, Er2) showed slight deviations from HW expectations ($P = 0.04$ and $P = 0.03$, respectively) but significance disappeared after sequential Bonferroni corrections. HW proportions calculated for each locus across populations did not deviate significantly from expectations in Britain or continental Europe. No LD was found for any locus combinations in British populations (data not shown). Among the continental populations, only four out of 122 testable locus combinations showed LD. Because these involved different locus combinations, it was conceded that loci are not linked. Identical multilocus genotypes (genets) were only observed in the six most northern British populations (two to four plants per genet, nine genets in total). These populations were very monomorphic ($0.18 < H_e < 0.26$, $1.57 < A < 2.14$) showing HW expectations and no linkage disequilibria across populations ($N_{individuals} = 60$). In one case, fewer identical multilocus genotypes were found than expected based on random mating (two observed, 3.6 expected); in six incidences, a single plant more was observed than expected (five cases: two observed, 0.4–1 expected; one case: four observed, three expected) and, in two incidences, two more plants were observed than expected (three observed, 0.5–0.7 expected).

Population differentiation

Genetic differentiation calculated locus by locus (analysis of molecular variance) revealed similar levels of among-population differentiation in Britain ($F_{ST} = 0.20$, $R_{ST} = 0.25$) and continental Europe ($F_{ST} = 0.28$, $R_{ST} = 0.24$) (Table 2). Divergence among all 17 populations was influenced by substantial regional differentiation ($F_{RT} = 0.25$, $R_{RT} = 0.24$), which in turn was shaped by locally evolved alleles ($R_{ST} > F_{ST}$). By contrast, no significant substructure caused by infested versus non-infested plants was found among all populations and in continental Europe. In Britain, a slight infestation related substructure was estimated by $R_{PT} = 0.15$ ($P = 0.02$) but not by $F_{PT} = 0.01$ ($P = 0.44$). The discrepancy was due to the combination of isolation by distance (see below) and that infested populations are found only in the north and have little genetic polymorphism.

Isolation by distance

Significant IBD was found among British populations ($P = 0.025$, $y = -0.251 + 0.092x$, $R^2 = 0.17$) and among all continental populations ($P < 0.01$, $y = -0.065 + 0.076x$, $R^2 = 0.14$), and for central continental populations with geographic distances equivalent to those in Britain (French populations omitted, $P = 0.047$, $y = -0.05 + 0.08x$, $R^2 = 0.11$).

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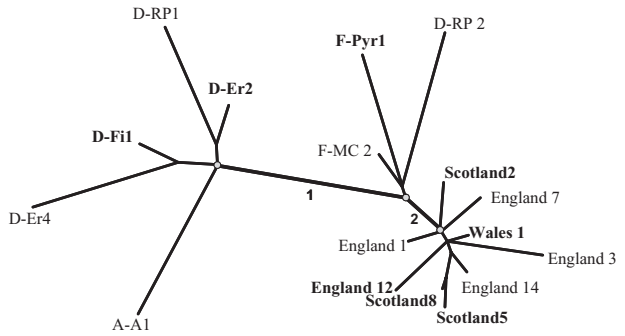

Figure 2. Maximum likelihood analysis of population relationships based on microsatellite allele frequencies showing two significant geographical partitions. Infestation was observed at localities written in bold. Full locality names are presented in Table 1.

Phylogenetic relationships

Maximum likelihood analysis (CONTML in PHYLIP; tree length = 0.48191, Ln log = 1229.21266) divided *C. palustre* populations into two genetic geographically ordered clusters: (1) an eastern group consisting of eastern Germany and the Alps (branch 1, length: 0.06049, 95% CI = 0.03629–0.08938) and (2) a western group consisting southern France and Britain (branch 2, length: 0.01390, 95% CI = 0.00569–0.02369) (Fig. 2). The two western German populations each belonged to one of the groups: the eastern most population to the eastern group and the western most population to the western group. The 95% CI for the branch to southern French and West German RP2 populations was not significantly different from zero. Population RP2 showed allelic affinity both to French and eastern German/Alps populations. Short branch lengths within the British cluster reflected the very little genetic variability in this region (see above).

ITS sequence analysis of *C. palustre*

No ITS sequence variation was found among the 17 plants analysed (one plant per population) (EU143252-68); thus, the analysis was abandoned. The sequences were identical to ITS found in *C. palustre* by Segarra-Moragues *et al.* (2007) (accession number DQ875390).

Introgression with *C. heterophyllum* at site Sc8

Cirsium heterophyllum at Sc8 was highly clonal, exhibiting only two multilocus genotypes (genets) in 20 plants. *Cirsium palustre* and *C. heterophyllum* did not share alleles at any of the five loci analysed in both species. However, for analysis, we pooled two alleles at the locus 05 (allele length 164 in *C. palustre* versus 165 in *C. heterophyllum*) to prevent bias in divergence estimation. There was no sign of introgression: Structure analysis identified individual plants with 99.5% likelihood to the respective species.

DISCUSSION

The infestation of *C. palustre* by *T. conura* showed a profound localized pattern in Scotland and northern England. In the present study, we confirmed a 20-year-old observation that infestation of *C. palustre* is high in Scotland and northern England (Romstöck & Arnold, 1987), and provide the first quantitative data demonstrating that infestation is almost lacking in areas of sympatry with *C. heterophyllum* in Central Europe where *T. conura* is common.

Genetic diversity and breeding system

Infestation in Britain coincided highly with reduced genetic diversity in northern populations. Several processes, including changes in breeding system (from out-crossing to selfing) and genetic drift, the latter either in isolated populations without gene flow or in small propagules during range expansion, may reduce genetic diversity. Selfing in itself does not deplete genetic variation within populations, where genetic diversity is partitioned between individuals (Templeton & Read, 1994), but differential survival of selfed groups will. Selfing may be inferred from a concomitant increase of identical multilocus genotypes, LD and deviations from HW proportions. No populations showed LD or deviated from HW, and only the six northern English and Scottish populations, five of which were infested by *T. conura*, had identical multilocus genotypes. In these six genetically impoverished populations, generally only one more plant was found with identical multilocus genotypes per population than expected. Admittedly, sample sizes of ten plants per population are low and hamper a rigorous test for deciding if this pattern is explained by a general change in mating system or by genetic drift. Continuous selfing is expected to retain variability and increase intrademic variance. This was not observed in *C. palustre*. Instead, British populations experienced a gradual latitudinal loss of genetic diversity coupled with IBD. The reduction of genetic variability was higher for allele number than for heterozygosity, which is expected in repeatedly bottlenecked populations (Nei, Maruyama & Chakraborty, 1975). The data thus imply a loss of variability with some gene flow between patches, entailing genetic depletion during colonization/range expansion as the most parsimonious explanation, rather than mating system change or isolated patches with interrupted gene flow. The assumption is

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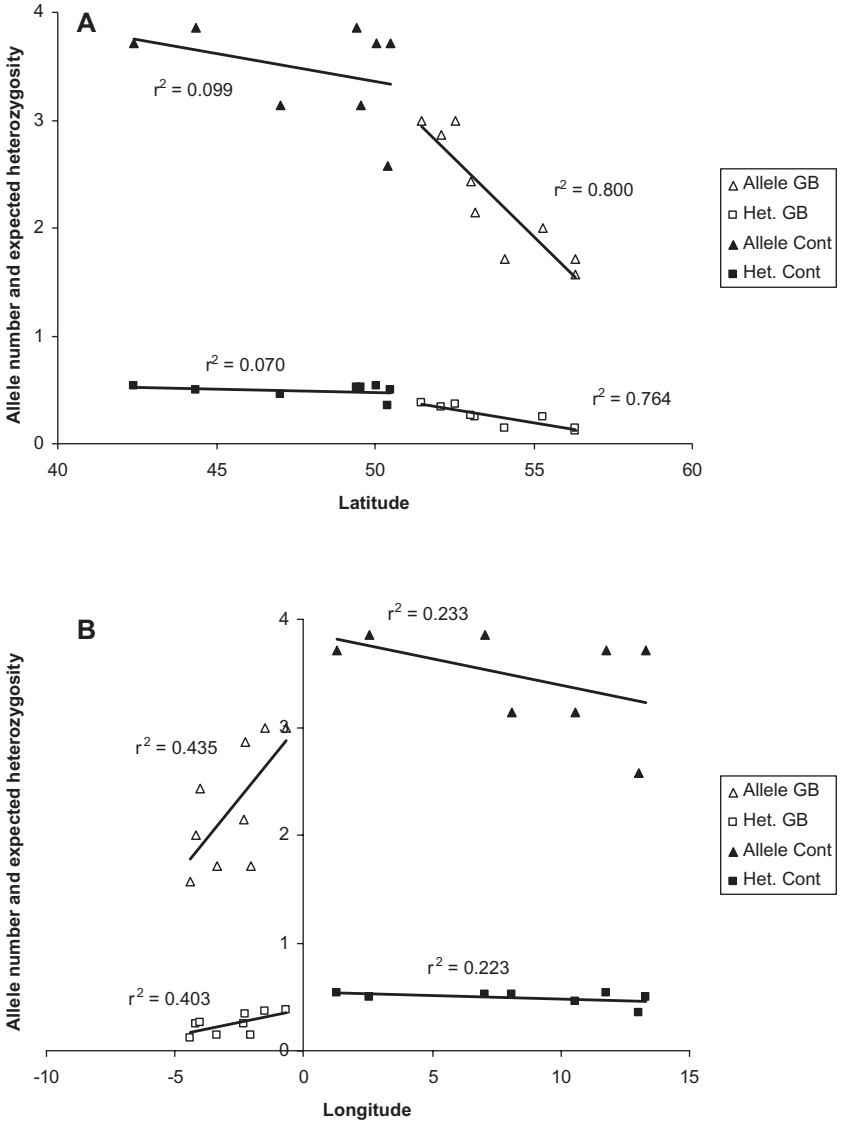

Figure 3. Genetic variability in *Cirsium palustre* in Britain (GB) and continental Europe (Cont) along latitudinal (A) and longitudinal (B) gradients. Het, expected heterozygosity; Allele, mean number of alleles per population. Populations in Britain experience a linear decrease in variability away from continental populations.

supported by an inverse relationship between genetic variability and population density in Britain (Romstöck & Arnold, 1987). The pattern of genetic decline was not related to small populations at range edges but appeared to be related to historical stepwise range expansion, as also observed in *C. acaule* in England (Jump *et al.*, 2003).

PHYLOGEOGRAPHY

Phylogeographic analyses may be used to locate an origin of diversification (Berlocher, 1998; Berlocher & Feder, 2002). In the context of diversifying evolution, this provides a chance to distinguish geographic range expansion from isolation as a major cause for initiating host shifts in phytophagous insects. In the case of *C. palustre*, we investigated whether infestation by *T. conura* followed a trajectory determined by allopatric lineages. Phylogenetic analysis of *C. palustre* revealed two western European lineages. The east–west partition was confirmed using genetic distance analyses (results not shown). All British *C. palustre* belonged to the western lineage, hence non-infested western continental and southern English *C. palustre* belonged to the same lineage as infested British *C. palustre*. Thus, infestation was unrelated to lineage. However, because infestation in Britain coincided with a continuous northward loss of

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allelic diversity, this bias caused significant genetic differentiation between infested and non-infested populations estimated by ordered alleles ($R_{PT} > 0$). An allozyme study of *C. palustre* (Steinert, 1992) indicated phylogeographic divergence between Scottish and central European populations, which was caused by reduced heterozygosity (but not loss of alleles) in Scotland. However, Steinert (1992) did not analyse British populations outside Scotland. Our results imply that distinct Scottish populations are caused by bottlenecked populations within one lineage at a range border. The observations that *C. palustre* is infested only in northern Britain, that *C. palustre* in the present study belongs to a lineage that, outside northern Britain, is not infested, and that *C. palustre* invaded Scotland post-glacially, all provide supportive evidence for local *de novo* host-range expansion.

Diegisser *et al.* (2006b) hypothesized that host-race evolution of *T. conura* on *C. heterophyllum* and *C. oleraceum* was achieved in peripatry, and this may very likely include short phases of sympatry. A localized host shift may, following the extinction or reciprocal loss of ability to use one or the other of two ancestral hosts, be accomplished in geographic separation. The genetic imprint of sympatry under peripatric conditions may be weak if the time for attaining independence is short relative to the time after the event. *Cirsium heterophyllum* and *C. palustre* occur sympatrically in Scotland, but can be thought of as peripatric isolates relative to their main distributions. In this sense, the present study supports the notion that peripatry and sympatry are not mutually exclusive as geographic speciation scenarios but may be part of an continuum in host-race evolution of phytophagous insects (Feder *et al.*, 2003; Diegisser *et al.*, 2006b; Jordal, Emerson & Hewitt, 2006; Michel *et al.*, 2007).

HYBRIDIZATION

In accordance with the rareness of morphological hybrids (A. Jump, pers. comm.; Stöhr, 2006) we found no evidence for hybridization at locality Sc8. This finding was hampered by the high clonality of *C. heterophyllum* at Sc8, which reduced the number of alleles available for detecting introgressive hybridization. Comparisons of allele frequencies of ten Scottish *C. heterophyllum* populations (Jump *et al.*, 2003) with *C. palustre* at six loci used in both studies found allele size overlap but common *C. heterophyllum* alleles did not occur in *C. palustre*. *Cirsium heterophyllum* were far more polymorphic per population ($H_e = 0.432$; $A = 2.80$) than *C. palustre* ($H_e = 0.194$, $A = 1.97$). If introgression were common, the extreme lack of variability in Scottish *C. palustre* is not expected because *C. heterophyllum* retains variability in local stands (Jump *et al.*, 2003); hence, hybridization should raise *C. palustre* variability in a mosaic fashion, which it did not. There was no evidence for admixture of ITS sequences (3% sequence divergence, J. Johannesen, unpubl. data). Thus, the genetic data corroborate the finding that hybrids probably are very rare in Britain and we conclude that there is no general evidence for widespread current or ancestral hybridization between *C. palustre* and *C. heterophyllum* in Scotland that could explain infestation.

CIRSIUM PALUSTRE AND INFESTATION BY *T. CONURA*

Infested Scottish *C. palustre* were genetically deprived but infestation was only observed in areas with *C. heterophyllum*, thus implying that infestation could be merely a by-product of co-occurrence of the two plants. Comparing the infestation pattern in Scotland to that in regions in Germany and Austria where the plants co-occur and *T. conura* is abundant on *C. heterophyllum*, continental populations had virtually no infestation, and infested and non-infested *C. palustre* were genetically similar. From this comparison, the possibility that infestation in Scotland is only a result of co-occurrence with *C. heterophyllum* cannot be excluded but it can be concluded that circumstances allowing infestation are unique to Scotland. Indeed, Scottish flies have changed morphologically by reducing the length of the oviscape (Romstöck & Arnold, 1987; Tritsch, 2005), which can be explained as an adaptation to smaller flower heads of *C. palustre* (Romstöck & Arnold, 1987). By contrast, morphological analyses of *T. conura* from infested continental populations imply that sporadic infestation here is due to egg-laying mistakes by *C. heterophyllum* flies (Tritsch, 2005).

The question remains why Scottish flies on *C. palustre* have not (yet) expanded southwards. There are several possibilities, all which remain speculative and require further study. First, the fragmented population structure in southern England might prevent southward range expansion of *T. conura*. However, dispersal data from continental Europe with similar patchy habitat structure to that of *C. palustre* in England indicate that dispersal propensity should not hinder range expansion (Diegisser *et al.*, 2006a). Second, Romstöck & Arnold (1987) found higher predation rates of *T. conura* on *C. palustre* than on *C. heterophyllum*. They suggested that *C. palustre* act as suboptimal sink populations, such that lasting infestation is only possible in *C. heterophyllum* surroundings. However, suboptimal is a relative measure, and survival may actually be high on *C. palustre* as demonstrated by emergence data. Thus, predation alone appears not to determine the lack of infestation in southern English populations;

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competition with insects found in the south but not in the north could play a role, but data are completely lacking in this respect. Finally, no infestation in southern England could be related to genetic diversity in *C. palustre* that impedes invasion from flies specialized on northern monomorphic (inbred) plants susceptible to herbivory. Comparison of secondary compounds between northern inbred and southern polymorphic plants will shed light on this option. Interestingly, data from *T. conura* might indicate a co-evolutionary feedback loop with *C. palustre* explaining infestation. The 'host-race gene' hexokinase, whose alleles separate host races on *C. oleraceum* and *C. heterophyllum* (Diegisser *et al.*, 2006a), has an allele unique to Scotland (T. Diegisser *et al.*, unpublished). The allele is found in flies on *C. palustre* and *C. heterophyllum* at equal low frequencies (approximately 10%) and may be involved (possibly by linkage) in the host shift. Hexokinase variation in *T. conura* has been related to host preference (Diegisser *et al.*, 2006a) and temperature selection in *Drosophila* (Beckenbach & Prakash, 1977). Selection on this or on a linked locus will change the genetic makeup of *T. conura* in Scotland and may interact with lowered genetic diversity in *C. palustre*.

CONCLUSION

The present study shows that infestation of *C. palustre* by *T. conura* in Scotland coincides with the loss of genetic variability in *C. palustre*. The data obtained cannot explain why *C. palustre* became infested it can reject the idea that infestation is related to historical phylogeographic lineages and hybridization with *C. heterophyllum* and, with high probability, rule out changes in the mating system. By studying plant genetic structure, the present study strongly supports Scotland as the geographic centre of a recent host-plant expansion of *T. conura*, and draws attention to the notion that peripatric and sympatric geographic speciation scenarios are not mutually exclusive in host-race evolution of phytophagous insects.

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