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Genetic differentiation in Scottish populations of the pine beauty moth, *Panolis flammea* (Lepidoptera: Noctuidae)

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Abstract

Pine beauty moth, *Panolis flammea* (Denis & Schiffermüller), is a recent but persistent pest of lodgepole pine plantations in Scotland, but exists naturally at low levels within remnants and plantations of Scots pine. To test whether separate host races occur in lodgepole and Scots pine stands and to examine colonization dynamics, allozyme, randomly amplified polymorphic DNA (RAPD) and mitochondrial variation were screened within a range of Scottish samples. RAPD analysis indicated limited long distance dispersal ($F_{ST} = 0.099$), and significant isolation by distance ($P < 0.05$); but that colonization between more proximate populations was often variable, from extensive to limited exchange. When compared with material from Germany, Scottish samples were found to be more diverse and significantly differentiated for all markers. For mtDNA, two highly divergent groups of haplotypes were evident, one group contained both German and Scottish samples and the other was predominantly Scottish. No genetic differentiation was evident between *P. flammea* populations sampled from different hosts, and no diversity bottleneck was observed in the lodgepole group. Indeed, lodgepole stands appear to have been colonized on multiple occasions from Scots pine sources and neighbouring populations on different hosts are close to panmixia.

Keywords: allozymes, colonization, dispersal, host-shift, mitochondrial DNA, *Panolis flammea*, *Pinus sylvestris*, *Pinus contorta*, RAPD

Introduction

The pine beauty moth, *Panolis flammea* (Denis & Schiffermüller) (Lepidoptera: Noctuidae), is a native British moth that historically has been associated with the native Scots pine, *Pinus sylvestris* L. (Pinaceae) (Stoakley, 1977). The abundance of *P. flammea* on Scots pine is never great enough to cause severe defoliation or tree mortality (Barbour, 1987; Watt & Leather, 1988), and is in contrast to the situation in continental Europe, where *P. flammea* has caused many outbreaks on Scots pine dating back to the 18th century (Schwenke, 1978; Klimetzek, 1979). In more recent times in

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Britain, *P. flammea* has been observed feeding on lodgepole pine, *Pinus contorta* Douglas (Pinaceae), mainly in the Scottish Highlands. Lodgepole pine is an introduced tree species from north-western North America that was planted extensively in the Highlands during the 1950s and 1960s (Lines, 1976). Since 1976, severe outbreaks of *P. flammea* have occurred regularly on this tree species and became so severe that intervention with chemical insecticides has been required (Hicks *et al*., 2001). Indeed, *P. flammea* is considered the United Kingdom's most serious lepidopteran pest of established forests (Day & Leather, 1997). A change in host plant for *P. flammea*, however, also offers the opportunity to examine the issue of potential host race evolution.

The ability of phytophagous insects to switch to a novel host or habitat has been proposed to promote genetic divergence of populations (Jaenike & Holt, 1991; Stone *et al*., 2001; Via, 2001; Dres & Mallet, 2002; Rokas *et al*., 2003), and can lead to incipient sympatric speciation (Emelianov *et al*., 1995; Filchak *et al*., 2000; Abrahamson *et al*., 2001; Dres & Mallet, 2002). Sympatric speciation, where genetic differentiation occurs between populations that are within dispersal distance of each other, has, however, proven difficult to verify (Butlin, 1989; Butlin & Ritchie, 1994), but recent research has challenged the view that species arise by allopatry alone (Dieckmann & Doebeli, 1999; Kondrashov & Kondrashov, 1999; Tregenza & Butlin, 1999; Berlocher & Feder, 2002; Kirkpatrick & Ravigne, 2002). The term host-race has been used to describe partially isolated, conspecific populations that exhibit host-associated adaptations (Dres & Mallet, 2002). They differ from true species by the fact that some interbreeding between populations of the different races is possible. However, host preference is not believed to be the sole cause of sympatric speciation in phytophagous insects (Dres & Mallet, 2002), and it is important to establish whether there has been a reduction in gene flow between populations on different hosts, and to identify potential reproductive isolating mechanisms. Mechanisms of sympatric reproductive isolation for phytophagous insects include differences in the levels of competition, natural enemy attack, plant secondary compounds and host phenology, and all may act to temporally isolate populations (Dres & Mallet, 2002). If reproductive isolation is complete then population genetic theory predicts that mutations will accumulate that differentiate the two host races, and should be detectable by neutral genetic marker analysis. Even if sufficient time has not elapsed for drift to occur or new mutations to accumulate, if the shift to a novel host involves a limited number of founders, genetic bottlenecks should reduce diversity and increase differentiation in the derived host race relative to ancestral populations. However, if the new host has been colonized on multiple independent occasions and/or if gene flow continues between new and old host populations then no difference in genetic diversity or differentiation will be observed, and the likelihood of establishment of separate host races and species is much reduced.

Molecular techniques have proved powerful tools for examining a range of ecological and evolutionary entomological questions (Loxdale & Lushai, 1998), and have resolved host-associated differentiation at the genetic level. For example, investigations of larch and pine races of *Zeiraphera diniana* Guenée (Lepidoptera: Tortricidae) (Emelianov *et al*., 1995) and apple and hawthorn races of *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae) (Filchak *et al*., 2000) using allozyme analysis showed clear genotypic differentiation. The gall-inducing fly, *Eurosta solidaginis* (Fitch) (Diptera: Tephritidae), has genetically differentiated and reproductively isolated host races on two species of goldenrod (*Solidago* sp.) (Abrahamson *et al*., 2001). Using a combination of mitochondrial and nuclear markers can allow the dating of host shifts, as well as estimation of the number of times such shifts have occurred (for example, see Stone *et al*., 2001).

Wainhouse & Jukes (1997) determined a geographical cline between English and Scottish populations of *Panolis flammea* using allozymes. They concluded that *P. flammea* was not a highly dispersive species, as their overall estimate of population structuring was high for a lepidopteran ($F_{ST} = 0.109$), and found no direct evidence of host-associated genetic differentiation. Their Scottish specimens were sampled only from lodgepole pine, and the English samples were all sampled from Scots pine. It is thus possible that the different allele frequencies observed at the 6*Pgd* locus within English and Scottish populations may indicate host differentiation. Wainhouse & Jukes (1997) however attributed the observed cline to co-varying latitudinal environmental variables, and a potentially selective influence.

The known and theoretical host shift dynamics of *P. flammea* in Scotland, and previous work on dispersal and genetic differentiation between English and Scottish populations of this species (Wainhouse & Jukes, 1997), generate further questions. Within this paper genetic variation is compared within the mitochondrial (sequencing COI locus), nuclear (allozymes) and total (RAPD) genomes of populations of *P. flammea* sampled from the two host species, and at different scales and locations across Scotland, to examine three main questions: (i) How dispersive is *P. flammea*; (ii) What is the origin of *P. flammea* in Scotland; and (iii) Is there any evidence for host-associated *P. flammea* races?

Materials and methods

Insect sampling

Two sampling strategies were adopted to address the objectives of the study. Firstly, samples of *P. flammea* were collected from across Scotland (regional sample) in order to examine the range of variation within Scottish samples relative to a European outgroup (from Germany) and to estimate the general dispersive ability of *P. flammea* (questions (i) and (ii), dispersal and origin). Insects were collected from the two host species and thus were used to assess evidence for host races (question (iii), host races). Randomly amplified polymorphic DNA (RAPD) and mtDNA sequencing was used to screen individuals of the regional sample. Secondly, intensive sampling of four populations occurred on the two hosts from the Moray Firth area, and was sampled over a restricted area (less than 50 km, local sample). This group of samples were used to identify gene flow between populations and any host specificity on a local scale (i.e. questions (i) and (iii), dispersal and host races). Allozyme analysis was used to screen individuals of the local sample.

Regional sample

During June and July 1998, nine forests (three lodgepole pine, LP, and six Scots pine, SP) were sampled in Scotland (fig. 1). Sampling consisted of beating the tree branches

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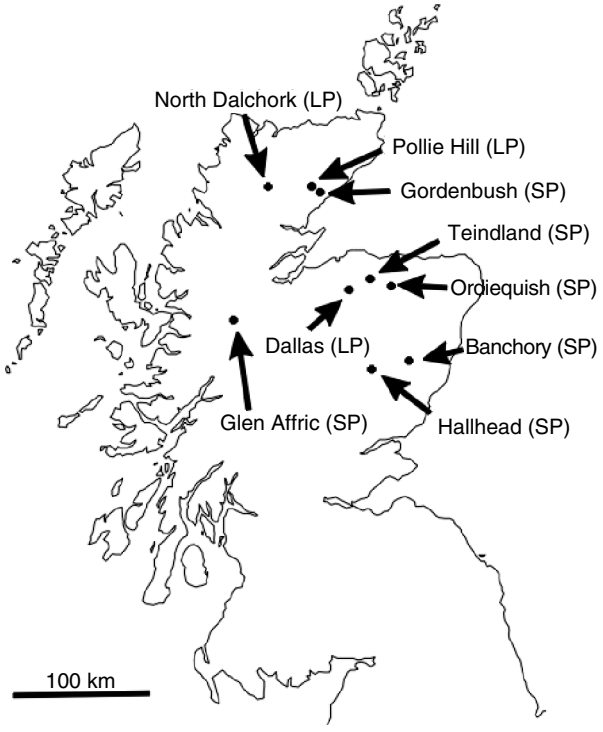

Fig. 1. Locations of the *Panolis flammea* sampling sites in Scotland during 1998–2000. LP, lodgepole pine, SP, Scots pine.

close to the ground and collecting larvae that fell onto sheets placed beneath trees. In total, 221 larvae were collected (95 LP and 126 SP). The larvae were taken to the laboratory and reared to adult eclosion. Adults were frozen at −80°C upon natural death until required for DNA extraction. Twenty-seven pupae from four locations in the northern Bavaria region of Germany were also obtained for out-group comparison. These pupae were reared to adult eclosion and promptly frozen at −80°C.

Local population sample

Sampling of larvae occurred during 1999 (table 1). The sampling strategy (i.e. beating tree branches close to the ground) was similar to the 1998 collection. Sampling locations were in the forest stands of Ordiequish forest (fig. 1) as this forest contained several stands of pure Scots and lodgepole pine but also some stands of mixed (SP/LP) species (total area = 600 ha). The larvae were reared in the laboratory until the pupal stage and then frozen to −80°C. To supplement the 1999 larval collection for allozyme analysis, adults were sampled during 5–9 April 2000, by pheromone traps. The traps were placed at the same sites within Ordiequish forest that larvae were collected during the previous summer. In addition, Dallas (LP) (10 km from Ordiequish) and Teindland (SP) (5 km from Ordiequish and 5 km from Dallas) forests were sampled. Two funnel traps (AgriSense-BCS, Mid Glamorgan), were baited with a lure containing 25 µg Z-9-tetradecenyl acetate + 2.5 µg Z-11-tetradecenyl acetate, and placed in each forest stand for a total of two nights. Each morning the traps were emptied of the live adults. The adults were kept in a cool box (5°C) until they could be transported back to the laboratory and deep frozen at −80°C.

Table 1. *Panolis flammea* sample sizes and site information for the local population sampling in three forests around the Moray district, Scotland during 1999 and 2000 used for allozyme analysis.

Location	Tree species	No. larvae collected 1999	No. adults collected 2000	Total
Dallas	LP	4	19	23
Teindland	SP	–	26	26
Ordiequish 1	LP	17	8	25
Ordiequish 2	SP	2	8	10
Ordiequish 3	SP	–	3	3
Ordiequish 4	SP	5	7	12
Ordiequish 5	LP/SP	14 (4-LP; 10-SP)	7	21
Ordiequish 6	LP/SP	5 (4-LP; 1-SP)	43	48

LP, lodgepole pine; SP, Scots pine.

Molecular marker experimental protocols

Allozymes

The protocol used for detecting allozyme variation in *P. flammea* was modified from Wainhouse & Jukes (1997). The head and prothorax of adults that were collected as larvae in 1999 or as adults in 2000 (from the local population sample, see table 1) were individually macerated in 25 µl of homogenizing buffer (15% sucrose). Wicks (2 mm × 6 mm) were cut from filter paper circles and placed in the liquid sample. Wicks were inserted into the starch gel and samples run at 60 mA for 5 h in a refrigerated incubator (5°C). After running, gels were sliced horizontally into two, 3 mm thick layers. Both layers were used for staining. The stains for individual enzymes were made fresh as required (following Cheliak & Pital, 1984). Stained gels were incubated in the dark at 37°C until dark bands appeared. The banding pattern was noted and photographs were taken.

Eight enzyme loci were initially screened on a sub-sample of insects using a number of buffer systems (Clayton & Tretiak, 1972). The loci tested (with associated buffer system in parenthesis) were: *Mdh*, malate dehydrogenase (morpholine/citrate pH 6.1); 6-*Pgd*, 6-phospogluconate dehydrogenase (morpholine/citrate pH 6.1); *Idh*, isocitrate dehydrogenase (H-buffer pH 7.0); *Pgm*, phosphoglucomutase (H-buffer pH 7.0); *Lap*, leucine-amino peptidase (LiOH pH 8.2); *Got*, glutamate oxaloacetate transaminase (LiOH pH 8.2); *Sdh*, shikimic acid dehydrogenase (Soltis buffer pH 7.0); and *Est*, esterase (H-buffer pH 7.0).

DNA extraction and RAPD profiling

Total genomic DNA was extracted and purified from frozen adult moths that were collected as larvae in 1998 (regional sample, see table 3) and the German adults, using a modified DNA ethanol precipitation procedure (Metcalfe, 1997). Polymerase chain reaction (PCR) conditions for RAPD are similar to those used by Lowe *et al*. (2000), and fragments were resolved on 1% agarose gels and sized against a 1 Kb

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ladder (Gibco). Initially, 40 RAPD primers were screened for polymorphism using a sub-sample of adults ($n = 10$) from different localities representing two of the nine populations. Five of the primers proved to be highly polymorphic and were used to screen the entire collection. The five primers used for the full survey were: OPC-04 (CCGCATCTAC), OPC-08 (TGGACCGGTG), OPH-12 (ACGCGCATGT), OPH-13 (GACGCCACAC), OPH-14 (ACCAGGTTGG) (Operon Technologies, Inc.).

MtDNA sequencing

The cytochrome oxidase subunit I gene (COI) was amplified by PCR using the primers Jerry (CI-J-2183) (5′ to 3′ CAACATTTATTTTGATTTTTTGG) and Pat (TL2-N-3014) (5′ to 3′ TCCAATGCACTAATCTGCCATATTA) (Simon *et al.*, 1994). Three PCR products per sample were run in adjacent wells on 1% agarose gels so as to yield enough gene product for sequencing. Cytochrome oxidase I bands of the correct length were excised from the gel and DNA extracted using QIAquick gel extraction kit (QIAgen, catalogue number 28704) into standard volumes of supplied buffer. The concentration and success of the extraction was estimated by running a DNA ladder of known concentration alongside products. Sequencing was carried out on an ABI 377 automated sequencer following the manufacturer's protocol (Applied Biosystems).

Data analysis

Gene flow estimates using allozymes

Allelic and genotypic frequencies were calculated separately for each population and locus. Genotypic frequencies for loci at each site were tested for compliance to Hardy-Weinberg expectations using an exact test corrected for multiple comparisons (FSTAT; J. Goudet, UNIL, Lausanne, Switzerland). Population differentiation statistics based on allele frequencies (F_{ST}) were used to examine genetic partitioning amongst Scottish populations. The sample at Ordiequish constituted collections from several sites within the forest. Each of these was treated as a separate sample population when examining differentiation amongst populations. For the three loci shared with the Wainhouse & Jukes (1997) study (*Mdh*, *6-Pgd* and *Idh*), allelic frequencies from the original paper and the study populations sampled here were used to generate genetic distance between Scottish, English and German sample sites according to Nei's unbiased estimate (Nei, 1978) using BIOSYS (Swofford & Selander, 1981). Distance values were used to construct a UPGMA tree within the NEIGHBOR program, part of the PHYLIP 3.5c package suite (Felsenstein, 1995).

Population diversity, differentiation and isolation by distance tests using RAPD

A binary matrix of presence and absence for each RAPD fragment was produced. Population diversity level and structure were analysed following the approach of Lynch & Milligan (1994) with removal of all loci showing very low allele frequency (i.e. less than 0.05). Levels of genetic diversity, represented by the expected heterozygosity (H), were determined within populations (H_S) and for the data set as a whole (H_T) using the program AFLP-SURV (Vekemans *et al.*, 2002), where allele frequencies were estimated by Bayesian method with non-uniform prior distribution of frequencies. Standard error (s) was calculated for all diversity estimates following the approach of Lynch & Milligan (1994), i.e. the square root of the variance of the diversity estimate; 95% confidence intervals were then obtained as $+/- 1.96(s/\sqrt{N})$, where N is the number of loci. F_{ST} was then estimated between all populations.

Pairwise differentiation of populations was further examined following the AMOVA model (Phi_{ST}, Excoffier *et al.*, 1992). The significance of partitioning of genetic variation amongst Scottish populations (both from the regional and local samples) and amongst host tree species was calculated from the nested genetic distance variance components using an analysis of molecular variance (WINAMOVA 1.5, L. Excoffier, University of Zurich). Pairwise genetic similarities were calculated from the presence/absence data matrix using Excoffier's algorithm (Excoffier *et al.*, 1992). Although AMOVA was originally developed for use with Euclidean metric distances, it can be used with other genetic distance indices with little adverse impact (Huff *et al.*, 1993). Significance values were assigned to variance components based on 1000 random permutations of individuals, assuming no genetic structure. Two forms of partitioning were tested: a two-level analysis amongst all Scottish populations, and a three-level analysis splitting populations by host tree (i.e. Scots pine vs. lodgepole pine). For AMOVA, individuals which had missing data were removed (this included all individuals from Gordenbush) and individuals from the different German populations were pooled.

A neighbor-joining tree based on pairwise Phi_{ST} values between populations was constructed using the NEIGHBOR program in the PHYLIP 3.5c package (Felsenstein, 1995). To assess for isolation by distance, a Mantels test of autocorrelation was applied to the matrices of pairwise Phi_{ST} differentiation and geographic distances between populations, using both total population samples and grouping samples by host, using TFPGA (Miller, 1997).

Phylogenetic analysis of mtDNA sequence data

Sequences of 849 bp were aligned and edited using CLUSTAL-W (Thompson *et al.*, 1994). Final COI sequences were found to have no stop codons when translated, and therefore were not pseudogenes. All contained the same reading frame. Phylogenies were generated by maximum likelihood (ML) using PAUP (v. 4.0b3; Swofford, 1998). Maximum Likelihood ratio tests incorporated in Modeltest 3.0 (Posada & Crandall, 1998) were used to identify the most appropriate substitution model for the data. This approach identified the most appropriate model as the Hasegawa-Kishino-Yano model, incorporating a gamma distribution of changes (HKY + G; Hasegawa *et al.*, 1985). The following parameters returned by Modeltest were used in PAUP: transition to transversion (Ti/Tv) ratio = 2.0182; base frequencies A = 0.3146, C = 0.1450, G = 0.1330, T = 0.4073; proportion of invariable sites = 0. Changes are gamma distributed with a shape parameter of 0.0098. All trees were generated using 100 random additions in a heuristic search, using the tree bisection-recognition (TBR) algorithm of PAUP. A 50% majority rule consensus tree was generated and subjected to bootstrap analysis using full heuristic searches for 100 replicates. Branches with less than 50% bootstrap support were collapsed.

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Table 2. Allozyme allele frequencies at four loci of *Panolis flammea* populations from Scotland and Germany.

Locality		Dallas	Teindland	Ordiequish							Germany	Scotland[a]	England[a]
				O1	O2	O3	O4	O5	O6	All			
Host		LP	SP	LP	SP	SP	SP	LP/SP	LP/SP	LP/SP	SP	LP	SP
Locus[b]													
Mdh[c]	1	0.543	0.558	0.440	0.550	0.500	0.500	0.524	0.458	0.479	0.630	0.387	0.562
	2	0.457	0.442	0.560	0.450	0.500	0.500	0.476	0.542	0.521	0.370	0.613	0.438
n		23	26	25	10	3	12	21	48	118	27	278	308
6-*Pgd*[c]	1	0.174	0.154	0.188	0.250	0.000	0.083	0.150	0.117	0.142	0.620	0.132	0.557
	2	0.826	0.846	0.813	0.750	1.000	0.917	0.850	0.883	0.858	0.380	0.867	0.443
n		23	26	24	10	3	12	20	47	115	25	200	184
Idh	1	0.087	0.039	0.000	0.500	0.000	0.083	0.000	0.010	0.017	0.037	0.083	0.063
	2	0.891	0.942	1.000	0.950	1.000	0.917	0.929	0.969	0.962	0.963	0.905	0.937
	3	0.022	0.019	0.000	0.000	0.000	0.000	0.071	0.021	0.021	0.000	0.012	0.000
n		23	26	25	10	3	12	21	48	118	27	304	257
Pgm	1	0.174	0.115	0.065	0.050	0.000	0.125	0.275	0.135	0.134	0.000	–	–
	2	0.804	0.885	0.913	0.900	1.000	0.792	0.725	0.813	0.828	1.000	–	–
	3	0.022	0.000	0.022	0.050	0.000	0.083	0.000	0.052	0.039	0.000	–	–
n		23	26	23	10	3	12	20	48	115	27	–	–

[a]Results included for comparison, and are averaged from allozyme profiles in Wainhouse & Jukes (1997). Scottish populations include all three samples and English populations include three most southerly sampled populations; [b]Alleles ordered toward origin (i.e. allele 1 fast, allele 2 slow); [c]Allozyme movement is cathodal.
LP, lodgepole pine, SP, Scots pine.

Results

Gene flow estimates based on allozymes

Four enzyme loci were either monomorphic (*Lap*, *Got*, *Sdh*) or stained poorly (*Est*), and were not included in the analysis. The other four enzyme loci (*Mdh*, 6-*Pgd*, *Idh*, *Pgm*; table 2) were polymorphic, and a total of ten alleles could be scored. Most loci and populations showed no significant departure from Hardy-Weinberg (HW) expectations. The two exceptions were for 6-*Pgd* at Dallas ($P = 0.038$) and 6-*Pgd* at one of the Ordiequish sites (OM5; $P = 0.043$). Gene frequencies observed for German samples are different to those from Scottish populations for all loci. A dendogram constructed from Nei's genetic distance calculated using population allele frequency and pooling the Ordiequish sample, places German populations far from the Scottish populations (fig. 2). Comparison to allele frequencies published by Wainhouse & Jukes (1997) indicates that German samples cluster with southern English samples whereas Scottish samples cluster with those from northern England (table 2). Population differentiation was further examined amongst three Moray *P. flammea* populations (Dallas, Teindland and Ordiequish) and found to be very low ($F_{ST} = 0.032$). In addition, population differentiation amongst the different samples taken within Ordiequish forest also showed similarly low genetic structure ($F_{ST} = 0.0344$). To test whether sampling strategy may have biased the results, pheromone trapped adults were removed from the analysis and only those individuals that were collected directly from trees as larvae were considered. In this case, F_{ST} was slightly higher (0.0454) but no population structure was apparent. No difference in allele frequency was apparent between samples collected from Scots pine or lodgepole pine host stands.

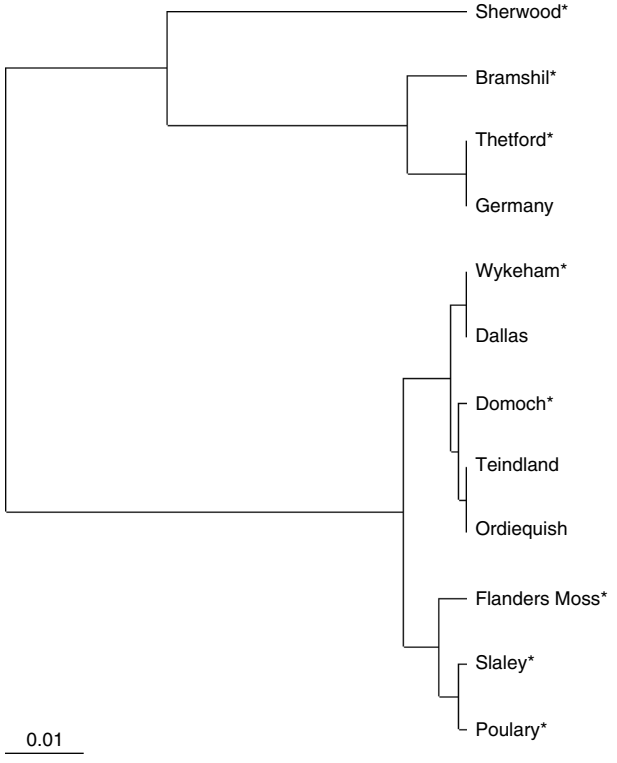

Fig. 2. UPGMA dendogram based on Nei's (1978) unbiased genetic distance calculation for populations of *Panolis flammea* using allozymes. Data are based on three allozyme loci, *Mdh*, 6-*Pgd* and *Idh* and data for populations marked with an asterisk are taken from Wainhouse & Jukes (1997). Three populations were sampled from Scotland (Dornoch, Poulary and Flanders Moss) and the remaining five are English (Slaley, Wykeham, Sherwood, Thetford and Bramshill), and are listed in decreasing order of latitude.

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Table 3. Nei's (1973) genetic diversity for Scottish and German populations of *Panolis flammea* based on RAPD analysis.

Location		Gene diversity	S.D.	*n*
Scotland	Glen Affric	0.216	0.1814	12
Scots pine	Banchory	0.2195	0.1672	21
	Gordenbush	0.2606	0.2024	7
	Hallhead	0.1216	0.1831	5
	Ordiequish	0.1611	0.1687	15
	Teindland	0.2156	0.1542	32
Scotland	Dallas	0.2075	0.16	34
Lodgepole pine	North Dalchork	0.1562	0.1459	31
	Pollie Hill	0.2267	0.1977	10
Germany	Eisenhüttenstadt	0.0791	0.1549	5
Scots pine	Neuzelle	0.0974	0.1783	5
	Lieberose	0.0585	0.1442	5
	Müllrose	0.1669	0.2053	6

Population diversity, differentiation and isolation by distance based on RAPD

The five random decamer primers generated a total of 38 scorable RAPD bands. An examination of Nei's (1973) genetic diversity across all the sites studied showed that the sampled German populations harboured considerably lower diversity than Scottish populations (table 3). Genetic diversity estimates were not notably different for *P. flammea* sampled from Scots pine or lodgepole pine populations. Population differentiation across all Scottish populations according to Lynch & Milligan's (1994) criteria was $F_{ST} = 0.099$.

An AMOVA test of the level of variance partitioned according to host tree only explained 5.6% of the observed variance and was not significant ($P = 0.080$; table 4). Most variance was partitioned within populations (68.8%), with a lesser but still significant proportion partitioned between populations (25.6%, once host tree had been considered). Pairwise Phi_{ST} values were used to construct a dendogram of population differentiation (fig. 3). A Mantel's test indicated a significant relationship between population differentiation, according to Phi_{ST}, and geographic distance ($P < 0.05$), and therefore the sample showed evidence of isolation by distance due to restricted gene flow between populations. When pairwise genetic and geographic distances were plotted (fig. 4) and host population was considered, no difference in the slope of spatial autocorrelation for population pairs sampled between the different hosts was evident.

Table 4. Results of nested analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) for individuals of *Panolis flammea* within Scottish populations according to tree species from which they were collected.

Source of variation	d.f.	MSD	Variance component	*P*-value	% of total
Among LP and SP hosts	1	46.78	0.291	0.08	5.6
Among populations within hosts	6	20.48	1.339	< 0.001	25.6
Within populations	107	3.6	3.56	< 0.001	68.8

LP, lodgepole pine; SP, Scots pine.
PhiST, 0.312; PhiSC, 0.271; PhiCT, 0.056.

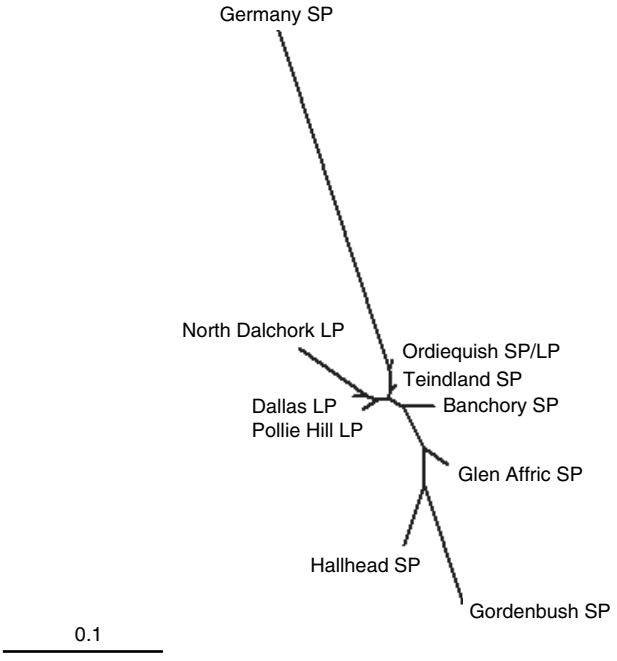

Fig. 3. Neighbor joining dendogram based on PhiST genetic differentiation estimates for Scottish and German populations of *Panolis flammea* using RAPD. LP, lodgepole pine, SP, Scots pine.

Phylogenetic analysis of mtDNA haplotypes

Of the 35 individual sequences, 33 different haplotypes were identified. Nucleotide divergence amongst all 33 haplotypes ranged from less than 0.0001% to 0.185%. Of the 849 bp of the COI locus amplified, 822 were constant across all sequences. Of the 27 variable sites, 14 were parsimony informative. Fifteen nucleotides differed at the third base position, eight at the first base position and only four at the second base position.

The ML 50% majority rule phylogeny (fig. 5) indicates that neither the Scottish nor the German sequences formed monophyletic groups. Scottish sequences resolved into two major groupings; one clade (clade 1) contains the majority of the Scottish haplotypes and a single German haplotype (Eisenhuttenstadt 1), while a second clade (clade 2) contains most of the German haplotypes and the remaining four Scottish haplotypes (Glen Affric 3, 4 and 5, and Gordenbush 6). Clade 1 is well supported by bootstrap analysis, but nesting of the smaller Scottish group within clade 2 is not strongly supported. The German haplotype in clade 1 is placed deep within the major Scottish lineage, and is separated from its nearest geographical neighbour by four bootstrap values above 80% (fig. 5). Within clade 2, the only other German haplotype that shows significant divergence from its neighbours is that of Nuezelle 9, which is separated by a bootstrap value of 83%. Sequences within the

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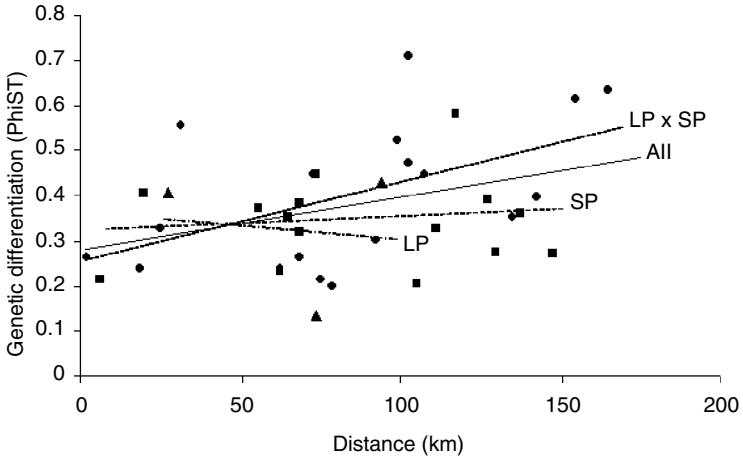

Fig. 4. Correlation between geographic and genetic differentiation (as measured by PhiST) between populations of *Panolis flammea*. ◆, between Scots pine stands (SP × SP); ■, between Scots and lodgepole pine stands (LP × SP); ▲, between lodgepole pine stands (LP × LP). A significant positive correlation (r = 0.3490, $P = 0.037$) was found between the two distance matrices using Mantel's test on the entire dataset. No significant correlation was found using the SP × SP (r = 0.0969, $P = 0.4070$), LP × LP (r = −0.1650, $P = 0.4790$) and LP × SP (r = 0.4812, $P = 0.0580$) datasets separately, although all slopes are indicated.

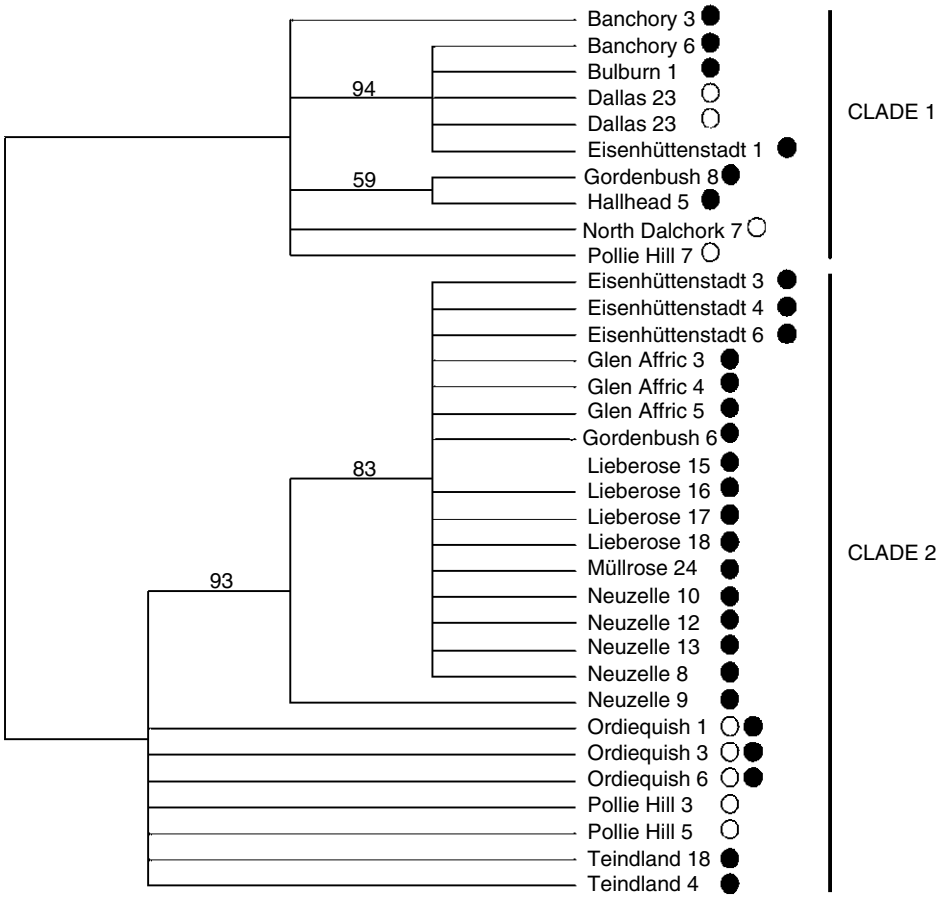

Fig. 5. A 50% majority-rule consensus phylogram generated using PAUP* and maximum likelihood, using parameter estimates for the HKY+G model returned by Modeltest. The tree has been rooted using the midpoint option in PAUP*. ●, Scots pine site; ○, lodgepole pine site; ○●, mixed site.

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largest Scottish grouping had maximal haplotype divergence of 0.02245%, whereas that between the smaller groups is less than 0.001%.

Discussion

Dispersive ability of P. flammea

Population differentiation between Scottish populations of *P. flammea* based on RAPD analysis was low but significant ($F_{ST} = 0.099$). This estimate was slightly lower than, that found by Wainhouse & Jukes ($F_{ST} = 0.109$; 1997) who used allozymes to estimate differentiation across a sample of English and Scottish populations. The slight difference between estimates could be due to the larger scale of sampling employed in the previous study, the potential selective bias of the *6-Pgd* locus and inherent differences in statistical comparison between marker types, although the two estimates are comparable. The level of differentiation found in the Scottish range of material of this study is high for a lepidopteran species in general (Wainhouse & Jukes, 1997), and suggests that that there is not much effective long distance gene flow. The RAPD results also indicated a significant isolation by distance effect (fig. 4) further suggesting limited long distance dispersal in *P. flammea*.

Whilst long distance dispersal may be rare, there appears to be frequent local dispersal over distances of up to 50 km, at least in some regions. Allozyme analysis of Moray populations recorded an F_{ST} value of only 0.032, suggesting much more extensive gene flow at this location and scale compared to the scales of Scotland (RAPD results) or Great Britain (Wainhouse & Jukes, 1997). The mtDNA sequence data were also suggestive in this matter and individuals from some populations around the Moray district (i.e. Teindland and Ordiequish), cluster together in the phylogeny (fig. 5), and could share a recent common ancestor. In other locations (e.g. Gordenbush, Pollie Hill and to a lesser extent, Banchory), mtDNA types are distributed throughout the phylogeny, with no clear recent common ancestor. In these locations, initial colonization was most probably by multiple individuals, each originating from a different geographic location. For example, the sequences from Pollie Hill individuals are divided between groups, each separated by 0.0930% sequence divergence. At other locations there appears to be little sharing of haplotypes between proximate population samples. For example Dallas and Teindland are less than 8 km apart, but they do not share haplotypes, although better population sampling would be required to fully support the above observations. The observed low level of gene flow between disparate Scottish populations corroborates the field study of Watt *et al*. (1989). These authors observed low dispersal abilities of adults when adults dispersed from heavily defoliated areas to local areas where no defoliation had occurred before.

Origin of Scottish P. flammea

Allozyme and RAPD analyses indicate considerable genetic divergence between *P. flammea* of Scottish and German origin (figs 2 and 3). The previous allozyme survey of Wainhouse & Jukes (1997) also showed that English *P. flammea* are significantly differentiated from Scottish populations. When these data were included in the current survey (fig. 2), Scottish populations remained significantly differentiated from both English and German samples, and material from the later two origins were very similar (table 2). MtDNA sequencing (fig. 5) indicated that Scottish *P. flammea* do not represent a monophyletic group. Instead, Scottish and German individuals are shared amongst the two major haplotype clades. These data suggest that at least some German and Scottish haplotypes are derived from the same ancestral population, and that similarities between Scottish and German sequences represent the results of sorting, and limited divergence, of ancestral polymorphism. RAPD analysis also found that Scottish populations were more diverse than the German sample, when populations of similar sample size were compared (table 3). This pattern argues against a significant founding effect during colonization of *P. flammea* in Scotland. Lower diversity in Germany could be due either to sampling limitations, or perhaps the augmentation of diversity in Scotland by genotypes from a discrete northern refuge for *P. flammea*.

The pattern of genetic diversity and divergence amongst British and European *P. flammea* may reflect the post-glacial colonization history of Scots pine, the moth's natural host. Monoterpene and isozyme analyses indicate that some Scottish Scots pine populations probably originated from a northwestern refugium in Ireland (Forrest, 1980; Kinloch *et al*., 1986; Bennett, 1995; Ennos *et al*., 1997). Further analysis of mitochondrial DNA (Sinclair *et al*., 1999) demonstrated that Scottish Scots pine populations were composed of two mitotypes: one corresponding to central European populations suggesting a Continental origin, probably via France; the second was present in some western Scottish populations, and together with pollen and microfossil evidence supported the presence of a northern refugium located to the west of Scotland (Ireland or the continental shelf, a so called 'cryptic' refugium which has also been suggested for other species; Stewart, 2003). It is therefore possible that a proportion of the Scottish *P. flammea* populations survived the last glacial maximum associated with an Irish/Scottish Scots pine refugium, and that there has been a more recent colonization of *P. flammea* into Scotland (and England) from a genetically differentiated source on the Continent (although not necessarily Germany). Under such a scenario, the largest Scottish grouping would represent material from this northern cryptic refugium that diverged from the continental lineage long before the last ice age. This is a working hypothesis and would need to be substantiated by much more extensive phylogeographic analysis and population sampling from across the British Isles and Continental Europe.

Host-association in P. flammea

Sequence divergence among the mtDNA haplotypes of lodgepole moths and their nearest Scots pine neighbours almost certainly pre-date the first appearance of *P. flammea* on lodgepole pine (25 years ago). The presence of lodgepole moths within the larger Scots pine grouping (fig. 5) indicates that many *P. flammea* matrilines have colonized lodgepole pine. RAPD and allozyme analyses also did not reveal significant differentiation between *P. flammea* from Scots or lodgepole pine hosts. In addition, there was no evidence

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for reduced genetic diversity (and, by inference, host shift founder effects) within lodgepole pine-reared moths. On the contrary, allozyme data suggest extensive intermixing between populations within the Scots and lodgepole pine habitats at the local scale within the Moray region. Under such conditions of apparent panmixia, separate races or even species are not expect to establish on the different hosts, and it appears most likely that *P. flammea* has just extended its ecological range to incorporate a new dietary opportunity.

It remains possible that host-associated population subdivision exists, but has not been detected in the current study. A relatively low number of loci (allozyme $n = 4$; RAPD $n = 42$) and populations were surveyed compared to other studies (e.g. Emelianov *et al*. (1995) used 19 allozyme loci), and certainly these aspects could be improved in future studies. The temporal scale of host shift is very recent and it could be argued that sufficient time has not elapsed for differentiation to take place. However, the distribution of mitotypes between host populations and comparable diversity estimates show no evidence for a founding bottleneck, usually a prerequisite for such a model of host divergence, and cannot be supported based on the current data.

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