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SHORT COMMUNICATION

AFLP reveals cryptic population structure in migratory European red admirals (*Vanessa atalanta*)

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Abstract. 1. The genetic differentiation in a migratory butterfly, the red admiral (*Vanessa atalanta*), was investigated to discern patterns of migratory routes used across Europe. AFLP profiles showed significant differences between almost all sampled locations, but there was no clear pattern of isolation-by-distance.

2. Using the software STRUCTURE 2.2, we found two distinct genotype clusters present in different frequencies at all study sites. The frequencies of these genotypic clusters varied significantly between years within the same site. Remarkably few individuals were of mixed ancestry, indicating that some isolating mechanisms are present. Twenty-seven mtDNA haplotypes were identified but they showed no geographic structure, nor were they related to either of the two genotype clusters identified in the AFLP data.

3. Most field observations of migrating red admirals suggest a regular north–south migration pattern in Europe. Our data indicate both long-distance migration and a more variable pattern in orientation, since the composition of the two genotypic clusters shows dramatic variation between sites and years in the northern part of the distribution range.

Key words. AFLP, migration, mtDNA, phylogeography, population structure, *Vanessa atalanta*.

Introduction

To understand the biology of annually migrating animals, selection pressures and mortality have to be estimated in the different geographical areas frequented by them, including those passed during migration (Sillett *et al*., 2000). Linking together winter and summer ecology requires knowledge of migration routes and population structure (Webster *et al*., 2002) but most available methods are only suitable for rather large animals. Insects make up a substantial proportion of animals that migrate (Dingle, 1996; Holland *et al*., 2006), but being rather small, fragile, and short lived, tagging projects are difficult. To date, large-scale mark–recapture studies have been successful only in one migratory insect, the monarch butterfly (*Danaus plexippus*) (Linnaeus, 1758) (e.g. Urquhart & Urquhart, 1978).

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The red admiral (*Vanessa atalanta*) (Linnaeus, 1758) is a migratory butterfly distributed in North America, Europe and the western part of Asia (Eliasson *et al*., 2005). Each spring the red admirals spread out northwards, leaving the southern areas where they have spent the winter season, and produce at least one new generation in the areas inhabited during the summer. The new generation returns south during autumn and recent studies in the Mediterranean region suggest that they do reproduce during winter in this areas (Stefanescu, 2001; Brattström, 2006). This means that the return migration due north next spring is performed by a newly hatched generation produced during winter. Even though red admirals are regarded as migratory, since they are only rarely found in the northernmost part of their distribution during winter, we still know very little about the actual migration routes.

Webster *et al*. (2002) reviewed several possible methods for studying migratory connectivity. If a migratory species is found over a large area and different populations use different migration routes each season, it is likely that genetic differences will be found between the subpopulations that

use different routes. Recent mitochondrial DNA (mtDNA) studies of Lepidopteran species with distribution ranges in Europe have reported no or low levels of genetic structuring in relation to distance between sample sites (e.g. Vandewoestijne *et al*., 2004; Margaritopoulos *et al*., 2007). Red admirals are much more mobile than these species. It is therefore unlikely that mtDNA analyses alone will be sufficient to discriminate between individuals from sites across its European range. We therefore decided to combine mtDNA sequencing with extensive nuclear genotyping using the AFLP method (Vos *et al*., 1995) to investigate the genetic structure of European red admirals.

Materials and methods

Collection of samples

Adult red admirals were collected in the field during 2004 and 2005. Sampling was carried out by the first author (OB) and by volunteers; in total we collected 277 individuals from nine different locations in Europe (Table 1). Most samples were from the late summer generation except for Italy, where we sampled separately in both spring and autumn. After capture, the butterflies were euthanised using ethyl acetate and the heads were stored in 99.9% ethanol until extraction of DNA was performed, using a standard phenol chloroform protocol (Sambrook *et al*., 1989).

Sequencing of the mtDNA CO-I region

We amplified 1200 bp of the CO-I gene using the primers VanCOIF (5′-TGA GCA GGA ATA GTA GGA ACT TC-3′) and VanCOIR (5′-TCG TCG AGG TAT TCC AGC TAA-3′). Polymerase chain reactions (PCRs) were performed in volumes of 25 μl and included 10 ng of total genomic DNA, 0.125 mM of each nucleotide, 1.5 mM $MgCl_2$, 0.6 μM of each primer and 0.5 U AmpliTaq polymerase. The PCRs were run using the following conditions: 30 s at 94°C, 30 s at 57°C, 60 s at 72°C (35 cycles). Before the cyclic reactions the samples were incubated at 94°C for 2 min, and after completion at 72°C for 10 min. The PCR products were precipitated (NH_4Ac and ethanol), dissolved in water and then used for sequencing (BigDye sequencing kit, Applied Biosystems, Foster City, California) in an ABI Prism® 3100 capillary sequencer (Applied Biosystems). We initially screened 72 individuals by sequencing the complete fragment, and then decided to investigate only the most variable 5′ region (460 bp) by sequencing all of the collected individuals with the internal primer F774 (5′-GCT ATA ATA GCA ATT GGA TTA TTA G-3′).

Table 1. Red admiral sampling locations and number of individuals analysed during 2004–2005. All individuals were collected in late summer, except for the Italian sample from 2005, which was divided into two groups since we sampled both in early spring and late summer from two clearly different generations.

	Coordinates		Number of individuals	
Sampling location	Latitude	Longitude	2004	2005
Denmark, Ølsted	55.92°N	12.07°E	10	9
Estonia, Karilatsi	58.07°N	26.55°E	15	—
Italy, Capri	40.55°N	14.23°E	15_{autumn}	15_{spring}/ 15_{autumn}
Russia (Kaliningrad), Rybachy	55.16°N	20.84°E	42	42
Poland, Czestochowa	50.81°N	19.12°E	—	29
Sweden, Kullaberg	56.30°N	12.47°E	15	—
Sweden, Ottenby	56.20°N	16.40°E	—	45
Sweden, Sandhammaren	55.38°N	14.20°E	15	—
Spain, Vilagarcía	42.57°N	8.76°W	10	—
Total			122	155

Generating AFLPs

We used amplified fragment length polymorphism (AFLP) to generate a large number of polymorphic markers randomly distributed throughout the genome. This method is useful for studies of population differences and requires no previous knowledge of the genome of the study species. We used the AFLP protocol described in Vos *et al*. (1995), and modified according to Bensch *et al*. (2002). Fragments were separated in 6% polyacrylamide gels and scanned with a Typhoon 9200 (Amersham Biosciences) after 70 min, and then again after an additional 60 min. We used three combinations of selective primers: A ($E_{TGA} \times M_{CAG}$), B ($E_{TGA} \times M_{CGA}$), and C ($E_{TCG} \times M_{CAA}$).

Data analysis

mtDNA sequences were aligned using the software BioEdit 7.0.9.0. (Hall, 1999) and pruned to a length of 433 bp, which gave unambiguous readings from all the successfully sequenced individuals. The data were then analysed using the software Arlequin 3.1 (Excoffier *et al*., 2005) to estimate genetic population structure. In the analysis we kept all the sample locations and samples from different years/seasons from the same location as separate groups.

AFLP gels were analysed manually, and only clearly visible bands that could be scored easily for presence/absence in all gels were included in the analysis. Arlequin 3.1 (Excoffier *et al*., 2005) was used to calculate pair-wise F_{ST}-values, as well as performing an AMOVA to calculate a global F_{ST}-value for differences between all sampled groups. We used a Mantel test to estimate *P*-values for correlations in the isolation-by-distance analyses for geographical distance between sites. We used the software STRUCTURE 2.2 (Pritchard & Wen, 2003; Falush *et al*., 2007) in order to estimate the most likely number of genetic populations, disregarding information on collection site. For individuals having the presence allele, the dominant nature of the AFLP method can make it difficult to separate between heterozygotes and homozygotes (Bensch

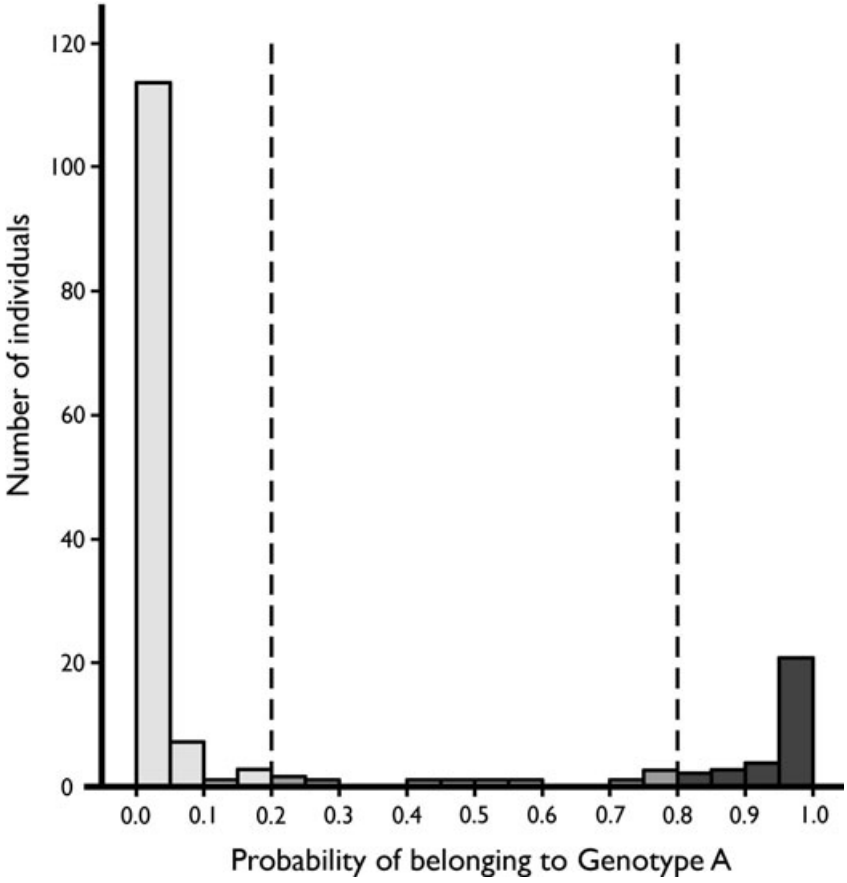

Fig. 1. Histogram showing the frequency of individuals of red admirals belonging to two suggested genotype clusters. Probability of belonging to genotype A was estimated by STRUCTURE 2.2 software using an admixture model and two suggested populations. The three groups (A, mixed ancestry, and B) used in Fig. 2 are marked with the same shade of grey as used in Fig. 2. The arbitrary limits set by us, for being assigned to each group are marked with dashed lines.

& Åkesson, 2005). STRUCTURE 2.2 takes this into account and considers all individuals with presence for a band as possibly hetero- or homozygote for that specific allele. Each run had a burn-in of 10 000 iterations, followed by 10 000 iterations of data collection. We ran the analysis for up to 10 possible populations ($K = 1–10$) with 20 different runs at each level. We used an admixture model, providing no prior information about the origin of our samples. To determine the most likely number of populations, we analysed the STRUCTURE 2.2 output using the methods described in Evanno *et al*. (2005). Finally, we tested whether the groups identified by STRUCTURE 2.2 were different with regard to mtDNA haplotypes.

Sex determination

To ensure that sexual differences would not bias our AFLP results, a total of 82 individuals were dissected to determine sex. This enabled us to exclude genetic markers that are exclusively present in only one sex, making local differences in sex ratios appear as population differences. This is important, since the sex ratios in a sample of red admirals from a single location can be extremely skewed (Brattström, 2006). We also performed a χ^2-test to study if sex was equally distributed within the groups identified by STRUCTURE 2.2.

Results and discussion

mtDNA sequences for the 433-bp long fragment were obtained from 249 individuals. In total we found 27 different haplotypes within our data set. Most individuals belonged to one main haplotype ($n = 168$) (GenBank GU229026-229193) and three medium-sized groups ($n = 21$, 19, and 10) (GU229194-229243) with only one base-pair difference from the main haplotype. The rest of the individuals ($n = 31$) belonged to a range of smaller groups containing one to four individuals (GU229244-229274). There was no significant difference between sample sites, and the highest (non-significant) F_{ST} value was 0.176 between Capri (autumn 2004) and Denmark (2005).

All 277 sampled individuals were included in the AFLP analysis. In total we identified and scored 199 AFLP loci, of

Fig. 2. Circular graphs showing the relative proportion of sampled red admirals belonging to two different genotypes (dark (A) and light (B) grey) or being of mixed ancestry (medium grey, less than 0.8 probability of belonging to either of the two main genotypes) from 2004 and 2005. Both genotypes are present each year and individuals with mixed ancestry are rare.

which 169 were polymorphic (30 for primer combination A, 69 for primer combination B and 70 for primer combination C).

The AMOVA revealed a significant genetic difference between the sampled groups ($F_{ST} = 0.048$, $P < 0.001$). The pair-wise F_{ST}-values ranged from 0.00024 to 0.17, and most of these (73 of 78) were significant. From the three sites where we had samples from both years (two samples each for Denmark and Russia, and three different samples from Italy), the pair-wise F_{ST}-values were significant in four of the five comparisons ranging from 0.03 to 0.10. The correlation between pair-wise F_{ST}-values and geographic distance was not significant (Mantel test; $r = 0.138$, $P > 0.05$), showing no evidence of an isolation-by-distance effect.

Using STRUCTURE 2.2 and following the method for calculating ΔK described in Evanno *et al.* (2005), two populations were most likely to have produced the given dataset [Ln$P(D)$ was highest at two populations ($K = 2$) and the variation was also smallest at this level]. When examining the results from STRUCTURE 2.2 at the level of individuals, it appeared that most individuals (96%) could be assigned with a high probability (>0.8) to either of the two suggested genotype clusters (Fig. 1).

Among the 82 dissected specimens (43 females and 39 males) no single marker was present exclusively in one sex, and the sex ratios of individuals in the two identified genetic clusters were similar ($\chi^2_{1,80} = 1.459$, $P = 0.23$). Hence we can conclude that the suggested clustering was not a result of sexual difference.

The geographic distribution of the individuals from the two different clusters, and those of mixed ancestry can be seen in Fig. 2. We found no clear difference in distribution of individuals from the two clusters at our sample sites, but variation was greatest in northern Europe, even between successive years at the same site. The selected limit of minimum probability for being assigned to either of these clusters (0.8) is arbitrary, but it is clear from Fig. 1 that altering the cut-points will only marginally change the proportion between the clusters.

The distribution of mtDNA haplotypes did not differ between the two main AFLP groups [F_{ST} −0.0065 (not significant $P = 0.52$)].

Our results show substantial genetic differentiation between red admiral populations within Europe, and there is also significant variation at the same sites between two consecutive years. We found no correlations between genetic and geographic distances. Despite this, most (94%) pair-wise comparisons between sampling sites show significant F_{ST}-values within the range 0.013–0.17. The reason for these patterns appears to be the occurrence of two different clusters of genotypes, whose frequencies varies significantly between sites and years (Figs 1 and 2). The patterns of genetic variation among individuals captured at our sample locations show that the migration of European red admirals is most likely to cover long distances, but not in a regular fashion as previous field observations have suggested (e.g. Williams, 1951; Benvenuti *et al.*, 1996; Hansen, 2001; Mikkola, 2003). We see a large variation in observed frequencies of the two genotype clusters between years at our northern sites, suggesting that the origin of red admirals varies between years. Despite the co-occurrence of these genotype clusters, we find remarkably few (13%, calculated as mean proportion among sites) individuals of mixed ancestry. Given the distribution of our sample sites and the fact that it is very hard to get a long-term balanced data set of migrating butterflies, we cannot fully explain this pattern. Still,

the occurrence of two distinct genotype clusters suggests that there are two genetic populations of red admirals in Europe that do not interbreed to a large extent. Most migrating insects seem to initiate reproductive activities soon after a migratory flight (Dingle, 1972), so if we have two different source populations in the wintering areas, they would only interbreed occasionally in the summer areas if the northward migration is initiated at slightly different times. To understand the patterns we have seen in the present study, samples are needed from more locations, especially the potential wintering areas in the eastern part of the distribution range. We expect that future studies combining biometric analyses and with molecular data will be most rewarding when disentangling the enigmatic migration behaviour of the red admiral.

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