Heredity **76** (1996) 437–448 *Received 24 May 1995*

A hierarchical analysis of genetic structure and variability in patchily distributed coexisting *Chiastocheta* species (Diptera: Anthomyiidae)

JES JOHANNESEN* & VOLKER LOESCHCKE

Department of Genetics and Ecology, University of Aarhus, Ny Munkegade, Building 540, DK-8000 Aarhus C, Denmark

The pattern of genetic variation in four coexisting fly species of the genus *Chiastocheta* was studied by allozyme electrophoresis. The fly species are confined to patches of one plant, *Trollius europaeus*, and thus experience very similar habitat fluctuations. Collection sites were chosen in a hierarchical fashion and *F*-statistics were estimated at three levels: intraregion, inter-region and total population. Population characteristic genetic parameters were compared within and among species and were related to the hierarchical level. The species were used as replicate experiments for inference of habitat history, and the hierarchical levels were used as inference for specific gene flow patterns. Genetic variability was related to relative species abundance but not to local population size. The species divided into two heterogeneity classes, *C. dentifera*/*C. trollii*, and *C. inermella*/*C. abruptiventris*, where the former pair had more genetic variation than the latter. However, among all species no differences in the average number of alleles per locus were found. The amount of variability was not related to interspecific phylogeny, and the species could thus be analysed independently for genetic structure. Species-specific genetic patterns were found, but for all species the amount of genetic differentiation was related to regional geography. For three species, differentiation within regions was often higher than at the total population level whereas the differentiation among regions was negligible. Differentiation of the fourth species, *C. inermella*, increased with geographical scale indicating an isolation-by-distance genetic structure. We suggest that within species the amount of genetic differentiation need not be related to intraspecific ancestry, as high differentiation was not necessarily associated with low expected geographical structure.

Keywords: *Chiastocheta*, coexistence, *F*-statistics, gene flow patterns, genetic variability, population structure.

Introduction

Habitat fluctuations and/or fragmentation influence the genetic structure of populations. Indirect estimates of the genetic structure and relatedness of populations are generally based on equilibrium assumptions (Slatkin, 1985, 1993), but ancestry may be obscured by drift followed by founder events and by habitat fluctuations. The latter is particularly important for the structure of phytophagous insect populations which may be strongly influenced by host plant structure (McCauley, 1991). The genetic variability of insect populations is determined by population size, migration rate and response to habitat conditions. Upon fragmentation the genetic variation of a continuous population may decrease relative to the half-life of large fragments, and the effective metapopulation size may be two orders of magnitude lower than if estimated only locally (Gilpin, 1991). Environmental stochasticity can reduce metapopulation persistence time, and stochasticity at the regional level may reduce it even further (Hanski, 1991).

Gene flow will influence genetic variance, and if local extinctions occur, re-immigration may have a strong effect on variation (Slatkin, 1977, 1993; Wade

*Correspondence: Present address: Institute of Zoology, Department of Population Biology, University of Mainz, Saarstrasse 21, D-55099 Mainz, Germany.

& McCauley, 1988; Whitlock & McCauley, 1990). The variance may increase as a result of colonization from only one population if the extinction rate exceeds the migration rate (Maruyama & Kimura, 1980). In an extinction/colonization study of a monophagous beetle, McCauley (1989) estimated the extinction rate at 0.02 per generation, which could either halve or double an F_{ST} estimate depending on the migration pattern (Wade & McCauley, 1988). The rate of migration may depend on habitat conditions or species interactions and may be a strategy leading to persistence of polyspecies systems (Pulliam, 1988). On the other hand, persistence time is a function of habitat stability. How and when species respond to habitat changes is probably a consequence of the structuring level that influences a species the most.

In this paper we examine four herbivorous coexisting and congeneric fly species of the genus *Chiastocheta*. All four are confined to patches of their common host plant *Trollius europaeus*, and all thus experience similar habitat fluctuations. Furthermore, as all species are confined within the same patches the distance to neighbouring patches will be similar for each. Comparatively we use these conditions in a hierarchical analysis of genetic structure to examine the influence of patch scale and population size on genetic variability and structure. The two main causes for species differences of genetic structure are habitat history (e.g. the network of habitat patches) and specific gene flow patterns. For the former we use the species as replicate samples to infer regional habitat history. For the latter, we use the hierarchical levels as replicates to reveal specific gene flow patterns that influence genetic structure.

Background

The genera *Trollius* (Ranunculaceae: 18 spp.) and *Chiastocheta* (Anthomyiidae: 17 spp.) have coevolved to a varying extent a pollinator–seed parasite dependency. The most highly coevolved flower–fly system is that of *T. europaeus* and its pollinators (Pellmyr, 1992). Four species of the genus *Chiastocheta* (*C. dentifera*, *C. inermella*, *C. trollii* and *C. abruptiventris*) occur sympatrically in Denmark. [The species *C. abruptiventris* (Pellmyr, 1992) is identical to that referred to as *C.* sp. aff. *rotundiventris* by Pellmyr (1989).] All are associated exclusively with a perennial host plant *T. europaeus*, on which larval development must take place. *Trollius europaeus* is distributed in discrete patches. Flowering starts in early May and lasts for 4–5 weeks. After emerging in spring flies move within a patch, from flower to flower, seeking mates. Mating and oviposition take place in the flower heads leading to pollination of the flowers. The larvae act as seed parasites. As the remaining seeds ripen the carpels burst and the larvae crawl out, drop to the ground, and pupate.

Pellmyr (1989) argued that flowers and flies experience an obligatory mutualistic interaction. He also suggested that *C. inermella*, *C. trollii* and *C. abruptiventris* contribute to pollination whereas *C. dentifera* does not. The latter species was described as purely parasitic. The larval mining patterns of the four species are stereotyped and interactions between larvae are minimized suggesting resource partitioning (Pellmyr, 1989), which may contribute to stability of coexistence. In a study of fly species distribution and composition within patches, Johannesen & Loeschcke (in press) found that population sizes of all species were highly linearly correlated with patch size. At patch sizes above *c.* 2500 flower heads relative abundances were fairly constant with *C. dentifera* constituting *c.* 30–40 per cent, *C. abruptiventris* 10 per cent, *C. inermella* 20 per cent and *C. trollii* 30–40 per cent of the *Chiastocheta* species. At smaller patch sizes *C. dentifera* was often missing, *C. inermella* became the dominant species and the abundance of *C. trollii* declined. Missing for the first 1–2 weeks of the flowering season *C. dentifera* showed the only pronounced seasonality.

However, the abundance and distribution of these *Chiastocheta* may also be affected by the recent fragmentation of *T. europaeus* patches, and the tendency of *T. europaeus* to flower irregularly between years. At smaller patch sizes the impact on the flies of stochastic flowering is increased. When combined, these effects may cause *Chiastocheta* to experience complex habitat fluctuation patterns.

Materials and methods

Sampling and species identification

Collections were made from nine *T. europaeus* patches on the Jutland peninsula, Denmark, where all four *Chiastocheta* species were present. The patches were discrete entities as no other patches of *T. europeaus* were found within a radius of about 1 km of these collection sites. Collection sites were chosen in a triangular fashion with three patches in each corner, the corners being *c.* 100 km apart. Each corner is termed a region, and all nine patches together are considered to be the total population. In this way a hierarchical analysis of genetic variation could be conducted. Patches were situated in

western Jutland, W1[Ormstrup], W2[Mølstedvej], W3[Ølgryde], average distance between collection sites 11 km; in eastern Jutland, E1 (Søften), E2 (Yderup), E3 (Tilst), 4 km average separation; and northern Jutland, N1 (Store Vildmose), N2 (Fyrkat), N3 (Blåkilde), 30 km average separation. Patch size was defined by the number of *T. europaeus* flower heads (Johannesen & Loeschcke, in press). Collections were made in May and June 1991, from the start to the end of flowering, at intervals of one week. Sampling was performed by vacuuming flower heads at random throughout a patch. Typically one or two flies per flower which contained flies were caught, but more (up to eight) were obtained in rare cases. The collection effort was similar at all sites, and thus the sample sizes reflect population sizes.

Both sexes of *C. inermella*, *C. trollii* and *C. abruptiventris*, and male *C. dentifera*, were identified using the diagrams of Collin (1954) and the nomenclature of Michelsen (1985 & unpublished). *Chiastocheta dentifera* females were verified by Michelsen. Males were identified by abdominal and genital morphology, whereas females were determined by ovipositor morphology.

Electrophoresis

Analyses were performed by horizontal starch gel (12 per cent Connaught) electrophoresis. Individuals were returned to the laboratory alive and frozen at −70°C. Prior to electrophoresis, flies were homogenized in 20 μL PGM buffer (Harris & Hopkinson, 1978) with 1 per cent polyvinylpyrrolidone and centrifuged for 1.5 min. Eighteen enzyme systems representing 21 loci could be scored consistently in all four species: EC 5.4.2.2 (*Pgm-1, Pgm-2*); EC 1.1.1.42 (*Idh*); EC 5.3.1.8 (*Mpi*); EC 2.7.1.1 (*Hk*); EC 1.1.1.8 (*Gpd*); EC 1.1.1.204 (*Xdh*); EC 1.1.1.43 (*Pgd*); EC 5.3.1.9 (*Gpi*); EC 3.1.1.1 (*Est UV-1*); EC 2.7.4.3 (*Ak*); EC 1.8.1.4 (*Dia-1, Dia-2, Dia-3*); EC 2.7.3.3 (*Ark*); EC 4.1.2.13 (*Ald*); EC 1.1.1.40 (*Me*); EC 1.1.1.37 (*Mdh-1*); EC 3.4.11/13 (*Pep-A, Pep-B*) and EC 4.2.1.2 (*Fum*). Three buffer systems were used: (1) Tris-citrate, pH 7.0 (buffer C of Ayala *et al.*, 1972); *Pgm-1, Pgm-2, Idh, Mpi, Hk, Ak, Me, Pep-B*; (2) Tris-citrate, pH 8.0 (buffer 5 of Selander *et al.*, 1971); *Gpd, Xdh, Pgd, Gpi, Est UV-1, Ark, Ald, Pep-A, Fum* and (3) discontinuous dipotassium hydrogen phosphate citrate, buffer pH 6.7, gel pH 7.0 (buffer 8 of Selander *et al.*, 1971); *Dia-1, Dia-2, Dia-3, Mdh-1*. Buffer system 1 was run for 2 h at 350–400 V, 100 mA; buffer 2 for 4 h at 200 V, 100 mA; and buffer 3 for 4 h at 100 V, 100 mA; all at 5°C. Three gels could be run for each individual and thus nine enzyme systems were scored for each. Each gel included individuals from three different patches to exclude possible scoring errors caused by different gel running conditions. In order to determine interspecific variation, all four species were run simultaneously.

Data analysis

All loci showed a banding pattern consistent with autosomal inheritance. Therefore, data for males and females were pooled. If staining revealed only one band, the locus was considered to be monomorphic. All polymorphic loci showed banding patterns consistent with the known enzyme quaternary structure (Richardson *et al.*, 1986). The number of alleles was scored conservatively, with microvariation in mobility interpreted as a single allele. A test for linkage disequilibrium was not performed as all enzyme combinations could not be scored for the same individual (hence independent). To obtain the largest sample size enzyme combinations were often changed; thus the data did not divide into two sample sets.

All populations were analysed for deviations from Hardy–Weinberg expectation by the *G*-test, which is approximately χ^2-distributed (Sokal & Rohlf, 1981). When more than two alleles were found at a locus, all except the most common were pooled. To avoid loss of information by pooling alleles we complemented the *G*-test with the Louis-Dempster exact test (Louis & Dempster, 1987) for loci where three or more alleles were detected. The expected heterozygosity, H_e, was calculated for all loci and averaged across the loci in all populations. To analyse the possible effects of bottlenecks on allele frequency distributions, we compared the observed distribution with that expected under the infinite alleles model (Kimura & Crow, 1964). For this model the number of alleles between x and $x+dx$ is given by $\Phi(x)dx = 4Nv(1-x)^{4Nv-1}x^{-1}dx$, where $4Nv = H_e/(1-H_e)$ (Kimura, 1983). Pairwise *G*-tests for heterogeneity between populations were used to test for significant differences in allele frequencies. *G*-tests for heterogeneity of loci in the total population were also performed to test for panmixis. For heterogeneity tests, at most three alleles were used, and if more alleles were found, these were pooled with the least common third allele. In all *G*-tests, a minimum expected number of 1 was required.

Genetic differentiation among populations was investigated at all hierarchical levels and quantified with the estimates of F_{IS}, F_{IT} and F_{ST} (Weir & Cockerham, 1984). Following the suggestions of Weir &

Cockerham (1984) the estimates of F_{IS}, F_{IT} and F_{ST} were also calculated using an unbiased jackknife estimator, which reduces the importance of a deviant population. Standard deviations were obtained using the jackknife procedure. Heterozygote deficiency was tested against the calculated fixation index (Swofford & Selander, 1989). A hierarchical F_{ST} analysis was performed to determine the amount of variance attributable to regional substructure (Wright, 1978; Swofford & Selander, 1989). Multilocus genetic distances were calculated using the 'coancestry distance' measure for distances between subpopulations (Reynolds *et al.*, 1983). Nei's (1972) genetic distance was estimated to compare intraspecific data with existing literature and to derive species ancestry. An unrooted tree based on maximum likelihood estimates was derived using the program CONTML from the PHYLIP software package (Felsenstein, 1985). The CONTML algorithm assumes divergence through drift only, and branch lengths are relative and not comparable among species. We measured the average genetic distances within and between regions, and compared species-specific structure using the Mantel test of morphological and environmental correlation (Mantel, 1967). This procedure tests the degree of randomness of the pairwise parameters. A total of 2000 permutations of the morphological matrix on the environmental matrix were performed.

Nonparametric tests were employed in all intraspecific analyses because of the bimodality of the data. Interspecific data fitted a Gaussian distribution (normality plot) and showed homoscedasticity of variances among species (F_{max}-test), except for tests of heterozygous loci per species and tests for average genetic distance between *C. trollii* and the other *Chiastocheta* species. The former were analysed with *t*-tests and the latter with Mann–Whitney *U*-tests. All significance levels are presented as table-wide significances for multiple tests within species or within character comparisons using the sequential Bonferroni technique as outlined by Rice (1989).

Results

Genetic variability

Across the three collection sites in each of the three regions of Jutland, average species sample sizes were similar, though differences were marginally significant (ANOVA, $F_{3,32} = 2.87$, $P = 0.052$) because of the small sample sizes for one species. Of the 21 loci only two, *Gpd* and *Dia-1*, were monomorphic (allele frequency table available from the authors on request). *Gpd* had the same mobility in all four species and *Dia-1* was diagnostic in *C. abruptiventris*. Heterozygosity was high for the enzymes *Pgm-1, Pgm-2, Ak, Est UV-1* and *Pep-B* in all species, though some alleles were species-specific. There were between three and five alleles in *Pgm-2* and *Pep-B* (except in *Pep-B* of *C. abruptiventris*, which had two) in all species. High levels of heterozygosity were found for *Mpi* in *C. dentifera*, for *Idh* in *C. dentifera* and *C. trollii* and for *Hk* in *C. abruptiventris*. Genotype frequencies for all populations of all four species showed no significant deviations from Hardy–Weinberg proportions. Between populations within species there were no differences in either heterozygosity or the mean number of alleles per locus.

Genetic variability measures are presented in Table 1. *Chiastocheta dentifera* populations did not show differences in the variability measures although variability was low at W2, where sample size was smallest. *Chiastocheta inermella* had only three loci, *Pgm-2, Est UV-1* and *Pep-B*, that were polymorphic in all populations, and it had the lowest heterozygosity estimate of the four species. Variability within *C. inermella* and *C. abruptiventris* was larger in west Jutland than in the other two regions. *Chiastocheta abruptiventris* was characterized by lacking loci having many alleles, and at only four loci was heterozygosity high (*Pgm-1, Pgm-2, Hk, Est Uv-1*) and at nine others heterozygosity was low. For *C. abruptiventris*, single locus heterozygosity was extremely bimodal. Six out of 13 polymorphic loci in *C. trollii* had a high degree of heterozygosity (*Pgm-1, Idh, Pgd, Est UV-1, Ak* and *Pep-B*). These were polymorphic in all populations. The locus *Pgm-2* expressed atypical variation as the 0.90 and 0.94 alleles were lacking in west Jutland and the less common allele 0.94 was nearly fixed at N3.

None of the species showed a significant correlation between local population sizes (estimated by Johannesen & Loeschcke, in press) and (i) number of alleles or (ii) heterozygosity (all species 7 d.f., W1 omitted as the population size was not estimated; all *P*-values >0.20). Under the infinite alleles model deviations from the expected allele frequency distributions were not found (Kolmogorov–Smirnov tests; Sokal & Rohlf, 1981). However, a pattern did emerge. *Chiastocheta inermella*, and to a lesser extent, *C. abruptiventris*, had more very rare alleles (frequency between 0 and 0.05) than expected, and *C. inermella* and *C. abruptiventris* also had an excess of alleles at intermediate frequencies.

Table 1 Genetic variability measures of *Chiastocheta* spp.

Patch	n	H_e	Polymorphic loci	Mean no. alleles/locus
C. dentifera				
E1	29	0.160	13	1.91
E2	26	0.136	11	1.62
E3	46	0.106	11	1.76
W1	37	0.177	14	1.81
W2	9	0.107	6	1.29
W3	24	0.127	10	1.57
N1	50	0.165	14	1.91
N2	16	0.116	9	1.57
N3	23	0.152	9	1.57
Mean		0.132	10.78	1.67
SD		0.026	2.64	0.12
C. inermella				
E1	41	0.095	7	1.57
E2	33	0.098	6	1.52
E3	25	0.077	5	1.33
W1	17	0.124	8	1.62
W2	31	0.118	7	1.57
W3	13	0.115	8	1.38
N1	38	0.088	6	1.48
N2	19	0.074	5	1.43
N3	29	0.102	6	1.62
Mean		0.099	6.33	1.50
SD		0.018	1.32	0.10
C. abruptiventris				
E1	11	0.082	6	1.33
E2	24	0.112	9	1.48
E3	21	0.082	7	1.38
W1	23	0.124	10	1.57
W2	32	0.119	11	1.57
W3	22	0.105	10	1.52
N1	20	0.093	7	1.38
N2	19	0.097	6	1.33
N3	20	0.110	9	1.48
Mean		0.103	8.33	1.45
SD		0.015	1.76	0.10
C. trollii				
E1	36	0.152	10	1.48
E2	43	0.144	9	1.76
E3	44	0.108	10	1.57
W1	28	0.135	10	1.57
W2	24	0.148	10	1.62
W3	43	0.106	8	1.57
N1	41	0.118	9	1.52
N2	39	0.146	10	1.71
N3	17	0.100	7	1.38
Mean		0.129	9.22	1.58
SD		0.021	1.05	0.12

n, mean sample size per locus.

Table 2 Summary of significance levels of interspecific pairwise comparisons. All measures are means based on population estimates. The mean genetic distance between populations is based on Reynolds *et al.* (1983) genetic distance

	Chiastocheta species		
	inermella	*abruptiventris*	*trollii*
C. dentifera			
No. polymorphic loci/popn	**	NS	NS
No. alleles/locus	NS	NS	NS
Heterozygosity	**	**	NS
Genetic distance	**	**	NS
C. inermella			
No. polymorphic loci/popn		NS	**
No. alleles/locus		NS	NS
Heterozygosity		NS	**
Genetic distance		NS	**
C. abruptiventris			
No. polymorphic loci/popn			NS
No. alleles/locus			NS
Heterozygosity			**
Genetic distance			**

**$P<0.01$.

The summary statistics of the between-species genetic variability measures are presented in Table 2. *Chiastocheta dentifera* and *C. trollii* had significantly more polymorphic loci per population than *C. inermella* (*C. dentifera*, $z = -3.09$, $P<0.01$; *C. trollii*, $z = -3.22$, $P<0.01$) (Tables 1 and 2). The difference between *C. abruptiventris* and *C. inermella* table-wide was marginally significant ($P = 0.035$). Also marginally significant was the difference between *C. dentifera* and *C. abruptiventris* ($P = 0.058$). Among species no significant differences in the average number of alleles per locus were found (Tables 1 and 2). *Chiastocheta inermella*, which had the lowest number of polymorphic loci, had more alleles per locus than *C. abruptiventris*. Many low frequency alleles were present at some loci in *C. inermella*, whereas, with the exception of one locus, *C. abruptiventris* possessed no more than two alleles per locus. The average heterozygosity divided the species into two groups: *C. dentifera*/*C. trollii* and *C. inermella*/*C. abruptiventris*. Within groups there were no significant differences in average heterozygosity, whereas differences between groups were all significant ($P<0.01$).

Table 3 Estimated *F*-statistics (Weir & Cockerham, 1984) of the *Chiastocheta* populations at all hierarchical levels. SDs were obtained using the jackknife procedure. Estimates are given for all nine populations (total), among regions (inter), and within each of the three regions (west, east, north). Unbiased jackknife estimates of F_{IT} and F_{ST} for *C. dentifera* (west) and *C. trollii* (north) are shown in parentheses. All other unbiased estimates did not show substantial deviations from the standard estimation

Level	F_{IS}	F_{IT}	F_{ST}
C. dentifera			
Inter	0.156	0.180	0.028
West	0.152	0.249	0.115
		(0.189)	(0.044)
East	0.104	0.217	0.126
North	0.071	0.111	0.044
Total	0.104	0.183	0.089
SD	0.015	0.038	0.035
C. inermella			
Inter	0.061	0.097	0.038
West	0.042	0.074	0.033
East	0.066	0.072	0.006
North	0.038	0.090	0.054
Total	0.051	0.101	0.053
SD	0.012	0.021	0.018
C. abruptiventris			
Inter	0.035	0.057	0.023
West	−0.036	−0.009	0.026
East	0.034	0.102	0.070
North	0.065	0.074	0.010
Total	0.008	0.050	0.042
SD	0.026	0.024	0.015
C. trollii			
Inter	0.130	0.167	0.043
West	0.034	0.073	0.038
East	0.084	0.188	0.113
North	0.048	0.220	0.181
		(0.129)	(0.083)
Total	0.059	0.166	0.114
SD	0.030	0.042	0.033

Genetic differentiation

At the total population level within Jutland (Table 3), *C. trollii* was most differentiated ($F_{ST} = 0.114 \pm 0.033$), with an inbreeding estimate of $F_{IS} = 0.059 \pm 0.030$. *Chiastocheta dentifera* was the second most differentiated ($F_{ST} = 0.089 \pm 0.035$), and consistently had the highest single locus F_{IS} estimates of the four species; the inbreeding estimate ($F_{IS} = 0.104 \pm 0.015$) was the largest of all the species (*t*-test; $P < 0.001$). Population differentiation was low for *C. inermella* ($F_{ST} = 0.053 \pm 0.018$) and *C. abruptiventris* ($F_{ST} = 0.042 \pm 0.015$), both of which exhibited homogeneous single-locus variances. The inbreeding estimate for *C. abruptiventris* was low ($F_{IS} = 0.008 \pm 0.026$). Nine out of 13 single-locus estimates showed heterozygote excess. *C. inermella* had a significantly higher inbreeding coefficient ($F_{IS} = 0.051 \pm 0.012$) than *C. abruptiventris* (*t*-test, $P < 0.01$) but relatively lower positive single-locus F_{IS} values than *C. abruptiventris*. Significant single-locus heterozygote deficiency ($P < 0.05$) was found for only two loci of *C. dentifera*, *Est UV-1* at E3 and *Pgm-1* at N1.

The within-region F_{ST} estimates (Table 3) showed a high degree of isolation in east Jutland, with the differentiation higher within the region than at the total population level for two species and similar for a third. Here only *C. inermella* had a low F_{ST} value. Differentiation in north and west Jutland was low. In one population of *C. dentifera* in west Jutland and one population of *C. trollii* in north Jutland low heterozygosity and unusual allele frequencies were observed. This caused the differentiation of these two species in these regions to become higher than at the total population level. However, calculating F_{ST} using the unbiased jackknife estimator revealed much lower F_{ST} values for *C. dentifera* in the west ($F_{ST} = 0.044$) and for *C. trollii* in the north ($F_{ST} = 0.083$) than the standard measures presented previously. No other estimates were significantly changed using this procedure. As at the total population level, *C. dentifera* and *C. trollii* were more

Table 4 Hierarchical *F*-statistics and variance components (Wright, 1978) for *Chiastocheta* spp. with two levels of subdivision (S population; R region)

Species	F_{SR}	VC	F_{ST}	VC	F_{RT}	VC
C. dentifera	0.116	0.389	0.094	0.309	−0.024	−0.080
C. inermella	0.040	0.087	0.053	0.118	0.014	0.031
C. abruptiventris	0.036	0.083	0.042	0.096	0.006	0.013
C. trollii	0.134	0.402	0.134	0.402	0.000	0.000

VC, variance component.

differentiated within regions than were *C. inermella* and *C. abruptiventris*. The within-region F_{IS} estimates followed the total population estimates, with *C. dentifera* having higher F_{IS} values than the other species.

For both single-locus and total variance estimates differentiation among regions (Table 3) was smaller than at the total population and regional levels. At the inter-regional level differentiation of all species was similar. Only *C. inermella* showed a tendency to be more differentiated at the larger scale examined. These findings were supported using the hierarchical analysis of Wright (1978) (Table 4), which showed that regions had no effect on the variance component for *C. dentifera* and *C. trollii*. Only 14 per cent of the variance found among populations for *C. abruptiventris* was caused by regional subdivision, but the corresponding figure for *C. inermella* was 25 per cent.

Heterogeneity *G*-tests for pair-wise significance of allele frequencies between populations (data not shown) revealed division of species into two heterogeneity classes: *C. dentifera*/*C. trollii* and *C. inermella*/*C. abruptiventris*. The former group was more differentiated than the latter. Pairwise comparisons of the relationship between populations revealed no consistent within-region homogeneity for *C. dentifera* populations where only one (N1/N2) of a total of three nonsignificant pairs was within a region. Population N1, which was included in all nonsignificant pairs, exhibited an average allele frequency. The only nonsignificant *C. trollii* pair was W1/W2. This pair also was homogeneous for *C. inermella* and *C. abruptiventris*. *Chiastocheta inermella* had nine nonsignificant pairs. Within east Jutland two nonsignificant pairs were found and one pair was significant at the 5 per cent level. Otherwise there was no consistent within-region homogeneity for *C. inermella*. *Chiastocheta abruptiventris* had 11 nonsignificant pairwise comparisons. Within north Jutland two nonsignificant pairs and one significant pair at the 5 per cent level were observed, and within west Jutland, two nonsignificant pairs were found. East Jutland had one nonsignificant pair and one pair at the 5 per cent level.

Genetic distances and phylogenetic trees

The interspecific genetic distances based on Nei's (1972) distance measure ordered *C. dentifera*, *C. inermella* and *C. trollii* in one cluster, with *C. abruptiventris* as an outgroup (Table 5).

The average intraspecific distances (Nei, 1972; Reynolds *et al.*, 1983) are presented in Table 6. The variance in mean genetic distance between populations was homoscedastic for *C. dentifera*, *C. inermella* and *C. abruptiventris*. On the other hand, the among-population genetic distances for *C. trollii* had a significantly higher variance than did those of *C. inermella* and *C. abruptiventris*. This difference was caused by the *C. trollii* population at N3 with its almost fixation of the *Pgm-2* 0.94-allele. The average genetic distances between populations of *C. dentifera* and *C. trollii* were significantly higher than those of *C. inermella* and *C. abruptiventris* (Tables 2 and 6).

The average genetic distance among populations was not significantly different within or between regions for any species (*C. dentifera*, $P = 0.59$; *C. inermella*, $P = 0.11$; *C. abruptiventris*, $P = 0.29$; *C. trollii*, $P = 0.57$). Similarly, neither were any of the Mantel tests of morphological and environmental correlation significant: *C. dentifera* $g = -1.05$, $P = 0.85$, $g > 12.2$ per cent of the randomized distributions; *C. inermella* $g = 1.23$, $P = 0.11$, $g > 88$ per cent; *C. abruptiventris* $g = -0.43$, $P = 0.67$, $g > 38$ per cent and *C. trollii* $g = 0.65$, $P = 0.25$, $g > 81$ per cent.

None of the CONTML phylogenetic trees was in complete accordance with expectation from geography (Fig. 1). *Chiastocheta abruptiventris* showed the most concordant geographical structuring. In *C. trollii*, the western populations clustered together, as did N2 and N3. The between-population divergence was higher in east Jutland than in the other regions which contributed to the distorted setting of the eastern populations. For *C. inermella* high divergence in northern populations caused N3 not to cluster with N1 and N2. *Chiastocheta dentifera* showed no correlation between genetic and geographical structure.

Discussion

Genetic variability

Comparing the four species we found that the amount of genetic variability reflected overall species abundance but not population size within a

Table 5 Genetic distances of *Chiastocheta* spp., calculated over 21 loci by Nei's (1972) distance measure

Species	*C. inermella*	*C. abruptiventris*	*C. trollii*
C. dentifera	0.366	0.753	0.236
C. inermella		0.796	0.256
C. abruptiventris			0.731

Table 6 Summary of all genotypic measures of *Chiastocheta* spp. Measures are presented as relative values and do not necessarily translate to absolute significances

Category	*C. dentifera*	*C. inermella*	*C. abruptiventris*	*C. trollii*
Population size	high	medium	low	high
F_{ST}	medium	low	low	high
F_{IS}	high	low	low	medium
H_e	high	low	low	high
No. polymorphic loci	high	low	medium	medium
Mean no. alleles/locus	medium	medium	medium	medium
Within/between regional fit	low	high	medium	low
Mantel fit	low	high	low	medium
CONTML	low	medium	high	medium
Genetic distances:				
Reynolds *et al.*(1983)	high (0.123 ± 0.071)	low (0.078 ± 0.047)	low (0.072 ± 0.038)	high (0.150 ± 0.113)
Nei (1972)	medium (0.034 ± 0.020)	medium (0.026 ± 0.017)	low (0.015 ± 0.008)	high (0.062 ± 0.088)

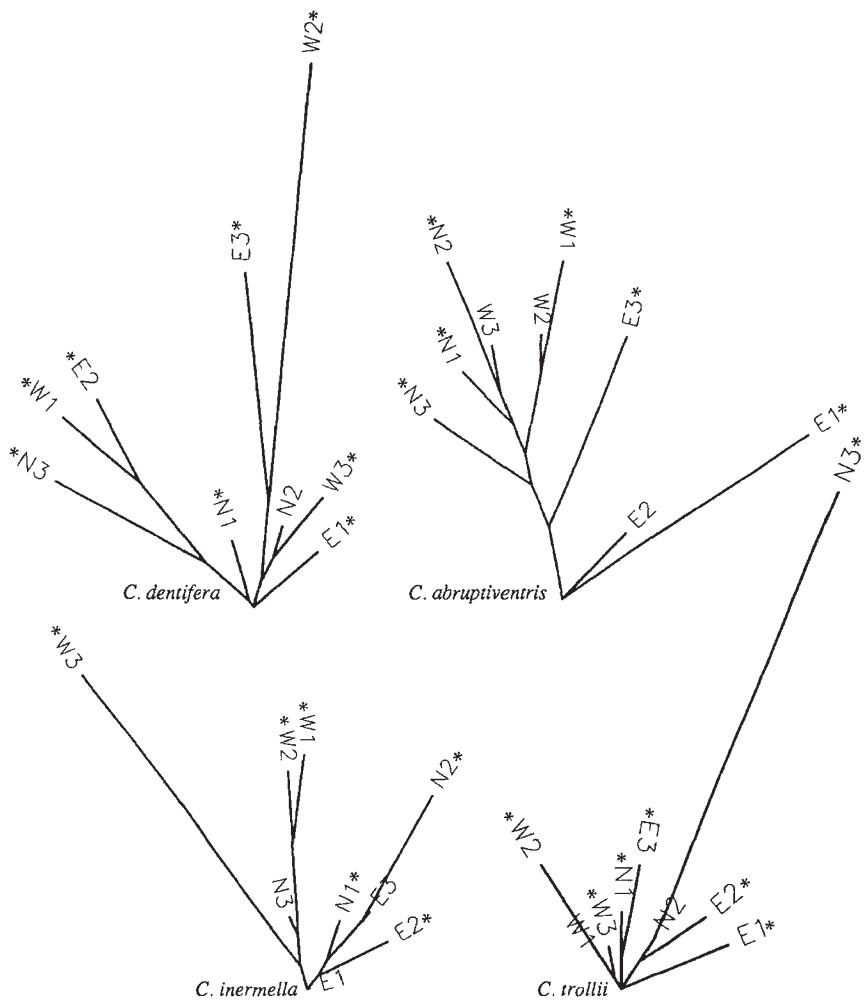

Fig. 1 Unrooted trees of population divergence for *Chiastocheta* spp. based on maximum likelihood estimation, calculated from allele frequencies. Branch lengths are relative and not comparable between species. *Significant branch length based on confidence intervals.

species. Thus, there were significant differences in the amount of genetic variability among species and among populations within species. The heterozygosities found in the *Chiastocheta* species studied ($0.099 < H_e < 0.132$) were generally within the ranges found in other Diptera (e.g. Powell, 1975; Bryant *et al.*, 1981; Black & Krafsur, 1985; Krafsur, 1993), though lower than found for the Horn fly (estimated in Krafsur, 1993). Interspecific differences were not the result of different sample sizes and there was no significant difference in the average number of alleles per locus for any of the species.

The hierarchical analysis indicated that migration did not greatly influence the genetic composition of the populations (see also next section, second paragraph). If neutrality of alleles is assumed, as indicated by the allele distribution, the number of alleles in constant-sized patches should correlate positively to patch size, partly as a result of higher mutation input and a lower loss of variability through drift (Wright, 1931; Nei *et al.*, 1975). Such correlations, however, were not found, and although population sizes were positively correlated with patch size (Johannesen & Loeschcke, in press), population size did not reflect the genetic composition of these populations. The low correlation between intraspecific genetic variability and population size may be a consequence of single patch history. Inference of a unique patch event was gained at E3, where heterozygosity was low in all species despite large population sizes. Perhaps a recent increase in host plant abundance at E3 may have caused fly population sizes to increase but rendered from only a few founders. The nonsignificant intraspecific differences in heterozygosity were partly a consequence of rare alleles in some populations, which caused a bimodal distribution of single-locus data and gave high between-locus variance. Nonparametric tests were required and these are conservative (Archie, 1985). Therefore, intraspecific genetic variation may have been present.

Although no correlations between population sizes and variability parameters were found within any of the species, regional habitat history, in the manner of the locality subdivision, may play an important role in preserving variation. In west Jutland, where habitat fragmentation has occurred most recently (during the past 15 years; personal observation), and additional patches were found between those sampled, three species had homogeneous allele frequency distributions between patches W1 and W2, suggesting that these two were originally part of one larger patch. In this region heterozygosity was highest for the two species with low genetic variability and small population sizes, *C. inermella* and *C. abruptiventris*. Migration between semi-isolated patches would give rise to a peak at intermediate allele frequencies, as observed for these two species. The observed excess of rare alleles of *C. inermella* especially may be ascribed to bottleneck events. Alternatively, rare alleles may result from slightly deleterious alleles, but if true, one would expect an excess of rare alleles in larger populations (Chakraborty *et al.*, 1980). As the opposite pattern was found, the excess therefore was most likely to have been caused by bottlenecks within patches. Compared to *C. inermella* and *C. abruptiventris* the amount and distribution of variability in *C. dentifera* and *C. trollii* seemed to be influenced more by single-patch events than by the regional network of patches, because of lower apparent dispersal ability. Consequently, geographical scale probably had different effects on genetic variability in the groups *C. dentifera*/*C. trollii* and *C. inermella*/*C. abruptiventris*.

Genetic differentiation and population structure

By combining a hierarchical analysis with an across-species analysis, we found that the amount of differentiation among populations was primarily a feature of regional biogeography, whereas the population structure is more likely to be related to species-specific gene flow patterns. The amount of variability and the general population structure did not reflect phylogenetic constraints, and the species could be viewed as distinct evolutionary lineages and analysed independently. For example, the low variability species *C. inermella* grouped phylogenetically to the two high variability species, and the highly differentiated *C. trollii* showed an intraspecific tree morphology similar to the two low variability species. Assuming neutrality of genotypes, the similarity among populations should depend on geographical distance and gene flow only. In this sense we used the hierarchical levels for inference of specific gene flow patterns, and the species as replicate experiments for inference of habitat history. However, these two analyses are not independent, as specific gene flow patterns may override a local influence of geography on population structure.

The lack of hierarchical population structure in *C. dentifera*, *C. trollii* and to a lesser degree in *C. abruptiventris* resulted in the network of habitat patches being important for the degree of differentiation of these species. All three showed low differentiation in north and west Jutland, compared to the eastern populations where no additional

patches were found between those sampled. The lack of hierarchical structure suggests isolation at the local level; the intraregional differentiation is higher than that at the total population level. The cause for lack of inter-regional differentiation is more difficult to interpret as within-region patches were pooled; patches that in many cases were found to differ significantly in allele frequency (*G*-tests). Loci that were differentiated intraregionally became homogeneous inter-regionally when pooled. This homoscedasticity indicates that differentiation found among populations is caused primarily by drift, as local patches act as discrete entities with little migration, and that selection at the regional level cannot explain the differentiation among populations at the total level.

Only *C. inermella* became more differentiated at larger scales, indicating an isolation-by-distance genetic structure. This was true not only for the hierarchical analysis, but also for the mean distance among populations within regions, and resulted in a conflicting estimate among species for the fragmentation of east Jutland relative to the other two regions. However, the intraspecific distances of the four discretely distributed *Chiastocheta* species were relatively large compared to those found for the more continuously distributed nature of *Drosophila melanogaster* (Singh & Rhomberg, 1987).

The effect of gene flow patterns on the amount of differentiation was exemplified by *C. trollii* and *C. dentifera* which had the lowest gene flow estimates in all regions and at the total population level. However, the differentiation of the *C. dentifera* populations may have been overestimated, and hence the level of gene flow underestimated. Intraspecific genetic distances of *C. dentifera* were not different from *C. inermella* when compared by Nei's (1972) distance measures, but were significant when calculated by the measure of Reynolds *et al.* (1983). Rare alleles were present in many populations. The discrepancy arises because Nei's measure assumes differentiation caused by mutation only, whereas Reynolds *et al.* assume differences caused only by drift (Tomiuk & Loeschcke, 1995). Increased migration may be caused by reduction of patch size (McCauley, 1991 and references therein). *Chiastocheta dentifera*'s stochastic distribution, coupled to its pronounced temporal occurrence, could be an indication of migration caused by sensitivity to patch fluctuations. If migration is higher than implied by the F_{ST} value and if local extinction is great, a directional gene flow could mediate increased genetic variance (Slatkin, 1977) and could also mediate a tree morphology unrelated to geography. Also, *C. dentifera* had the most species-specific alleles (10) compared to diagnostic loci (one only). Mutation with subsequent drift in small populations may have established new alleles at the species level. *Chiastocheta inermella* had, with an even relative abundance and high rate of gene flow, a relationship of 2.5:1. Based on the data, *C. dentifera* should react more strongly to regional habitat alterations than *C. inermella*. The above examples imply differences in gene flow patterns between *C. dentifera* and the other three species. Previously, lack of inter-regional differentiation, with low levels of gene flow among regions, has been related to different migration patterns of central and border populations in the butterfly *Parnassius mnemosyne* (Descimon & Napolitano, 1993; Napolitano & Descimon, 1994).

For the cluster analyses, our only expectation of tree morphology was that regions should cluster. Our cluster analyses showed that regions were generally well defined for *C. abruptiventris*, *C. trollii* and *C. inermella*. It was observed that the amount of differentiation need not necessarily predict the relationship among populations. Despite a higher differentiation among populations, the phylogenetic tree of *C. trollii* populations was closer to the expected tree based on geography than the tree for *C. dentifera*, and *C. trollii* had a phylogenetic tree similar to that of *C. inermella*, where populations within two regions clustered as expected from geography, though the regions differed. The eastern populations of *C. abruptiventris* clustered together despite the great within-regional variance compared to the among-regional variance. The most likely reason for random clustering was population size fluctuations. The differences in the amount of differentiation of *C. trollii*, *C. inermella* and *C. abruptiventris* were determined by the dispersal probability, and by the network of habitat patches at a regional level.

Acknowledgements

We are grateful to Bob Krebs, Jürgen Tomiuk, Jørn Madsen, Mikkel Schierup, Christian Damgaard, Jens Mogens Olesen and Hans Siegismund for helpful discussions and comments on the manuscript, to Verner Michelsen for help with the identification of flies and for providing an unpublished revision of the genus *Chiastocheta*, and to Kirsten Petersen and Doth Andersen for technical assistance. Hans Siegismund also provided advice on data analysis using his statistical package G-Stat. The study was supported by grants from the Danish National Science Research Council (Nos 11–9025–1 and 11–9639–1) and Mag. Art. Marcus Lorentsens Mindelegat.

References

ARCHIE, J. W. 1985. Statistical analysis of heterozygosity data: independent sample comparisons. *Evolution*, **39**, 623–637.

AYALA, F. J., POWELL, J. F., TRACY, M. L., MAORAOC, A. AND PERÉZ-SALAS, S. 1972. Enzyme variability in the *Drosophila willistoni* group. IV. Genic variation in natural populations of *Drosophila willistoni*. *Genetics*, **70**, 113–139.

BLACK, W. C., IV, AND KRAFSUR, E. S. 1985. Electrophoretic analysis of genetic variability in the house fly (*Musca domestica* L.). *Biochem. Genet.*, **23**, 193–203.

BRYANT, E. H., VAN DIJK, H. AND VAN DELDEN, W. 1981. Genetic variability of the face fly, *Musca automalis* De Geer, in relation to a population bottleneck. *Evolution*, **35**, 872–881.

CHAKRABORTY, R., FUERST, P. A. AND NEI, M. 1980. Stastistical studies on protein polymorphism in natural populations. III. Distribution of allele frequencies and the number of alleles per locus. *Genetics*, **94**, 1039–1063.

COLLIN, J. E. 1954. The genus *Chiastocheata* Pokorny (Diptera: Anthomyiidae). *Proc. R. Ent. Soc. Lond. B*, **23**, 95–102.

DESCIMON, H. AND NAPOLITANO, M. 1993. Enzyme polymorphism, wing pattern variability, and geographical isolation in an endangered butterfly species. *Biol. Conserv.*, **66** 117–123.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.

GILPIN, M. 1991. The genetic effective size of a metapopulation. *Biol. J. Linn. Soc.*, **42**, 165–175.

HANSKI, I. 1991. Single-species metapopulation dynamics: concepts, models and observations. *Biol. J. Linn. Soc.*, **42**, 17–38.

HARRIS, H. AND HOPKINSON, D. A. 1978. *Handbook of Enzyme Electrophoresis in Human Genetics. Supplement*. North-Holland Publishing Company, Amsterdam.

JOHANNESEN, J. AND LOESCHCKE, V. Distribution, abundance and oviposition patterns of four coexisting *Chiastocheta* species (Diptera: Anthomyiidae). *J. Anim. Ecol.* (in press).

KIMURA, M. 1983. *The Neutral Theory of Molecular Evolution*. Cambridge University Press, Cambridge.

KIMURA, M. AND CROW, J. F. 1964. The number of alleles that can be maintained in a finite population. *Genetics*, **49**, 725–738.

KRAFSUR, E. S. 1993. Allozyme variation in stable flies (Diptera: Muscidae). *Biochem. Genet.*, **31**, 231–240.

LOUIS, E. J. AND DEMPSTER, E. R. 1987. An exact test for Hardy–Weinberg and multiple alleles. *Biometrics*, **43**, 805–811.

MANTEL, N. 1967. The detection of disease clustering and a generalised regression approach. *Cancer Res.*, **27**, 209–220.

MARUYAMA, T. AND KIMURA, M. 1980. Genetic variability and effective population size when local extinction and recolonization of subpopulations are frequent. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 6710–6714.

McCAULEY, D. E. 1989. Extinction, colonization, and population structure: a study of a milkweed beetle. *Am. Nat.*, **134**, 365–376.

McCAULEY, D. E. 1991. The effect of host plant patch size variation on the population structure of a specialist herbivore insect, *Tetraopes tetraophthalmus*. *Evolution*, **45**, 1675–1684.

MICHELSEN, V. 1985. A revision of the Anthomyiidae (Diptera) described by J. W. Zetterstedt. *Steenstrupia*, **11**, 37–65.

NAPOLITANO, M. AND DESCIMON, H. 1994. Genetic structure of French populations of the mountain butterfly *Parnassius mnemosyne*, L. (Lepidoptera: Papilionidae). *Biol. J. Linn. Soc.*, **53**, 325–341.

NEI, M. 1972. Genetic distance between populations. *Am. Nat.*, **106**, 283–291.

NEI, M., MARUYAMA, T. AND CHAKRABORTY, R. 1975. The bottleneck effect and genetic variability in populations. *Evolution*, **29**, 1–10.

PELLMYR, O. 1989. The cost of mutualism: interactions between *Trollius europaeus* and its pollinating parasites. *Oecologia*, **78**, 53–59.

PELLMYR, O. 1992. The phylogeny of a mutualism: evolution and coadaptation between *Trollius* and its seed-parasitic pollinators. *Biol. J. Linn. Soc.*, **47**, 337–365.

POWELL, J. R. 1975. Protein variation in natural populations of animals. *Evol. Biol*, **8**, 79–119.

PULLIAM, R. H. 1988. Sources, sinks and population regulation. *Am. Nat.*, **132**, 652–661.

REYNOLDS, J., WEIR, B. S. AND COCKERHAM, C. C. 1983. Estimation of the coancestry coefficient: basis for a short-term genetic distance. *Genetics*, **105**, 767–779.

RICE, W. R. 1989. Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.

RICHARSON, B. J., BAVERSTOCK, P. R. AND ADAMS, M. 1986. *Allozyme Electrophoresis. A Handbook for Animal Systematics and Population Studies*. Academic Press Inc., San Diego.

SELANDER, R. K., SMITH, M. H., YANG, S. Y., JOHNSON, W. E. AND GENTREY, J. B. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse (*Peromyscus polionotus*). Studies in Genetics IV. Univ. Texas Publ. no. 7103, 49–90.

SINGH, R. S. AND RHOMBERG, L. R. 1987. A comprehensive study of genic variation in natural populations of *Drosophila melanogaster*. I. Estimates of gene flow from rare alleles. *Genetics*, **115**, 313–322.

SLATKIN, M. 1977. Gene flow and genetic drift in a species subject to frequent local extinctions. *Theor. Pop. Biol.*, **12**, 253–262.

SLATKIN, M. 1985. Gene flow in natural populations. *Ann. Rev. Ecol. Syst.*, **16**, 393–430.

SLATKIN, M. 1993. Isolation by distance in equilibrium and non-equilibrium populations. *Evolution*, **47**, 264–279.

SOKAL, R. R. AND ROHLF, F. J. 1981. *Biometry*, 2nd edn. W. H. Freeman and Co., New York.

SWOFFORD, D. L. AND SELANDER, R. B. 1989. BIOSYS-1: a Fortran program for the comprehensive analysis of electrophoretic data in population genetics and system-

atics. *J. Hered.*, **72**, 281–283.

TOMIUK, J. AND LOESCHCKE, V. 1995. Genetic identity combining mutation and drift. *Heredity*, **74**, 607–615.

WADE, M. J. AND MCCAULEY, D. E. 1988. Extinction and recolonization: their effects on the genetic differentiation of local populations. *Evolution*, **42**, 995–1005.

WEIR, B. S. AND COCKERHAM, C. C. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

WHITLOCK, M. C. AND MCCAULEY, D. E. 1990. Some population genetic consequences of colony formation and extinction: genetic correlations within founding groups. *Evolution*, **44**, 1717–1724.

WRIGHT, S. 1931. Evolution in Mendelian populations. *Genetics*, **16**, 97–159.

WRIGHT, S. 1978. *Evolution and the Genetics of Populations*, vol. 4, *Variability Within and Among Natural Populations*. University of Chicago Press, Chicago.