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Ecomorphological and genetic divergence between lowland and montane forms of the *Pieris napi* species complex (Pieridae, Lepidoptera)

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The present study aimed to investigate the morphological and genetic differentiation of lowland and montane populations of the *Pieris napi* species complex in Norway and to make inferences about the subspecific status of *Pieris napi adalwinda* and *Pieris napi napi*. We measured 22 morphological characters on 228 individuals from eight populations in Central Norway (20–1100 m a.s.l.). In addition, lowland and mountain animals were reared at a high altitude locality. Half the animals from either locality were reared on mountain plant, and the other half on a lowland plant. Finally, a fragment of the mitochondrial cytochrome *b* gene was sequenced for individuals from Central and South-eastern Norway and Germany. Principal component analysis of morphological characters showed a zone of abrupt change from lowland to mountain morphological character states between populations at 506 m and 730 m a.s.l., respectively. The transplant experiment showed that the morphological differences have a genetic basis and that food plants have no impact on morphology. Limited, but significant, molecular genetic differentiation was found between lowland and mountain animals, but the phylogenetic analysis, however, showed that the lowland form (*P. n. napi*) is paraphyletic and the montane form (*P. n. adalwinda*) is monophyletic. Further study is required before taxonomic recognition can be applied.

ADDITIONAL KEYWORDS: adaptation – altitude gradient – cline – Lepidoptera – melanization – morphology – mtDNA.

INTRODUCTION

Taxonomists defining species and subspecies make taxonomic decisions based on inferences about population processes, particularly (reproductive) isolation and adaptation (Mayr, 1963). Difficulties arise when two or more phenotypically distinct but closely-related populations replace each other along an environmental gradient. The geographical range limits of these populations can be affected by genetic differentiation and phenotypic plasticity. If sufficient genetic variation is present, selection can create genetic differentiation in fitness-related traits across the gradient. Gene flow from the central to marginal populations and genetic drift in the marginal populations can constrain this selection-mediated range expansion (Endler, 1977; Hoffman & Blows, 1994; Case & Taper, 2000). In alpine landscapes, the short distance between clearly different biotopes bring taxa with supposedly different habitat preference into close contact (Porter, 1997). Closely-related taxa that come into contact may show clinal variation over medium to long distances, form hybrid zones over short distances or not interbreed at all, well within the range that individuals can move within their lifetime (Mayr & Ashlock, 1991). The source of phenotypic variation, whether it be phenotypic plasticity, hybridization or genetic adaptation, is of great impor-

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tance when assessing subspecies status. However, sources of variation have often not been taken into consideration in the past, leading to the naming of numerous subspecies based on poor data (Mayr, 1982; Frost & Hillis, 1990; Manier, 2004).

The systematics and taxonomy of the family Pieridae (Lepidoptera) have been studied extensively, but there are divergent views when it comes to the relationships and status of several taxa at different hierarchical ranks, from subfamilies to subspecies (Geiger, 1980). Problems at the species and subspecies level are very pronounced in the *Pieris napi* species complex and the theories of this group's evolution are many and uncertain (Petersen, 1949; Bowden, 1972; Geiger & Shapiro, 1992). The *P. napi* complex contains taxa that are distributed over the Holarctic region (Geiger & Scholl, 1985). In Europe, the relationships between the *P. napi* (Linnaeus, 1758) and *Pieris bryoniae* Ochsenheimer, 1808 have been well studied (Drosihn, 1933; Müller & Kautz, 1939; Petersen & Tenow, 1954; Bowden, 1979; Geiger & Scholl, 1985; Porter & Geiger, 1995). *Pieris napi* is a widespread species in Europe and occurs at elevations below approximately 1200 m. *Pieris bryoniae* occurs in the Alps and the Carpathians at elevations above 1200 m (Porter, 1997).

In Scandinavia, there is an analoguous situation, where the differences are not only seen across the altitude gradient, but also across the latitude gradient from south to north. In Scandinavia, the two forms usually are described as subspecies based on morphological differences. At low altitude and latitude, *Pieris napi napi* (Linnaeus, 1758) is widespread and, at higher altitudes and latitudes, *Pieris napi adalwinda* Fruhstorfer, 1909 is found (Petersen, 1947). In the northern parts of Scandinavia, *P. n. adalwinda* is found all the way from the coastline to the mountains and *P. napi napi* is not present (Petersen, 1947). These lowland and montane/arctic forms, both in Scandinavia and Central Europe, differ in several ways. The most striking differences are found in wing morphology, especially the amount of melanization and the ground colour on the wing surface of the females. The *P. n. adalwinda* and *P. bryoniae* females have yellow ground colour on the ventral surface and a broad and dark, almost coherent melanization along the veins, whereas the *P. n. napi* and *P. napi* females are white with only minuscule amounts of melanization, but with a pronounced dark apical spot and discal spots (Müller & Kautz, 1939; Petersen, 1947; Varga & Toth, 1978). There are also differences in life-history traits, mainly those involved in the control of pupal diapause. The montane forms are strictly univoltine, and always produce one generation per year. The lowland form seems to be facultative uni- or multivoltine and produces one or more generations dependent on photoperiod and temperature. The difference in melanization between the montane/arctic (dark) and lowland forms (white) resembles the seasonal polyphenism observed in *P. napi* (Bowden, 1978) and several other butterfly taxa (*Bicyclus* spp., Roskam & Brakefield, 1996; *Araschnia* spp., Fric, Konvicka & Zrzavy, 2004; *Polygonia c-album*, Wiklund & Tullberg, 2004), with the first-generation animals having a broader melanic pattern than the second generation (Shapiro, 1976).

Differences in behaviour (Petersen, Tornblom & Bodin, 1951) and habitat preference (Müller & Kautz, 1939; Petersen, 1947; Petersen & Tenow, 1954) have also been found between the lowland and mountain forms. However, some traits that often vary among closely-related butterfly taxa show no variation between these forms. The genitalia of *P. napi* and *P. bryoniae* are indistinguishable (Drosihn, 1933). Analysis of allozymes shows high genetic diversity within *P. napi* and *P. bryoniae*, but only a slight genetic differentiation between them, and gene flow between the lowland and mountain forms is supposed to occur both in Scandinavia and Central Europe (Porter & Geiger, 1995). Studies of a narrow hybrid zone in the Alps showed little or no differential selection on host use between *P. napi* and *P. bryoniae* (Porter, 1997).

The Norwegian fjord and mountain landscape, with a steep gradient in altitude, gives a unique possibility to study clines in morphology, ecology and genetics along altitude differences within an area of limited size. The present study aimed to investigate the morphological and genetic differentiation of lowland and montane populations of the *P. napi* complex in Norway and to make inferences about the subspecific status of *P. n. adalwinda* and *P. n. napi*.

To resolve this problem, we performed a multivariate analysis of morphological characters across an elevational gradient, a population transplant experiment where lowland animals were reared under mountain conditions, and sequencing of a molecular marker, the cytochrome *b* gene of the mitochondrial DNA.

MATERIAL AND METHODS

Sampling and sample area

Adult butterflies were collected from nine sample sites in Central Norway, two in South-Eastern Norway (Fig. 1A, B) and one in Northern Germany. Details of sample size, geographical position and altitude of each sample site are given in Table 1. Eight of the localities were located in the Sunndalen-Drivdalen valley system where the elevation rises from 0–1100 m a.s.l. over a distance of approximately

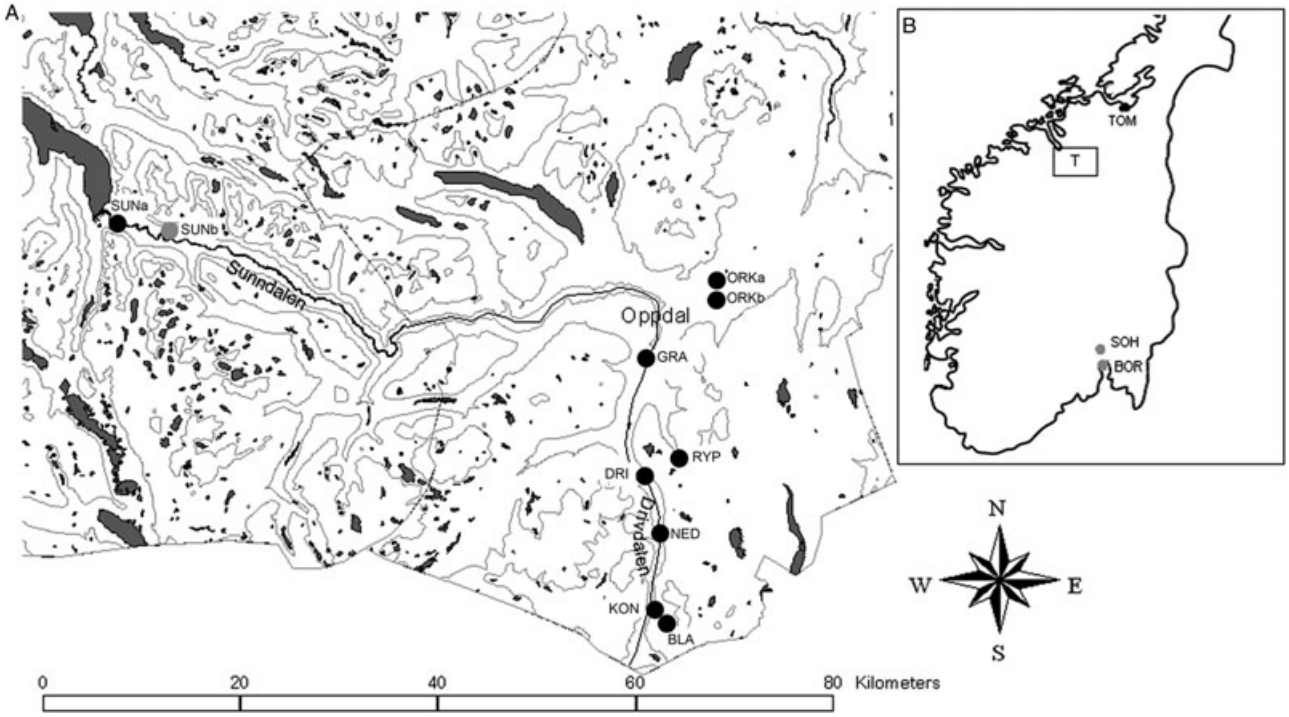

Figure 1. A, map over the Sunndalen-Drivdalen valley system showing the locations of the sample sites in the altitude gradient. B, map showing the location of the valley system (box denoted T) and the location of the remaining sample sites. In sites represented with a black dot, only first generation animals were sampled and, in sites represented with a grey dot, only second-generation animals were sampled.

150 km. The annual mean temperature drops from 6.7 °C at sea level to –0.4 °C at 900 m a.s.l.

Morphological analysis

The selected morphological characters were known to be distinguishing from the literature (Müller & Kautz, 1939; Petersen, 1947; Eitschberger, 1983). The sexes had to be measured independently because of profound sexual dimorphism. For each individual, ten characters were measured manually and 12 characters by digital image analysis. An additional six characters were derived from these characters (Table 2, Fig. 2A, B). Only wing characters were chosen because only wings were available for several individuals. The morphology of a total of 152 males and 59 females from the eight populations in the Sunndalen-Drivdalen valley system was compared to infer the structure of the morphological change; whether a cline, hybrid zone or total separation was present. A standardized principal components analysis (PCA) was performed, to test the null hypothesis of clinal morphological variation in the elevational gradient. A one-way analysis of variance (ANOVA) followed by Tukey's test was carried out, to see whether individual principal component scores differed significantly between populations. The PCA was performed using CANOCO for Windows 4.5 (Ter Braak & Smilauer, 2002) and the univariate analyses were performed using S-Plus, version 6.2 (Venables & Ripley, 1999).

Population transplant experiment

Seven females captured at Sunndalsøra (SUN), at sea level, were transported to Kongsvoll (KON), 900 m a.s.l. In addition, three females caught at Kongsvoll were used in the experiments. The females were induced to oviposit individually in transparent plastic boxes containing *Cardamine amara* L. and kept outdoors at Kongsvoll. Upon hatching, half the larvae from each brood were transferred to plastic boxes containing a lowland plant, *C. amara* L., and the other half to boxes containing a montane plant, *Arabis alpina* L. All plants were wild and searched for predators, eggs and other larvae before given to the larvae. The boxes were covered with a small-meshed net to keep the temperature approximately the same as the outdoor air temperature and to prevent larvae from escaping. The larvae were fed until they pupated in late July and early August 2003, and the pupae were then individually transferred to glass jars for over-wintering.

Table 1. Summary of all populations including locality, sample size, haplotype and nucleotide diversity

Population	Locality	Latitude/ longitude	Altitude (m)	Total sample size	Morphology sample size	mtDNA sample size	Number of haplotypes	Haplotype diversity	Nucleotide diversity
SUN	Sunndalen/Central			34	33	10	2 (Pier2, Pier4)	0.56 ± 0.09	0.0045 ± 0.0033
	(a) Hoven	62°39′N, 8°33′E	20	26	26	5	2 (Pier2, Pier4)		
	(b) Bjørnhjell	62°38′N, 8°33′E	50	8	7	5	2 (Pier2, Pier4)		
ORK	Orkeldalsveien/ Central			35	30	5	2 (Pier1, Pier3)	0.60 ± 0.18	0.0051 ± 0.0041
	(a) Stølen	62°36′N, 9°43′E	560	10	10	–			
	(b) Orkeldalsveien	62°35′N, 9°43′E	560	25	20	5	2 (Pier1, Pier3)		
GRA	Granmo/Central	62°32′N, 9°37′E	506	17	17	10	3 (Pier1, Pier2, Pier5)	0.64 ± 0.15	0.0061 ± 0.0042
DRI	Drivstua/Central	62°25′N, 9°37′E	713	5	5	5	2 (Pier1, Pier2)	0.40 ± 0.24	0.0011 ± 0.0014
RYP	Ryphusan/Central	62°25′N, 9°42′E	1100	44	39	9	1 (Pier2)	0.00	0.0000
NED	Nedstavoll/Central	62°23′N, 9°38′E	730	35	31	10	3 (Pier1, Pier2, Pier10)	0.60 ± 0.13	0.0036 ± 0.0028
KON	Kongsvoll/Central	62°25′N, 9°36′E	900	46	41	10	3 (Pier1, Pier2, Pier8)	0.64 ± 0.10	0.0021 ± 0.0019
BLS	Blæsbekken/Central	62°17′N, 9°37′E	1100	10	9	9	2 (Pier1, Pier2)	0.56 ± 0.09	0.0016 ± 0.0016
TOM	Tomseth/Central	63°23′N, 10°26′E	175	34	33	8	3 (Pier2, Pier3, Pier4)	0.75 ± 0.10	0.0058 ± 0.0041
BOR	Borøya/South-Eastern	59°34′N, 10°32′E	0	41	39	9	4 (Pier3, Pier4, Pier5, Pier6)	0.78 ± 0.11	0.0073 ± 0.0048
SOH	Søhol/South-Eastern	60°04′N, 10°12′E	100	18	18	9	4 (Pier3, Pier4, Pier5, Pier7)	0.78 ± 0.11	0.0083 ± 0.0054
PLN	Plön/Northern Germany	54°10′N, 10°25′E	0	5	5	3	2 (Pier3, Pier9)	0.67 ± 0.31	0.0075 ± 0.0068

In the Locality column, Central indicates Central Norway and South-Eastern indicates South-Eastern Norway. All populations in Central Norway, except Tomseth, are part of the studied altitude gradient. Populations (a) and (b) were joined together in the analyses.
mtDNA, mitochondrial DNA.

Table 2. The measured discrete, continuous and derived morphological characters

Character	Unit/character description
Discrete	
Ventral side	
Fore wing	
A1 Apical spot	1 = Absent, 2 = very weak, 3 = weak, 4 = clear, 5 = strong, 6 = very strong
A2 1. Discal spot	1 = Absent, 2 = weak, 3 = clear, 4 = strong
A3 2. Discal spot	1 = Absent, 2 = weak, 3 = clear, 4 = strong
A4 Dorsal line	1 = Absent, 2 = weak, 3 = clear, 4 = strong
A5 Propagation of melanic markings	1 = White, dark suffusion on veins absent. 2 = Veins with slightly dark suffusion. 3 = Veins with dark suffusion. 4 = Some dark suffusion also outside of veins. 5 = Dark suffusion also outside of veins. 6 = Broad dark suffusion outside of veins. 7 = Dark suffusion almost coherent. 8 = Dark suffusion coherent.
A6 Color of melanic markings	1 = Black, 2 = brown
Hind wing	
A7 Shade of ground color	1 = White, 2 = trace of yellow, 3 = weak yellow, 4 = clear yellow, 5 = intense yellow, 6 = very intense yellow.
A8 Median spot	1 = Absent, 2 = weak, 3 = clear, 4 = strong
Dorsal side	
Fore wing	
A9 2. Discal spot	1 = Absent, 2 = weak, 3 = clear, 4 = strong
Hind wing	
A10 Shade of ground color.	1 = White, 2 = trace of yellow, 3 = weak yellow, 4 = clear yellow, 5 = intense yellow, 6 = very intense yellow.
Continuous	
Dorsal side	
A11 Total area left fore and hind wing	mm^2
A12 Melanic area	mm^2
A13 Mean darkness of melanic areas	150–255
A14 Maximum darkness of melanic areas	150–255
Ventral side	
A15 Melanic area	mm^2
A16 Mean darkness of melanic areas	150–255
A17 Maximum darkness of melanic areas	150–255
Hind wing	
A18 Wing length from wing base to end of third median vein	mm
A19 Length of suffusion from wing base along third median vein	mm
A20 Wing width from first radial to third median vein	mm
A21 Width of suffusion where third median vein branches off from median vein	mm
A22 Width of suffusion 3 mm from wing margin on third median vein	mm
Derived	
A23 A12/A11	
A24 A15/A11	
A25 A12/A15	
A26 A19/A18	
A27 A20/A18	
A28 A21/A22	

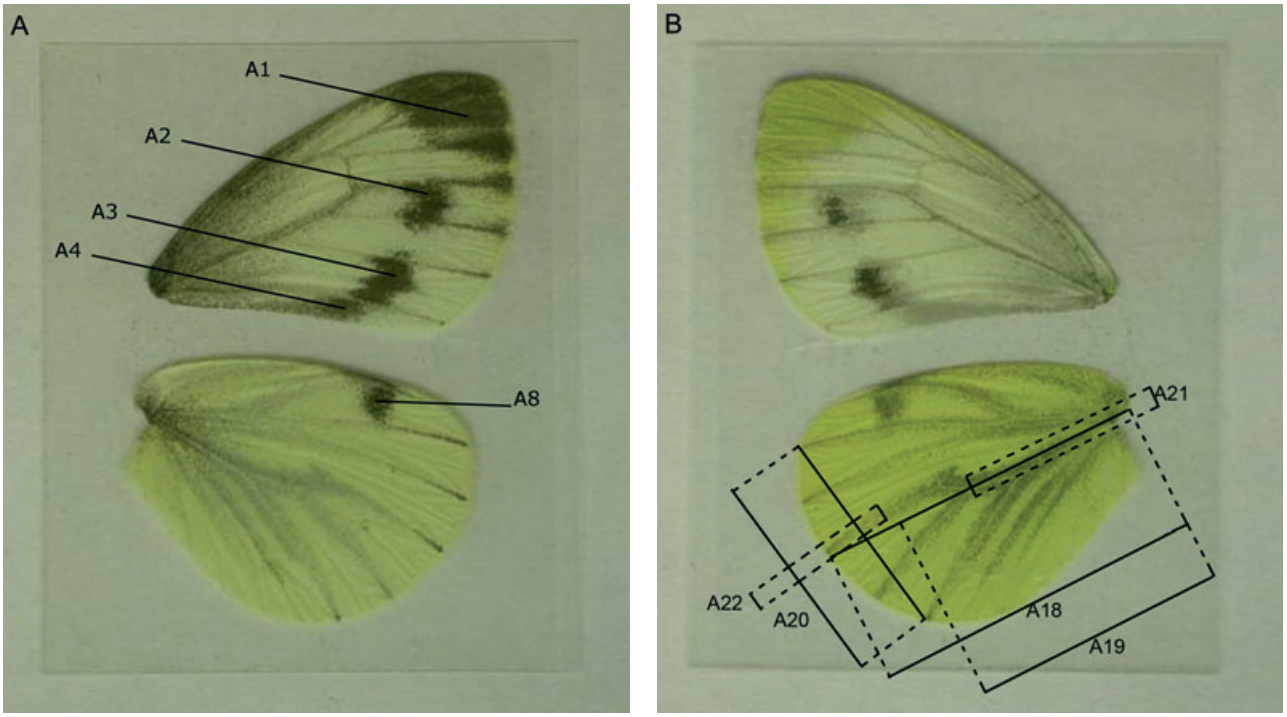

Figure 2. A, some of the qualitative morphological characters measured on the dorsal wing surface. B, some of the quantitative morphological characters measured on the ventral wing surface.

The number of first-instar larvae, pupae and emerged adults were counted for each treatment, and the total survival rate defined as the ratio of emerged adults to first-instar larvae. An ANOVA was performed on the two effects controlled for [effects of origin (KON/SUN) and food plant (*C. amara*/*A. alpina*)] to test whether there was a difference in survival rate for the animals with different origin and for the animals getting different food plants. Food plant was nested within origin. The sequential Bonferroni technique corrected for multiple comparisons (Rice, 1989).

A standardized PCA was performed using the morphological characters, comparing the two factors controlled for: origin and food plant. As a control, another standardized PCA was carried out, comparing the morphology of the animals from Kongsvoll and Sunndalsøra growing up in the wild and in captivity. This should reveal any eventual confounding effects of growing up in captivity versus in the wild.

Analysis of mitochondrial DNA

A fragment of the cytocrome *b* mitochondrial gene was extracted from a total of 98 individuals from 13 populations; 12 from south and Central Norway and one from Northern Germany (populations 1–8 and 16–17 in Table 1). Mitochondrial DNA (mtDNA) was chosen because its rapid evolution makes it a good choice when the relationships of populations within species and closely-related species are investigated (Avise, 2000; Sperling, 2003).

DNA was extracted from abdominal tissue using the Chelex 100 (Bio-Rad) method (Walsh, Metzger & Higuchi, 1991) and this was used as template for the polymerase chain reaction (PCR). PCR amplification [cycle: 1 min at 96 °C; (30 s at 96 °C, 15 s at 50 °C, 4 min at 60 °C) × 30; 4 °C] and sequencing were achieved using invertebrate specific primers CB-J-10933 (TATGTACTACCATGAGGACAAATATC) and CB-N-11367 (ATTACACCTCCTAATTTATTAGGAAT) (Simon *et al*., 1994). Negative controls for PCR showed no contamination. PCR products were purified (Qiaquick PCR purification kit) and sequenced by the dye-labelled termination method (ABI PRISM Big Dye Terminator cycle sequencing Ready reaction kit), producing a 400-bp unambiguous sequence. The sequences were aligned by eye and if necessary corrected with BioEdit, version 7.0.1. (Hall, 1999). The reading frame determined by Aagaard *et al*. (2002) was used. The individuals from sample site SUNa and SUNb were joined together to site SUN, and ORKa and ORKb were joined together to site ORK, in all further analyses. The sequences have been deposited in the EMBL Nucleotide Sequence Database under accession numbers AM236002 to AM236011, and EF165025 to EF165027.

Haplotype diversity (h; Nei, 1987), and nucleotide diversity (π; Tajima, 1983), were calculated using Arlequin, version 2.00 (Schneider, Roessli & Excoffier, 2000). Linear regression analysis was then used to test for correlation between nucleotide diversity (π) and latitude and altitude, respectively, within the Sunndalen-Drivdalen valley. A Mantel test (Mantel, 1967; Manly, 1991) implemented in NTSYSpc, version 2.10b (Rohlf, 1997) was used to test whether geographical and genetic distance between pairs of populations are correlated (i.e. testing for significant isolation by distance) (Wright, 1943; Slatkin, 1993). Genetic distance between pairs of populations was expressed as Nei's net number of nucleotide differences between populations (D_A) (Nei & Li, 1979) and geographical distance as the Euclidean distance of the geographical coordinates. Nei's net genetic distance corrects for within-population distance when calculating the between-population distance. The significance of genetic distances was tested by permuting the haplotypes between populations (500 permutations). Nei's genetic distance measure was also used to estimate an unweighted pair-group method of arithmetical averages (UPGMA) topology (Sneath & Sokal, 1973) of population relationships using NTSYSpc.

Analysis of molecular variance (AMOVA; Cockerham, 1969) was applied to partition haplotypic diversity into within population (sample site), between population within group and between groups of population components, without regard of taxonomic status. AMOVA estimates genetic structure indices using sequence information of haplotypes, as well as their frequencies (Excoffier, Smouse & Quattro, 1992). Different groupings of populations were tested to see which groupings explained most of the variation between the groups (F_{CT}). Significance was tested using a permutation test with 1000 permutations.

A minimum spanning tree was computed from the matrix of pairwise distances between all pairs of haplotypes and, additionally, the frequency of the haplotypes in the different populations was calculated. Minimum spanning tree and AMOVA were calculated using Arlequin and the frequency was calculated by hand.

A Bayesian analysis (Rannala & Yang, 1996; Mau, Newton & Larget, 1999) was carried out using MrBayes, version 3.1.2. (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) with the general time reversible model of substitution with gamma-distributed rate variation across sites and a proportion of invariable sites ($GTR + I + \Gamma$).The Markov chain Monte Carlo run was monitored for 250 000 generations, resampling trees every 100 generations. The first quarter of the trees was discarded as burn-in and the remaining trees were imported into PAUP, version 4.0b10 (Swofford, 2001) and a 50% majority-rule consensus tree was produced to obtain posterior probabilities of the clades.

In addition, a maximum parsimony analysis using heuristic search, random taxon addition, tree-bisection-reconnection and branch swapping was performed using PAUP. *Gonopteryx rhamni* (Linnaeus 1758) was chosen as outgroup for the phylogenetic analyses and *Pieris brassicae* (Linnaeus 1758) and *Pieris rapae* (Linnaeus 1758) were included for comparison. An estimate for support of the branches was provided using bootstrap (Felsenstein, 1985) with 1000 replications.

RESULTS

MORPHOLOGY

Females

The first four principal components explained approximately 61% of the morphological variation among females (Fig. 3A). PC1 and PC3 showed significant difference between populations (PC1, $F_2 = 7.68$, $P < 0.001$; PC3, $F_2 = 7.41$, $P < 0.001$). These two axes accounted for 29% and 10% of the variation, respectively. The first principal component illustrated that the high-altitude populations at Kongsvoll (KON, 900 m) and Nedstavoll (NED, 730 m) differed significantly from the three lower-altitude populations at Granmo (GRA, 506 m), Orkeldalsveien (ORK, 560 m), and Sunndalsøra (SUN, 20–50 m) at the $P < 0.05$ to $P < 0.01$ levels (Table 3). Also, the population at Drivstua (DRI, 713 m) differed significantly ($P < 0.05$) from the population at Granmo and Sunndalsøra. PC3 showed a significant difference between the Ryphusan (RYP, 1100 m) population and all the other populations in the gradient, except for the population at Nedstavoll. PC1 was correlated with six of the nine characters associated with intensity or amount of melanization on the ventral wing surface and also with six of the nine characters associated with melanization on the dorsal wing surface. In particular, the weighted total melanization on both the dorsal and ventral showed a high correlation with this component ($r = -0.88$ for both); the melanization increases with increasing altitude. PC3 did not show a correlation above $r = 0.50$ for any character. There is little overlap between the lowland and mountain populations, but one individual from Granmo is clearly showing mountain characteristics.

Males

The first four principal components accounted for approximately 56% of the morphological variation among males (Fig. 3B), and all differed significantly between populations (PC1, $F_2 = 3.94$, $P < 0.001$; PC2, $F_2 = 13.05$, $P < 0.001$; PC3, $F_2 = 2.51$, $P = 0.02$; PC4, $F_2 = 2.36$, $P = 0.03$).

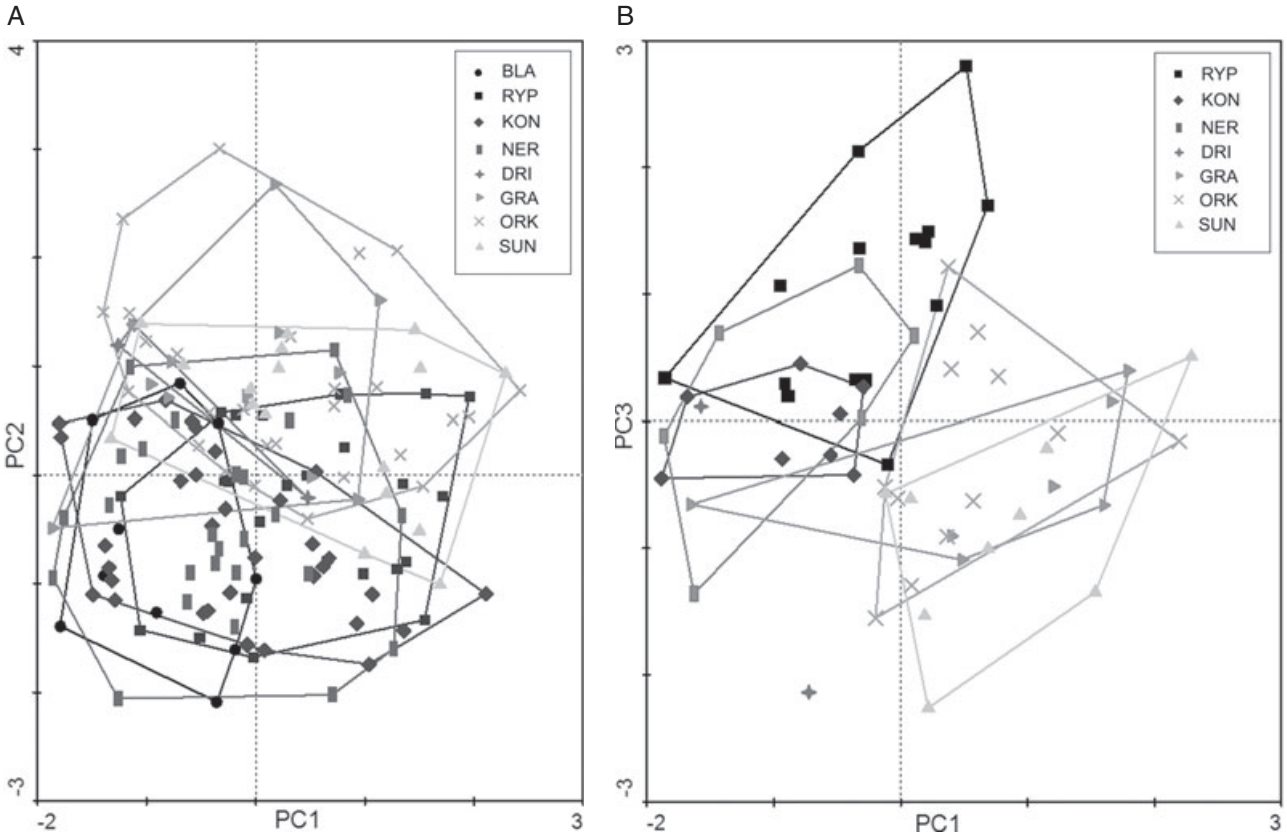

Figure 3. A, ordination of males by morphological characters (principal components analysis, PCA) when comparing the populations in the altitude gradient. B, ordination of females by morphological characters (PCA) when comparing the populations in the altitude gradient.

Table 3. Tests of morphological differentiation on the principal components for females for the populations in the elevation gradient (Tukey's test)

	KON	RYP	NED	DRI	GRA	ORK	SUN
KON	–	NS	NS	NS	**	**	**
RYP	*	–	NS	NS	NS	NS	*
NED	NS	NS	–	NS	**	**	**
DRI	NS	*	NS	–	*	NS	*
GRA	NS	**	NS	NS	–	NS	NS
ORK	NS	**	NS	NS	NS	–	NS
SUN	NS	***	NS	NS	NS	NS	–

Only significantly different principal components are shown.
NS, nonsignificant.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. PC1 above the diagonal and PC3 below.
For population descriptions, see Table 1.

However, after correction for multiple comparisons, only PC1 and PC2 were significant. These two axes explained 21% and 16% of the variation, respectively. The results of the Tukey–Kramer test performed on PC1 and PC2 showed a clear trend (Table 4). PC1 only differed significantly between Blæsbekken (BLS, 1100 m) and the three populations at RYP, ORK, and SUN. PC2 differed significantly between the four populations at highest altitude (NED, KON, RYP, and BLS) and the three populations at lowest altitude (GRA, ORK, and SUN). From the ordination diagram it is apparent that the populations at Ryphusan and

Table 4. Tests of morphological differentiation on the principal components for males for the populations in the elevation gradient (Tukey's test)

	BLS	KON	RYP	NED	DRI	GRA	ORK	SUN
BLS	–	NS	**	NS	NS	NS	*	**
KON	NS	–	NS	NS	NS	NS	NS	NS
RYP	NS	NS	–	NS	NS	NS	NS	NS
NED	NS	NS	NS	–	NS	NS	NS	NS
DRI	NS	NS	NS	NS	–	NS	NS	NS
GRA	***	****	**	***	NS	–	NS	NS
ORK	****	****	****	****	NS	NS	–	NS
SUN	**	***	*	**	NS	NS	NS	–

Only significantly different principal components are shown.
NS, nonsignificant.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. PC1 above the diagonal and PC2 below.
For population descriptions, see Table 1.

Nedstavoll may consist of mixtures of lowland and mountain males. Seven of the eight characters related to either amount or intensity of melanization on the dorsal surface of the wings were correlated with PC1 ($r > 0.50$). PC2 suggested that the apical and median spots, and also the total amount of melanization on the ventral side, decreased with increasing altitude. The colour of the melanization also changed from blackish to brownish with increasing altitude.

Population transplant experiment

In the rearing study at Kongsvoll, all animals emerged during 3 June to 2 July 2004, except one Sunndalsøra female that emerged on 15 August 2003. This individual clearly exhibited the characteristics of a second-generation animal and was excluded from all further analyses.

Survival and development

No difference in survival from first instar larvae to emerged adults was found (Table 5), either between origins ($F = 1.13$, $P = 0.31$) or between food plant nested within origin ($F = 0.19$, $P = 0.83$). Animals from Sunndalsøra developed from egg to pupae in 48 days on average and those from Kongsvoll in 35 days. Thus, on average, the lowland animals required 13 days longer to develop from egg to pupae than the mountain animals, when reared under montane conditions.

Origin and food plants as sources of morphological variation

For the females, the principal components analysis showed significant difference for PC1 ($F = 51.16$, $P < 0.001$) between all the Kongsvoll and Sunndalsøra populations, irrespective of food plant (Fig. 4A, Table 6). PC1 accounted for 39.8% of the variation. The first four principal components explained approximately 69% of the variation. PC4 ($F = 3.03$, $P = 0.04$) was not significant after a sequential Bonferroni correction. Of the 21 characters in the analysis, 14 showed a correlation of $r = 0.50$ or higher with the first principal component. Of these, six were related to melanization on the ventral surface, seven to melanization on the dorsal surface, and one to the ground colour of the ventral surface. The weighted total melanization on both the dorsal ($r = –0.89$) and ventral ($r = –0.95$) surface showed an especially high correlation with PC1; the amount of melanization increased with increasing altitude. In addition, on average, the dorsal line ($r = –0.80$) was larger and the apical spot ($r = 0.79$) smaller in the high altitude populations. This is also indicated by the ordination diagram (Fig. 4A), where the characters with a fit equal to 75% or higher to the ordination space are shown. Character A25, the relative difference between the melanic area of the dorsal and ventral surface, contributed to a large amount of the within population variance.

For the males (Table 7), only PC1 ($F = 5.85$, $P = 0.007$) showed a significant difference between the origin/food plant combinations. PC1 explained 25.9% of the variation and only distinguished between the Kongsvoll and Sunddalsøra populations receiving *Arabis* ($P < 0.05$). Character A22, the width of the melanic suffusion on the median vein, showed a correlation of 0.82 with PC1.

Control: wild and reared animals from the same population

PC1 showed significant differences ($F = 63.90$, $P < 0.001$) between all populations originally from Kongsvoll and those originally from Sunndalsøra,

Table 5. Number of eggs, larvae, pupae and adults in population transplant experiment at KON.

Nr	Locality	Origin	Food plant	Eggs	First-instar larvae	Pupae	Adults	Sex
589	KON	SUN	*Cardamine*	13	4	2	1	1♀
			Arabis		4	3	3	2♂ 1♀
591	KON	SUN	*Cardamine*	23	4	4	4	1♂ 3♀
			Arabis		3	1	1	1♀
592	KON	SUN	*Cardamine*	35	10	5	3	3♀
			Arabis		9	3	3	2♂ 1♀
587	KON	SUN	*Cardamine*	9	4	1	1	1♀
			Arabis		0			
593	KON	SUN		0				
586	KON	SUN	*Cardamine*	47	3	2	2	2♀
			Arabis		11	4	4	1♂ 3♀
590	KON	SUN	*Cardamine*	4	0			
			Arabis					
528	KON	KON	*Cardamine*	58	15	11	6	5♂ 1♀
			Arabis		17	13	9	3♂ 6♀
530	KON	KON	*Cardamine*	77	34	23	17	9♂ 8♀
			Arabis		31	23	20	8♂ 12♀
536	KON	KON	*Cardamine*	23	1	1	1	1♂
			Arabis		7	7	6	3♂ 3♀

Nr, serial number of the females ovopositing the eggs.
KON, Kongsvoll; SUN, Sunndalsøra.

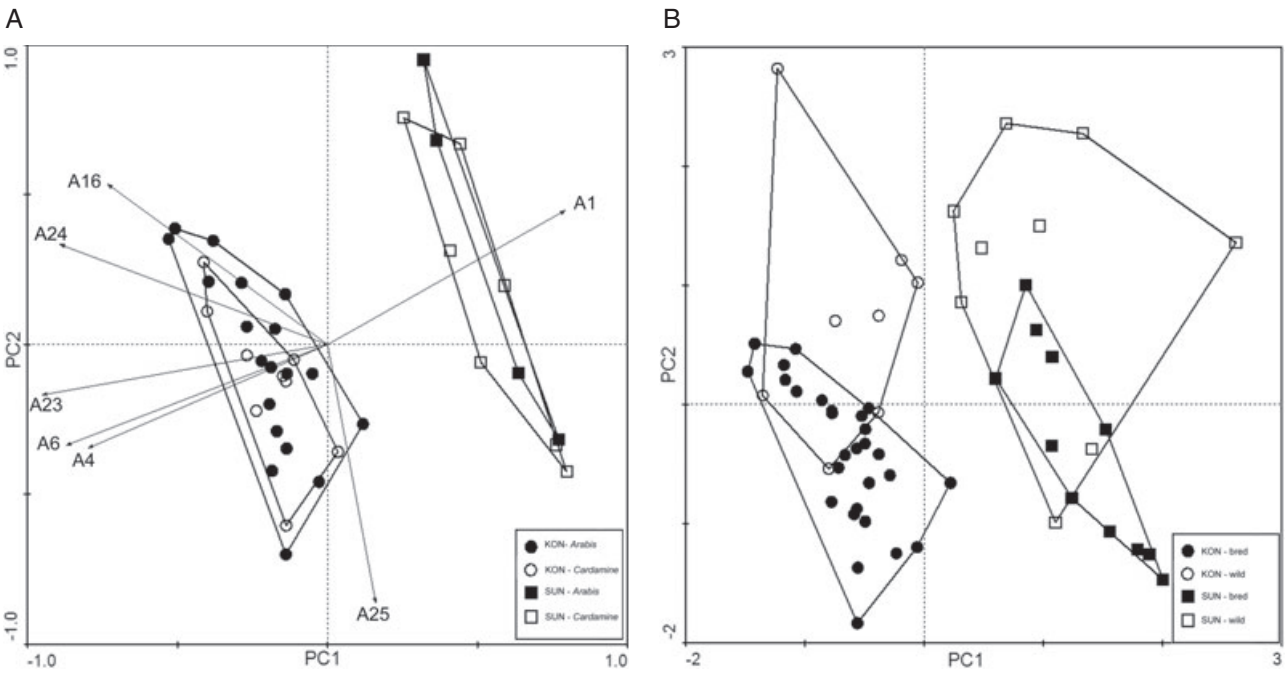

Figure 4. A, ordination of females by morphological characters (principal components analysis, PCA) for the transplant experiment, when comparing populations receiving different food plants. The arrows are the characters with a fit to the ordination space equal 75% or higher. B, control: ordination of females by morphological characters (PCA) for the transplant experiment, when comparing the wild and reared populations, irrespective of food plant.

Table 6. Tests of morphological differentiation on the principal components for the females of the population transplant experiment (Tukey's test)

Origin/food plant†	PC1	Control‡	PC1	PC2
KON *Arabis*–KON *Cardamine*	NS	KON reared–KON wild	NS	**
KON *Arabis*–SUN *Arabis*	****	KON reared–SUN reared	****	NS
KON *Arabis*–SUN *Cardamine*	****	KON reared–SUN wild	****	****
KON *Cardamine*–SUN *Arabis*	****	KON wild–SUN reared	****	*
KON *Cardamine*–SUN *Cardamine*	****	KON wild–SUN wild	****	NS
SUN *Arabis*–SUN *Cardamine*	NS	SUN reared–SUN wild	NS	***

†Test for origin and food plant (*Arabis alpina* or *Cardamine amara*).
‡Control, test for differences between wild and reared animals. Only significantly different principal components are shown. NS, nonsignificant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.
NS, nonsignificant; KON, Kongsvoll; SUN, Sunndalsøra.

Table 7. Tests of morphological differentiation on the principal components for the males of the population transplant experiment (Tukey's test)

Origin/food plant†	PC1	Control‡	PC1	PC2	PC3
KON *Arabis*–KON *Cardamine*	NS	KON reared–KON wild	***	***	**
KON *Arabis*–SUN *Arabis*	*	KON reared–SUN reared	*	NS	NS
KON *Arabis*–SUN *Cardamine*	NS	KON reared–SUN wild	NS	NS	****
KON *Cardamine*–SUN *Arabis*	NS	KON wild–SUN reared	****	NS	NS
KON *Cardamine*–SUN *Cardamine*	NS	KON wild–SUN wild	****	**	NS
SUN *Arabis*–SUN *Cardamine*	NS	SUN reared–SUN wild	NS	NS	NS

†Test for origin and food plant (*Arabis alpina* or *Cardamine amara*).
‡Control, test for differences between wild and reared animals.
Only significantly different principal components are shown. NS, nonsignificant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.
NS, nonsignificant; KON, Kongsvoll; SUN, Sunndalsøra.

irrespective of being wild or reared (Fig. 4B, Table 6). PC2 ($F = 11.10$, $P < 0.001$), however, suggests that there also is some difference between the wild and reared animals from the same population. PC4 ($F = 3.36$, $P = 0.03$) also showed significant difference between the combinations, but was not significant after a sequential Bonferroni correction. These three principal components accounted for 34.1%, 18.1%, and 6.4% of the variation, respectively. The first four principal components explained approximately 64% of the variation. Six and three characters associated with melanization on the ventral and dorsal surface, respectively, and the intensity of the ground colour on the ventral wing surface correlated with PC1. The characters mainly responsible for this difference shown on PC2 are the ratio of melanization on the ventral to dorsal surface ($r = –0.82$), the mean intensity of melanization on the dorsal surface ($r = 0.69$), and the ground colour on the dorsal surface ($r = –0.69$).

The same trend was apparent for the males (Table 7). PC1 ($F = 16.56$, $P < 0.001$) and PC3 ($F = 10.27$, $P < 0.001$) combined, show that there was significant difference between the KON–SUN combinations, irrespective of wild or reared. But PC1, PC2 ($F = 7{,}82$, $P < 0.001$), and PC3 all also showed some difference between wild and reared animals (Table 7). The first four principal components accounted for 56.5% of the variation, PC1 24.5%, PC2 14.3%, and PC3 9.9%, respectively.

Population genetic analysis

In the sequenced segment of the cytochrome *b* gene, 11 polymorphic sites were detected of which six were phylogenetically informative under the parsimony criterion. All substitutions were transitions (A>G, C>T) and none resulted in an amino acid substitution. Nucleotide diversity (Table 1) ranged from 0.00 at Ryphusan to 0.0061 at Granmo within the Sunndalen-Drivdalen valley, and to 0.0083 at Søhol (SOH) elsewhere. The mean number of pairwise differences between haplotypes was 1.17 within the Sunndalen-Drivdalen valley and 2.12 including all

Table 8. Nei's net number of nucleotide differences between populations

	BLS	KON	RYP	NED	DRI	GRA	ORK	SUN	TOM	BOR	SØH	PLN
BLS												
KON	–0.06											
RYP	0.28*	0.22*										
NED	0.01	–0.01	0.07									
DRI	0.06	0.02	0.00	–0.05								
GRA	0.28*	0.22*	0.22	–0.01	0.14							
ORK	0.53*	0.52*	0.70**	0.41	0.54	0.40						
SUN	0.68**	0.62**	0.40	0.23	0.40	–0.10	0.62*					
TOM	0.88**	0.83**	0.61**	0.40	0.61	0.00	0.26	–0.09				
BOR	2.20**	2.12**	2.17**	1.61**	2.08**	0.74*	1.09*	0.61*	0.27			
SOH	1.79**	1.73**	1.64**	1.31**	1.59**	0.77*	0.25	0.71*	0.16	0.25		
PLN	1.61**	1.56**	1.33**	1.07*	1.33**	0.56	0.03	0.40	–0.14	0.09	–0.36	

*$P < 0.05$ after 500 permutations, **$P < 0.01$ after 500 permutations.
For population descriptions, see Table 1.

samples. Haplotype diversity (Table 1) ranged from 0.00 at Ryphusan to 0.64 at Granmo and Kongsvoll within the Sunndalen-Drivdalen valley, and to 0.78 at Borøya (BOR) and Søhol.

In the valley system alone, the correlation between altitude and the nucleotide diversity of the populations was significant ($F = 7.08$, $P = 0.04$); the nucleotide diversity decreased with increasing altitude in the gradient. The correlation between nucleotide diversity and latitude, including all sample sites, was not significant ($F = 4.08$, $P = 0.07$).

There was significant pairwise genetic distance between lowland and mountain populations, but few significant differences within these groupings (Table 8). The pairwise genetic distance between the populations was not significant for any combinations of the populations from Tomseth (TOM), Borøya (BOR), Søhol (SOH), and Plön (PLN, Table 8). There was also no significant pairwise genetic distance between Tomseth, Sunndalen, Orkeldalsveien, and Granmo, apart from for the genetic distance between Orkeldalsveien and Sunndalen, which was significant. Except for Ryphusan, which was significantly different from both Blæsbekken and Kongsvoll, none of the populations from Drivstua to Blæsbekken had significantly different pairwise genetic distances, but they were significantly different from most of the remaining populations.

Nei's net genetic distance (Table 8) and the geographical distance was significantly correlated when studying all the Norwegian populations ($r = 0.63$, $P = 0.01$), but not when including Plön ($r = 0.29$, $P = 0.08$) or studying the valley system only ($r = 0.40$, $P = 0.14$). The UPGMA tree of the populations estimated from Nei's net genetic distance (Fig. 5) is composed of two major clades, one with the lowland populations including Orkeldalsveien at 560 m and Granmo at 506 m and the other with the populations from Drivstua at 713 m to Blæsbekken and Ryphusan at 1100 m. All the populations in the clade consisting of high altitude populations contained both haplotype Pier1 and Pier2 except Ryphusan which only contained Pier2.

The minimum spanning tree (Fig. 6) displays a line-like pattern where the maximum number of nucleotide differences between any pair of haplotypes is 9 (Pier1 and Pier5, Pier8, and Pier5). This tree shows some geographical structure; the three haplotypes found in the mountainous area of Central Norway in typical *P. n. adalwinda* specimens (Pier1, Pier2, and Pier8) are placed at one end. Most of the haplotypes found either just in South-Eastern Norway or Plön are mixed with haplotypes found in lowland habitats in most regions (Table 9).

In the AMOVA, the grouping of populations into Central and South-Eastern Norwegian-German explained more of the variation among groups of populations ($F_{CT} = 0.37$) than the grouping of populations into *P. n. napi* and *P. n. adalwinda* (based on morphology) ($F_{CT} = 0.29$), or into *P. n. adalwinda*, central and south-eastern German *P. n. napi* populations ($F_{CT} = 0.33$). In the latter, the variation among populations within groupings was the smallest ($F_{SC} = 0.05$). All groupings of populations were significant ($P < 0.05$), indicating that the groups of populations are differentiated from the total population.

PHYLOGENETIC ANALYSIS

The Bayesian and parsimony analysis gave almost identical trees, so only the Bayesian tree is shown (Fig. 7). Parsimony analysis gave six most parsimo-

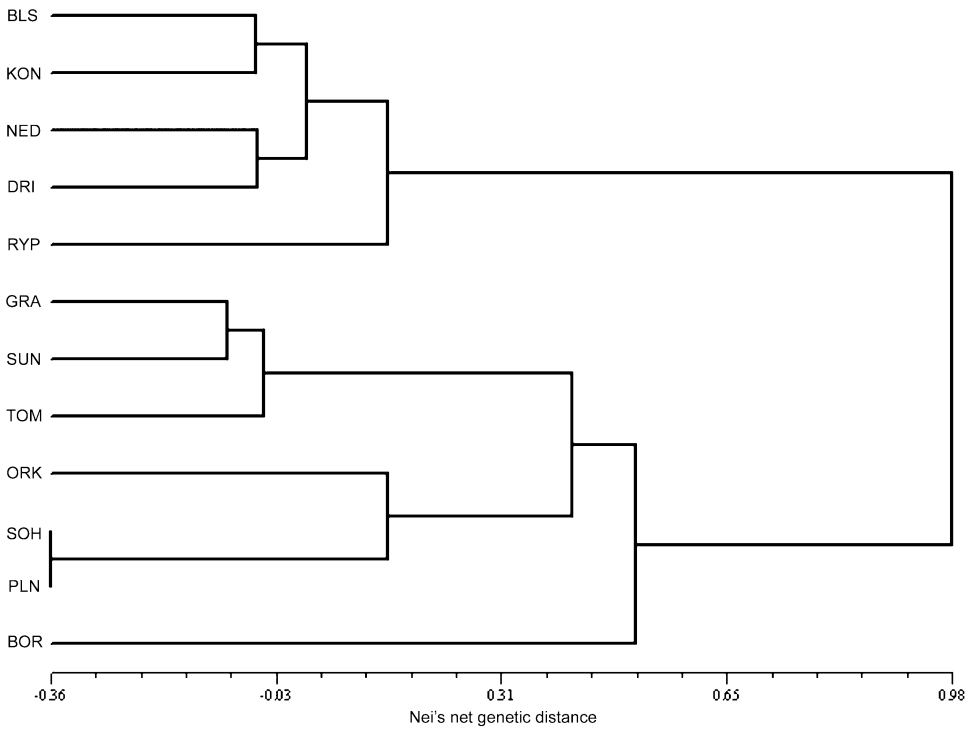

Figure 5. Unweighted pair-group method of arithmetical averages tree of the populations estimated from Nei's net number of nucleotide differences between populations.

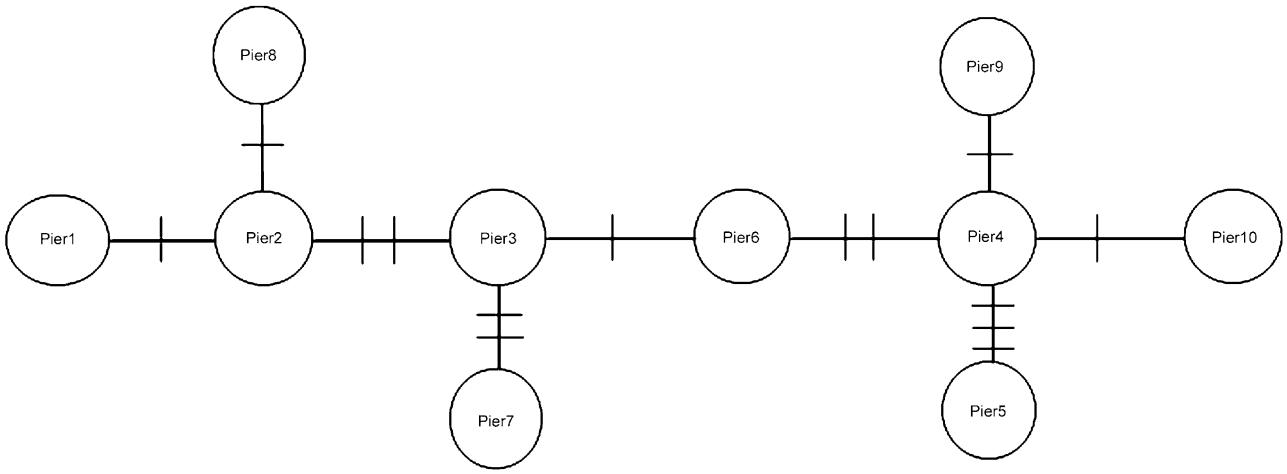

Figure 6. Minimum spanning tree showing the relationships between the haplotypes. Each crossbar represents one substitutional difference.

nious trees which only differed in their placement of haplotypes Pier3, Pier6, and Pier7, which either appeared as a sister group to Pier1, Pier2, and Pier8 or with Pier6 as a sister group to a group consisting of all the five other mentioned haplotypes. The Bayesian analysis agreed best with the latter, although with low posterior probability. In the parsimony analysis, *P. brassicae* is sister to *P. napi* with high support; in the Bayesian analysis, the relationship between *P. brassicae* and *P. napi* is unresolved. When translating haplotypes to subspecies, haplotypes Pier1, Pier2, and Pier8 (*P. n. adalwinda*) form a monophyletic clade, and the *P. n. napi* haplotypes (Pier3 to Pier10, excluding Pier8) form a paraphyletic assemblage basal to *P. n. adalwinda*. Bootstrap values for the parsimony analysis were generally low, except for the placement of *P. napi* as a sister species to *P. brassicae*, which has strong

Table 9. The number of individuals from each population with the different haplotypes

	Haplotype									
Population	1	2	3	4	5	6	7	8	9	10
BLS	5	4								
KON	5	4						1		
RYP		9								
NED	3	6								1
DRI	1	4								
GRA	1	6		2	1					
ORK	2		3							
SUN		6		4						
TOM		3	2	3						
BOR			2	4	2	1				
SOH			4	2	1		2			
PLN			2						1	
SUM	17	42	13	15	4	1	2	1	1	1

For population descriptions, see Table 1.

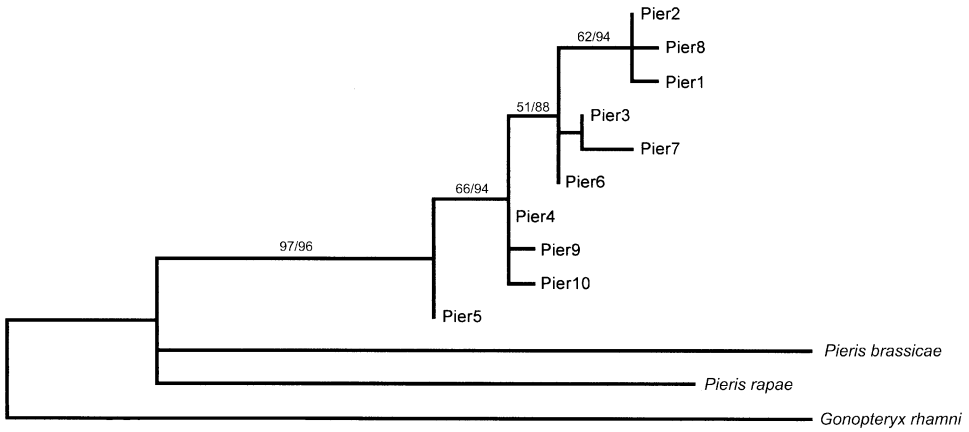

Figure 7. Fifty percent majority-rule consensus tree from the Bayesian analysis of the 354-bp fragment of the cytochrome *b* gene. The number on the branches is the bootstrap value for that branch for the maximum parsimony tree (before the slash) and the posterior probability of the clades in the Bayesian analysis (after the slash).

support. Posterior probabilities generally were somewhat higher.

DISCUSSION

Lowland and mountain forms

Significant morphological differences are found between the lowland and mountain populations both for the males and females. For both sexes, it is evident that there is a change in morphology towards more melanized individuals between the locality Granmo at 506 m and Nedstavollen at 730 m a.s.l. in Drivdalen. In general, the mountain females are also more yellow on the ventral hind wing than the lowland females. The geographical distance between Granmo and Nedstavollen is approximately 19 km, so a hybrid zone or a steep cline can possibly exist between these localities. When looking at the ordination diagrams, there is a slight difference between the sexes. There is very little overlap between lowland and mountain forms for the females. For the males, the distinction between lowland and mountain populations is less clear. The single individual female showing mountain characteristics in the Granmo population can have drifted to this location from the mountains as an egg or pupae, on the nearby river. There was severe flooding in the area both in August 2003 and April 2004, bringing vegetation and debris from the mountains down the valley system. Adult dispersal appears less likely, as the females are largely stationary whereas the males can be more migratory in their search for females (Petersen *et al*., 1951).

The differences in melanization between lowland and mountain animals resemble the difference in melanization observed between the two generations produced during the summer by the lowland animals where the first generation has a greater amount of melanization than the second. The length of the diapause is believed to be a factor causing the differences (Shapiro, 1976). The second generation emerges without diapausing whereas the first generation is in diapause from August to May the following year, and the montane and arctic populations are in diapause from July to June or July the following year.

The first lowland generation and the montane populations live in a generally colder environment than the second lowland generation. The higher amount of melanization of the former is an adaptation to the environment (Watt, 1968; Kingsolver, 1987); dark wing surface is more effective in absorbing energy than pale wing surface. A study of the butterfly *Colias eurytheme* showed that melanized females had a higher egg maturation rate at cold temperatures, which again may increase the reproductive output (Ellers & Boggs, 2004). The same patterns of diapause length and amount of melanization are observed as one goes from south to north in Scandinavia (Petersen, 1947) and also in Britain (Lees & Archer, 1980).

Hybridization between *P. napi* and *P. bryoniae* has been observed in the Alps (Porter, 1997) and crossing of lowland and mountain animals from Sweden has given viable and reproductive offspring (Petersen, 1963). It would have been an advantage to study several altitude gradients (e.g. in Scandinavia and in the Alps) to investigate whether the pattern found is general. This would also eliminate eventual confounding factors that differ between the high and low altitude populations, such as the amount of suitable habitat. It would also be an advantage to include more characters, mainly characters not related to wing morphology, because some of the wing characters are correlated in genetic control, and possibly are responsive to only a restricted set of environmental pressures, which differ between lowland and mountain habitats (W. B. Watt, pers. comm.).

Effects of rearing environment

The morphological differences between the two lowland generations have obviously no genetic basis, but the transplant experiment showed that the differences between lowland and montane forms have a genetic basis; it was not possible to induce the melanism by rearing animals in a different environment or by rearing them on different plants, at least not in just one generation. There was also no difference in the survival of lowland or mountain animals reared at Kongsvoll or between animals reared with different food plants. This last result agrees with the findings of Porter (1997) who found a broad overlap in the range of suitable host plants in a hybrid zone in Switzerland, but not with Varga's (1967) supposition that the lowland and mountain forms are specialized to use different host plants. On average, the lowland animals required 13 more days in their development than the mountain animals when reared under montane conditions, implying lower reproductive success for lowland animals under short montane summer conditions.

The food plant has no effect on morphology and the difference found between wild and reared animals with the same origin, seen most clearly in females, is correlated with PC2, whereas the differences between animals of different origin are correlated with PC1. Most of the characters in females correlated with PC2 are related to intensity of either melanization or the yellow ground colour on the dorsal surface. This lack of effect also applies to males, whose morphological differences in total are less pronounced than for the females, and therefore are more masked by differences between reared and wild animals. These differences probably arise because the wing scales of wild animals are gradually worn off and the wings thereby fade as the butterflies age. The reared animals were killed at most 1 day after unfolding their wings, before such fading of wing colour could take place.

Only one animal from Sunndalsøra hatched without diapausing at Kongsvoll, but second generation animals are frequently observed at Sunndalsøra. This suggests that the Sunndalsøra population must be facultatively bivoltine, producing one or two generations dependent mostly on temperature because photoperiod is the same for the two localities. The amount of data was limited for some populations and thus the results have to be interpreted according to this limitation.

Genetic relationships

The mtDNA cytochrome *b* gene provides evidence of significant genetic differentiation between lowland and mountain populations.

The nucleotide diversity was moderate and the haplotype diversity was quite high compared to other studies based on different mtDNA markers (Vandewoestijne *et al*., 2004). Moderate to high haplotype frequency and low nucleotide diversity is the signature of rapid demographic expansion from a small effective population size (Hundertmark *et al*., 2002). High haplotype diversity may also indicate secondary contact of populations from isolated glacial refugia in Southern Europe (Fry & Zink, 1998), suggesting that *P. napi* may have colonized Norway several times.

This is further indicated, although weakly, by the structure of the minimum-spanning tree. The haplotypes common at higher altitudes (Pier1, Pier2, and Pier8) are direct ancestors with only one substitutional difference, but two or more substitutional differences away from the more typical lowland haplotypes (Pier3 to Pier10, except Pier8).

During rapid colonization, pioneers quickly expand to fill new areas, and the genes of successful pioneer individuals will dominate the new population genome as their progeny extend the population into new areas (Hewitt, 1999). These pioneers would expand and fill the area rapidly before significant numbers of other dispersers could arrive, meaning that one or a few haplotypes would be expected as the most common allele in populations (Joyce & Pullin, 2001), as found in the altitude gradient in the present study.

The nucleotide diversity decreased with altitude in the gradient. Higher genetic differentiation was associated with greater geographical separation, suggesting some isolation by distance (Wright, 1943; Slatkin, 1993) between the South-eastern and Central Norwegian populations, but not within the altitude gradient.

The UPGMA tree estimated from Nei's net genetic distance shows that the greatest genetic distance can be found between lowland populations and montane populations. Based on this tree, the lowland populations and montane populations form two clear clusters. A study of the allozymes of *P. napi* populations from Central and South-eastern Norway further supports this assessment. Little genetic differentiation between lowland populations from either side of the Norwegian mountain ranges was found, but a marked differentiation between the mountain and lowland populations was detected (K. Aagaard *et al*. unpubl. data).

In the AMOVA, on the other hand, the most variance among groups of populations was explained by separating populations into South-eastern-German and Central Norwegian groups. The AMOVA and the UPGMA tree estimated from Nei's net genetic distance give different results. This may suggest that the genetic differentiation, as inferred from the mitochondrial cytochrome *b* gene, between the lowland and mountain form is quite weak.

The phylogenetic analysis showed that the mountain haplotypes which makes up *P. n. adalwinda* are monophyletic, but that the rest of the haplotypes (*P. n. napi*) form a paraphyletic group, indicating no subspecific relationships between the two forms. Weak support for some nodes is a consequence of closeness of sequence identity rather than character conflict. Chew & Watt (2006) had the same problem in their study of the American *P. napi* group. Traditional phylogenetic methods such as maximum parsimony are not very well suited for estimating genealogical relationships among genes at the population level (Posada & Crandall, 2001). These methods assume that ancestral haplotypes are no longer in the population, an assumption invalid at the population level. Coalescent theory predicts that ancestral haplotypes will be the most frequent sequences sampled in a population study (Watterson & Guess, 1977; Donnelly & Tavaré, 1986). The genealogical information associated with population level divergences is also frequently nonbifurcating, making trees inferred from maximum parsimony or Bayesian implementation often highly unresolved with little information (Crandall & Templeton, 1993), as seen in the present study.

Ecological and/or morphological differentiation with little or no genetic differentiation has been found in several studies. Two good examples can be found in Vandewoestijne *et al*. (2004). In a study of the butterfly *Papilio polyxenes colouro* with ecological divergence but no mitochondrial divergence, the poor correspondence was explained by faster evolution of ecological differentiation than mtDNA, and by the idea that mtDNA is comparatively free of genes coding for ecological differences with alleles bound to local conditions (Sperling & Harrison, 1994). In song sparrow, *Melospiza melodia*, two explanations were given for the poor correlation of morphology and mtDNA; either the faster evolution of the highly heritable morphological traits than mtDNA, or the idea that gene flow is an historical and not a contemporary event (Fry & Zink, 1998). The hypothesis currently favoured is 'that ecological and morphological differentiation occurs more quickly than mtDNA evolution as, although morphological, physiological and behavioural changes can produce subspecies and species at almost any time, genome divergences proceeds over millions of years' (Vandewoestijne *et al.*, 2004). mtDNA is not suited to alone detect hybridization because it is inherited maternally. Results from allozymes or nuclear genes should be included to obtain a better picture of possible hybridization and genetic divergence, and samples from other parts of Europe would also help clarify the results.

CONCLUDING REMARKS

In the present study, it is demonstrated that there is a great deal of variation in wing morphology and melanization between the lowland and montane form of the *P. napi* complex in Norway and that this variation is not related to food plant differences. It is also shown that the differences in wing morphology have a genetic basis because the montane and lowland forms retain their morphology in the next generation when reared in a different environment. The population genetic analyses shows that there is a weak genetic

divergence between mountain and lowland populations but, in the phylogenetic analysis, the lowland form, *P. n. napi* is paraphyletic, giving no support for subspecific status of the two forms. As seen in the present study, as well as other studies (Chew & Watt, 2006), investigation of the *P. napi* complex is a complex problem. The results from different analyses are often conflicting, making any unanimous conclusion difficult.

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