Master of Science Thesis

Population genetic structure of aposematic alpine wood tiger moths (*Parasemia plantaginis)*

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ABSTRACT

Alpine landscape with natural fragmentation restricts gene flow among populations and causes spatio-genetic structuring (high genetic differentiation) in species living there. Consequently, alpine habitat fragmentation and dispersal barriers should make isolated populations such as wood tiger moth (*Parasemia plantaginis*) populations prone to lose genetic diversity by local adaptation and fixation of fittest phenotype in each local population. This species is also known to be aposematic. Yellow colour on the males' hind wings in wood tiger moth is presumed to work more efficiently against visual hunting predators due to increased conspicuousness. In addition, in field experiments with wood tiger moth, females with red hind wings were avoided more than those with orange hind wings. Therefore, yellow and red phenotypes are expected to reach fixation by directional selection in each given local population. Alternatively, uniform stabilizing selection imposed by predators can lead to constant allele frequencies throughout their distribution range. However, this species are very variable across its entire distribution both locally and regionally. Despite this dramatic variation for *P. plantaginis* throughout its range, it is not clear if observed phenotypic variation matches genetic divergence among populations. The main objective of the thesis was to investigate the spatio-temporal population genetic structure and differentiation of *P. plantaginis* population over two consecutive years in different Alpine regions in Italy, Austria, and Switzerland. The investigation was conducted using the pattern of variation at 10 microsatellite loci and in a 664 bp portion of the COI gene in the mtDNA. Expected heterozygosity was high and similar within all regions. Bayesian analyses revealed just one *P. plantaginis* population as a whole. This was supported by pairwise FST value, AMOVA and COI results, which indicated no differentiation among populations during two successive sampling years 2009-2010. Hence, high genetic diversity and weak population differentiation suggest high gene flow and/ or high population density in *P. plantaginis*. We speculate that in spite of extensive gene flow, this species remain morphologically polymorphic because the homogenizing effect of high gene flow has been compensated by differential environmental and ecological selective forces along the altitudinal gradient. However, given high gene flow (both current and past), and lack of strong post-settlement selection pressure, selective forces due to altitude are not strong enough to significantly differentiate the studied population in terms of microsatellites and mtDNA.

Contents

1. INTRODUCTION

Understanding species population genetic structure is one effective way to infer their biogeography, local adaptations, and interconnectivity among populations. Genetic structure refers to any pattern in the genetic makeup of individuals within a population. Populations can be genetically stratified because of geographic, ecological or behavioural factors (Hedrick 2000).

It is known that habitat fragmentation can affect genetic structure (Templeton et al., 1990). Physical barriers such as a river or mountain can divide a species into separate populations where selection favours specific phenotypic characteristics adapted to the prevailing environmental conditions. Habitat fragmentation can decrease genetic diversity of populations due to its effects on gene flow by restricting dispersal (Johansson et al. 2007). Habitat fragmentation will also significantly increase the effects of genetic drift (loss of genetic variation) due to small local population sizes (Frankham et al. 2002). Differences in environmental conditions between fragmented populations may also give rise to local adaptation by introducing novel selective pressures. Local adaptation is known as a mechanism to maintain phenotypic divergent (Kawecki & Ebert 2004) in fragmented populations. Natural selection eliminates deleterious alleles from populations and carrying advantageous alleles to fixation. Therefore, selection within a population will reduce genetic diversity, as selection will remove poorly adapted genotypes. Consequently, entire population may become fixed for a single morph, resulting in genetically differentiated populations (Lenormand 2002). In other words, local adaptation should lead to the exclusion of all but one phenotype in any locality. In the context of local adaptation, every species occupies an ecological niche based on its tolerance for abiotic and biotic factors in the environment.

However, the survival probability of species in small, isolated subpopulations depends, in part, on their ability to disperse. One consequence of dispersal is gene flow between populations. Gene flow, defined as the movement of gametes or individuals from one place to another and incorporation of the genetic material into the recipient population, influences both the population structure and geographic distribution of a species, as well as the adaptation of populations to their local environments (Slatkin, 1987). Gene flow caused by dispersal can create demographic and genetic connectivity between neighbouring and distant locations, influencing structure and ultimately the persistence of populations. For example, dispersal among habitat fragments also may provide sufficient gene flow to maintain the genetic diversity within fragments. Genetic diversity is essential for evolution because it provides raw material on which selection operates and it protects species from extinction or dangerous bottle necks (Hamilton 2009). In the absence of dispersal limitations and environmental variability, gene flow can prevent natural selection to establish and maintain optimal genotypes. However, the effects of gene flow on local adaptation can be difficult to predict. On one hand, gene flow can prevent local adaptations by genetic admixture of populations with different selection pressures (Slatkin, 1987, Lenormand 2002). In theory one effective migrant per generation is sufficient to avoid the deleterious loss of genetic drift in small populations and reduce the chances at population differentiation (Slatkin 1985; Mills & Elmendorf 1996). On the other hand, adaptive differentiation can occur even in the face of gene flow if selection is strong (Endler 1977). In concert, geographical genetic structure among populations is determined by balance between selection and gene flow among populations with different selection regime.

Individuals may differ in their phenotypes because of genetic differences, environmental differences or both. Colour polymorphism as a visible character provides a

link between a genotype and an externally expressed phenotype, and is ideal for studying the evolutionary process. The evolutionary and ecological factors that favour particular colours can be related to where the animals live, how they mate, how they escape from predators or how they hunt (Hill & McGraw 2006a, 2006b). Coloration plays important roles including: prey-predator interactions, mate choice, intersexual competition, thermoregulation and communication (Poulton 1890). In addition to inter- and intra-species interactions, coloration can be affected by habitat, diet, and lighting conditions (Beddard, 1892). In general, the existence of different morphs has been attributed to selective factors when the polymorphic character is directly advantageous. Selective factors that have been proposed to maintain phenotypic diversity are frequency-dependent selection, density-dependent selection, disruptive selection, heterozygous advantageous, sexual selection, and negative assortative mating. Furthermore, random factors such as gene flow**,** heterogeneous environment**,** and genetic drift account for polymorphism (Review by Gray & McKinnon 2006; See also Stevens and Ruxton 2012.).

Butterflies and moths comprise a suitable model system to study colour polymorphism because of their variability in coloration between and within species (McMillan et al.2002; Brown & Benson 1974). Wood tiger moth *Parasemia plantaginis* (Arctidae) exhibits phenotypic and genotypic warningly coloured variation therefore they have been used to study genetically and environmental constrain for colour pattern formation (Lindstedt et al. 2008, 2009). However, practically nothing is known regarding their genetic population structure. So far, the only valuable information we have about population structure of this species has come from wood tiger moth subspecies from Japan. Okano and Katayamya (1976) described that there are different subspecies and forms of wood tiger moth species in Japan who's about 80% of the country is covered with mountains. Adult females have continuous colour variation from red to orange with black pattern (melanin) on their hind wings and body. Adult males are either black and white or black and yellow (Lindstedt et al. 2008, Nokelainen et al.2012), species is shown to be aposematic (Lindstedt et al. 2008, 2011). Aposematic is a strategy taken by some animals and plants to advertise their unprofitably by different forms of warning signals to keep predators away from them. Red, black, yellow often characterize a warning coloured organisms (Cott 1940) because these colours are high contrasted against background, resistant to shadow, chromatic contrasted, luminance contrasted and distance-dependent (Stevens & Ruxton 2012).

Natural selection is expected to favour monomorphic warning signals rather than polymorphic because prey would benefit from low mortality cost by sharing warning signals (Müller theory). In general, aposematic animals are expected to be under positive frequency dependent selection in which the predator attacks the rarer form of the prey more frequently than expected (Marples & Mappes 2011). In other words, existing aposematic signals should be under uniform stabilizing selection to remain the same and safe from being eaten by predators. Uniform stabilizing selection must be favouring a similar phenotype among geographically isolated populations due to purifying selection among population (Wojcieszek & Simmons 2012). Yellow colour on the males' hind wings in wood tiger moth is presumed to work more efficiently against visual hunting predators due to increased conspicuousness (Nokelainen et al.2012). In addition, in field experiments with wood tiger moth, females with red hind wings were avoided more than those with orange hind wings (Lindstedt et al. 2011). Therefore, yellow and red phenotypes are expected to reach fixation by directional selection in each given local population.

The Alpine environment due to a pronounced mountainous topography and associated abiotic heterogeneity has created a variety of unique natural fragmented

habitats with pronounced temporal shifts in elevation (Körner 2003). Habitat fragmentation and dispersal barriers should also make isolated wood tiger moth populations prone to lose genetic diversity by local adaptation and fixation of fittest phenotype in each local population. Consequently, all local population may reflect same local optima if directional selection by predators is the prevailing force of aposematic coloration evolution. If so, wood tiger moth species is expected to show uniform colour distribution within and among population.

However, Preliminary phenotypic analysis showed intra- and inter-population polymorphism in hind wing colour and melanization degree both across *P. plantaginis* distribution range in the Alps (Galarza et al. in prep., Hegna et al. in prep.). Despite this dramatic variation for *P. plantaginis* throughout its range, it is not clear if observed phenotypic variation matches genetic divergence among populations. In my thesis, I study genetic composition of the *P. plantaginis* populations in three different locations over two consecutive years. In the present study, the genetic structure of *P. plantaginis* from alpine area was assessed using the pattern of variation at 10 microsatellite loci and in a 644 bp portion of the COI gene in the mtDNA. Microsatellites are neutral nuclear markers that provide estimates of gene flow and other population processes (drift/dispersal/ fragmentation) independent of mtDNA.

My goals were to answer these question using both mitochondrial and nuclear genetic markers: (i) Is there any correlation between molecular divergence and phenotypic divergence among populations? (ii) What is the level and pattern of genetic diversity present in populations of *P. plantaginis* within our sampling area in the Alps? (iii)How does quickly evolving nuclear microsatellite variation compare with that of mtDNA in *P. plantaginis*?

2. METHODS

2.1 Study species and sample collection

Wood tiger moth can be found throughout northern Europe, northern Asia, and western regions of North America. They fly during the day, especially the males. The larvae feed from July until April of the following year on a wide variety of herbaceous plants. In other words, they are polyphagous herbivore (Lindstedt et al. 2008). It overwinters as a larva. Larvae are hairy and hairiness is not effective against predators but hairs have other important functions including: thermoregulation, protection against injuries and parasites, increasing prey's survival through increasing handling time (Lindstedt et al. 2008). Adults do not feed at all (capital breeders; Tammaru & Haukioja 1996) therefore adults' chemical defences (i.e. iridoid glycosides) is probably acquired as larvae. They produce orange warning signal by depositing both diet-derived flavonoids and trace levels of synthetic eumelanin in their hairs. Pigment of adult wings is unknown but the most likely candidate is monochromes and flavonoids (J. Mappes pers.). As mentioned before, the wood tiger moth displays an extraordinary degree of polymorphism and sexual dimorphism in their coloration (Figure 1).

A total of 116 wood tiger moth (*P. plantaginis*) individuals were collected from 3 localities within the Alps, of which we genotyped 112 individuals (Table 2). Samples were collected from Italy, Austria, and Switzerland (Fig. 2). All samples for this study were collected in 2009- 2010 except for Switzerland where data is not available for 2010.We used nets and pheromone traps containing live females to catch males and looked for females by eye.

Figure 1. Colour variation in wood tiger moth.

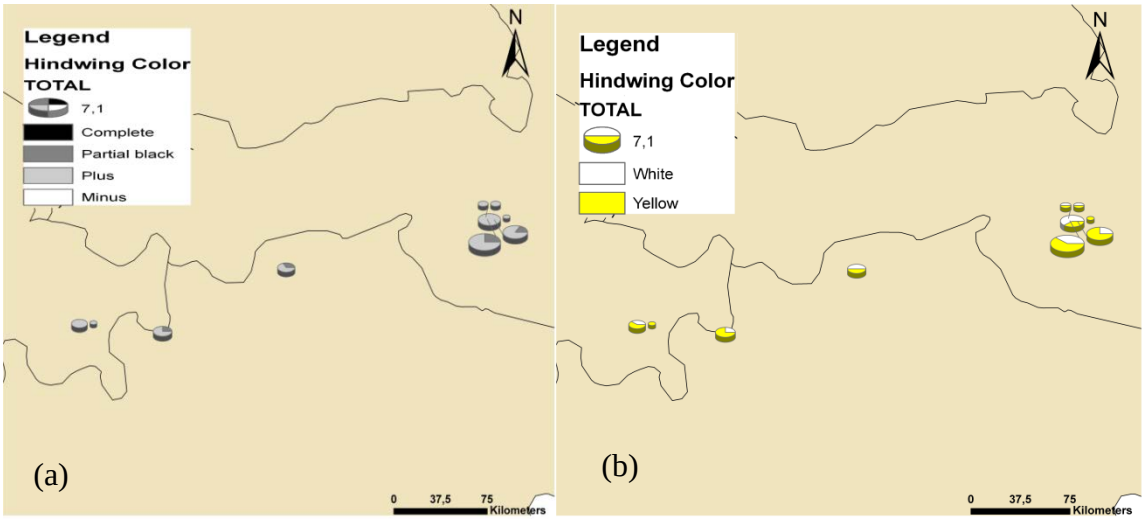

Figure 2. Polymorphism in (a) melanization degree and (b) hind wing colour across *P. plantaginis* distribution range in the Alps. (Figure re-drawn from Hegna et al. with permission).

2.2 DNA extraction and microsatellite data analysis

We extracted DNA from one or two legs per ethanol-preserved individual using a DNAeasy blood+ Tissue extraction kit (Qiagen) through a robot (Kingfisher) and concentrated via ethanol precipitation. A total of 116 individuals were genotyped at ten microsatellite loci (Plan107,109,279,313,317,323,363,414,439,382) designed for wood tiger moth (*P. plantaginis*) using primers and protocols described by Galarza et al. (2010). Amplified PCR products were run on an ABI 3130xl Genetic Analyser (Applied Biosystems) using forward primers labelled with 6-FAM, NED, PET and VIC (Applied Biosystems). Allele scoring was carried out using GENEMAPPER V.4.0 software (Applied Biosystems).

The total number of alleles per locus and sample was obtained using GENETIX v. 4.01 (Belkhir et al. 1997). Observed and expected heterozygosities were calculated using the software package ARLEQUIN (version 3. 01; Schneider et al. 2000). Allelic richness (Rs) was calculated using FSTAT 2.9.3.2 (Goudet 1995). Deviations from Hardy-Weinberg equilibrium (HWE) were performed by the GENEPOP Package (Raymond and Rousset 1995). The program performs a probability test (sub-option 3) using a Markov Chain (dememorization 10,000, batches 100, iteration per batch 1000). The significance level was adjusted using the Bonferroni correction (Rice 1989). I used Micro-Checker 2.2.3 (Van Oosterhout et all.2004) to detect null alleles that account for the most probable cause for departures from HWE.

Genetic linkage disequilibrium was estimated between all locus pair using GENEPOP (version 4). Furthermore, to investigate the distribution of genetic variance, I used hierarchical analyses of molecular variance (analysis of molecular variance [AMOVA]; Excoffier et al. 1992) in ARLEQUIN (version 3. 01; Schneider et al. 2000) to compute

percentage of differentiation among sets of defined group (2010 and 2009), among populations (Italy, Austria, and Switzerland) within group (2009 and 2010), and within populations. I grouped population according year (2009, 2010). Each group consists of samples from the same year and populations represent sampling locations. Finally I used GENETIX v. 4.01 (Belkhir et al. 1997) to calculate pair wise FST values and their estimated probabilities (by 10 000 permutations) according to Weir and Cockerham (1984) and Bonferroni corrected for multiple comparisons (Rice, 1989). The software package GENELAND (Guillot 2008; Guillot et al. 2008) was used to distinguish genetically distinct clusters using spatial Bayesian clustering models. GENELAND makes no a priori assumptions about pre-defined populations. To determine the most probable number of clusters (i.e. populations), two independent runs with 100000 iterations (thinning = 100) were performed, allowing the number of possible populations (K) to vary from 1 to 7.

2.3 Phylogenetic analysis

All samples were subjected to mitochondrial sequencing to reconstruct phylogenetic relationships among them. Amplification of mitochondrial Cytochrome Oxidise Subunit I (COI) was achieved using universal primers LCOI490 and HCO2198 described by Folmer (1992). Each primer was labelled with a standard M13 tail to increase the yield of PCR products and facilitate the high throughput sequencing process (Table 1).

All polymerase chain reaction (PCR) amplifications used for sequencing were performed in 20µL total volume. The specific volumes were 2 µL DNA template, 12 µL dd H2O, 3.75 µL 10xPCR buffer, 1.5 µL 25 mM MgCl2, 0.5 µL dNTPs (10 mM each),1.5 µL of 20 µM forward and reverse primers and 1 unit of Taq DNA polymerase. The temperature profile for the amplification of the gene fragments included an initial denaturation step of 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1.5 min, and a final extension step at 72°C for 10 min.

The amplified products were examined by electrophoresis in 2% agarose gels in TAE buffer and purified by ExoSAP. PCR products were sequenced in both directions 5' and 3' using the BigDye Terminator cycle-sequencing ready reaction kit (Applied Biosystems, Inc.). Cycle sequencing products were purified using Ethanol/EDTA/Sodium Acetate Precipitation and sequenced on an ABI 3130 Genetic Analyser (Applied Biosystems). Sequences were edited using both SeqScape version 2.1 and Sequencher version 4.0 software (Gene Codes Corp., Ann Arbour, Mich, USA).

I used both strains to ensure the reproducibility of the alignment result and to generate contiguous sequences. Nucleotide sequences were aligned according to respective amino acid sequences aligned with Clustal W implemented in MEGA 4. We used MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) for Bayesian analyses. I used HKY model which was identified by the program JModelTest (Posada 2008). The analysis was run for 3 million generations, with trees sampled every 30,000 generations. FigTree ver.1.2.1 (Rambaut, 2008) was used to display resulting tree.

Table 1. Oligonucleotide primers used to PCR-amplify COI.

Universal primer M13 F	GTAAAACGACGGCCAGT
Universal primer M13 R	AACAGCTATGACCATG
Universal primer LCO (+M13 F)	GGTCAACAAATCATAAAGATATTGG
Universal primer HCO (+M13 R)	TAAACTTCAGGGTGACCAAAAAATCA

3. RESULTS

3.1. Microsatellites

The loci Ppla 107 and Ppla 382 were moderately polymorphic and only showed variation in the AUS2009, AUS2010 and/or ITA2010 samples which is concordant with the finding of a previous study (Galarza et al. 2011). Ppla 279 and Ppla 317 were the most polymorphic loci.

The mean number of alleles per locus per population ranged from 5.3 (Italy 2009) to 13.90 (Austria 2009) (Table 2). Observed heterozygosity averaged over all loci for each population ranged from 0.433 (Italy 2010 & Austria 2009) to 0.518(Austria 2009). Expected heterozygosity, He, for the 10 loci scored in all five populations were high, ranging from 0.724 (Austria 2009) to 0.653 (Switzerland 2010).

Results from Microcheker indicated 7 out of ten loci were prone to null alleles. Null alleles are non-amplified alleles in a PCR, because the PCR conditions are not ideal, the primer-binding region contains mutations that inhibit binding, or DNA template is degraded. In general, null alleles are a common cause of heterozygote deficit (Homozygote excess; Dakin and Avise 2004, Chapuis & Estoup 2007). Homozygote excess can be due to population substructuring or admixture, inbreeding, selection at or near a microsatellite locus, selection against heterozygotes, null alleles. Inbreeding, selection and admixture are unlikely to provide the underlying reasons for significant Fis value because expected heterozygosity is so high. Furthermore, most significant Fis values occurred randomly throughout the populations and years and thus, no loci were discarded at this point.

In 33 of 50 possible tests, significant deviations from HWE were observed after the Bonferroni corrections (Table 2). Loci Ppla 363,439,323 showed significant departures from HWE across all sampling locations. This is most likely due to technical issues such as mis-scoring or poor amplification. Therefore we excluded these loci from further analysis. Also, we excluded the 2 less informative loci Ppla107 and Ppla382 that showed low variability across all populations.

The average allelic richness per locus varied from 4.86 in Italy 2009 to 5.31 in Austria 2009 (Table 2). No linkage-disequilibrium was found between any of the locus pairs in any of the locations. Bayesian analysis in GENELAND showed that all individuals could most likely be assigned to one single genetic cluster. This was supported by pairwise Fst calculations, which demonstrated no significant differentiation (ranging from -0.00572 to 0.00671) between locations (Table 3).

Additionally, the AMOVA showed that more than 99% of the molecular variation was found within populations, while there was no variation (-0.3%) associated with differentiation according to the sampling year (2009 and 2010). Only a small proportion of variation (1.2%) was attributable to variation among populations within the sampling years (Table 4).

3.1. Mitochondrial sequences (COI)

Using mitochondrial DNA from same individuals used in Microsatellites, did not detect any genetic structure either. The phylogenetic analysis recognized a single monophyletic clade, including all individuals in 3 sampling sites with the exception of one cluster containing two individuals from the 2010 Italy population due to singleton variable site (Figure3).

Table 2. Variability of ten microsatellite loci in *Parasemia plantaginis* populations from Austria (AUS), Italy (ITA), Switzerland (SWI). **Note:** N, number of individuals; A, alleles' number; Ho, observed heterozygosity; He, expected heterozygosity; Rs, allelic richness; F_{is}, inbreeding coefficient (Weir and Cockerham). Asterisks pre sent significant Fis at α=0.05, significant values after Bonferroni corrections are presented in bold α=0.005.

	2009	**2009**	**2010**	**2010**	**2010**
LOCUS	AUS	ITA	SWI	AUS	ITA
Ppla107					
N	42	6	10	34	17
A	3	1	1	1	2
Rs	1.773	1	1	1	1.508
He	0.195	0	0	0	0.114
Ho	0.214	0	0	0	0.117
Fis	-0.095	monomorphic	monomorphic	monomorphic	-0.032
Ppla 109					
N	43	6	10	35	18
A	20	6	10	21	14
Rs	7.256	5.485	6.966	7.397	7.076
He	0.922	0.848	0.915	0.923	0.912
Ho	0.906	0.833	1.000	0.914	0.888
Fis	0.017	0.019	-0.097	0.0105	0.026
Ppla 279					
N	43	6	10	35	17
A	23	11	12	24	17
Rs	8.196	9.318	7.711	8.074	7.988
He	0.954	0.984	0.942	0.949	0.948
Ho	0.883	0.833	0.800	0.714	0.823
Fis	0.075	0.166	0.157*	**0.250***	0.135
Ppla 414					
N	42	6	10	35	18
A	13	7	8	9	8
Rs	6.024	6.000	5.872	5.267	5.681
He	0.862	0.772	0.863	0.837	0.850
Ho	0.761	0.666	0.600	0.657	0.611
Fis	0.118	0.148	0.316*	**0.217***	0.287*
Ppla 363					
N	41	6	10	34	15
A	6,000	2	4	6	3
Rs	3.595	2,000	2.995	3.819	2.714
He	0.604	0.484	0.573	0.661	0.480
Ho	0.219	0,000	0.200	0.235	0.200
Fis	**0.639***	1.000*	**0.663***	**0.6478***	**0.592***
Ppla 317					
N	43	6	10	35	18
A	20	7	9	19	17

Table2.(*Continued*)					
Rs	7.064	6.455	6.667	7.628	8.192
He	0.893	0.811	0.850	0.917	0.916
Ho	0.674	0.666	0.500	0.542	0.625
Fis	**0.262***	0.285	0.464*	**0.423***	**0.287***
Ppla 382					
N	42	6	10	35	16
A	4	2	1	2	1
Rs	1.642	1,000	1,000	1.375	1,000
He	0.149	0,000	0,000	0.079	0,000
Ho	0.167	0,000	0,000	0.086	0,000
Fis	-0.057	monomorphic	monomorphic	-0.030	monomorphic
Ppla 439					
N	37	6	8	35	16
A	11	5	6	9	7
Rs	5.124	4.803	5.214	5.021	4.431
He	0.816	0.833	0.825	0.817	0.735
Ho	0.405	0.500	0.375	0.371	0.187
Fis	**0.506***	0.423	**0.562***	**0.549***	**0.751***
Ppla 313					
N	40	6	8	35	16
A	23	7	11	21	13
Rs	7.759	6.591	7.804	7.347	6.979
He	0.937	0.924	0.941	0.92	0.913
Ho	0.500	0.666	0.75	0.457	0.437
Fis	**0.469***	0.298*	0.215	**0.506***	**0.529***
Ppla 323					
N	42	5	10	33	18
A	16	6	7	15	8
Rs	6.373	6.000	5.446	6.192	5.345
He	0.880	0.888	0.836	0.847	0.834
Ho	0.452	0.400	0.400	0.454	0.444
Fis	**0.489***	0.578*	**0.535***	**0.467***	**0.474***
Over All Loci Per Population					
	2009	2009	2010	2010	2010
	AUS	ITA	SWI	AUS	ITA
N	43	6	10	35	18
A	13,9	5,3	6,9	12,7	8,9
Rs	5.312	4.860	4.948	5.117	5.091
He	0.741	0.654	0.653	0.695	0.670
Ho	0.518	0.456	0.463	0.443	0.443

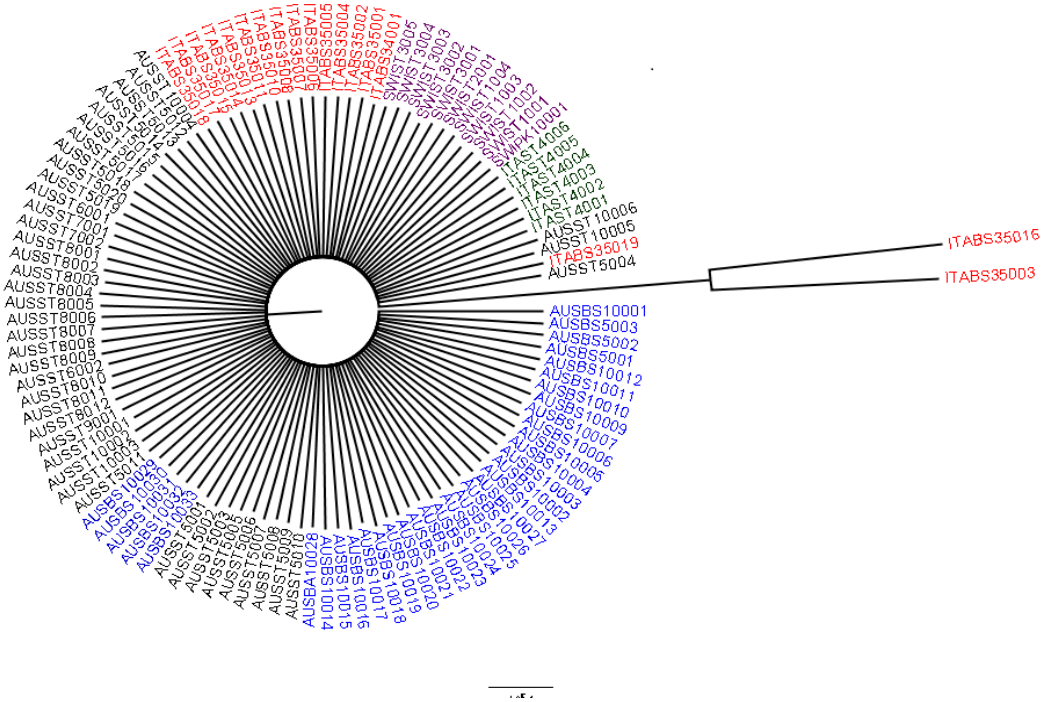

Figure 3. Phylogenetic relationships of tiger moth inferred from Bayesian analysis COI mtDNA. Red: Italy 2010, Green: Italy 2009, Purple: Switzerland 2009, Black: Austria 2009, Blue: Austria 2010.

Table 3. Pairwise FST values among populations. There is no significant value after Bonferroni corrections α=0.005.

	FST	FST; Without (Ppla 323, 439,382,363,107)
AUS 10 vs. AUS 09	0.0041	0.0012
AUS 10 vs. ITA 09	-0.0140	-0.0035
AUS 10 vs. SWI 09	-0.0002	0.0053
AUS 10 vs. ITA 10	0.0088	0.0067
AUS 09 vs. SWI 09	0.0041	-0.0045
AUS 09 vs. ITA 09	-0.0119	-0.0039
AUS 09 vs. ITA 10	0.0047	0.0060
SWI 09 vs. ITA 09	-0.0158	-0.0114
SWI 09 vs. ITA 10	0.0074	-0.0057
ITA 09 vs. ITA 10	-0.0219	-0.0150

Table 4. Analysis of Molecular Variance (AMOVA) using 10 microsatellites loci, partitioning genetic variation among sampling years and within and among samples of *Parasemia plantaginis* from three sites (Italy, Austria, and Switzerland) on the Alpine region.

Source of variation	Sum of squares	Variance components	% variation
Among groups (2010 & 2009)	5.170	-0.013	-0.370
Among (ITA/AUS/ SWI) within groups (2010 & 2009)	9.972	0.044	1.260
Among individuals within populations	682.156	3.522	99.110
Total	697.298	3.554	

4. DISCUSSION

The present study examines the spatial and temporal population genetic composition of aposematic but polymorphic *Parasemia plantaginis* employing both microsatellite and mitochondrial markers in the Alps including Italy, Switzerland, and Austria.

Analyses of two molecular marker types showed almost no genetic structure in *P. plantaginis* across the sites sampled in the Alps for this study. More specifically, *P. plantaginis* populations breeding in alpine area were not differentiated from each other even over far distances.

This result was surprising because the Alps have acted as a dispersal barrier, and thus represents a region of great diversity in terms of the flora and fauna (Hewitt 2000). This diversity is a product of spatial variation in patterns of selection and limited gene flow. Hegna et al. (in prep) highlighted the impact of physical barriers on the *P. plantaginis* population structure in Japan using microsatellites. They found genetic differentiation among populations situated on Japan's mountains, despite close geographic proximity. Consequently, the Alpine landscape with natural fragmentation is expected to restrict gene flow among populations and causes spatio-genetic structuring by one of the underlying mechanisms such as genetic drift or local adaptation. While genetic differentiation among sampling sites was expected to be high, individuals from different sampling sites (Italy, Switzerland, and Austria) appear to be a panmictic population in this study. Therefore, the differences observed in phenotypes cannot be attributed to a different genetic composition or temporal genetic variation.

Dispersal estimates obtained in the field (e.g., from mark-and-recapture studies) can be difficult to apply to certain types of organisms such as insects and they are also limited in space and time. However, the rate of gene flow is inferred from the genetic structure of a species indirectly is averaged over thousands of generations or more and integrated geographically over many populations. Gene flow can be inferred from population genetic structure (e.g. from Fst), greater genetic differentiation implies lower gene flow and vice versa (Wright 1951, Slatkin 1985). On the other hand, FSTs reflect a combination of the amount of genetic drift and gene flow among populations. Our finding of low and non-significant FSTs excludes genetic drift from contributing to colour differentiation between populations. Therefore, extensive gene flow may account for low genetic differentiation among our study populations. It has been shown that species with high dispersal rate (high gene flow) often exhibit low level of genetic differentiation among populations (Procházka et al. 2010).

The results determined by microsatellite markers also showed that there was relatively high genetic diversity within wood tiger moth populations in spite of the fact that directional local selection by predators. The spread of an advantageous allele, together with selection against disadvantageous alleles, is expected to lead to a loss of genetic variation. Since expected heterozygosity was similar within all populations, it could be inferred that extensive gene flow could homogenize the distribution of genetic diversity along sampled populations (Salehi Shanjani et al. 2011). Alternatively, larger populations typically retain more genetic diversity (Frankham 1995). Thus, high genetic diversity may suggest high gene flow and/ or high population density in wood tiger moth.

Unexpected high genetic diversity and low genetic differentiation among sampling locations in the Alps was indeed similar to some other butterfly species. The butterfly *Pararge aegeria* (Nymphalidae) is also characterized by high gene flow and population density, despite localized European fragmented landscapes including the Netherlands and Belgium (Vandewoestijne & Van Dyck 2010). Yet, the failure to detect genetic structure across alpine area contrasts with results observed in some other species like adders *Vipera*

berus (Ursenbacher et al. 2009). The genetic differentiation among *V. berus* populations appears to be large as a result of limited gene flow in adders.

Low genetic differentiation among wood tiger moth populations might also be accounted for either by a recent population expansion or by the presence of alleles shared due to the common ancestry (e.g., Grant et al. 2005), rather than by recurrent gene flow. For example, the high rates of gene flow inferred from the FST's are probably an artifact of the range expansion rather than a true reflection of effective migration. In this study, estimates of genetic diversity were similar and high among populations, which would support a gene flow explanation instead of a recent expansion. However our results cannot rule out this option completely. Range expansions generally lead to a loss of genetic diversity (Excoffier et al, 2009). However, a high, multi-directional, on-going gene flow can remove any loss of diversity caused by range expansion (Pruett et al. 2009).

On the other hand, mtDna results were consistent with nuclear (microsatellites) DNA. All sequenced individuals for COI also shared just two haplotypes, one of the two haplotypes shared almost among all individuals except two individual form Italy that shared second haplotype that was a singleton variable. Nuclear and mitochondrial genomes are differentially susceptible to variation in gene flow. MtDNA is maternally inherited and has one-fourth the effective population size of a nuclear locus (Avise 2000), therefore mtDNA markers are expected to be less sensitive than nuclear genes to the homogenizing effects of gene flow among populations and detecting population structure (Hale & Singh 1991). Consequently, potential problems and possible technical considerations (e.g. lower marker power) due to applying just one of these will be ruled out. Our results indicates that level of gene flow appear sufficient to completely homogenize mtDNA haplotype frequencies. Nonetheless, the mode of gene flow is unknown. The fact that wood tiger moth's range extends across the entire northern hemisphere indicates that individuals of this species likely disperse great distances. Large geographic distribution was accounted for effective dispersal rate and high gene flow for example in old world butterfly (Lohman et al.2008). Therefore, wood tiger moth species may be capable of dispersing long distances from their origin site directly and maintain connectivity over large geographical areas. Alternatively, this species may be able to disperse over a large area by step-by-step migration of individuals between neighbouring populations which maintains species' homogeneity. For example, Hoareau (2007) reported that step-by-step migration is the only way for red-tailed goby species to disperse across remote archipelagos such as the Mascarene and Comoros islands. Finally, wood tiger moths migration could be considered to be a type of stratified dispersal (Hengeveld 1989) allowing long-distance dispersal (wind and human transportation) as well as short distance connections by flight. Furthermore, consistent pattern between the mitochondrial and microsatellite suggests that there is no sex-biased dispersal in wood tiger moth across our sampling sites in the Alps, which is surprising because females are known to fly only little during the oviposition. However, only future studies dedicated to extensive mark-recapture can clarify a more accurate picture about gene flow in this species.

Using both mitochondrial and nuclear markers allows us to better understanding of the relative roles that historical and contemporary events have had on population structure (Howes et al. 2006). Mitochondrial DNA are the most suitable markers for studies of ancient population dynamics because of its haploid, non-recombining mode of inheritance, relatively rapid mutation rate, and because it is usually considered to be selectively neutral (Avise 2004). MtDNA may not evolve fast enough, in terms of mutation, to resolve evolutionary histories in recent times (Angers and Bernatchez 1998). Therefore, we used microsatellites to estimate contemporary gene flow. As mentioned

before, we found one common and widespread haplotype. Therefore, very low levels of mitochondrial sequence divergence may suggest that substantial level of gene flow have occurred historically among wood tiger moth. On the other hand, weak genetic structuring indicates on-going high gene flow across wood tiger populations in a highly fragmented Alpine landscape. Congruence between mtDNA and microsatellite implies the both types of markers could be subject to the same evolutionary forces. Thus the present -day wood tiger moth population across sampling sites may bear the genetic signature of both historical and on-going high gene flow.

Although absence of population structuring in *P. plantaginis* may be a consequence of extensive gene flow, gene flow alone cannot explain the establishment of observed phenotypic differences along sampled populations. In general, several factors may maintain morphological differentiation in face of high gene flow. One possibility is phenotypic plasticity in response to local habitat features. *P. plantaginis* exhibit low degree of plasticity (Ojala et al. 2007) and their phenotypic plasticity is limited in the range of environments they can respond to (Lindstedt et al. 2009). Phenotypic plasticity is an unlikely explanation for morphological divergence observed in our study, because of the high heritability of signal (different colour pattern on wings such as orange) size. A second is that tiger moth actively move to areas that better suit their morphology (i.e., habitat selection)—but only dispersal pattern can clarify exact answer for this possibility to see if there is any phenotype-biased dispersal. A third possibility is genetic differentiation in quantitative traits is possible even when neutral marker genes indicate no differentiation at all. Therefore, it is possible selection maintain differentiation at selected loci despite much lower differentiation at unlinked neutral loci (Wu 2001).

While wood tiger moth individuals can be highly mobile within their geographic range, they may be restricted to that distribution because they could be unable to physiologically tolerate the extreme environmental conditions (e.g. temperature) beyond their tolerance via local adaption. On the other hand, shifts in predator regimes may restrict gene flow in aposematic species because of predator-induced mortality on new immigrants. Instead, our results are congruent with observations that neutral gene flow can be high even in the presence of substantial phenotypic differentiation. This paradox may be explained as follows. The homogenizing effect of high gene flow has been compensated by differential environmental and ecological selective forces along the altitudinal gradient. Consequently, the selectively neutral markers may not capable of expressing the genetic differentiation among populations that can be detected by quantitative analyses.

The coloration of species can have multiple functions, such as predator avoidance and sexual signalling, thermoregulation that directly affect fitness. A consequence of having one character affect multiple performances is the potential for functional trade-offs (Endler & Mappes 2004). For example, trade-offs between being protected and other fitness-related traits such as thermoregulation (Lindstedt et al. 2009), and mating success (Nokelainen et al. 2012) has been suggested to maintain variation in aposematic coloration in this species.

Occurrence of white and yellow male morphs in wood tiger moth regardless of their aposematic colouration has been demonstrated to be the result of trade-off between survival selection and sexual selection researchers have explained that more conspicuous morph (yellow) have a selective advantage compared with a less conspicuous morph (white) but females prefer wither morphs (Nokelainen et al. 2012). Differential predation pressure along geographical gradient can lead to different pattern in trade-offs between survival selection and mating success.

Since aposematic signals are combine with melanin based pattern, aposematic

coloration in wood tiger moth may also be affected by environmental and ecological factors along alpine elevations thorough the opposing selection pressures. One explanation for variation in melanin pattern along geographical gradient has been explained by thermal melanism hypothesis i.e. TMH predicts that dark individuals are at an advantage in cool climates as they heat faster and reach higher equilibrium temperatures than lighter individuals (Watt 1968, Clusella-Trullas et al. 2008). Alternatively, Common garden experiment has been shown that in warmer climates butterflies can overheat, so lighter individuals have an adaptive advantage over melanic ones in high temperatures (Nice& Fordyce 2006). Ellers and Boggs (2004) demonstrated that geographic distribution in the melanin pattern of Colias butterflies reflects adaptation to different temperature regimes, namely the higher temperatures in low elevation region. Therefore, local adaptation to the abiotic environment may constrain local adaptation to predators under contrasting selection pressures, resulting localized interactions that vary in outcome depending on local condition such as temperature (Mochida 2011).). In *Parasemia plantaginis* Hegna et al. (in prep) found that more melanized males were better able to warm up, though they were also more prone to predation. Melanin in the wood tiger moth is a heritable trait (Lindstedt et al. in prep). Therefore, some evidence exists that geographic variation in the phenotype of *P. plantaginis* is influenced by environmental based selection.

In both cases, stabilizing selection imposed by predators may differ among different habitat along elevations leading to inter-population colour variation in this species. However, given high gene flow (both current and past), and lack of strong post-settlement selection pressure, selective forces due to altitude are not strong enough to significantly differentiate the studied population in terms of microsatellites and mtDNA. Moreover, exchange of individuals adapted to alternative environment (gene flow) may increase intra-individual colour variation within each locality.

Our results corroborate previous findings that showed that local adaptations may be maintained despite high levels of gene flow when selection pressure is high (Endler 1977) and has been demonstrated in several species such as, fly (*Drosophila montana*) due to adaptation to seasonal changes(Tyukmaeva 2011), Dominican anole (*Anolis oculatus*) exposed to different rainfall and vegetation type (Stenson 2002), Galapagos lava lizard (*Microlophus albermarlensis*) with different anti-predator behaviour, and Trinidadian guppy populations (*Poecilia reticulata*), under different in predation pressure (Magurran 1998).

In conclusion, the results from our analysis using the Bayesian clustering method in GENELAND suggest that gene flow among populations over sampling sites in Italy, Switzerland, and Austria are sufficiently high to group them together as a single population. Our results showed that weak neutral genetic differentiation does not necessarily mean phenotypic resemblance. Even very high rates of gene flow among population separated with physical barriers do not appear to prevent morphological divergence in characters important to survival. Our results offer evidence that extensive gene flow (historically and / or contemporary) along with abiotic and biotic contrasting selection pressures contributes to the maintenance of colour polymorphism in this species.

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