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Unprecedented long-term genetic monomorphism in an endangered relict butterfly species

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Running title: Long-term genetic monomorphism in *Parnassius apollo vinningensis*

1 **Abstract**

2 Multi-locus monomorphism in microsatellites is practically non-existent, with one notable
3 exception, the island fox (*Urocyon littoralis dickeyi*) population on San Nicolas island off the
4 coast of southern California, having been called “the most monomorphic sexually reproducing
5 animal population yet reported” (Aguilar et al., 2004). Here, we present the unprecedented
6 long-term monomorphism in the highly endangered *Parnassius apollo* butterfly which is
7 protected by CITES and classified as “threatened” by the IUCN. The species is disjunctly
8 distributed throughout the western Palaearctic and has occurred in several small remnant
9 populations outside its main distribution area since the last ice age. We screened 78
10 individuals from one such relict area (Mosel valley, Germany) at 16 allozyme and six
11 microsatellite loci with the latter known to be polymorphic in this species elsewhere. From
12 the same area, we also genotyped 55 museum specimens sampled from 1890 to 1989 to
13 compare historical and present levels of genetic diversity. However, none of all these
14 temporal populations yielded any polymorphism. Thus, present and historical butterflies were
15 completely monomorphic for the same fixed allele. This is the second study to report multi-
16 locus monomorphism for microsatellites in an animal population and the first one to prove
17 this monomorphism not to be the consequence of recent factors. Possible explanations for our
18 results are a very low long-term effective population size and/or a strong historic bottleneck
19 or founder event. Since the studied population has just recovered from a recent population
20 breakdown (second half of 20th century) and no signs of inbreeding depression have been
21 detected, natural selection might have purged the population of weakly deleterious alleles,
22 thus rendering it less susceptible to the usual negative corollaries of high levels of
23 homozygosity and low effective population size.

24

25 Keywords: butterfly, microsatellites, allozymes, monomorphism, purging, collection samples,
26 climate change

27

1 **Introduction**

2 After the last glacial period xerothermophilic elements (re)colonized Central Europe from the
3 Mediterranean region (Hewitt 1996). Organisms with highly specific ecological demands only
4 survived at isolated habitat patches in some parts of Central Europe with especially hot and
5 dry conditions where they have persisted over long periods of time to the present (De Lattin
6 1967). Living in isolation enhances processes of population dynamics like population
7 fluctuations (den Boer 1981), population stochasticity (Holzhauer et al. 2005) and the loss of
8 genetic diversity in local sites through drift, which cannot be compensated by immigration
9 from other populations (Hanski 1999), and many theoretical and experimental studies have
10 analysed these effects (e.g. Ellis et al. 2006). Due to their high mutation rate, microsatellite
11 loci are a suitable analytical tool for the detection of genetic diversity and differentiation of
12 isolated and fragmented populations because they more often show polymorphisms than other
13 molecular markers (e.g. Zachos et al. 2008).

14 In the present study, we analysed populations of the butterfly *Parnassius apollo*. This for
15 Central Europe highly endangered species, listed in Red Data Books, the Appendix IV of the
16 Flora Fauna Habitat Directive and the CITES-Convention, survived in isolation in some
17 marginal localities far away from its main distribution areas in mountainous regions. One
18 relict population survived on the rocky slopes of the Mosel valley in Western Germany,
19 classified as the endemic subspecies *P. apollo vinningensis*. Since low genetic diversity was
20 expected for this taxon, we chose 16 allozyme and 6 microsatellite loci the latter of which are
21 known to be polymorphic in this species within its main distribution range (Petenian et al.
22 2005; Meglecz et al. 2004). To test if there has been a detectable reduction in genetic diversity
23 due to the intensification of viticulture and a recent bottleneck in this region over the last
24 decades, extant samples were compared with samples from museum collections dating back
25 to 1890. The use of such collections has been shown to be a valuable approach to comparing
26 past and present levels of genetic diversity in a wide array of taxa (for a review see Wandeler

1 et al. 2007). In this study, we tempt to answer the questions how is the genetic diversity of
2 this relict population of *Parnassius apollo*, and which conclusions can be drawn for
3 conservation biology?

4

1 **Material and Methods**

2 *Study species*

3 The butterfly *Parnassius apollo ssp. vinningensis* (Stichel 1899) is an endemic subspecies of
4 the xeromontanic butterfly *Parnassius apollo* (Linnaeus 1758), patchily distributed from
5 Spain to southern Fennoscandia and the Balkan Peninsula including the northwestern
6 Peleponnesos (Kudrna 2002). The species is divided into many subspecies distributed over
7 Europe, and some of these subspecies are restricted to isolated regions of Central Europe
8 (Tolman and Lewington 1997).

9 *Parnassius apollo* has univoltine populations flying from June to August (Tolman and
10 Lewington 1997). The habitats of *Parnassius apollo vinningensis* are rocky slopes with the
11 larval foodplant *Sedum album* (Kinkler et al. 1987). Habitat destruction due to widespread
12 spraying of pesticides and fallow land led to a severe collapse during the 1970s, and this
13 formerly widespread and common butterfly of the Mosel valley between Trier and Koblenz
14 declined strongly to some few remnants during this period (Kinkler et al. 1987; Löser and
15 Rehne 1983). However, strict conservation programmes of habitat reconstruction combined
16 with reduced usage of pesticides resulted in a recovery of the population. At present,
17 *Parnassius apollo* is classified as one of the most endangered butterflies in Europe (IUCN
18 1996), listed in the European Red Data Book (Van Swaay and Warren 1999), the Appendix II
19 of the Habitat Directive (EEC 92/43/EWG) of the European Union and the CITES-
20 Convention.

21

22 *Sampling and genetic analysis*

23 Seventy-eight samples of *Parnassius apollo* were taken from four sites of the Mosel valley,
24 including the westernmost and the easternmost area of occurrence (Figure 1). Butterflies were
25 sampled from the beginning of June to the end of July 2004. The individuals were netted in
26 the field and one leg was dissected per individual and stored in liquid nitrogen until analysis.

1 To avoid recaptures of individuals, all sampled butterflies were marked before release. As
2 analytical tools we used allozyme electrophoresis and microsatellite markers. The sampled
3 animal tissue of one leg per individuuum was sufficient for both molecular approaches as a
4 nonlethal method. We completed the samples by 55 museum specimens from 1895 to 1989
5 (five samples of each year: 1895, 1897, 1898, 1904, 1909, 1932, 1946, 1953, 1968, 1979,
6 1989), by analysing these samples exclusively for the six microsatellite loci.

7 For the allozyme analysis, the femur of each sample was homogenised in Pgm-buffer (Harris
8 and Hopkinson 1978) with ultrasound and centrifuged at 17,000 g for 5 minutes. The
9 remaining tibia and tarsus were stored for DNA extraction. We ran electrophoreses on
10 cellulose acetate plates (Hebert and Beaton 1993) and analysed 16 enzyme systems (running
11 conditions Table 1).

12 For microsatellite markers, DNA was extracted from the remaining tibia using the Qiagen
13 DNeasyTM Tissue Extraction Kit (Hilden, Germany), following the manufacturers' protocol.
14 PCR reactions were carried out in a thermal cycler (Corbett Research CG1-96). Microsatellite
15 loci were amplified from 50-100ng diluted DNA in a Thermozym Mastermix (Molzym,
16 Bremen, Germany). The samples were screened and genotyped for six microsatellite loci
17 (PA35, 45, 56, 79, 82, 85). The forward primer of each pair was 5' end-labelled with the
18 fluorescent phosphoramidites FAM. Primer sequences and PCR conditions were taken from
19 Meglecz et al. (2004) and optimized (for details see Table 2). PCR products were visualised
20 by electrophoresis on a 2.4% agarose gel stained with ethidium bromide as a control before
21 scoring the microsatellites using an automated sequencer with the Megabace software (GE
22 Healthware, USA).

23

1 **Results**

2 All allozyme and microsatellite loci for both methods were monomorphic, showing the same
3 fixed allele. This was similarly true for the historical samples suggesting that the fixation of
4 one allele at each of the 22 loci occurred prior to 1890.

5

1 **Discussion**

2 *The most monomorphic butterfly*

3 In accordance with our a priori expectations based on the relict status of the Mosel valley
4 populations, no high genetic diversity was detectable. However, the complete lack of genetic
5 diversity in all individuals analysed from different localities all over the Mosel distribution
6 range spanning a time frame of more than 100 years was unexpected. The four local sampling
7 sites therefore do not show any differentiation among each other, but must be considered as
8 belonging to one homogeneous gene pool.

9 While monomorphism at allozyme loci has been found before (e.g., Zachos et al. 2006) the
10 multi-locus monomorphism detected for six microsatellites is indeed exceptional. Although
11 the presence of additional rare alleles still cannot be completely excluded, our reasonably
12 large sample sizes add credibility to our results. When and how the genetic depletion of *P.*
13 *apollo vinningensis* occurred, cannot be deduced with our data set but most probably it is best
14 explained by repeated bottlenecks and/or founder events during the postglacial periode
15 enduring since some thousands of years, possibly going back until the first colonisation of the
16 area after the last glacial period. The geographically restricted distribution of this butterfly is
17 likely to have enforced environmental and demographic stochasticity (Frankham et al. 2002)
18 as well as concomitant population fluctuations, bottlenecks combined with genetic drift and
19 finally the complete loss of alleles.

20 Genetic depletion has been found in several other butterfly species with similar biotic and
21 abiotic demands (Bereczki et al. 2005; Debinski 1994; Figurny-Puchalska et al. 2000;
22 Gadeberg and Boomsma 1997; Schmitt and Seitz 2004), but all of them show at least some
23 genetic diversity within and among populations, making *P. a. vinningensis* the most
24 monomorphic butterfly taxon known to science. To our knowledge, the only other known
25 case of multi-locus monomorphism at microsatellite loci is the island fox (*Urocyon littoralis*)
26 population on San Nicolas Island off the southern coast of California (Aguilar et al. 2004).

1 Consequently, these foxes have been called “the most monomorphic sexually reproducing
2 animal population yet reported” (Aguilar et al. 2004). Except for the endangered damselfly
3 species *Coenagrion mercuriale* (Watts et al. 2006) and the Chilingham cattle *Bos tarus*
4 (Visscher et al. 2001), where part of the analysed microsatelit loci showed no variability, in
5 all other thoroughly studied cases of isolated relicts or highly endangered populations
6 microsatellites showed reduced levels of polymorphism with occasionally fixed alleles at
7 single loci but no multi-locus monomorphism (e. g., Kawamura et al. 2007; Zachos et al.
8 2007).

9 The high stability of deoxyribonucleic acid and effective DNA extraction protocols allow the
10 comparison of extant populations with older collection specimens going back to the 18th
11 century (Mandrioli et al. 2006; Watts et al. 2007). By using such collections from the late 19th
12 and early 20th century we were able to show that the genetic depletion in *P. apollo*
13 *vinningensis* is not a result of recent anthropogenic impacts during the 20th century, but must
14 have occurred earlier, as the historical samples were monomorphic for the same alleles as the
15 extant specimens. On the contrary, other butterfly species like *Thymelicus acteon* (Louy et al.
16 2007), *Coenonympha hero* (Cassel and Tammaru 2004), *Speyeria idalia* (Williams et al.
17 2003), *Lycaena helle* (Finger et al., unpublished) or the burnet moth *Zygaena loti* (Habel et
18 al., unpublished) are also confined to isolated local populations, but still show unexpectedly
19 high genetic diversity. However, these species constantly survived in large metapopulation
20 networks and only very recently suffered isolation in the course of the changes in land use, so
21 that their genetic diversity is probably best explained as a remnant of a ‘better past’.

22

23 *How important is genetic diversity for the fitness of populations?*

24 *Parnassius apollo vinningensis*, which has probably been isolated from conspecific
25 populations in the Vosges, the Black Forest or the Schwäbische Alb for a very long time,
26 possibly thousands of years, has recovered well from a recent anthropogenic bottleneck. In

1 contrast to general theory in conservation genetics (Frankham et al. 2002, Reed and Frankham
2 2003; Schmitt and Hewitt 2004), no high level of genetic diversity was necessary; on the
3 contrary, this recovery occurred in a population without any diversity at all at two normally
4 polymorphic marker systems.

5 Given that this taxon was already genetically depleted more than a hundred years ago, the
6 subspecies has probably undergone more than one historic bottleneck event. If so, the
7 successful recent recovery of the population might be a consequence of purging effects:
8 recessive deleterious alleles might have become exposed to natural selection as a
9 consequence of an increase in homozygosity due to inbreeding. Thus, the populations
10 surviving bottleneck events might have had a reduced genetic load and therefore might be less
11 susceptible to inbreeding depression (caused by homozygous deleterious alleles). While
12 recent analyses have shown that the effects of purging generally seem to be limited and that
13 immunity to second bottlenecks cannot be expected (Frankham et al. 2001; Thévenon and
14 Couvet 2002), the case of *P. apollo vinningensis* might be a rare exception from the rule.
15 Future analyses of possible inbreeding depression in this taxon as compared with its
16 genetically more diverse conspecifics from other locations might turn out to be a fruitful
17 contribution to studying the importance of purging.

18

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9

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Long-term genetic monomorphism in *Parnassius apollo vinningensis*

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1 **Figure 1:** Geographic location of the four sample sites Calmont, Valwig, Dortebrachtal and
2 Winingen of *Parnassius apollo vinningensis* in the Mosel valley.

3

4

1 **Table 1:** Conditions of electrophoresis for different enzymes analysed for *Parnassius apollo*
 2 *vinningensis*.

3

Enzyme	EC-No.	Number of loci	Buffer	Homogenate applications	Running time (min.)
6PGDH	1.1.1.44	1	TC	2	50
GPDH	1.1.1.8	1	TM	3	45
MDH	1.1.1.37	2	TM	2	45
MPI	5.3.1.8	1	TC	3	30
AAT	2.6.1.1	1	TM	3	40
G6PDH	1.1.1.49	1	TM	3	45
FUM	4.2.1.2	1	TM	3	45
ME	1.1.1.40	2	TC	3	30
PK	2.7.1.40	1	TM	3	40
APK	2.7.3.3	1	TM	3	40
PGM	5.4.2.2	1	TG	3	35
GPI	5.3.1.9	1	TG	1	35
IDH	1.1.1.42	2	TC	2	50

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6 TC, Tris-citrate pH 8.2; Tris-glycine pH 8.5; TM, Tris-maleic acid pH 7.0 (adjusted from TM

7 pH 7.8). All buffers were run at 200 V.

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Long-term genetic monomorphism in *Parnassius apollo vinningensis*

1 **Table 2:** Characteristics of six microsatellite loci in *Parnassius apollo vinningensis* developed for *Parnassius apollo* by Meglecz et al. (2004),
 2 modified.

3 Abbreviations: F: forward primer; R: reverse primer; T_a: annealing temperature; individuals were analysed for each locus.

4

Locus	GenBank Accession no.	Primer sequence (5'-3')	Repeat motif	Size of sequenced allele (bp)	T _a (°C)
PA35	AY491887	F: CCCACGTCAATATCACTCTTTG R: CTGGGACGGATTGCTAGTTG	(TACA) ₅ TACG(TACA)(TG) ₂ (CA) ₄	240	54
PA45	AY491895	F: GCCTACATGTGAGGCGTCAT R: GCATGTAGATGTAAGTGTGCGTG	(TACA) ₅ ...(TACA) ₅	235	51
PA56	AY491906	F: ACTAGTCGGTCGACATAGTACC R: CCAAATGGAAGTCTGTAGTCTC	(TACA) ₆	158	51
PA79	AY491924	F: TGGTCCTGTAGCTCTGTATCAC R: CTATTAAGCGGCTCGTACATC	(TGTA) ₂₊ (TGTA) ₅	107	54
PA82	AY491926	F: TGTAGATGACGCCCCATAT R: GTCATCTACATACGGTACGCAT	(TGCG) ₃ GC(TGTA) ₇	164	54
PA85	AY491928	F: AATGCAGGCACATAACTAAGAC R: TCTATGTGGCGTTTTGTGG	(CA) ₃₇ TA(CA) ₂	212	54

5