universität wien

MASTERARBEIT

Titel der Masterarbeit

Tracing the radiation of *Maniola* (Lepidoptera: Nymphalidae: Satyrinae) butterflies: new insights from phylogeography hint at one single incompletely differentiated species complex

verfasst von

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angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2014

Studienkennzahl lt. Studienblatt:	A 066 831
Studienrichtung lt. Studienblatt:	Masterstudium Zoologie
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Contents

Abstract

Maniola butterflies show a peculiar distribution: *Maniola jurtina*, the most widely distributed species of this genus, can be found all over Europe, whereas the other six described species are restricted to the Mediterranean area - among them three species are island endemics on Sardinia, Cyprus and Chios, respectively. The species are almost indistinguishable morphologically and hybridization seems to occur occasionally. To clarify species boundaries and diversification history of the genus, I performed a phylogeographical study. I reconstructed the phylogeny with help of the genetic markers *COI*, *CytB*, *Elongation factor 1a* and *wingless*, analysed variation in mitochondrial and nuclear DNA, estimated divergence times, compared possible migration routes and inferred demographic developments from genetic models. The topology of the recovered phylogenetic tree is not consistent with accepted taxonomy, but rather reveals several haplotype clades that are incongruent with nominal species boundaries. This represents a rare case where several taxa earlier described by means of morphological traits become merged into fewer species (or even a single one). Usually more (cryptic) species are uncovered with genetic methods than have been taxonomically described using morphological characters. Instead of seven species, I recognized two major lineages which could have formed during an out-of-Africa scenario: one part of the *Maniola* genus migrating over the Strait of Gibraltar and the Iberian Peninsula to the west of Europe, and the other part wandering eastwards through Asia Minor and over the Bosporus into eastern Europe. Genetic diversity proved to be surprisingly high, even in island species. Furthermore, molecular data suggest that *Maniola* is a very young taxon (about 0.6 to 0.9 Mya old).

Keywords: *COI*; *Cyt-B*; *wingless*; *Elongation factor 1a*; speciation; endemism; migration routes; phylogeny; biogeography; integrative taxonomy; DNA barcoding; species delimitation

Zusammenfassung

Schmetterlinge der Gattung *Maniola* weisen eine eigentümliche Verbreitung auf: *Maniola jurtina*, der am weitesten verbreitete Vertreter der Gattung, kann in ganz Europa angetroffen werden, wohingegen die anderen sechs beschriebenen Arten auf das Mittelmeergebiet beschränkt sind - drei Arten sind sogar jeweils endemisch nur auf einer bestimmten Insel (Sardinien, Zypern und Chios). Dabei sind die Arten morphologisch fast ununterscheidbar, auch Hybridisation wurde schon beobachtet - interessante Voraussetzungen für phylogeografische Untersuchungen. Deshalb habe ich die Phylogenie dieser Gattung mittels der genetischen Marker *COI*, *CytB*, *Elongation factor 1a* und *wingless* rekonstruiert. Außerdem habe ich die genetische Variation analysiert, Divergenzzeiten geschätzt, mögliche Einwanderungsrouten verglichen und mittels genetischer Modelle Rückschlüsse auf demografische Entwicklungen gezogen. Die Topologie des erhaltenen Kladogramms entspricht nicht der gängigen Taxonomie der Gattung, sondern zeigt unterschiedliche Haplotypen-Gruppen, die nicht mit den herkömmlichen Artgrenzen zusammenfallen. Normalerweise werden mit genetischen Methoden mehr (kryptische) Arten gefunden als allein aufgrund morphologischer Merkmale. Mit dieser Arbeit präsentiere ich den selten anzutreffenden Fall, dass mehrere Arten eigentlich zu einer einzigen zusammenzufassen sind. Anstelle der sieben morphologisch beschriebenen Arten habe ich nur zwei Haupt-Abstammungslinien gefunden, die sich während eines “Out-of-Africa”-Szenarios geformt haben könnten. Ein Teil der afrikanischen *Maniola*-Population wäre dabei über Gibraltar und die Iberische Halbinsel in das westliche Europa eingewandert, der andere Teil ostwärts über Kleinasien und den Bosporus in das südöstliche Europa. Die genetische Diversität innerhalb der Populationen stellte sich als überraschend hoch heraus, sogar bei den Inselendemiten. Außerdem konnte ich herausfinden, dass *Maniola* ein sehr junges Taxon (etwa 0.6 bis 0.9 Millionen Jahre alt) ist.

Keywords: *COI*, *Cyt-B*, *wingless*, *Elongation factor 1a*; Artbildung; Endemismus, Migrationsrouten, Phylogenie, Biogeographie; Integrative Taxonomie; DNA Barcoding; Art-Abgrenzung

1 INTRODUCTION

Due to rapid developments in phylogenetic science, we are nowadays able to use molecular techniques routinely to study various aspects of evolution also in non-model organisms. As a consequence, putatively well-established taxonomic systems for many groups of organisms have become subject to constant, and often drastic, change due to continuous revisions. Newly described cryptic species and novel insights into relationships between taxa frequently overturn traditional systematics, even in comparatively well-known groups such as butterflies (e.g. Dincă et al., 2011; Talavera et al., 2013; Zahiri et al., 2011). Especially the use of so-called DNA-barcoding approaches has led to a very substantial increase of recognized species numbers during the last decade (Hebert et al., 2004; Hausmann et al., 2011). However, in this flood wave of newly described species, might the opposite - although surely a rare case - sometimes happen as well? Namely, that a group of species so far doubtlessly considered distinct has to be merged into a single species complex with a considerable amount of gene flow between single taxonomic units? Taxonomic mishaps like 'oversplitting' (the misinterpretation of individual variants as new specific entities) and 'lumping' (grouping several species into a single one) are well known problems in modern taxonomy and systematics that can probably only be solved through the combined approach of integrative taxonomy (Dayrat, 2005). The basic understanding of what a species is and how to delineate one from the other is crucial for biology, but both traditional taxonomy as well as the barcoding approach show limits in species delineation: traditional morphology-based taxonomy can only describe morphospecies, whereas DNA barcoding as a single-character system will hardly be capable of identifying species across (nearly) all life. The main advantage of integrative taxonomy is that it is able to overcome overlapping character variation through its multi-character system (Will et al., 2005).

Phylogeny and phylogeography have become well-established fields of study that contribute greatly to our comprehension of the evolution and diversification of life. Research in these fields has provided deep and novel insights into the evolution of animal groups such as birds (e.g. Cibois et al., 2014), amphibians and reptiles (e.g. Barlow et al., 2013; Honda et al., 2012), or mammals (e.g. Grill et al., 2009; Yannic et al., 2012). However, particularly among the invertebrates, the evolutionary history is often still poorly understood because of their sheer species richness. This is also true for butterflies, although research in this field is already well represented (e.g. Dapporto et al., 2011; Matos-Maraví et al., 2013; Jeratthikul et al., 2013). A great beginning has been made by Wahlberg et al. (2003, 2009) by bringing light into the phylogeny of nymphalid butterflies, supplemented by Peña et al. (2006) by investigating the phylogeny of the Satyrinae subfamily as well as the radiation of Satyrini butterflies (Peña et al., 2011). Further studies about subgroups within nymphalid butterflies have been following since (Nazari et al., 2010; Peña et al., 2010; Kondaramaiah et al., 2010; Seraphim et al., 2014). Nevertheless, we are far from a well-grounded knowledge of their origins and evolution and it is clear that a combined effort of scientists all over the world approaching different groups of butterflies with diverse modern phylogenetic and phylogeographical methods would be important for closing this gap in knowledge. Although the phylogenetic investigation of subgroups cannot contribute to the question of the origins of butterflies as a whole, they are of greatest importance to comprehend the numerous historical processes that have contributed to the multitude of extant species and their present geographical distributions. Understanding the biogeographic impact of climatic oscillations during the past at a continental scale requires phylogeographic investigations of taxa with large, pan-continental distributions (Barlow et al., 2013). Widely distributed over much of Europe, north-west Africa, the Canary Islands and eastwards to the Ural mountains and NW Iran (Tshikolovets, 2011), the satyrine butterfly *Maniola jurtina* (Linnaeus, 1758) provides an excellent model for phylogeographic studies. So far, some studies about population genetics and phylogeography of this butterfly have already been conducted (Dapporto et al., 2011; Grill et al., 2006a; Schmitt et al., 2005). But what makes the *Maniola* genus as a whole an enthralling subject for both phylogenetic and phylogeographic studies is the restriction of the other six described species to the Mediterranean region

(Grill et al., 2007; Grill et al., 2006a) with a special focus on the Aegean islands and Turkey. Three species of this genus are endemic to islands: *chia* Thomson, 1987 to the Greek island Chios, *cypricola* Graves, 1928 to Cyprus and *nurag* Ghiliani, 1852 to Sardinia and therefore belong to the rarest butterflies of Europe in terms of distribution areas (Grill et al., 2007; Kudrna et al. 2011). *M. chia* and *M. cypricola* are said to replace *jurtina* on their islands, whereas *nurag* and *jurtina* occur sympatrically on Sardinia and hybridize there at least occasionally. The remaining three species all inhabit parts of Turkey: *M. telmessia* (Zeller, 1847) can be found on Aegean islands, in southern Turkey from the Bosporus eastwards through Syria, Lebanon, Israel, Jordan and Iraq to SW-Iran (Hesselbarth et al., 1995; Tshikolovets, 2011). *M. halicarnassus* Thomson, 1990 occurs on the Turkish Bodrum peninsula as well as on the Aegean island Nissiros. The distribution area of *M. megala* (Oberthür, 1909) is in Greece (Lesbos island) and southern Turkey as far as the Syrian border (Tshikolovets, 2011). *M. halicarnassus* and *M. telmessia* share almost exactly the same habitat and hybridization between these two species has been recorded (Hesselbarth et al., 1995). Although neighbouring islands or the mainland would be in flight distance for all island endemics, the distribution areas of the *Maniola* species living on islands appear to be well restricted to the boundaries of their own island. *M. chia* is said to entirely replace *M. jurtina* and *M. telmessia* on Chios, both species that are generally found on the neighbouring islands and the Turkish mainland, which is only a few kilometres away from Chios. Considering the known distribution areas of the currently accepted *Maniola* species, the question arises how much speciation and colonization events in the past contributed to current distributional patterns. As the species delimitation and their distribution both appear questionable, an investigation with modern genetic methods becomes necessary. Furthermore, the seven taxonomically described species also strikingly resemble each other. For non-experts it is hardly possible to distinguish them, and even for experts it is a challenging task because of the great morphological variation and overlap in wing patterns and the shape of genitalia even within species and populations (Thomson, 1973; Olivier, 1993; Grill et al., 2004). *Maniola jurtina*, *telmessia*, *halicarnassus* and *megala* also show a sympatric geographic distribution in Turkey. Where they are occuring syntopically, they are said to be differentiated through disparate habitat preferences as well as times of emergence (Hesselbarth et al., 1995). This also applies to the sympatric occurrence of *jurtina* and *nurag* on Sardinia (Grill et al., 2006b).

In the last decade DNA barcoding has become a handy tool for resolving species identifications, especially in clades where morphological characterization of putative taxa is weak or inconsistent (Hebert et al., 2003b). Indeed genetic markers are not only helpful for identification and species delimitation (e.g. by the so-called barcoding gap: Wiemers & Fiedler, 2007; Meyer & Paulay, 2005; Hebert et al., 2003a), but also provide the possibility to gain deeper insight about migration routes, possible glacial refugia, and divergence times.

Since 45 Mya, climate on Earth became constantly cooler, though with fluctuations. From late Pliocene climatic oscillations occurred in 41 ky cycles and since Mid-Pleistocene (0.9 Mya), periodicity became 100 ky, thereby producing the recent series of ice ages with short warm interglacial periods (Hewitt, 2011). In southern Europe, the big mountain ranges of the Pyrenees, the Alps, Carpathians and the Caucasus were covered with large ice sheets during the cold. Between the northern polar ice sheet and these mountain glaciers, vast plains of permafrost, tundra and cold steppe spread. Many species which now range across Europe could not cope with the harsh climate during the ice ages and were probably limited to milder refugia in the south, although more recent research shows evidence, that during the last glacial maximum the central and eastern European scenery was indeed partly covered by taiga/montane woodland, which also enclosed isolated pockets of temperate trees in some regions (Willis & Andel, 2004). When the climate warmed, these species would expand from their refugia and spread north rapidly (termed as 'leptokurtic expansion' or 'leading-edge colonization') - some from just one refugium, others from several. The three main refugia are associated to the three big peninsulas in the Mediterranean region: the Iberian, the Apennine and the Balkan Peninsula (Hewitt, 1996; 1999). Three

post-glacial recolonization routes out of these refugia have been named 'grasshopper', 'hedgehog' and 'bear', respectively, after paradigm species patterns. The grasshopper *Chorthippus parallelus* populated Europe from a Balkan refugium, the hedgehog *Erinaceus* from all three refugia and the brown bear *Ursus arctos* colonized Europe from Iberia and the Balkan. Due to the rapid northwards expansion and related bottleneck effects, species often show a lower genetic diversity in northern populations, while southern populations in refugial regions show considerable diversity that probably accumulated over several ice ages (Hewitt, 1999; Hewitt, 2011).

According to a plausible, but untested scenario *Maniola jurtina*, as the farthest distributed species, would represent the basal group of Meadow Brown populations from which the others originated in the course of the ice ages. During cold periods, *Maniola* butterflies might have found refugia in the Mediterranean area and migrated back north when the climate became warmer again, leaving behind populations that became separate species in isolation on islands, when the sea-level rose again. Indeed, Schmitt et al. (2005) described two major genetic lineages (an eastern and a western one) for *Maniola jurtina* that are supported by allozyme data, wing patterns and genitalia morphology and evolved most likely in parallel with glaciation cycles. These lineages are not very clearly differentiated from each other and in taxonomic terms could represent subspecies or a so-called semispecies complex (Braby et al., 2012). On the other hand, Sardinian populations of both *M. jurtina* and *nurag* exhibit levels of genetic variation comparable with mainland populations of *M. jurtina*. Furthermore, Sardinian and central and western European *M. jurtina* populations did not follow an isolation-by-distance pattern (Grill et al., 2007; Schmitt et al., 2005). Another possible scenario concerning the origin and migration routes of the whole genus was suggested by Tauber (1970), who postulated two lineages (same as Hesselbarth et al. (1995), with small differences in species division), the western *M. jurtina* lineage and the eastern *M. telmessia* species complex, diverging from North Africa. According to Tauber, the *M. jurtina* lineage should have spread westwards over Gibraltar and the Iberian Peninsula while the *M. telmessia* complex has spread through Palestine, Lebanon and Syria to the east. This hypothesis coincides with the fact that *M. telmessia* is the only *Maniola* species occurring in Israel, Jordan, Lebanon and Syria (Tshikolovets, 2011). According to Tauber's hypothesis, the western *M. jurtina* complex is said to have spread over Europe and met the eastern *M. telmessia* complex in Asia Minor, giving rise to a sympatric occurrence of both lineages in the Eastern Mediterranean nowadays.

For other Palaearctic nymphalid butterflies an origin in Africa has already been reported (Aduse-Poku et al., 2009; Kodandaramaiah & Wahlberg 2007). So if the ancestor of extant *Maniola* had lived in Africa as well, the end of the last glacial maximum, when Europe became more hospitable for thermophilic species again, could have provided an incentive to shift their species range northwards. As there are no *Maniola* butterflies to be found in Libya, Egypt and the Arabic Peninsula nowadays (Tshikolovets, 2011), the beginning of the expansion of the Sahara desert about 6000 years ago (Kröpelin et al., 2008) could have erased populations in Northern Africa.

So, this study aims at (1) reconstructing the phylogeny of the genus *Maniola*, (2) comparing it with the currently accepted taxonomy, thereby looking for cryptic species or evidence that there are fewer species than originally described, (3) testing the usability of DNA barcoding for species identifications in *Maniola*, and (4) investigating if molecular data reveal information about the existence of refugia in the Mediterranean region and possible migration routes that have led to the current distribution of the species.

2 MATERIAL & METHODS

2.1 Sample collection, DNA extraction, amplification, sequencing & alignment

Butterfly specimens from various sites throughout the range of the genus *Maniola* (Fig. 10, Tab. 7) were provided and identified by experts (see Acknowledgements). They had either been dried or put into 99% alcohol after killing before they were sent to our lab. Samples were then stored at -20°C until DNA extraction. Whole genomic DNA was extracted from 138 specimens. Genetic markers could be amplified with varying success (Tab. 1), as the specimens had been collected between the years 1980 and 2013 and the older samples performed often worse in PCR than the more recent ones.

Tab. 1: Successfully amplified marker sequences.

	COI	*CytB*	*wgl*	*EF 1a*	mtDNA	combined
M. jurtina (n=51)	47	29	30	28	27	22
M. nurag (n=25)	19	12	12	11	8	9
M. chia (n=18)	16	7	14	17	6	6
M. megala (n=6)	5	5	5	3	5	3
M. cypricola (n=14)	12	14	11	9	12	9
M. telmessia (n=16)	11	16	14	13	11	11
M. halicarnassus (n=8)	8	8	5	5	8	5
total	118	91	91	86	77	65

As outgroups, I downloaded sequences of closely related satyrine butterfly species, especially from the closer periphery of *Maniola* to support the phylogeny of the genus, from GenBank (see Tab. 5) which I selected from the publication of Peña et al. (2006). But as they did not use *CytB* in their study, I additionally sequenced two specimens of *Pyronia cecilia* that were on hand at our lab to have at least one species available with complete gene sampling.

Total genomic DNA was extracted from two legs, respectively, or if missing, from thoracic muscle following a standard protocol (DNeasy Blood & Tissue Kit, Qiagen Inc., Valencia, CA, USA). DNA samples were extracted and amplified in a separate room exclusively dedicated to DNA extractions. Primer names, references and primer sequences are shown in Tab. 2.

Tab. 2: Primer sequences used in this study (F = forward, R = Reverse).

Gene	Primer name	References	Sequence (5'-3')
COI	*LepF*	Hajibabei et al.,	ATTCAACCAATCATAAAGATATTGG (F)
	LepR	2006	TAAACTTCTGGATGTCCAAAAAATCA (R)
CytB	*CB-J-10933*	Simon et al.,	TATGTACTACCATGAGGACAAATATC (F)
	CB-N-11367	1994	ATTACACCTCCTAATTTATTAGGAAT (R)
wgl	*LepWG1*	Brower &	GARTAYAARTGYCAYGGYATGTCTGG (F)
	LepWG2	Desalle, 1998	ACTICGCARCACCARTGGAATGTRCA (R)
Elongation	*EF51.9*	Monteiro &	CARGACGTATACAAAATCGG (F)
factor 1a	*EFrcM4*	Pierce, 2001	ACAGCVACKGTYTGYCTCATRTC (R)
	Starsky/M3	Cho et al., 1995	CACATYAACATTGTCGTSATYGG (F)
	Luke/rcM51-1		CATRTTGTCKCCGTGCCAKCC (R)

Gene fragments of the mitochondrial (*COI*, *CytB*) and nuclear DNA (*wgl*, *EF1a*) were amplified using polymerase chain reaction (PCR) in a thermal cycler (Eppendorf Mastercycler pro S vapo.protect). Negative (sterile water) and positive (samples with known genotypes) controls were always used. PCRs were performed in 25 µl volumes containing 1 µl of genomic DNA, 22.5 µl of ReddyMix®, 0.5 µl of the respective forward and reverse primer and 1 µl BSA. PCR conditions were optimized for each primer pair.

Protocol for *COI*: The PCR cycle consisted of 4 min initial denaturation at 94°C, 1 min cycle denaturation at 94°C, 1.5 min cycle at annealing temperature of 44°C, 1.5 min cycle extension at 72°C for 5

cycles. Afterwards another 35 cycles with 1 min at 94°C, 1 min 15 s at 46°C, and 1 min 15 s at 72°C followed. A final extension at 72°C for 7 min was conducted after all cycles had finished.

Protocol for the *CytB*: 5 min initial denaturation at 92°C, 1 min cycle denaturation at 92°C, 1 min 20 s cycle at 46°C annealing temperature, 1 min 20 s cycle extension at 72°C for 35 cycles. A final extension at 72°C for 5 min was done after all cycles had finished.

Protocol for *EF51.9/EfrcM4* primer pair: 5 min initial denaturation at 95°C, after that 40 cycles of 1 min denaturation at 94°C, 1 min 30 s annealing at 52.5°C, and 1 min 30 s of elongation at 72°C. A final elongation period of 10 min at 72°C followed.

Protocol for *Starsky/Luke* primer pair: 7 min initial denaturation at 95°C, after that 40 cycles of 1 min denaturation at 95°C, 1 min annealing at 54°C, and 1 min 30 s of elongation at 72°C. A final elongation period of 10 min at 72°C followed.

Protocol for *wingless*: The PCR cycle consisted of 4 min initial denaturation at 94°C, 1 min cycle denaturation at 94°C, 1.5 min cycle at annealing temperature of 48°C, 1.5 min cycle extension at 72°C for 40 cycles. A final extension at 72°C for 7 min occurred after all cycles had finished.

PCR-products were visualized on an agarose gel to verify amplification success. Afterwards, they were analysed using an ABI capillary sequencer (3730 DNA analyzer). Sequences contained no gaps or stopcodons and were aligned and edited by eye in BioEdit 7.1.3.0 (Hall, 1999) for each gene separately. DNA was tested for *Wolbachia* sp. infections as these arthropod parasites evolved various mechanisms for manipulating reproduction of their hosts, enabling them to promote rapid speciation by causing reproductive incompatibility between populations (Werren, 1997). Furthermore, they can decrease levels of mtDNA diversity as well as accelerate the rate of divergence among mtDNA lineages in infected host species (Shoemaker et al., 2004; Charlat et al., 2009; Smith et al., 2012).

2.2 Phylogenetic and haplotype networks analyses

I used Maximum Likelihood (ML) and Bayesian inference (BI) methods to reconstruct the phylogenetic trees. The combined genes trees were based on an alignment of 2427 base pairs. Additional sequences of outgroup species were taken from GenBank (see Tab. 5 for accession numbers). MtDNA trees included 77 sequences with 1089 bp length. Preliminary trees were reconstructed with MEGA 5.1 (Tamura et al., 2011) with 1000 bootstrap replications. The phylogenetic trees did not conflict strongly and all well-supported clades were similar across all trees (data not shown). The final ML analysis was performed with Treefinder (v. 2011; Jobb et al., 2004) with respect to the estimated substitution models.

Genetic variation was estimated as the numbers of variable sites (S), average numbers of nucleotide differences (k), haplotype diversity (h: Nei, 1987) and nucleotide diversity (π: Nei & Li, 1979) for each gene as well as species, using the software DnaSP v5 (Librado & Rozas, 2009).

I used the program JModelTest (v. 2.0) on the Phylemon-Server 2.0 (Sánchez et al., 2011) to estimate the best-fit models of nucleotide substitutions (out of three possible models) as judged by the corrected Akaike information criterion (AICc) implemented for ML and the Bayesian information criterion (BIC) for BI. Only the best-fit models were subsequently used for evaluations of tree topology.

Genetic distances (Kimura-2-distances) were calculated with MEGA 5.1 (Tamura et al., 2011).

BI analysis and relaxed clock analysis were performed with MrBayes v.3.2, considering the estimated best-fit substitution models (Huelsenbeck & Ronquist, 2001).

I generated median-joining networks (Bandelt et al., 1999) for several datasets using the program Network 4.6.1.1. (http://www.fluxus-engineering.com). The median-joining method is based on a maximum parsimony algorithm that searches for all shortest trees of a particular dataset (Bandelt et al., 1999).

2.3 Estimation of divergence times

Usually, divergence dates are estimated with help of fossils, but there are no known fossils of *Maniola* and generally very few fossils of butterflies (Gaunt & Miles, 2002; Grimaldi & Engel, 2005), although a study of Wahlberg (2006) was published, that used very good fossil records. Therefore, in the absence of a fossil record, I had to use the preliminary molecular clock for mtDNA, which was estimated for recently derived arthropods, with an evolutionary rate of 2.3% pairwise sequence divergence per million years (Brower, 1994), which is equivalent to a rate of 0.0115 substitutions per site per million years, for calibrating the divergence times of the nodes. Furthermore, by using the same approach, it was easier to compare my results with those of Grill et al. (2006a).

So, divergence dates among the main clades were estimated using a relaxed clock model in MrBayes v.3.2. To avoid overestimation of evolutionary time scales, all individuals were used, not only unique haplotypes (in accordance with Marino et al., 2011). For the analysis, only the mtDNA dataset was used. As underlying strict clock model, the uniform clock model was selected and for the variation of the clock rate across lineages the independent gamma rates model (IGR) was used. The clock rate prior was set to fixed (0.0115) and the treeage prior to exponential (0.01). The MCMC analyses were run for 1000000 generations using a burn-in of 25%, for both *COI* and mtDNA. Results were visualized using FigTree v.1.3.1 (Rambaut, 2009).

2.4 Historical demography

To reveal the demographic history of the *Maniola* butterflies, I employed Fu's *Fs* and Tajima's *D* neutrality statistics as an assessment of possible population expansion by deviation from neutrality. Large negative values of *Fs* reflect a number of rare alleles in a population, which indicates a recent increase in population size or positive selection. A significantly positive value of *D* can be interpreted as balancing selection, population subdivision or population contraction, while a significantly negative value indicates recent population size expansion (e.g. after a bottleneck or a selective sweep). Both statistics are expected to be close to zero in populations that have been stable over time. Furthermore, Ramos-Onsins and Rozas' R_2 statistic, which has more statistical power at small sample sizes, was calculated. A significantly positive value of R_2 reveals population growth. Additionally, to test possible population size changes, pairwise mismatch distribution analyses (Rogers & Harpending, 1992) were computed under a constant population expectation as well as under a population growth-decline model. In these analyses, demographic stability is shown by multimodal distributions, whereas sudden expansion events generate unimodal patterns (Slatkin & Hudson, 1991). All analyses were conducted in DnaSP v5 (Librado & Rozas, 2009).

3 RESULTS

3.1 Genetic diversity and species delimitation by DNA barcoding

For *COI* (657 bp), 118 sequences showed 37 different haplotypes with 48 variable sites. The 91 *CytB* sequences (432 bp) displayed 33 haplotypes and 43 variable sites. The 87 sequences of *Elongation factor* (1051 bp) showed 53 haplotypes and 66 variable sites and *wingless* (403 bp; 91 sequences) showed 34 haplotypes and 25 variable sites. In nuclear genes, variable sites accord to ambiguous base pairings, but apart from that they were rather conserved and showed almost no true mutations.

For all species and all genetic markers, the estimates of haplotype diversity (h) of all species were rather high, ranging from 0.333 to 0.996, whereas estimates of nucleotide diversity (π) were much lower (especially in nuclear genes), ranging from 0.00112 to 0.02804. Interestingly, the endemic species did not show lower haplotype and nucleotide diversity than their mainland congeners, *M. nurag* even showed the highest diversity of all as well as very high average numbers of nucleotide differences (k). The *Maniola megala* specimens all had exactly the same haplotype (see also Fig. 4) and *M. halicarnassus* revealed rather low numbers of variable sites. As two lineages could be detected in the phylogenetic trees (see subsection 3.2), they were also investigated: lineage A (comprising the taxa *cypricola*, *telmessia* and *halicarnassus*) generally had a lower nucleotide diversity than lineage B (all other species).

Of 29 tested *Maniola* specimens, only one *M. telmessia* individual from Israel was infected with *Wolbachia* sp., which indeed showed queer marker sequences in comparison to the other *telmessia* specimens. Molecular diversity indices are given in Tab. 3.

In Fig. 1 the intraspecific distances are shown for *COI*, *Elongation factor 1a* and the combined genes alignment. *COI* sequences showed rather high intraspecific distances whereas the nuclear gene *EF-1a* showed almost no distances at all. *Maniola nurag* exhibited the highest (above two percent for *COI*) intraspecific distance of all *Maniola* species.

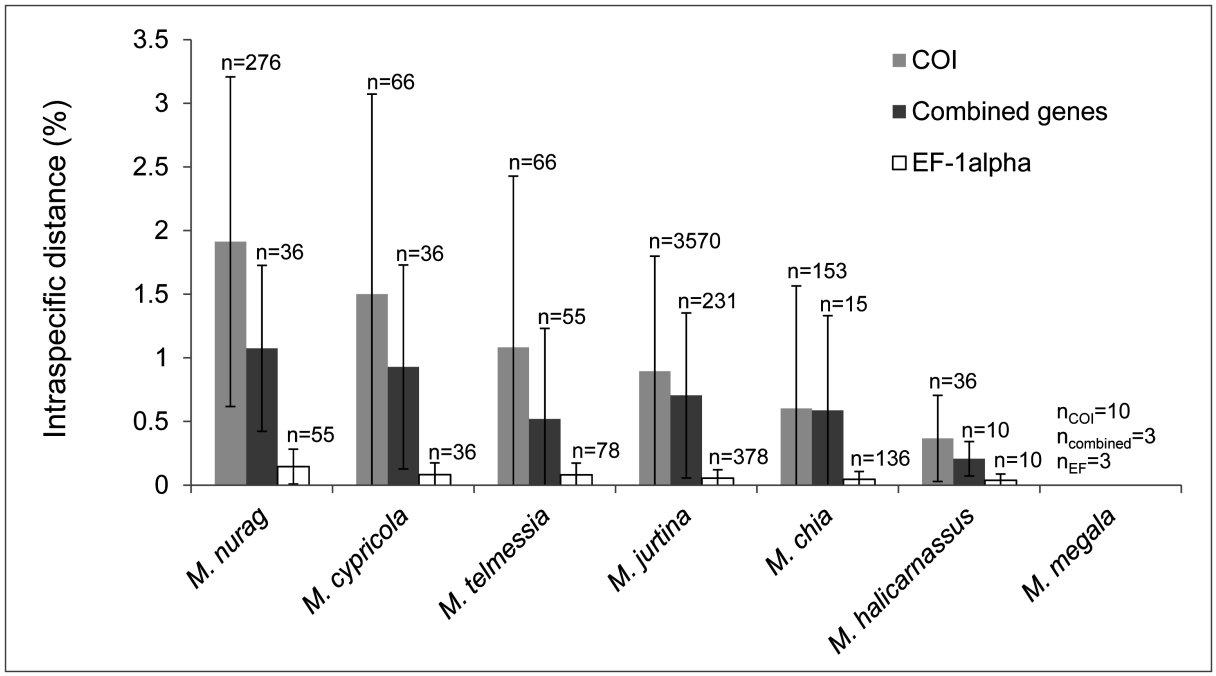

Fig. 1: Mean intraspecific distances (Kimura-2-distances) with standard deviation of *COI*, *Elongation factor 1a* and the combined gene alignment (n = number of pairwise comparisons). *COI* showed relatively high intraspecific distances, especially for the island endemic *M. nurag*. Nuclear markers showed almost no distances at all. The *megala* specimens were genetically absolutely identical and therefore showed no intraspecific genetic distance at all.

If 3% interspecific distance based on mitochondrial DNA are taken as threshold for species delimitation (Hebert et al., 2003a), only few species pairs (11 of 21) are separated properly through mitochondrial DNA (mainly species not belonging to the same lineage, as defined in section 3.2 below). Comparisons between species belonging to the same lineage, especially *jurtina-chia*, *cypricola-halicarnassus*, *cypricola-telmessia* and *halicarnassus-telmessia* showed very low interspecific distances (Fig. 2). Indeed, intraspecific genetic distances were often higher than interspecific ones.

In more recent studies, a 2% (Hausmann et al., 2011) or 2.2% (BIN System; Ratnasingham & Hebert, 2013) sequence divergence threshold is applied. In this case, only 4 species pairs cannot be delimited through their interspecific genetic distances.

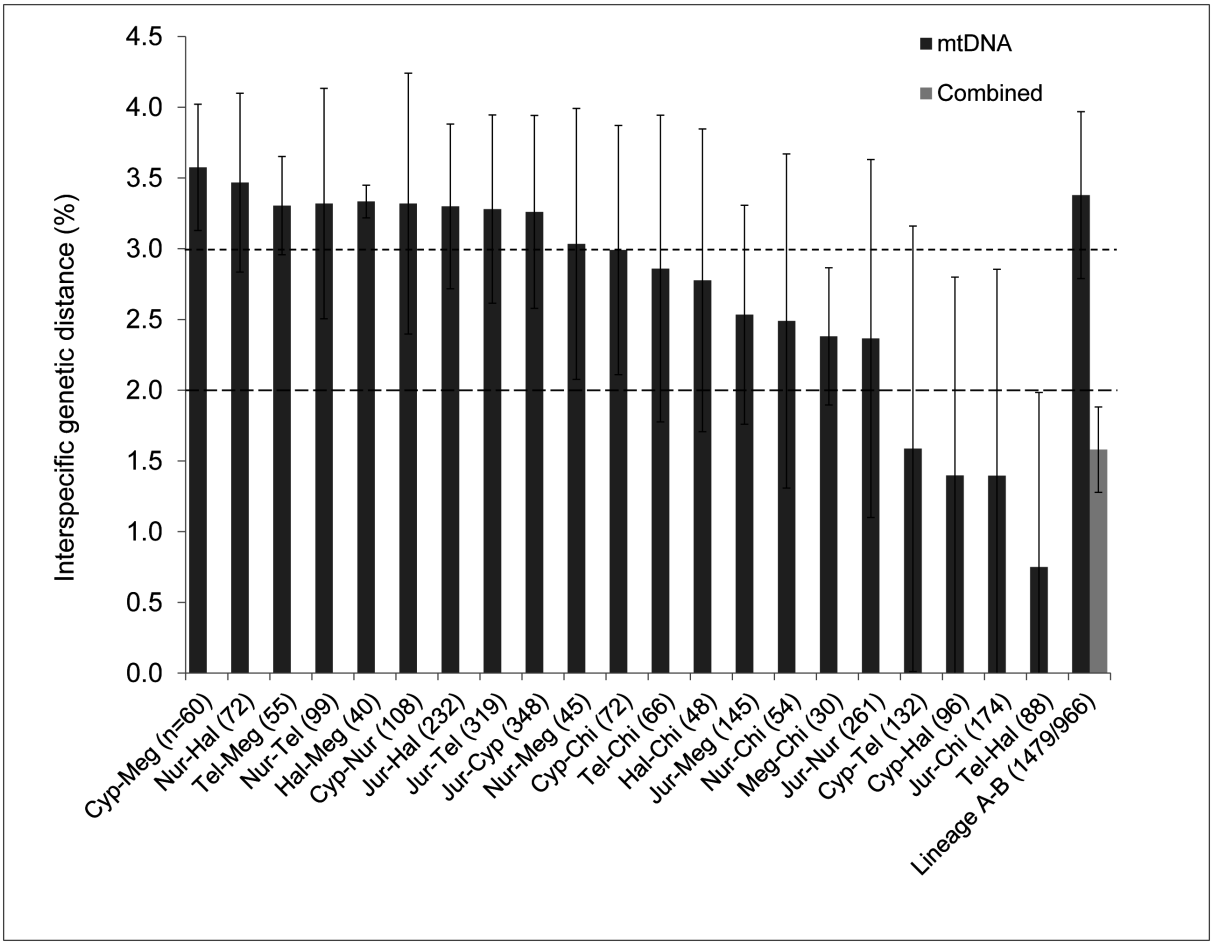

Fig. 2: Mean interspecific distances between species pairs with standard deviation; number of pairwise comparisons in brackets. At 2% and 3% interspecific distance, a line is drawn to show the used thresholds for species delimitation for mtDNA (Hebert et al., 2003a; Hausmann et al., 2011). Only about half of the species pairs reach the 3% line, but most reach the 2% threshold. Especially low interspecific distances show the species pairs *cypricola-telmessia*, *cypricola-halicarnassus*, *jurtina-chia* and *telmessia-halicarnassus* (all of them intra-lineage pairs). The highest interspecific distance is shown between *cypricola* and *megala*. Lineage A (*telmessia*, *halicarnassus* and *cypricola*) and lineage B (remaining species) show 3.4 percent interspecific distance, whereby most of the high distances between species pairs can be explained.

In order to use the barcoding region for species identification, a distinct barcoding gap should exist – separating intra- from interspecific distances. In the *Maniola COI* sequences, no such gap could be found, as intra- and interspecific distances were intermixing (see Fig. 3). Approximately 66% of the *COI* sequences showed two or more percent interspecific distance and most individuals (69%) showed negligible intraspecific distances (0-1% genetic distance). Nevertheless, about 35% of specimens showed low ($< 2\%$) interspecific and 14% showed high ($\geq 2\%$) intraspecific distances.

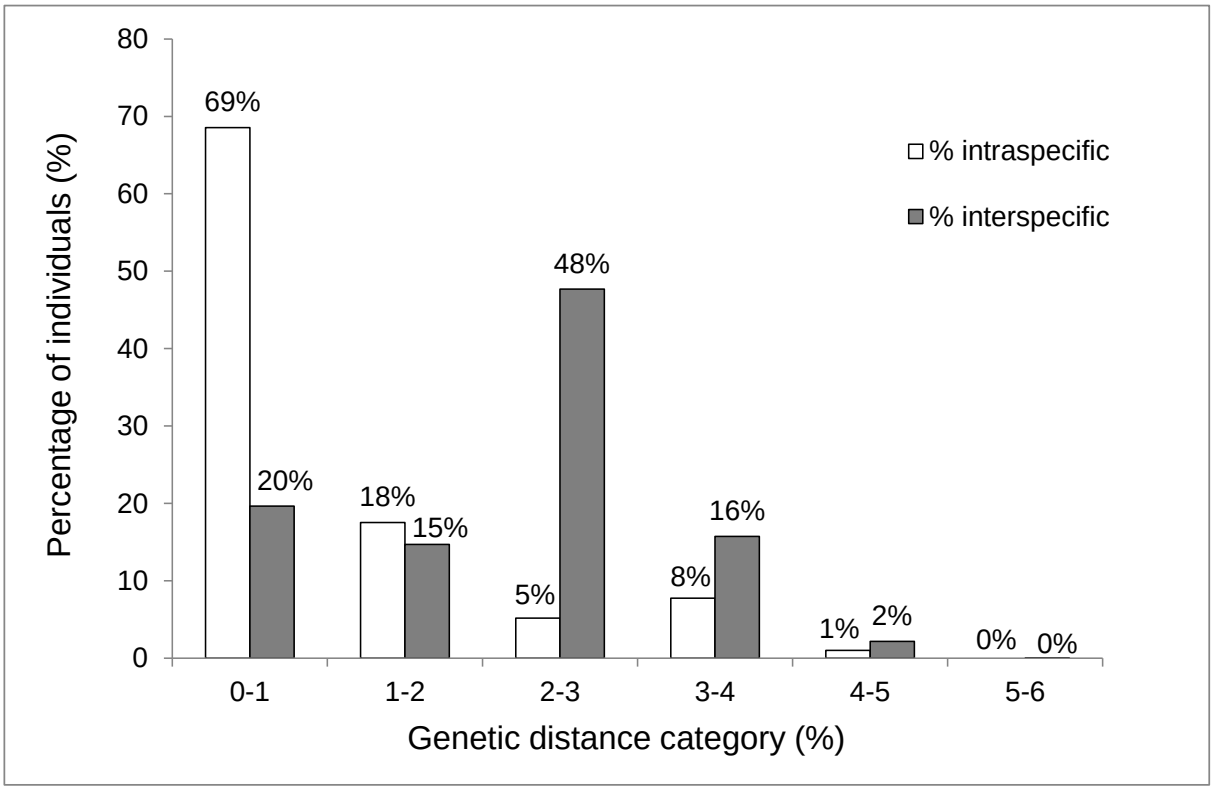

Fig. 3: Intra- and interspecific Kimura-2-distances of the *COI* sequences. To ensure the usability of DNA barcoding for species delimitation, a 'barcoding gap' should exist between these two data series. In *Maniola*, however, intra- and interspecific genetic distances intermix.

Tab. 3: Summary of molecular diversity indices of *COI*, *CytB*, *wgl*, *Elongation factor 1a* genes, mitochondrial DNA (combined *COI* and *CytB*), nuclear DNA (nDNA) and combined genes; sample size (n), number of haplotypes (no.), number of variable sites (S), average number of nucleotide differences (k), haplotype diversity (h), and nucleotide diversity (π) with standard deviation (SD). Nuclear genes have been doubled for analysis by the program DNAsp to avoid ambiguous sites.

Gene	Species	n	no.	S	k	h (±SD)	π (±SD)
COI	*jurtina*	47	20	40	6.29	0.834±0.048	0.01065±0.00185
	nurag	19	8	33	10.60	0.871±0.044	0.01849±0.00213
	megala	5	1	-	-	-	-
	cypricola	12	6	26	8.86	0.848±0.074	0.01497±0.00461
	chia	16	5	15	2.26	0.65±0.108	0.00474±0.00283
	telmessia	11	7	30	6.47	0.873±0.089	0.00985±0.00491
	halicarnassus	8	4	7	2.61	0.750±0.139	0.00397±0.00114
	lineage A	31	14	17	3.71	0.890±0.036	0.00627±0.00059
	lineage B	87	23	42	7.27	0.864±0.025	0.01585±0.00138
	all samples	118	37	48	8.84	0.919±0.015	0.01926±0.00099
CytB	*jurtina*	29	14	30	5.48	0.894±0.040	0.01305±0.00404
	nurag	12	7	32	11.67	0.909±0.056	0.02804±0.00382
	megala	5	1	-	-	-	-
	cypricola	14	8	25	7.82	0.868±0.068	0.01867±0.00563
	chia	7	4	24	8.48	0.714±0.181	0.02173±0.00831
	telmessia	16	7	24	4.00	0.792±0.089	0.00952±0.00490
	halicarnassus	8	5	6	1.79	0.786±0.151	0.00413±0.00120
	all samples	91	33	43	10.54	0.906±0.021	0.02703±0.00106
wgl	*jurtina*	30 (60)	11	8	1.47	0.774±0.041	0.00432±0.00044
	nurag	12 (24)	13	11	2.57	0.906±0.046	0.00754±0.00095
	megala	5 (10)	7	8	3.62	0.933±0.062	0.00899±0.00106
	cypricola	11 (22)	6	7	1.05	0.671±0.077	0.00281±0.00066
	chia	14 (28)	9	5	1.83	0.831±0.051	0.00537±0.00051
	telmessia	14 (28)	6	3	1.18	0.741±0.067	0.00348±0.00040
	halicarnassus	5 (10)	3	4	0.96	0.511±0.164	0.00281±0.00137
	all samples	91 (182)	34	25	2.48	0.904±0.011	0.00731±0.00033
EF 1a	*jurtina*	28 (56)	25	24	1.85	0.881±0.032	0.00183±0.00021
	nurag	12 (24)	19	52	9.65	0.975±0.021	0.00952±0.00349
	megala	3 (6)	2	1	0.33	0.333±0.215	0.00032±0.00021
	cypricola	9 (18)	7	7	1.87	0.784±0.085	0.00183±0.00035
	chia	17 (34)	11	10	1.43	0.811±0.052	0.00143±0.00020
	telmessia	13 (26)	7	9	1.39	0.689±0.088	0.00134±0.00035
	halicarnassus	5 (10)	4	5	1.16	0.533±0.180	0.00112±0.00046
	all samples	87 (174)	53	66	2.72	0.851±0.021	0.00285±0.00060
mtDNA	*jurtina*	29	14	38	8.21	0.899±0.036	0.01389±0.00244
	nurag	9	5	26	12.61	0.861±0.087	0.01974±0.00250
	cypricola	12	10	51	17.59	0.955±0.057	0.01740±0.00519
	chia	6	4	35	12.13	0.867±0.129	0.01159±0.00688
	telmessia	11	7	54	11.45	0.873±0.089	0.01052±0.00570
	halicarnassus	8	6	13	4.39	0.893±0.111	0.00403±0.00106
	lineage A	29	19	29	6.13	0.921±0.041	0.00606±0.00078
	lineage B	51	17	37	9.63	0.904±0.022	0.01776±0.00153
	all samples	80	30	43	10.81	0.946±0.011	0.01994±0.00095
nDNA	*jurtina*	26 (52)	33	31	3.40	0.962±0.015	0.00248±0.00022
	nurag	11 (22)	21	29	6.41	0.996±0.015	0.00457±0.00029
	megala	3 (6)	4	8	3.93	0.867±0.129	0.00276±0.00059
	cypricola	9 (18)	11	13	2.93	0.908±0.051	0.00208±0.00039
	chia	14 (28)	20	14	3.33	0.974±0.016	0.00245±0.00021
	telmessia	13 (26)	16	12	2.61	0.902±0.049	0.00186±0.00030
	halicarnassus	5 (10)	5	9	2.11	0.756±0.130	0.00148±0.00071
	all samples	81 (162)	90	62	4.42	0.976±0.005	0.00339±0.00014

3.2 Phylogeny and phylogeography

Phylogenetic trees computed with Maximum Likelihood and Bayesian Inference analysis gave similar topologies for all genes. Only small differences in tip clades were found. Moreover, as the number of available sequences varied across genetic markers, this resulted in minor differences between the trees as well. For ML trees, the best fitting evolution models under AICc were: [GTR + G] for *COI*; [HKY + I + G] for *CytB*; [GTR + I + G] for mtDNA, nDNA and all genes combined; [SYM + I] for *Elongation factor*; and [HKY + I] for *wingless*. For BI trees under BIC, the best-fit models were: [GTR+G] for *COI*; [HKY + I + G] for *CytB*; [GTR + I + G] for mtDNA and all genes combined; [K80 + I] for *Elongation factor* and nDNA; and [HKY + I] for *wingless*.

The topology of the Bayesian Inference tree of all *Maniola* samples (based on specimens for which all genetic markers were available) plus the selected outgroup species (Fig. 5) is not consistent with current taxonomy. Rather, these nominal species form mixed clades. Nevertheless specific clusters can be recognized: there are one *jurtina* (mixed with *chia*, *nurag* and *megala*) lineage (Lineage B in the lower part of the tree), another one containing *jurtina*, *nurag*, *cypricola* and one *telmessia* sequence (upper Lineage B) and one *telmessia* (together with *halicarnassus* and *cypricola*) lineage (Lineage A). The mixed island species clade containing *nurag*, *jurtina*, *cypricola* and a single *telmessia* sequence shows very high probability support. This *telmessia* specimen was positively tested for *Wolbachia* sp. infection. Otherwise, all *telmessia* specimens always clustered together in the same clade. This also applies to *halicarnassus* and *megala*. All three can only be found in one clade, respectively. Quite to the contrary, sequences of the island endemics never formed coherent clusters. All sequenced samples of *cypricola* and *nurag* are part of several different clades. Most individuals of *M. chia* are clustered with one *jurtina* lineage, but one individual appears in the same *telmessia*/*halicarnassus* cluster that also contains most *cypricola* individuals. Overall, the monophyly of *Maniola* is very well supported, whereas most clusters within *Maniola* had low support. Stable, well supported groupings were only the invariant samples from Lesbos and one cluster comprising three *nurag*, three *jurtina* from Crete, two *cypricola* and the single *Wolbachia*-infested *telmessia* ("mixed island species clade").

The tree based on the *COI* barcoding sequences shows similar groupings (Fig. 6). Its topology is basically similar to the tree of mtDNA (*COI*+*CytB*) sequences, but emphasizes the split of the haplotypes into two main lineages better: One lineage (lineage A; light grey) contains *Maniola telmessia*, *halicarnassus* and *cypricola*, whereas the other contains *jurtina*, *nurag*, *chia* and *megala* (lineage B; dark grey), although both with some exceptions (black arrows). Lineage A shows a probability value of 76% (87% bootstrap support in ML tree).

Median-joining networks were calculated for *COI*, mtDNA, nDNA and combined genes and resembled the phylogenetic trees largely, but in comparison, they show a clearer resolution of relationships between haplotypes. Networks of *COI*, mtDNA and combined genes were much alike: they showed the same underlying clustering, but the more genes were used, the more single haplotypes and mutational steps between clusters could be found. Only the COI network is shown (Fig. 4), as it is the most clearly arranged. The nDNA network was very complex and confused and did not show clear clustering. In the *COI* network, most haplotypes of *jurtina*, *nurag* and *chia* cluster together. Two different lineages contain the majority of *jurtina* individuals. *Maniola megala* is clearly separated from the rest. Another clade is formed mainly by *nurag* sequences and one *jurtina* sequence from Sardinia. *Maniola telmessia*, *halicarnassus* and *cypricola* dominate a further clade. Interestingly, a few individuals of *nurag*, *cypricola* and *jurtina* from Crete form an own group (together with the one *Wolbachia* infected telmessia of Israel).

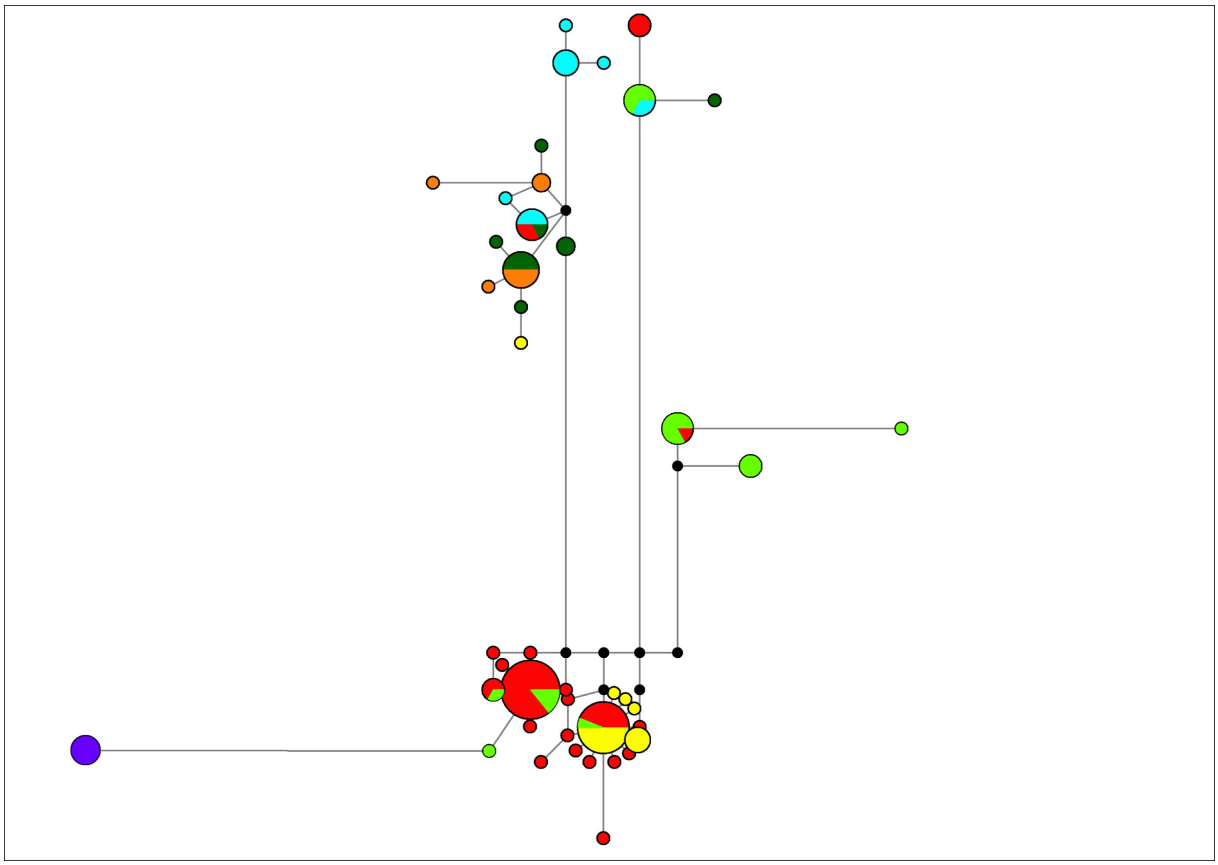

Fig. 4: Median-joining network of *COI* sequences. Size of circles is proportional to number of sequences with similar haplotypes, length of lines is proportional to number of mutational steps between haplotype clades. Five different haplotype clades can be recognized. Species colour codes: red = *jurtina*, light green = *nurag*, yellow = *chia*, violet = *megala*, blue = *cypricola*, dark green = *telmessia*, orange = *halicarnassus*. Down in the middle two different *Maniola jurtina* clades or lineages can be seen, left of them is the *megala* clade. To the right side, a *Maniola nurag* clade (with a single *jurtina* from Sardinia) can be found. On the top, there is the *telmessia*, *halicarnassus* and *cypricola* clade to the left and the mixed island species clade (*jurtina* from Crete, *nurag*, *cypricola* and one *telmessia* individual) to the right. Single haplotypes are common to all clades.

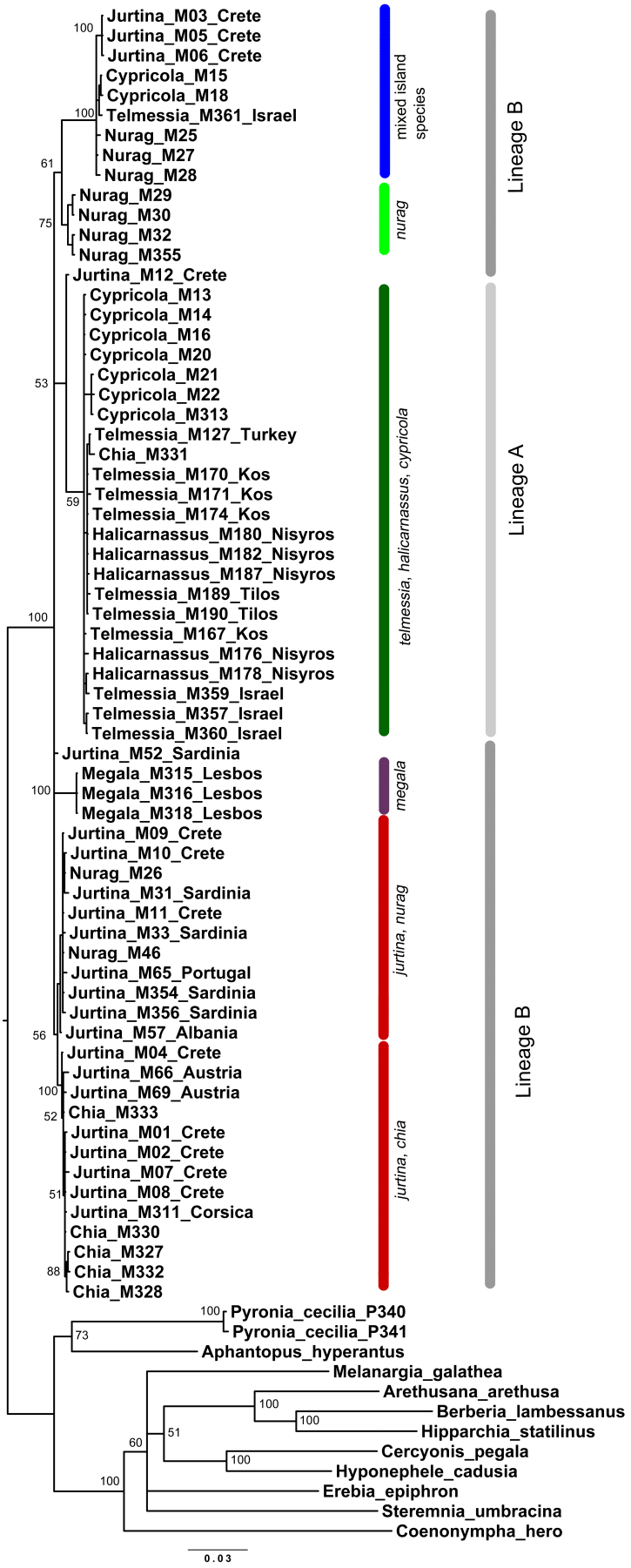

Fig. 5: Baysian Inference tree of combined genes dataset with probability values (%). Species do not form clades according to current taxonomy, but genetic lineages can be roughly defined: one lineage (A) containing *telmessia*, *halicarnassus* and *cypricola* (59 % prob.; 84 % bootstrap in ML tree) and another lineage (B) containing the remaining species. Only *megala* and *halicarnassus* do not occur in several clades.

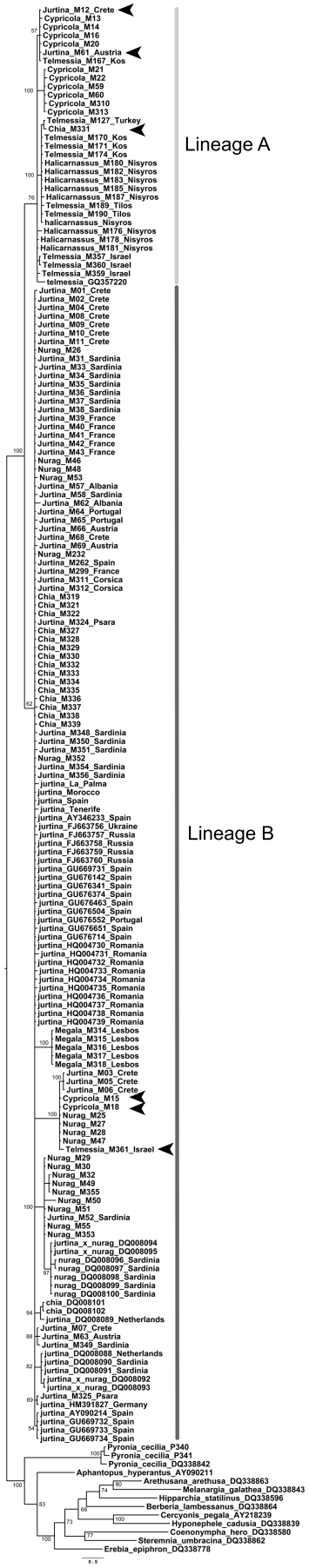

Fig. 6: Bayesian Inference tree of *COI* sequences with support values. Nominal species form intermixed clades that are not consistent with current taxonomy. Also each of the island endemics spreads through several clades. The only monophylum coinciding with a described species is represented by *M. megala*. The tree shows two main branches (lineages A and B).

3.3 Possible origin of *Maniola* butterflies and divergence times of single species

Divergence time analysis with an uncorrelated lognormal relaxed clock run of mtDNA sequences (*COI* and *CytB*) in MrBayes produced a tree with a topology similar to those of the Maximum Likelihood and BI analyses (Fig. 7). However, this tree shows a much weaker resolution of branches, although one can see again that the island endemics are not monophyletic. Furthermore, in relation to the other species, they seem to be neither especially young nor older than other clades. The time scale is quite short, so the different lineages are about 0.6 million years old. According to this tree, lineage A and B separated approximately 0.58 Mya ago. The split *Maniola-Pyronia* is dated back to 0.62 million years. Clades of lineage A are a bit younger by trend than those of lineage B. The *jurtina* (plus *chia*) sequences are 0.52, *nurag* 0.45, lineage A 0.48 (0.4 for some *cypricola* sequences) and *megala* 0.42 Mya old.

It is noteworthy that I also calculated an analysis of the *COI* dataset, but only showed the dated tree of mtDNA here because of its smaller size. Anyway, the topologies of both datasets were almost the same with the one difference that the *COI* dataset contains far more *M. jurtina* sequences. The really interesting fact in the dated *COI* tree is the extended time scale: According to these results, the genus *Maniola* diverged from its sister taxon *Pyronia* approximately 0.9 million years (Myr) ago during early Pleistocene. Again, lineage B is a bit older than lineage A. The *jurtina* (plus *chia*) sequences are approximately 0.89, *nurag* 0.71, lineage A 0.67 (0.45 for some *cypricola* sequences) and *megala* 0.34 Mya old.

3.4 Historical demography and possible migration routes

Values of Tajima's D were overall close to zero, indicating stable population sizes over time. In a few cases, however, this test statistic showed significantly negative values, indicating a recent population size expansion (e.g. after a bottleneck or a selective sweep). This applied for *Maniola chia* (*COI*: -1.96033), *M. telmessia* (*CytB*: -1.82291; mtDNA: -1.79228), *M. jurtina* (*Elongation factor*: -2.12539), and for all species together (*Elongation factor*: -2.41463, nDNA: -1.95314). The value of Fu's *Fs* was close to zero as well for all species for the mitochondrial genes, indicating stable populations. For the nuclear genes, *Fs* values were large and negative for some samples (*wgl*, all samples (-23.159); *Elongation factor*, *jurtina* (-25.073), all samples (-57.947); and nDNA for *jurtina* (-32.197), *nurag* (-15.938), *chia* (-15.236) and all samples (-128.846)), indicating a recent increase in population sizes or positive selection. The small positive values of R_2 were never significant, so no population growth can be inferred (for details, see Tab. 4). In summary, all population expansion test statistics suggest more or less stable populations.

Results of the mismatch distributions are difficult to interpret. Mitochondrial DNA showed to be very biased and unclear, with many observed peaks (multimodal distributions) which do not match the expected curve, neither for the assumption of constant population sizes nor for assumption of population size changes, and are therefore describing fluctuations around a demographic equilibrium. Even lineage A and B do not show any demographic trends. In Fig. 8, mismatch distributions are shown for all species of the respective gene, as mismatch distributions of single species are all quite similar. Nuclear genes show unimodal distributions and therefore suggest population size changes for almost all species (*cypricola*, *halicarnassus* and *telmessia* actually show one big and one small peak and are therefore not perfectly consistent with a strict unimodal distribution (data not shown)). In summary, nuclear genes rather suggest population growth, whereas the mitochondrial genes rather indicate stable population sizes.

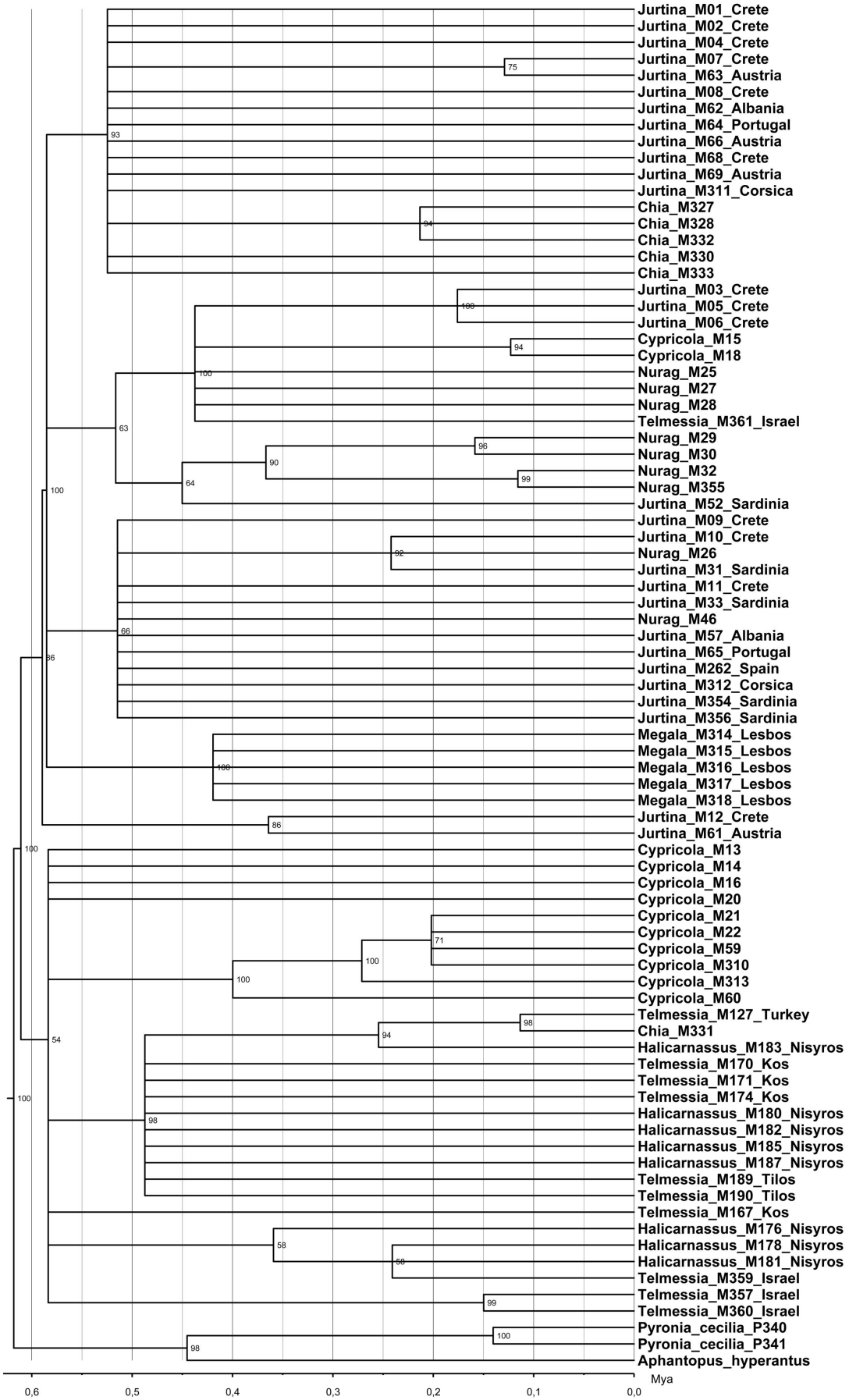

Fig. 7: Estimation of divergence times, based on a relaxed clock analysis of the mtDNA dataset (1000000 generations; $p < 0.05$). According to this tree, the *Maniola* genus diverged from *Pyronia* a bit more than 0.62 Myr ago. Most *telmessia* and *halicarnassus* seem to be younger than 0.5 Myr. *Megala* is even younger, whereas *jurtina* seems to be slightly older than *telmessia*.

Tab. 4: Summary of population expansion test statistics of *COI*, *CytB*, *wgl*, *Elongation factor 1a* genes, mtDNA (combined *COI* and *CytB*), nuclear DNA (nDNA) and combined genes; sample size (n), Tajima's *D*, Fu's *Fs* and Ramos-Onsins and Rozas' R_2.

Gene	Species	n	*D*	*Fs*	R_2
COI	*jurtina*	47	-1.16457	-3.642	0.0751
	nurag	19	0.35675	3.458	0.1506
	megala	5	-	-	-
	cypricola	12	0.13174	2.728	0.1647
	chia	16	-1.96033*	0.632	0.1944
	telmessia	11	-1.70566	0.289	0.2101
	halicarnassus	8	-0.16319	0.702	0.1833
	lineage A	31	-0.43417	-3.905	0.1005
	lineage B	87	-0.40647	-1.645	0.0847
	all samples	118	-0.11253	-6.733	0.0910
CytB	*jurtina*	29	-1.02626	-2.190	0.0845
	nurag	12	0.45575	2.315	0.1723
	megala	5	-	-	-
	cypricola	14	-0.02005	0.823	0.1514
	chia	7	-0.76245	2.993	0.1726
	telmessia	16	-1.82291*	0.291	0.1647
	halicarnassus	8	-1.06428	-1.403	0.1496
	all samples	91	0.78180	-4.006	0.1205
wgl	*jurtina*	30 (60)	-0.64715	-3.965	0.0913
	nurag	12 (24)	-0.43744	-6.467	0.1119
	megala	5 (10)	1.21188	-1.541	0.2172
	cypricola	11 (22)	-1.44703	-1.949	0.0952
	chia	14 (28)	0.53398	-2.713	0.1793
	telmessia	14 (28)	1.28170	-1.243	0.1971
	halicarnassus	5 (10)	-1.24468	0.390	0.2293
	all samples	91 (182)	-1.37987	-23.159	0.0484
EF 1a	*jurtina*	28 (56)	-2.12539*	-25.073	0.0355
	nurag	12 (24)	-1.29520	-5.794	0.0884
	megala	3 (6)	-0.93302	-0.003	0.3727
	cypricola	9 (18)	-0.68229	-1.642	0.1271
	chia	17 (34)	-1.27626	-5.745	0.0753
	telmessia	13 (26)	-1.31859	-1.851	0.0844
	halicarnassus	5 (10)	-1.38818	-0.575	0.1373
	all samples	87 (174)	-2.41463**	-57.947	0.0198
mtDNA	*jurtina*	29	-0.71798	-0.473	0.1029
	nurag	9	1.34546	3.762	0.2386
	cypricola	12	0.19111	-0.077	0.1662
	chia	6	-1.32578	3.007	0.3518
	telmessia	11	-1.79228*	1.748	0.2229
	halicarnassus	8	-0.6271	-0.757	0.1491
	lineage A	29	-0.61698	-6.752	0.0943
	lineage B	51	0.37493	0.842	0.1288
	all samples	80	0.61343	-2.905	0.1244
nDNA	*jurtina*	26 (52)	-1.78076	-32.197	0.0476
	nurag	11 (22)	-0.74549	-15.938	0.0969
	megala	3 (6)	0.72300	0.730	0.2244
	cypricola	9 (18)	-1.05590	-4.591	0.0980
	chia	14 (28)	-0.46439	-15.236	0.1110
	telmessia	13 (26)	-0.57011	-10.714	0.1058
	halicarnassus	5 (10)	-1.47308	-0.479	0.2030
	all samples	81 (162)	-1.95314*	-128.846	0.0339

* $p<0.05$
** $p<0.01$

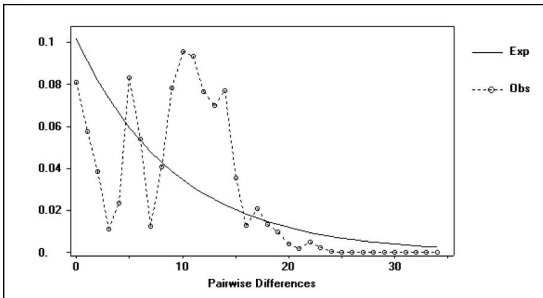

Fig. 1 COI all sequences - constant population

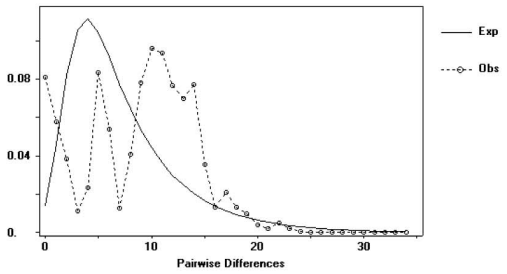

Fig. 2 COI all sequences - population change

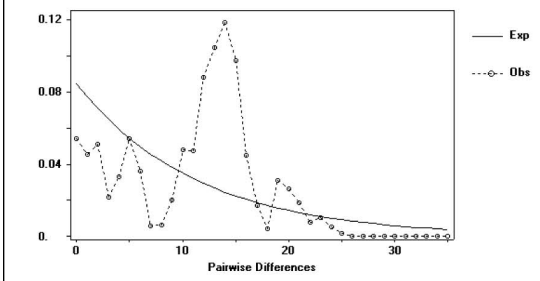

Fig. 3 mtDNA all sequences - constant population

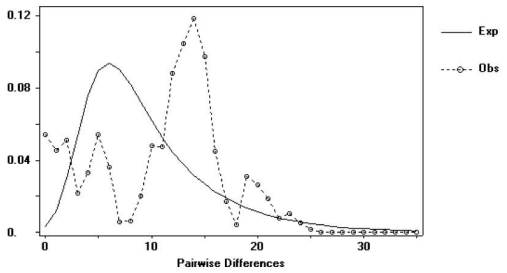

Fig. 4 mtDNA all sequences - population change

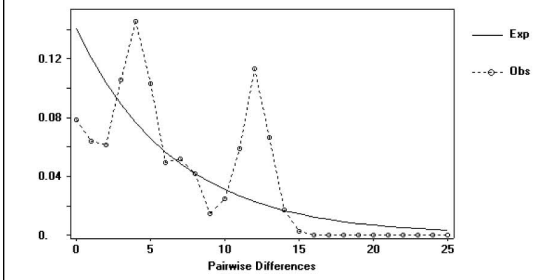

Fig. 5 mtDNA eastern lineage - constant population

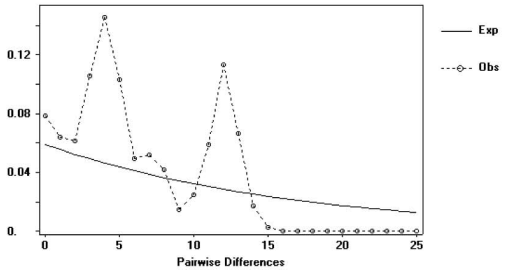

Fig. 6 mtDNA eastern lineage - population change

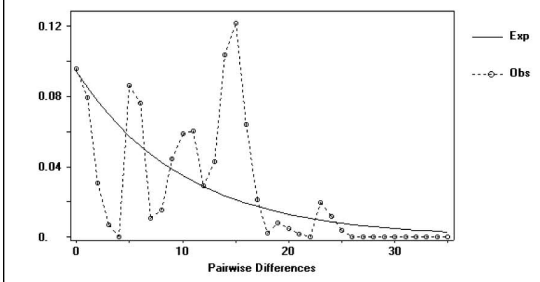

Fig. 7 mtDNA western lineage - constant population

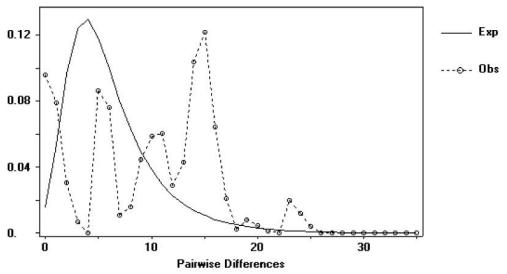

Fig. 8 mtDNA western lineage - population change

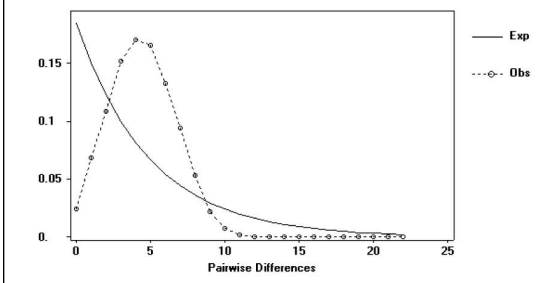

Fig. 9 nDNA all sequences - constant population

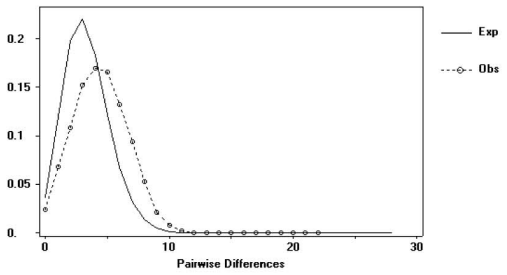

Fig. 10 nDNA all sequences - population change

Fig. 8: Some examples of the calculated mismatch distributions for *COI*, mtDNA and nDNA, showing the expected and observed frequency of pairwise nucleotide site differences. The mitochondrial genes are characterised through multimodal (i.e. several peaks) distributions, hinting at demographic stability, whereas nuclear genes show a unimodal distribution, suggesting sudden expansion events.

4 DISCUSSION

My data indeed show one of the - at least up to now - rare cases (cf. Vila et al., 2010), where the differentiation of several nominally described, but rather ambiguously characterized species did not receive stronger support and higher resolution of 'cryptic diversity' by use of genetic methods. Although one might have suspected the existence of even more cryptic species amongst *Maniola* because of their geographic situation around the Mediterranean Sea and its many islands, the opposite was found, viz. the whole genus is obviously represented by only one quite variable species with a considerable amount of gene flow between its constituent populations.

4.1 Genetic diversity and DNA barcoding

Haplotype as well as nucleotide diversity of the whole *Maniola* genus are comparable with those of a single widely distributed species (in Jeratthitikul et al. (2013) for *Tongeia fischeri* are given: haplotype diversity (h) ranging from 0.500 to 0.936 and nucleotide diversity (π) from 0.00020 to 0.00284). Especially the high genetic diversity of island species of *Maniola* is surprising, as island populations (Frankham, 1997), and isolated populations in general (Cassel & Tammaru, 2003), typically have less genetic variation than continental populations due to bottlenecks experienced by small founder populations. Nevertheless, these outcomes are in accordance with earlier results by Grill et al. (2007). This seems to show that severe inbreeding does not occur, as it is often reported for endemic or island species (Cianchi et al., 2003). In contrast, gene flow and hybridization events seem to be frequent among Meadow Browns. Hybridization between *nurag* and *jurtina* could possibly explain the high intraspecific genetic distances of *nurag*, though. Of course, the fact that sequences of endemic species do not cluster together but occur in several clades increases their intraspecific distances.

In the case of the various *Maniola* taxon, DNA barcoding cannot be reliably used for species delimitation, as there is no sufficient barcoding gap in the *COI* sequences. In contrast to the maximum likelihood tree based on all genes, the tree constructed from the barcoding region of the *COI* gene alone does not reveal different sublineages of *Maniola jurtina*. Instead it shows a clear split of two lineages, A and B, but with just moderate support. *Maniola megala* is the only species that is represented by a monophylum in the tree, but the available *megala* specimens were all from the island Lesbos. Individuals from the mainland populations might probably cluster otherwise (only a single *megala* individual collected in 1980 on the mainland was at hand - the successfully amplified *wgl*-sequence clustered with *jurtina*). Although two *Maniola jurtina* and one *chia* individual clustered with the *telmessia* clade, the opposite case did never happen (with the exception of the single *Wolbachia* infected *telmessia* specimen), so these two lineages seem to be rather well, though not unequivocally, supported.

4.2 Phylogeny and taxonomic implications

Analysis of four different sequence markers revealed that there is substantial genetic variation within and between *Maniola* populations, with sizeable numbers of different haplotypes being uncovered in all nominal taxa. Nevertheless, my findings indicate that the different genetic lineages of *Maniola* do not correspond to current taxonomy. The seven morphologically defined *Maniola* species are not well differentiated on the basis of the *COI* barcoding region.

Although interspecific distances (Fig. 2) give the impression of 'good' species, it is not quite as it seems. The phylogenetic trees revealed a missing monophyletic clustering of the species, so the different species intermixed. Two distinct lineages have been found instead. Of course, this leads to rather high interspecific distances, when a species belonging to the one lineage is compared with a species of the other one.

Addition of further markers did not enhance resolution of clades. Individuals invariably clustered according to a different pattern than expected from their taxonomic affiliation.

The results of this study suggest that, instead of accepting seven distinct 'good' species, it is more parsimonious to assign all Meadow Browns to just one single genetic group with considerable gene flow. One may distinguish the two lineages A and B as different genetic entities, with some weak further clustering of haplotypes. Remarkably, the BOLD Website (www.boldsystems.org) specifies only 3 BIN clusters (Ratnasingham & Hebert, 2013) as well for the *Maniola* taxon.

Of course, it is understandable that a taxon morphologically as variable as *Maniola* tempted taxonomists of the 19th and 20th century to describe new species, but from my point of view they only described variation, not speciation. Clearly, this can happen easily due to the high phenotypical plasticity of this genus, e.g. of wing patterns or genital morphology (Thomson, 1973; Grill et al., 2004), that is not only the genotype affecting appearance, but also environmental conditions influencing larval development. In Tab. 6, a survey of described morphological and ecological differences of the *Maniola* species is given.

Especially the two last described species *Maniola chia* and *M. halicarnassus* (only proposed as distinct taxa in 1987 and 1990, respectively) probably should have been investigated more critically before describing them as new species. For *M. chia* there is no reasonable evidence for recent speciation. Chios is very close to the mainland, so it would not be difficult for butterflies from Turkey to reach this island. Therefore, one can assume that gene flow is too frequent for Chios to bring forth an own endemic species (as a matter of fact I could only find the terrestrial isopod *Trachelipus buddelundi* to be endemic to Chios ("Only known from its original description. A doubtful species."; Alexiou & Sfenthourakis, 2013)). From the clustering patterns in the tree topologies, *M. chia* is hardly differentiated from typical *jurtina*. The same is true for *Maniola halicarnassus*. As there exists no recognizable geographic or ecological barrier between habitats populated by *halicarnassus* and *telmessia*, respectively, and since no clear genetic differences between these species could be found, they should both be referred to as *telmessia*, as this is the earlier described species. *M. halicarnassus* would therefore become a subjective synonym. The data of this study allow no conclusions about mainland *M. megala*, but at least the island population on Lesbos seems to be the only species that can be reliably identified based on morphology and shows a genetic monophyly in the phylogenetic tree. The individuals from Lesbos show smaller fore wings than the *megala* individuals from Turkey, and *Maniola megala* as a whole are said to be morphologically distinguishable from congeners (Grill et al., 2004). Obviously, this taxon would deserve an analysis based on a larger material from more different localities.

Interestingly, a dichotomy of the 'super-species' (or species complex) *M. jurtina* has been postulated multiple times. Starting from Tauber (1970), also Hesselbarth et al. (1995) recognized groupings that were assigned to the more western *M. jurtina* sensu stricto on the one hand, and the eastern *M. telmessia*, on the other. Later on, Schmitt et al. (2005) reported the existence of two genetic lineages of *Maniola jurtina* based on allozyme data. It is alluring to match the grouping uncovered by Schmitt with the dichotomy found in the BI tree of combined sequences (see Fig 5) as well as in the haplotype network (see Fig. 4), where one part of the *jurtina* sequences clusters together with *chia* and the other with *nurag*. Yet this seems not to be sufficient proof to support Schmitt's findings. Although, in my results, two *Maniola jurtina* individuals clustered with lineage A, the larger fraction of the *jurtina* sequences belonged to lineage B. Maybe Schmitt et al. had more *jurtina* sequences belonging to lineage A in their data. In this case, our results would fit together quite well. However, genetic lineages being connected to the main glacial refugia as postulated by Hewitt (1996; 1999) could not be detected within *Maniola*.

Theoretically, the lack of clustering in concordance with species boundaries could be caused by *Wolbachia* infection, as such infestations have recently been shown to distort barcode clustering patterns (Smith et al., 2012). I believe this, however, to be very unlikely, as most control samples did not show any signs of infection. Besides individual M361 (*telmessia* from Israel) neither the other species in its clade nor the other *telmessia* individuals from Israel were infected. So the remaining question is whether

the phylogeny of this taxon reflects speciation still in progress or introgression. Regarding the estimated divergence times observed in *Maniola*, the first option seems to be the more likely one.

4.3 Possible origin of *Maniola* butterflies and divergence times of individual species

In a first study examining the phylogenetic relationships among European *Maniola* species based on mitochondrial DNA (*COI* and *CytB*) sequences, the divergence time between *M. nurag* and *M. jurtina* was estimated between 1.1 and 1.2 Ma (Grill et al., 2006a). However, my results indicate that the whole taxon is even younger than that (about 0.6 to 0.9 million years). This discrepancy is founded on an arithmetical error that happened in the cross-multiplication of Grill's paper – instead of 1.1 to 1.2 million years it would correctly be about 0.87 My, what is decidedly closer to my estimates.

I do not attempt to resolve the phylogeny of *Maniola* butterflies with certainty in this study, nor can I assess how much the time scale would have changed if I could have used fossils as calibration point or a combined analysis with nDNA, but various studies have already proven the ability of *cox1* and other mitochondrial loci to form a robust molecular clock in insects (Gaunt & Miles, 2002), including a butterfly species (*Heliconius* sp.; Brower, 1994) and even Wahlberg (2006) found likewise rates of molecular evolution for *COI*.

My findings could not clarify which lineage is the older one, as all possible scenarios could be found in my data: one tree topology (BI of mtDNA) suggests lineage A to be the older one, from which lineage B derived. Several topologies support lineage B to be the older one, from which lineage A should have evolved. However, there are also three topologies (ML of mtDNA, BI of *COI* and relaxed clock of mtDNA) that show the two lineages as separated sister clades. So one cannot say for sure, which lineage is the older one, this maybe being the most plausible scenario.

The molecular clock tree showed that, in relation to the other species, the island endemic species seem to be neither especially young nor older than other clades. Thus, a single colonization event for each island appears to be unlikely.

The last glacial maximum (22000 to 19000 years ago; Yokoyama et al., 2000) did obviously not play an important role in speciation, as most clades are far older than that, but it could be an explanation for the sympatry of *jurtina* and *nurag* on Sardinia as Grill et al. (2006a) state. Neither did the desiccation of the Mediterranean Sea about 5 million years ago play a role like postulated by Grill, as the genus is too young. Although my estimates would fit together with the start of the 100 ky climatic cycles during Mid-Pleistocene (0.9 Mya), reported by Hewitt (2011) that produced the most recent series of ice ages and their short interglacial periods.

But, above all, one must not forget that calibration of phylogenetic trees based on molecular clocks is despite all already outdated again in phylogenetic research, so my results can only be seen as tentative estimates that need further examination with more up-to-date techniques.

4.4 Historical demography and possible migration routes

The population expansion test statistics as well as the mismatch distributions both give different evidence for nuclear and mitochondrial genes. Nuclear genes hint at population expansions or population size changes, whereas mitochondrial genes rather suggest stable populations. Conflicting patterns of mitochondrial and nuclear DNA diversity can sometimes hint at a selective sweep affecting the maternally inherited genes, as shown in Bensch et al. (2006) and Rato et al. (2010). But as nuclear genes are more conserved than mitochondrial ones, they could potentially also reflect signals of the population demography in the more distant past. So the nuclear genes might carry signatures of population size changes that took place a long time ago, whereas the mitochondrial genes show the more stable distributions of the recent past. It is currently not possible to disentangle these possibilities.

Although over forty years old, Tauber's (1970) "Out-of-Africa"-hypothesis seems to be not so unlikely, as other studies have already shown for butterflies as well (Weingartner et al., 2006; Kodandaramaiah & Wahlberg, 2009; Husemann et al., 2014; Habel et al., 2010). If the geographical origin of this taxon should have lain in Northern Africa (as it is for other nymphalid butterflies as well, see Aduse-Poku et al. (2009) and Kodandaramaiah & Wahlberg (2007)), *Maniola* butterflies could have spread north-westwards over Gibraltar and the Iberian peninsula and then eastwards all over Europe - but for this scenario, the eastern lineage (lineage A) should be younger than the western one (lineage B) (for example, like in Fig. 5). If the only migration pathway ran eastwards over the Bosporus and then over Europe, the western lineage must be younger. Both ways are possible, and I did find tree topologies that could be interpreted in both of those two directions. But the probably most convincing scenario is exactly what Tauber (1970) hypothesized on the grounds of morphological characters and paleo-ecological considerations: a split in the North African population, with one part wandering to the west and the other one to the east. This would probably result in a tree topology like in Fig. 6. Lineage A (*cypricola*, *telmessia* and *halicarnassus*) could have migrated along an eastern migration pathway, and lineage B (*jurtina*, *nurag*, *chia*, and *megala*) could have colonized Europe through a western migration route (Fig. 9). The few individuals that cluster with the wrong lineage could indicate a zone of rather recent intermixture in the Aegean Sea. Of course, to further investigate this hypothesis, haplotypes of Northern Africa must be examined.

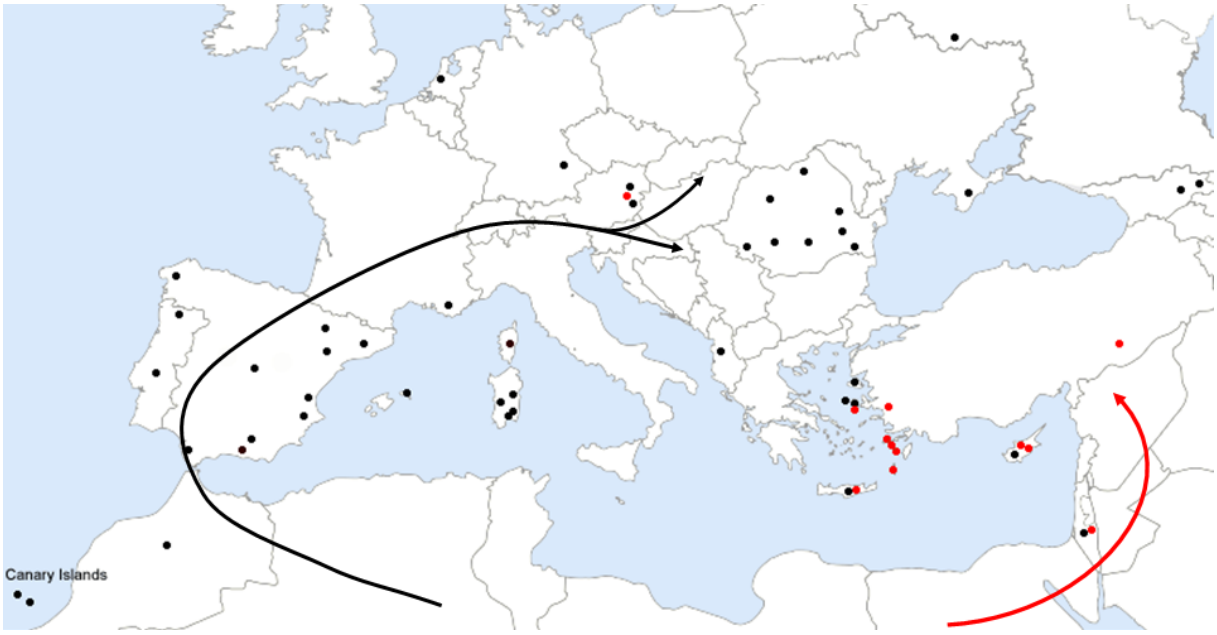

Fig. 9: Hypothetical migration routes of the two *Maniola* lineages A (eastern pathway; red) and B (western pathway; black). Changed after User:Madman2001/Wikimedia Commons/ CC-BY-SA-3.0.

Another fact fitting to an African origin of *Maniola* is their distribution : Although closely related taxa like *Hyponephele* sp. and *Aphantopus* sp. show a Eurasian distribution, the habitat of *Maniola jurtina* ends roughly at the Ural Mountains (Tshikolovets, 2011).

So, altogether I would suggest referring to the *Maniola*-complex as a kind of 'super-species' (Amadon, 1966) that contains several 'semi-species' (e.g. the eastern lineage A and western lineage B as well as maybe the mixed island species clade as a third one) that are only rudimentary differentiated from each other (e.g. through incomplete lineage sorting or gene flow) at present.

4.5 Outlook

As the genes *wingless* and *Elongation factor 1-a* seem to be nuclear genes with rather low variation it would probably be better for further research to use other markers like *Ldh* or *Rpl5* (see Jeratthitikul et al., 2013). Nevertheless, the results probably would not be different from the ones obtained in this study, as the mitochondrial genes will always contain more information about young Pleistocene radiations because of their more rapid evolution. Mitochondrial gene trees are more likely to represent the 'true' species tree than nuclear gene trees (Moore, 1995). Likewise, Zink & Barrowclough (2008) showed in a review about bird phylogeny that nuclear genes contradict mitochondrial ones only in very rare cases and that mtDNA patterns are robust indicators of population history and species limits. Still, nuclear genes are important for correct estimates of the depth of phylogenetic trees and usually give a better resolution of tree topology when combined with mitochondrial markers. To obtain a robust dated phylogeny it would be important to use an approach with calibration points through fossil records, like in Strutzenberger & Fiedler (2011) and Wahlberg (2006).

5 CONCLUSIONS

During the last decade, phylogenetic studies using sequence data revealed numerous examples for the unexpected discovery of many cryptic species. My study presents the not so common opposite case: various distinct nominal species melting together to a single species-complex. Obviously, genetic diversity through gene flow between populations or hybridization events is far too great in Meadow Brown butterflies to allow the formation of distinct species, so I did not find any proof for the existence of the seven morphologically defined species with the genetic methods used. Instead I found two distinct genetic lineages. As to the formation of these lineages, I suggest a hypothetical origin in Africa and different migration routes emerging from there, as postulated by Tauber (1970). Numerous studies in the past few years have strikingly uncovered similar "out of Africa" examples, lending further support to this idea as for the satyrine genus *Maniola*. Moreover, my results raise the controversial question whether over-splitting of species, despite all the contrasting evidence in recent barcode studies, might be more common than expected until now.

Acknowledgements

This research has been financed through the FWF (project V169-B17) and the Department of Botany and Biodiversity Research of the University of Vienna. I express my gratitude to M. Schurian, W. Eckweiler, W. ten Hagen, S. Cuvelier, A. Grill, E. Haeler, S. Schindler and D. Benyamini for providing butterfly samples, to Daniela Polic, who did the *Wolbachia* testing and to Harald Letsch for methodical advice. Martin Wiemers kindly supplied several COI sequences. I am deeply indebted to Konrad Fiedler and Andrea Grill for being great supervisors and to my beloved husband and family for their emotional support.

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6 Appendix

Tab. 5: Sequences from GenBank with accession numbers used in this study. All sequences from Peña et al. (2006), except *CytB* sequences.

Species	Locality	Species-ID	*COI*	*EF-1a*	*wgl*	*CytB*
Hyponephele cadusia	Iran	CP10-07	DQ338839	DQ338989	DQ338702	JQ996403
Cercyonis pegala	USA	EW8-2	AY218239	AY218259	AY218277	
Aphantopus hyperanthus	Sweden	EW2-1	AY090211	AY090177	AY090144	JQ924450
Erebia epiphron	France	EW24-3	DQ338778	DQ338921	DQ338638	EF545676
Coenonympha hero	Russia	CP-AC23-26	DQ338580	DQ338919	DQ338636	JQ924449
Melanargia galathea	France	EW24-17	DQ338843	DQ338993	DQ338706	JQ924453
Berberia lambessanus	Morocco	EW26-29	DQ338864	DQ339019	GQ357379	
Hipparchia statilinus	Greece	EW25-24	DQ338596	DQ339024	DQ338733	
Arethusana arethusana	Spain	CP11-06	DQ338863	DQ339018	DQ338728	
Steremnia umbracina	Peru	CP07-89	DQ338862	DQ339016	DQ338726	

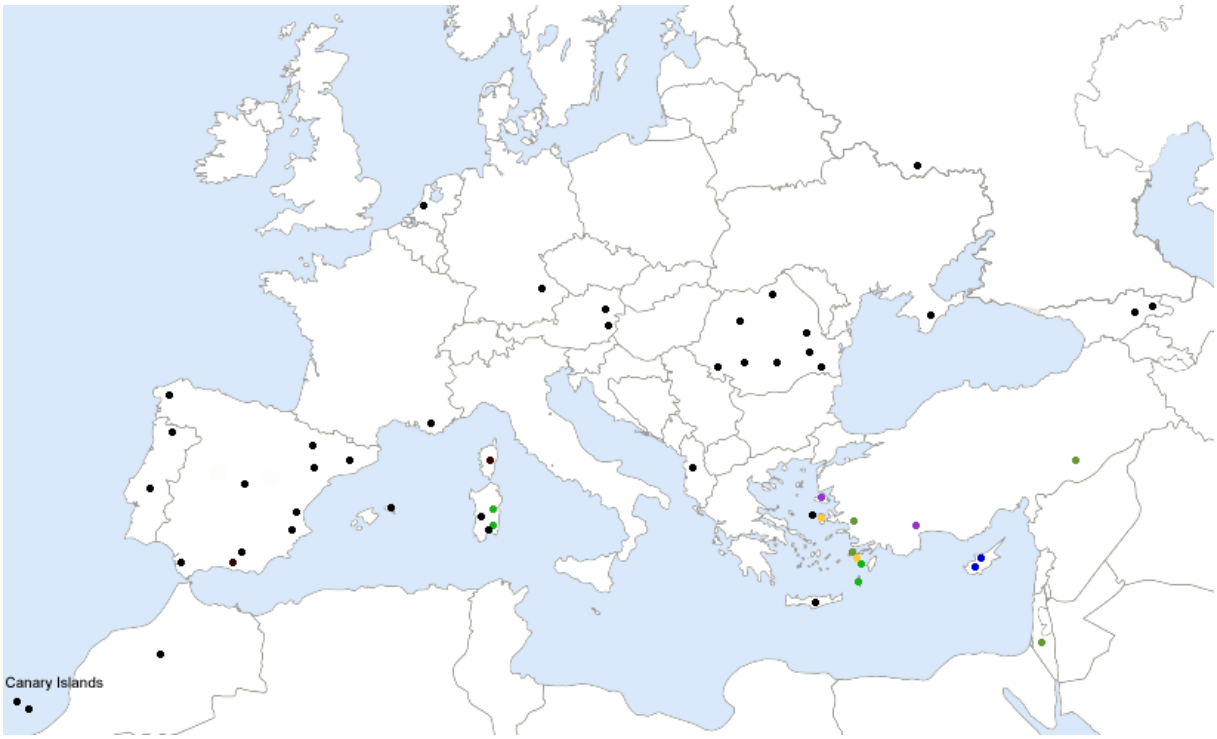

Fig. 10: Locations of sampled specimens. Colour code: *M. jurtina* = black, *nurag* = light green, *chia* = yellow, *megala* = violet, *cypricola* = blau, *telmessia* = darkgreen, *halicarnassus* = orange. Changed after User:Madman2001/Wikimedia Commons/ CC-BY-SA-3.0.

Tab. 6: Survey of described *Maniola* phenology (synapomorphies of this genus: broad valves and presence of Jullien Organ in males), from Hesselbarth et al. (1995), Olivier (1993), Tshikolovets (2011) and Tolman & Lewington (2008).

	M. jurtina	*M. nurag*	*M. chia*	*M. megala*	*M. telmessia*	*M. halicarnassus*	*M. cypricola*
Wingspan	male: 36-44 mm; female: 37-46 mm	male: 36-40 mm, smaller than *jurtina* ; female: 36-40 mm	male: 36-44 mm; female: 37-46 mm	male: 36-44 mm; female: 37-46 mm, larger than *jurtina*	male: 36-45 mm, smaller than *jurtina*; female: 37-46 mm	male: 42-47 mm; female: 42-47 mm, larger than *telmessia*	like *telmessia*
Elevation	up to 2700 m in Caucasus	500-1500 m	up to 800 m	up to 1000 m	up to 2000 m	up to 450 m	up to 1900 m
Flight period	May-September	May-early August	May-October	May-October	April-October	May-October	April-November
Habitat	grassy, bushy, often flowery places	grassy, flowery places amongst bushes and rocks	grassy, rocky and bushy places; cultivated ground	similar to *telmessia*; grassy areas including woody and cultivated areas	light woodlands, rocky scrublands	moist and shaded grassy places	everywhere on Cyprus
Ovum	11-21 longitudinal ribs, regionally variable	yellow-orange areas more extensive than in *jurtina*; better developed, more sharply defined in females; conspicious sex-brand in males	13-14 longitudinal ribs	19-21 longitudinal ribs	14-16 longitudinal ribs, very small eggs	9-18 longitudinal ribs	
Morphological differences			indistinguishable from *jurtina*; distinct genitalia and biochemical differences	hindwing outer margin averagely more undulate; underside markings darker; very short penis and spiny excrescences on basal part of gnathos	Apical ocellus always distinct and bigger than in *jurtina*, yellow-orange encircled	male sex-brand large, triangular; male genitalia distinguishable from *megala, chia* and *jurtina*; females more orange than all other *Maniola*	male forewings larger, more elongated and hairy than in *telmessia*, triangular sex-brand; female genitalia different from *telmessia*
No. larval instars	6				5	variable, 5 or 6	
Notes	morphologically very variable species			Lesbos: smaller fore wings, highest portion of individuals with bipupillary ocelli	emergence 2-4 weeks earlier than *jurtina*	emergence 2.5 weeks after *telmessia*, flies at cooler, shadier places than *telmessia*	larvae feed only on silicate poor grasses

Tab. 7: List of used specimens of this study and their localities.

Specimen code	Species	Region	Date
M01	*jurtina*	Crete	2009
M02	*jurtina*	Crete	2009
M03	*jurtina*	Crete	2009
M04	*jurtina*	Crete	2009
M05	*jurtina*	Crete	2009
M06	*jurtina*	Crete	2009
M07	*jurtina*	Crete	2009
M08	*jurtina*	Crete	2009
M09	*jurtina*	Crete	2009
M10	*jurtina*	Crete	2009
M11	*jurtina*	Crete	2009
M12	*jurtina*	Crete	2009
M13	*cypricola*	Cyprus	2011
M14	*cypricola*	Cyprus	2011
M15	*cypricola*	Cyprus	2011
M16	*cypricola*	Cyprus	2011
M18	*cypricola*	Cyprus	2011
M20	*cypricola*	Cyprus	2011
M21	*cypricola*	Cyprus	2011
M22	*cypricola*	Cyprus	2011
M25	*nurag*	Sardinia	2001
M26	*nurag*	Sardinia	2001
M27	*nurag*	Sardinia	2001
M28	*nurag*	Sardinia	2001
M29	*nurag*	Sardinia	2001
M30	*nurag*	Sardinia	2001
M31	*jurtina*	Sardinia	2001
M32	*nurag*	Sardinia	2001
M33	*jurtina*	Sardinia	2001
M34	*jurtina*	Sardinia	2001
M35	*jurtina*	Sardinia	2001
M36	*jurtina*	Sardinia	2001
M37	*jurtina*	Sardinia	2001
M38	*jurtina*	Sardinia	2001
M39	*jurtina*	France	2000
M40	*jurtina*	France	2000
M41	*jurtina*	France	2000
M42	*jurtina*	France	2000
M43	*jurtina*	France	2000
M45	*jurtina*	France	2000
M46	*nurag*	Sardinia	2001
M47	*nurag*	Sardinia	2001
M48	*nurag*	Sardinia	2001
M49	*nurag*	Sardinia	2001
M50	*nurag*	Sardinia	2001

Specimen code	Species	Region	Date
M51	*nurag*	Sardinia	2001
M52	*jurtina*	Sardinia	2001
M53	*nurag*	Sardinia	2001
M55	*nurag*	Sardinia	2001
M57	*jurtina*	Albania	2011
M58	*jurtina*	Sardinia	2001
M59	*cypricola*	Cyprus	2011
M60	*cypricola*	Cyprus	2011
M61	*jurtina*	Austria	2011
M62	*jurtina*	Albania	2011
M63	*jurtina*	Austria	2011
M64	*jurtina*	Portugal	2011
M65	*jurtina*	Portugal	2011
M66	*jurtina*	Austria	2011
M68	*jurtina*	Crete	2009
M69	*jurtina*	Austria	2011
M83	*megala*	Anatolia, Turkey	01.06.1980
M97	*jurtina*	Georgia	29.07.2007
M98	*jurtina*	Georgia	30.07.2007
M117	*chia*	Chios, Greece	23.05.2006
M124	*telmessia*	Turkey	16.05.2008
M127	*telmessia*	Turkey	08.05.2011
M128	*telmessia*	Turkey	08.05.2011
M167	*telmessia*	Kós, Greece	02.06.2011
M169	*telmessia*	Kós, Greece	02.06.2011
M170	*telmessia*	Kós, Greece	02.06.2011
M171	*telmessia*	Kós, Greece	03.06.2011
M174	*telmessia*	Kós, Greece	04.06.2011
M176	*halicarnassus*	Nísyros, Greece	05.06.2011
M178	*halicarnassus*	Nísyros, Greece	05.06.2011
M180	*halicarnassus*	Nísyros, Greece	05.06.2011
M181	*halicarnassus*	Nísyros, Greece	05.06.2011
M182	*halicarnassus*	Nísyros, Greece	05.06.2011
M183	*halicarnassus*	Nísyros, Greece	05.06.2011
M185	*halicarnassus*	Nísyros, Greece	05.06.2011
M187	*halicarnassus*	Nísyros, Greece	05.06.2011
M189	*telmessia*	Tílos, Greece	08.06.2011
M190	*telmessia*	Tílos, Greece	08.06.2011
M193	*telmessia*	Kárpathos, Greece	09.06.2011
M199	*telmessia*	Kárpathos, Greece	11.06.2011
M202	*nurag*	Sardinia	09.06.2000
M206	*nurag*	Sardinia	17.06.2000
M220	*nurag*	Sardinia	17.06.2000
M232	*nurag*	Sardinia	13.06.2000
M262	*jurtina*	Spain	13.05.2000
M299	*jurtina*	France	30.05.1999
M310	*cypricola*	Cyprus	2011

Specimen code	Species	Region	Date
M311	*jurtina*	Corsica	23.07.2000
M312	*jurtina*	Corsica	23.07.2000
M313	*cypricola*	Cyprus	2011
M314	*megala*	Lesbos, Greece	09.06.2012
M315	*megala*	Lesbos, Greece	07.06.2012
M316	*megala*	Lesbos, Greece	07.06.2012
M317	*megala*	Lesbos, Greece	09.06.2012
M318	*megala*	Lesbos, Greece	07.06.2012
M319	*chia*	Chios, Greece	26.05.2012
M320	*chia*	Chios, Greece	26.05.2012
M321	*chia*	Chios, Greece	26.05.2012
M322	*chia*	Chios, Greece	26.05.2012
M324	*jurtina*	Psara, Greece	29.05.2012
M325	*jurtina*	Psara, Greece	29.05.2012
M327	*chia*	Chios, Greece	09/2002
M328	*chia*	Chios, Greece	09/2002
M329	*chia*	Chios, Greece	09/2002
M330	*chia*	Chios, Greece	09/2002
M331	*chia*	Chios, Greece	09/2002
M332	*chia*	Chios, Greece	09/2002
M333	*chia*	Chios, Greece	09/2002
M334	*chia*	Chios, Greece	09/2002
M335	*chia*	Chios, Greece	09/2002
M336	*chia*	Chios, Greece	09/2002
M337	*chia*	Chios, Greece	09/2002
M338	*chia*	Chios, Greece	09/2002
M339	*chia*	Chios, Greece	09/2002
P340	*Pyronia cecilia*	Italy	01.06.2002
P341	*Pyronia cecilia*	Italy	01.06.2002
M342	*jurtina*	Sardinia	10.05.2001
M343	*nurag*	Sardinia	04.06.2012
M344	*nurag*	Sardinia	04.06.2012
M345	*nurag*	Sardinia	04.06.2012
M346	*cypricola*	Cyprus	2011
M347	*cypricola*	Cyprus	2011
M348	*jurtina*	Sardinia	06/2012
M349	*jurtina*	Sardinia	06/2012
M350	*jurtina*	Sardinia	06/2012
M351	*jurtina*	Sardinia	06/2012
M352	*nurag*	Sardinia	06/2012
M353	*nurag*	Sardinia	06/2012
M354	*jurtina*	Sardinia	09/2013
M355	*nurag*	Sardinia	09/2013
M356	*jurtina*	Sardinia	09/2013
M357	*telmessia*	Israel	09/2013
M359	*telmessia*	Israel	09/2013
M360	*telmessia*	Israel	09/2013

Specimen code	Species	Region	Date
M361	*telmessia*	Israel	09/2013
DQ008101	*chia*	Chios, Greece	2002
DQ008102	*chia*	Chios, Greece	2002
Wiemers1	*halicarnassus*	Nísyros, Greece	03.06.2009
Wiemers2	*jurtina*	La Palma	1996
Wiemers3	*jurtina*	Morocco	-
Wiemers4	*jurtina*	Spain	20.07.2001
Wiemers5	*jurtina*	Tenerife	22.07.1990
AY090214	*jurtina*	Spain	-
AY346233	*jurtina*	Spain, Galicia	-
DQ008088	*jurtina*	Netherlands, Amsterdam	2001
DQ008089	*jurtina*	Netherlands, Amsterdam	2001
DQ008090	*jurtina*	Italy, Sardinia	2001
DQ008091	*jurtina*	Italy, Sardinia	2001
FJ663756	*jurtina*	Ukraine	20.07.2000
FJ663757	*jurtina*	Russia	26.06.1991
FJ663758	*jurtina*	Russia	26.06.1991
FJ663759	*jurtina*	Russia	26.06.1991
FJ663760	*jurtina*	Russia	26.06.1991
GU669731	*jurtina*	Spain, Catalonia	16.09.2008
GU669732	*jurtina*	Spain, Catalonia	16.09.2008
GU669733	*jurtina*	Spain, Catalonia	02.08.2008
GU669734	*jurtina*	Spain, Catalonia	29.07.2008
GU676142	*jurtina*	Spain, Aragon	06.07.2008
GU676341	*jurtina*	Spain, Cadiz	07.09.2008
GU676374	*jurtina*	Spain, Alicante	22.08.2008
GU676463	*jurtina*	Spain, Granada	03.06.2008
GU676504	*jurtina*	Spain, Valencia	14.06.2008
GU676552	*jurtina*	Portugal	26.07.2008
GU676651	*jurtina*	Spain, Madrid	05.06.2008
GU676714	*jurtina*	Spain, Balears	02.06.2008
HM391827	*jurtina*	Germany, Bavaria	17.07.1996
HQ004730	*jurtina*	Romania	29.05.2008
HQ004731	*jurtina*	Romania	26.05.2008
HQ004732	*jurtina*	Romania	02.06.2008
HQ004733	*jurtina*	Romania	07.06.2008
HQ004734	*jurtina*	Romania	29.06.2004
HQ004735	*jurtina*	Romania	09.08.2007
HQ004736	*jurtina*	Romania	01.06.2007
HQ004737	*jurtina*	Romania	06.06.2007
HQ004738	*jurtina*	Romania	22.06.2006
HQ004739	*jurtina*	Romania	25.06.2006
DQ008092	*jur* x *nur*	Sardinia	2001
DQ008093	*jur* x *nur*	Sardinia	2001
DQ008094	*jur* x *nur*	Sardinia	2001
DQ008095	*jur* x *nur*	Sardinia	2001
DQ008096	*nurag*	Sardinia	2001

Specimen code	Species	Region	Date
DQ008097	*nurag*	Sardinia	2001
DQ008098	*nurag*	Sardinia	2001
DQ008099	*nurag*	Sardinia	2001
DQ008100	*nurag*	Sardinia	2001
GQ357220	*telmessia*	-	-
DQ338842	*Pyronia cecilia*	-	-

Curriculum Vitae - Angelina Kreuzinger

Personal data:

Name:	Angelina Kreuzinger, BSc (née Gallauner)

Education:

March 2011 – March 2014:	Studies of Master Zoology at the University of Vienna
October 2007 – February 2011:	Studies of Bachelor Biology at the University of Vienna
September 1999 – June 2007:	High School BG/BRG Klosterneuburg
September 1995 - June 1999:	Elementary school at Kritzendorf

Career:

March 2012 – today:	Teaching assistant at the Department of Integrative Zoology (1090 Vienna)
October 2008 – March 2012:	Receptionist at a doctor's office (Dr. Gallauner, 1100 Vienna)
July – September 2010:	Internship at the Wolf Science Centre (WSC), wild park Ernstbrunn
October 2005 – May 2007:	Sales assistance (School's canteen BG/BRG Klosterneuburg)

Rewards:

2011	Award for excellent study success at the University of Vienna
2010	Award for excellent study success at the University of Vienna
2007	Matura exam with excellent success

Professional activities/Field experience:

Summer 2013	XVIII European Congress of Lepidopterology (oral presentation); Blagoevgrad, Bulgaria
Spring 2012	Field work about *Maniola* butterflies; Sardinia
Summer 2011	Field work about Alpine newts (*Ichthyosaura alpestris*); Zillertaler Alps
Summer 2010	Field work about alpine pollinators; Hohe Tauern National Park

February 24th, 2014

Curriculum Vitae - Angelina Kreuzinger

Additional skills:

Languages:	German (Mother tongue), English (fluently), French and Latin (school level)
EDV-knowledge	MS-Office, LaTeX, SPSS, Statistica, The Observer XT, BioEdit, MEGA, IMARIS, Treefinder, MrBayes, Figtree, DNAsp, Network
Driving license	
First Aid Course	

Publications:

Sztatecsny Marc, Gallauner Angelina, Klotz Luisa, Schabetsberger Robert (2013): Benefits of high-energy prey in a low-productivity habitat: Anuran tadpoles increase the condition index of syntopic Alpine newts (*Ichthyosaura alpestris*) in oligotroph high altitude ponds. *Annales Zoologici Fennici* 5o: 209-215.

Kreuzinger Angelina, Fiedler Konrad, Letsch Harald & Grill Andrea (in prep.): Tracing the radiation of *Maniola* (Lepidoptera: Nymphalidae: Satyrinae) butterflies: new insights from phylogeography hint at one single incompletely differentiated species complex.

February 24th, 2014