

# Speciation in *Pararge* (Satyrinae: Nymphalidae) butterflies – North Africa is the source of ancestral populations of all *Pararge* species

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**Abstract.** The genus *Pararge* comprises three species: *P. aegeria*, distributed in Europe and North Africa; *P. xiphia*, endemic to Madeira; and *P. xiphioides*, endemic to the Canary Islands. Two subspecies are recognized in *P. aegeria*, *P. a. tircis* and *P. a. aegeria*, distributed in northern and southern Europe, respectively. In the 1970s, *P. aegeria* appeared on Madeira. However, despite the status of *P. aegeria* as a model species in ecological studies, the evolutionary history of *Pararge* remains unknown. We studied the phylogenetic relationships of the three *Pararge* species, using the mitochondrial gene *cytochrome oxidase subunit I* and the nuclear gene *wingless* to infer modes and times of speciation. On the basis of our analyses, *Pararge* forms a strongly supported monophyletic group, with the DNA haplotypes of the three species also forming well-supported monophyletic groups. We found that *P. xiphia* diverged first from the common ancestor a maximum of five million years ago, with *P. xiphioides* and *P. aegeria* being sister species that diverged a maximum of three million years ago. The two subspecies, *P. a. tircis* and *P. a. aegeria*, were not distinguishable on the basis of DNA haplotypes; instead, our data clearly distinguished between European specimens and those from North Africa. Madeiran *P. aegeria* has North African haplotypes and thus originated from there rather than from Europe. We hypothesize that the Mediterranean Sea forms a strong barrier to dispersal for *Pararge* butterflies, and has done so for approximately the past one million years.

## Introduction

The speckled wood butterfly, *Pararge aegeria* (Linnaeus, 1758), is one of the most frequently used butterfly species in ecological studies. It has been a model species in studies of behaviour and territorial defence (Davies, 1978; Wickman & Wiklund, 1983; Shreeve, 1984, 1987), life history traits (Gotthard *et al.*, 1994, 2000; Sibly *et al.*, 1997; van Dyck *et al.*, 1997; van Dyck & Wiklund, 2002; Stevens, 2004) and the impact of climate change on the species distributions (Hill *et al.*, 1999). The two subspecies, *P. a. tircis* (Godart, 1821) and *P. a. aegeria*, have been used commonly in comparative studies of life history traits

(Nylin *et al.*, 1993, 1995; Gotthard *et al.*, 1994, 2000) and thermoregulatory ability in different colour morphs (van Dyck & Matthysen, 1998). However, the evolutionary history of the genus remains unstudied. This paper focuses on the speciation processes in *Pararge* Hübner, 1819 to understand better the differences found between species and populations and to provide a phylogenetic framework for comparative studies.

The genus *Pararge* is considered presently to comprise three species: *P. aegeria*, *P. xiphia* (Fabricius, 1775) and *P. xiphioides* Staudinger, 1871. Previously, these were considered to form a single polytypic species with four clearly distinguished subspecies, three of which differed in their genitalia (Higgins, 1975). Today, *P. xiphia* and *P. xiphioides* have separate species status, but *P. a. aegeria* and *P. a. tircis* are treated still as two conspecific subspecies.

*Pararge aegeria* is distributed throughout Europe (east to the Urals), Asia Minor and the Middle East (Bozano,

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1999), and from the southern slopes of the High Atlas in northern Africa to Scandinavia. *Pararge aegeria tircis* and *P. a. aegeria* are distributed north and south of the Alps, respectively, with a zone of intermediates in France. The two subspecies are distinguishable clearly on morphology: *P. a. tircis* has a yellow ground colour and *P. a. aegeria* has an orange ground colour. Since 1976, *P. a. aegeria* has been recorded also from Madeira, where it is now well established (Owen *et al.*, 1986). Thus, the colonization of Madeira by *P. aegeria* offers a unique opportunity to study the interactions between the endemic *P. xiphia* and the newcomer (Owen *et al.*, 1986; Jones, 1992; Shreeve & Smith, 1992; Jones *et al.*, 1998).

The other two species, *P. xiphia* and *P. xiphioides*, are endemic to Madeira and the Canary Islands, respectively. *Pararge xiphioides* has been recorded from the Canary Islands of Gran Canaria, Tenerife, La Gomera and La Palma. With such species distributions, the speciation process in *Pararge* is likely to have taken place allopatrically.

As species of the genus *Pararge* frequently are used as models in ecological studies, it is of great importance to understand their evolutionary history. It has been suggested that Madeira was colonized first by individuals carried by strong air currents or, in the case of *P. aegeria*, perhaps deliberately or accidentally introduced by humans (Owen *et al.*, 1986). We hypothesize that the common ancestor of *Pararge* species colonized both Madeira and the Canary Islands, followed by speciation due to isolation. However, were Madeira and the Canary Islands colonized by individuals from North Africa or from the Iberian Peninsula? The colonization of Madeira by *P. aegeria* might offer a sufficiently recent event, in which it is still possible to find the same haplotype in populations on the mainland. This is one of the aims of this paper. In addition, we want to determine whether *P. aegeria* is divided into two genetically differentiated subpopulations corresponding to the two currently recognized subspecies, that is, are *P. a. aegeria* and *P. a. tircis* separate entities, with different mitochondrial haplotypes, as we can expect from morphology?

## Materials and methods

### Sampling

*Pararge aegeria* was sampled as widely as possible to maximize the number of represented haplotypes (Appendix 1). Both subspecies are represented by several populations: *P. a. aegeria* from populations in southern Portugal, central Spain, southern France and southern Greece; *P. a. tircis* from populations in Sweden, the UK and Belgium. *Pararge xiphioides* was sampled from three of the four islands where it is present, but unfortunately we could not obtain specimens from the island of Gran Canaria. *Pararge xiphia* was sampled from various localities on Madeira. As outgroup taxa, we chose representatives from other genera and species belonging to the same tribe, Parargini: *Kirinia roxelana* (Cramer, 1777),

*Lasiommata maera* (Linnaeus, 1758), *L. megera* (Linnaeus, 1767), *L. petropolitana* (Fabricius, 1787) and *Lopinga achine* (Scopoli, 1763). The origin and voucher identifications of the specimens used in this study are detailed in Appendix 1. Specimens are presently stored at  $-20^{\circ}\text{C}$  at the Department of Zoology, Stockholm University, Sweden, and will be deposited at the Swedish Museum of Natural History, Stockholm, Sweden.

### Molecular methods

We extracted DNA from two legs of the frozen or dried butterflies using a QIAGEN (Hilden, Germany) DNeasy Extraction Kit following the manufacturer's instructions.

We sequenced two gene regions: one mitochondrial gene, *cytochrome oxidase subunit I* (COI), of 1450 bp, and one nuclear gene, *wingless*, of 407 bp. COI was sequenced for all seventy-one individuals sampled. From these seventy-one individuals, we chose twenty-three individuals showing the most divergent COI sequences for which we then also sequenced *wingless*. Polymerase chain reactions (PCRs) were performed in a 20  $\mu\text{l}$  reaction volume. The protocol used was: 1  $\mu\text{l}$  DNA, 11.5  $\mu\text{l}$   $\text{H}_2\text{O}$ , 2  $\mu\text{l}$  10 $\times$  buffer, 2  $\mu\text{l}$   $\text{MgCl}_2$  (25 mM), 1  $\mu\text{l}$  of each primer, 0.4  $\mu\text{l}$  dNTP (10 mM) and 0.1  $\mu\text{l}$  Taq Gold. The cycling profile was 95  $^{\circ}\text{C}$  for 5 min, 35 cycles of 94  $^{\circ}\text{C}$  for 30 s, 47  $^{\circ}\text{C}$  for 30 s, 72  $^{\circ}\text{C}$  for 1 min 30 s and 72  $^{\circ}\text{C}$  for 10 min. We used the primers of Brower & DeSalle (1998) for *wingless* and of Wahlberg & Zimmermann (2000) for COI.

The same primers were used in the sequencing reaction. In addition, for COI, the internal primer 'Patty' (Wahlberg & Nylin, 2003) was used. The sequencing reactions were performed in a total volume of 20  $\mu\text{l}$ : 13.5  $\mu\text{l}$   $\text{H}_2\text{O}$ , 1.5  $\mu\text{l}$  10 $\times$  buffer, 2  $\mu\text{l}$  CEQ2000 Mix, 2  $\mu\text{l}$  primer and 1  $\mu\text{l}$  DNA. Sequencing was undertaken using a Beckman Coulter (Bromma, Sweden) CEQ8000 capillary sequencer. All sequence chromatograms were checked in the program BIOEDIT (Hall, 1999) and aligned by eye.

### Phylogenetic analyses

In total, three datasets were analysed: COI and *wingless* alone and both genes combined. For the combined analysis, we used only those individuals (twenty-three specimens) for which both genes had been sequenced (see Appendix 1).

The most parsimonious cladograms were searched for from the equally weighted and unordered data matrix using a heuristic search algorithm in the program NONA 2.0 (Goloboff, 1998) via the program WINCLADA 1.00.08 (Nixon, 2002). The heuristic searches were conducted with 200 random addition replicates using tree bisection-reconnection (TBR) branch swapping, with ten trees held during each step and a final swapping to completion. The robustness of the phylogeny was evaluated using bootstrap (Felsenstein, 1985)

and Bremer support values (Bremer, 1988, 1994). Bootstrap values were obtained using 1000 replicates. When calculating Bremer supports, TREEOT version 2 (Sorensen, 1999) was used in conjunction with PAUP 4.0b10 (Swofford, 2001) to define constraint trees for each node. The most parsimonious trees without a particular node were found in PAUP using a heuristic search with random additions of twenty replicates and one tree held during each step. Maxtrees was set to 500 and branch swapping was performed using TBR. To evaluate the contribution of each dataset to the combined result, we used partitioned Bremer support values (Baker & DeSalle, 1997; Gatesy *et al.*, 1999). Pairwise sequence divergences and base frequencies were calculated using MEGA version 3 (Kumar *et al.*, 2004).

#### Timing of divergences

For dating purposes, the most parsimonious tree from the combined analysis was pruned to contain one representative of each major *Pararge* lineage (i.e. one *P. xiphia*, one *P. xiphioides* from each island, one Moroccan *P. aegeria* and one *P. aegeria* from Europe), together with the outgroups. Branch lengths for this tree were estimated from the combined dataset using maximum likelihood and a GTR + G + I model. Branch lengths and parameter values were estimated in PAUP 4.0b10 (Swofford, 2001) with and without a molecular clock enforced. The evolution of the sequences in a clocklike manner was tested with the likelihood ratio test. If the likelihoods of the models are significantly different, the molecular clock can be rejected. Owing to practical problems with estimating branch lengths at the root node (see accompanying instructions of Sanderson, 2004), after the branch lengths had been estimated, two outgroup taxa (*Kirinia* Moore, 1893 and *Lopinga* Moore, 1893) were pruned from the tree, leaving *Pararge* and its putative sister genus *Lasiommata* Westwood, 1841.

To obtain reliable age estimates for clades, the ages of particular nodes need to be fixed or constrained using external information from fossils or geological events. In the present case, no fossils of either *Pararge* or its putative sister genus *Lasiommata* are known. There are two geological events that potentially can be used to calibrate our age estimates. The age of Madeira, at five million years (Geldmacher & Hoernle, 2000), can be used to place the maximum age of the divergence between the ancestor of *P. xiphia* and the other two *Pararge* species. However, this estimate is complicated by the fact that the island of Porto Santo is much older (fourteen million years; Geldmacher & Hoernle, 2000) and, although it does not support populations of *P. xiphia* now, it may have done so in the past. The second calibration age is a more certain maximum age, namely the age of the island of La Palma at two million years (Ancochea *et al.*, 1994). Current methods require that at least one node age be fixed. We therefore fixed the split between the La Gomera lineage and the La Palma lineage of *P. xiphioides* at two million years.

Dating of the phylogenies was made with the program r8s (Sanderson, 2004). We used three algorithms to estimate the ages of divergence: the Langley–Fitch method (which assumes a global molecular clock), nonparametric rate smoothing (Sanderson, 1997) and penalized likelihood (Sanderson, 2002). For the penalized likelihood method, we estimated the value of the smoothing parameter using a cross-validation procedure. In all cases, to prevent the algorithms from converging on a local optimum, the searches were begun at five different initial time estimates. The local stability of the solutions for each estimate was checked by perturbing them and restarting the search five times. We compared our age estimates with those obtained from the now standard constant mutation rate of 2.3% pairwise sequence divergence per million years (Brower, 1994).

Errors in age estimates resulting from the stochastic nature of substitution processes were assessed using a bootstrap resampling procedure, with the help of Perl scripts made available by Eriksson (2005). One hundred bootstrap replicates of the combined dataset were constructed using the SEQBOOT program (Felsenstein, 1993), and branch lengths were calculated using maximum likelihood and the GTR + G + I model for each replicate and then input to the r8s program. The divergence ages were estimated for the bootstrapped datasets using nonparametric rate smoothing and fixing the age of the split between the La Palma and La Gomera lineages of *P. xiphioides* at two million years. Bootstrap estimates of the standard deviation for each node were calculated for the age distribution estimates obtained.

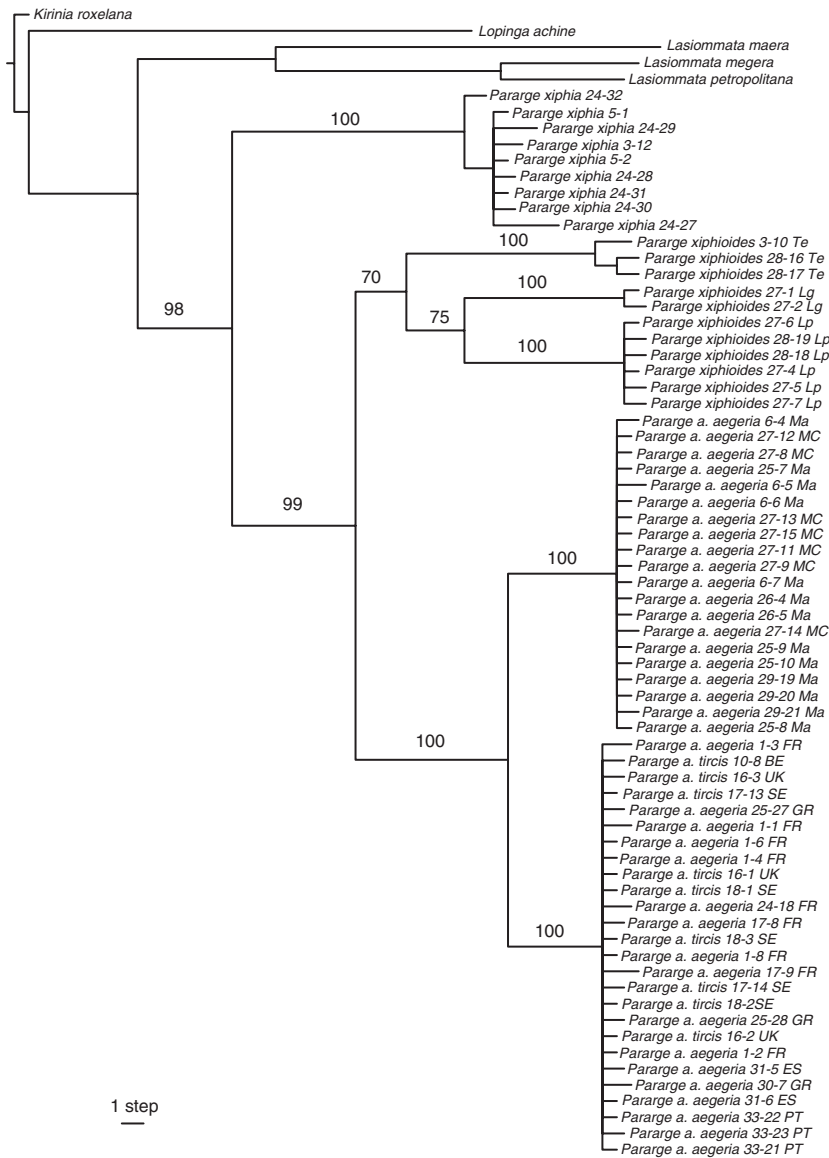
## Results

The COI dataset ( $n = 71$  individuals) consisted of 1450 base pairs, of which 235 were parsimony informative. The base frequencies were: T, 40.9%; C, 15.4%; A, 30.1%; G, 13.6%. In the heuristic search, 110 trees of length 604 steps were found (CI = 0.71, RI = 0.92). The strict consensus tree with bootstrap values is shown in Fig. 1.

The *wingless* dataset ( $n = 23$ ) consisted of 407 base pairs, of which thirty-three were parsimony informative. The base frequencies were: T, 19.2%; C, 28.4%; A, 22.6%; G, 29.7%. The analysis found a single most parsimonious tree of length seventy steps (CI = 0.91, RI = 0.94). The tree, with branch lengths, is shown in Fig. 2.

Analysis of the combined dataset ( $n = 23$ ) generated one most parsimonious tree (Fig. 3) with 647 steps (CI = 0.74, RI = 0.83). Of the 1857 characters, 260 were parsimony informative.

The genus *Pararge* is supported strongly as a monophyletic group by both the COI and *wingless* datasets (Figs 1 and 2). We find that *P. aegeria* is the sister species to *P. xiphioides* and that *P. xiphia* is sister to these two. In the COI analyses, the *P. xiphioides* populations on different islands are monophyletic with high bootstrap support values. The population from Tenerife is sister to those from La Gomera and La Palma. However, using *wingless*,



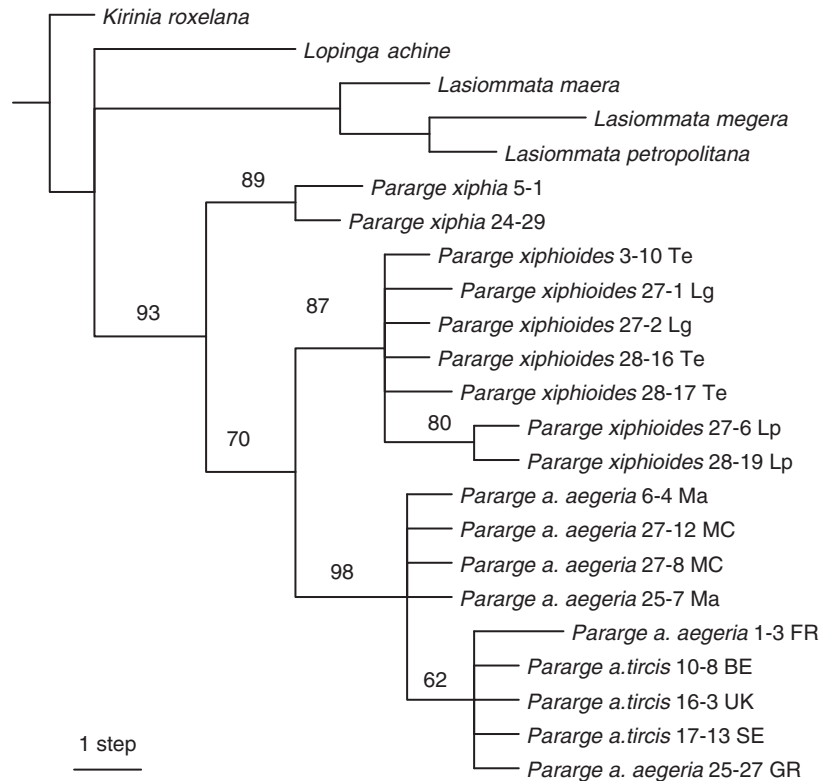
**Fig. 1.** Strict consensus tree of 110 most parsimonious trees of equal length, 604 steps, based on the *cytochrome oxidase subunit I* (COI) dataset ( $n = 71$  taxa). Bootstrap support is shown above the branches. Branch lengths are proportional to the number of changes inferred from parsimony. Geographic abbreviations: BE, Belgium; ES, Spain; FR, France; GR, Greece; Lg, La Gomera; Lp, La Palma; Ma, Madeira; MC, Morocco; SE, Sweden; Te, Tenerife; UK, United Kingdom.

only the population from La Palma is resolved as monophyletic and does not share haplotypes with the other islands. In contrast, the two subspecies of *P. aegeria* are not recovered as monophyletic. Instead, according to COI, the species is divided into two clades, with continental European specimens forming one group, and the African and Madeiran specimens forming the other. The analysis of *wingless* supports the European clade, but the African and Madeiran group is unresolved.

The genus itself receives high bootstrap support for both genes (Figs 1 and 2), as do *P. aegeria* and *P. xiphia*. However, the value is less for *P. xiphioides*, 70% for COI and 87% for *wingless*. The sister group relationship of *P. aegeria* and *P. xiphioides* is strongly supported by COI (bootstrap value of 99%), but less so by *wingless* (bootstrap value of 70%).

The phylogeny of the combined dataset unsurprisingly corresponds to the topologies of COI and *wingless* (Fig. 3). The monophyly of all three species receives moderate to strong Bremer support values, although this is mainly due to the COI partition. The sister group relationship of *P. aegeria* and *P. xiphioides* is supported strongly by COI data partition, but weakly by the *wingless* partition. Within *P. xiphioides*, the populations from different islands receive high support from the COI partition, but low or no support from the *wingless* partition. The highly supported division of *P. aegeria* into European and North African + Madeiran clades is due almost entirely to the COI partition.

Pairwise sequence divergences are shown in Table 1. For COI, an average of about 1.9% difference between the two major *P. aegeria* lineages is detected. The pairwise COI sequence divergence within the two lineages is close to 0%



**Fig. 2.** Most parsimonious tree found for the *wingless* dataset (tree length, 70 steps;  $n = 23$  taxa). Bootstrap support is shown above the branches. Branch lengths are proportional to the number of changes inferred from parsimony. Geographic abbreviations: BE, Belgium; ES, Spain; FR, France; GR, Greece; Lg, La Gomera; Lp, La Palma; Ma, Madeira; MC, Morocco; SE, Sweden; Te, Tenerife; UK, United Kingdom.

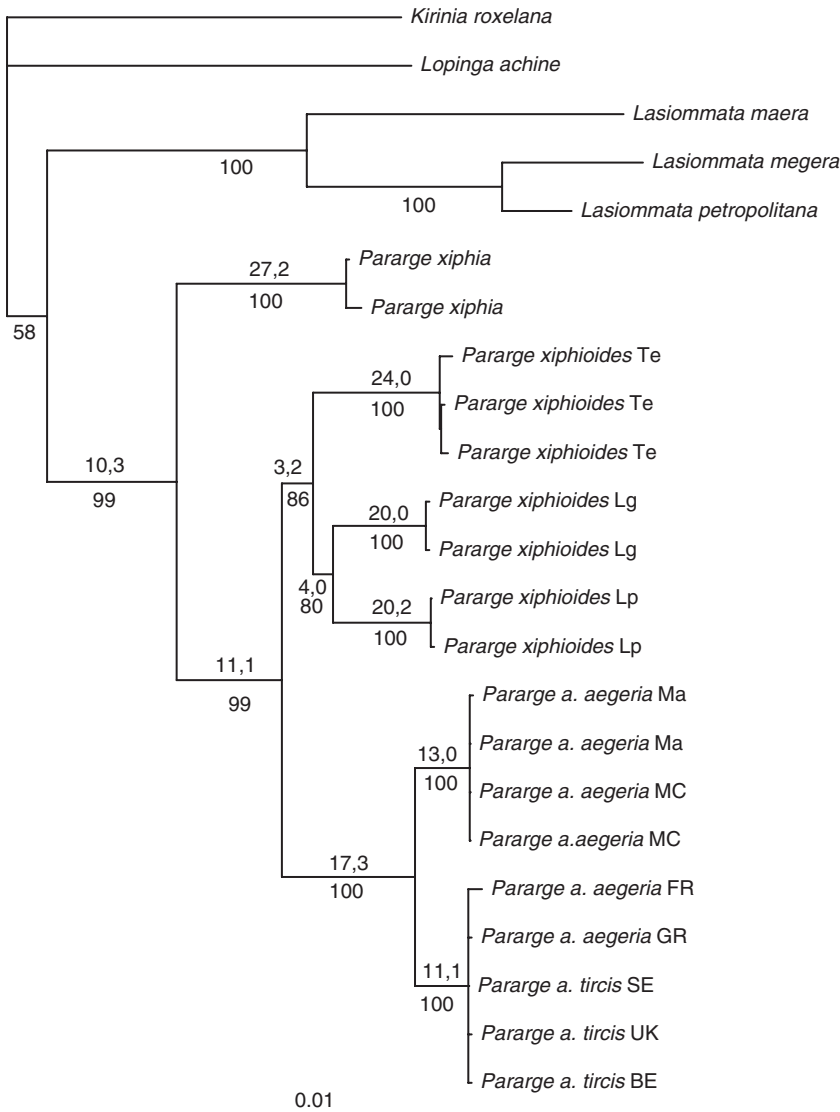
(i.e. haplotypes tend to differ by only one base pair out of 1450 sequenced). The most common haplotype in Europe also is the most widespread, being found in individuals collected in southern Portugal, central Spain, southern France, the UK and Sweden. Most haplotypes in Morocco and Madeira are identical to each other. The highest intraspecific diversity in COI is found within *P. xiphioides*, with a maximum of 4.4% difference between specimens. Haplotype diversity within the major lineages is very poor (close to 0%), suggesting repeated bottlenecks or selective sweeps purging the populations of mitochondrial haplotype diversity.

Clockwise evolution of the sequences in the combined dataset was rejected ( $P < 0.05$ ), and so we base our discussion on the nonclock results. However, the choice of algorithm did not change the age estimates very much (Table 2). Calibrating the split between the La Palma and La Gomera lineages of *P. xiphioides* at two million years provides us with a maximum estimate of the ages of the other splits in the tree (Fig. 4). The age of the split between *P. xiphia* and the rest of *Pararge* is estimated at a maximum of 5.6 million years with a standard deviation of 0.9 (Table 2). This age is consistent with the age of Madeira and suggests that the ancestor of *P. xiphia* may have reached the island soon after it emerged from the ocean. The age of the split between *P. xiphioides* and *P. aegeria* is estimated at a maximum of 3.1 million years ( $\pm 0.46$ ), while the split between North African populations and European populations of *P. aegeria* may have occurred around 0.87 million years ( $\pm 0.24$ ) ago. Interestingly, applying Brower's

(1994) molecular clock estimate of 2.3% pairwise sequence divergence per million years to *Pararge* gives similar ages for the younger splits, including the assumed two million years for the split between *P. xiphioides* on La Gomera and La Palma (Table 2).

## Discussion

Speciation in butterflies may be promoted by an expanded host plant range (Janz *et al.*, 2001; Weingartner *et al.*, 2006). However, species of the genus *Pararge* are specialists of grasses and show no evidence for an expanded host range. Nevertheless, host plant use could still be an important factor in the diversification process. Of the ten endemic butterfly species of the Canary Islands, five are grass-feeding satyrids (*Hipparchia wyssii* Christoph, 1889, *H. bacchus* (Higgins, 1967), *H. gomera* (Higgins, 1967), *H. tilosi* (Manil 1984) and *P. xiphioides*). Of the others, one is a strong flier (*Vanessa vulcanica* Godart, 1819; Nymphalidae), one is a hesperid (*Thymelicus christi* Rebel, 1894), which also feeds on grass, two belong to the family Pieridae (*Pieris cheiranthi* (Hübner, 1808), *Gonepteryx cleobule* (Hübner 1825)) and one is a lycaenid (*Cycleryx webbianus* (Brullé, 1840)). The dominance of grass feeders on the Canary Islands might reflect their good colonization ability. Grass is a resource available almost everywhere, and it might be easy to colonize new habitats even though the butterflies themselves are not very good dispersers.



**Fig. 3.** Most parsimonious tree of the combined dataset [*cytochrome oxidase subunit I* (COI) + *wingless*]. Tree length, 647 steps ( $n = 23$ ). Partitioned Bremer support values are shown above the branches. The first value refers to the support contributed by COI and the second to the support value of the *wingless* dataset. Bootstrap support is shown below the branches. Branch lengths have been estimated using maximum likelihood (GTR + G + I model). Geographic abbreviations: BE, Belgium; ES, Spain; FR, France; GR, Greece; Lg, La Gomera; Lp, La Palma; Ma, Madeira; MC, Morocco; SE, Sweden; Te, Tenerife; UK, United Kingdom.

In the case of *Pararge*, it thus appears that speciation happened through processes not directly related to host plants. Quite likely, *P. xiphia* and *P. xiphioides* speciated after their ancestors had colonized their respective islands.

Where these ancestors came from is thus of interest. The island of Madeira is about 850 km south-west of Portugal, about 540 km west of Morocco and about 450 km north of the Canary Islands. The island of Gran Canaria (the closest

**Table 1.** Average uncorrected pairwise distances of *cytochrome oxidase subunit I* (COI) haplotypes for each of the major lineages of *Pararge*.

Taxon	<i>Lasiommata</i>	<i>P. xiphia</i>	<i>P. xiphioides</i> (Te)	<i>P. xiphioides</i> (Lg)	<i>P. xiphioides</i> (Lp)	<i>P. aegeria</i> (Africa)	<i>P. aegeria</i> (Europe)
<i>Lasiommata</i>	0.061	0.089	0.098	0.093	0.093	0.095	0.092
<i>P. xiphia</i>		0.001	0.060	0.057	0.056	0.057	0.064
<i>P. xiphioides</i> (Te)			0.002	0.043	0.043	0.047	0.049
<i>P. xiphioides</i> (Lg)				0.001	0.032	0.048	0.045
<i>P. xiphioides</i> (Lp)					0.000	0.047	0.048
<i>P. aegeria</i> (Africa)						0.000	0.019
<i>P. aegeria</i> (Europe)							0.001

Lg, La Gomera; Lp, La Palma; Te, Tenerife.

**Table 2.** Estimated ages for splits within *Pararge*.

Node	Constant	Clock	NPRS	PL	Boot SD	Boot range
Root	4.1	9.07	9.96	9.88	1.59	4.26–12.50
<i>Pararge</i>	2.6	5.16	5.63	5.64	0.90	3.02–8.13
<i>P. aegeria</i> + <i>P. xiphioides</i>	2.1	3.09	3.06	3.08	0.46	2.14–4.56
<i>P. aegeria</i>	0.8	0.94	0.86	0.87	0.24	0.42–1.58
<i>P. xiphioides</i>	1.9	2.45	2.43	2.44	0.26	2.00–3.09
La Palma + La Gomera <sup>a</sup>	1.4	2.00	2.00	2.00	0.00	2.00

Constant, assuming a constant mutation rate of 1.2% per million years (Brower, 1994); NPRS, nonparametric rate smoothing; PL, penalized likelihood with a smoothing rate of 0.0032; Boot SD, standard deviation of bootstrapped datasets; Boot range, minimum and maximum ages estimated from bootstrapped datasets.

<sup>a</sup>Node fixed at two million years.

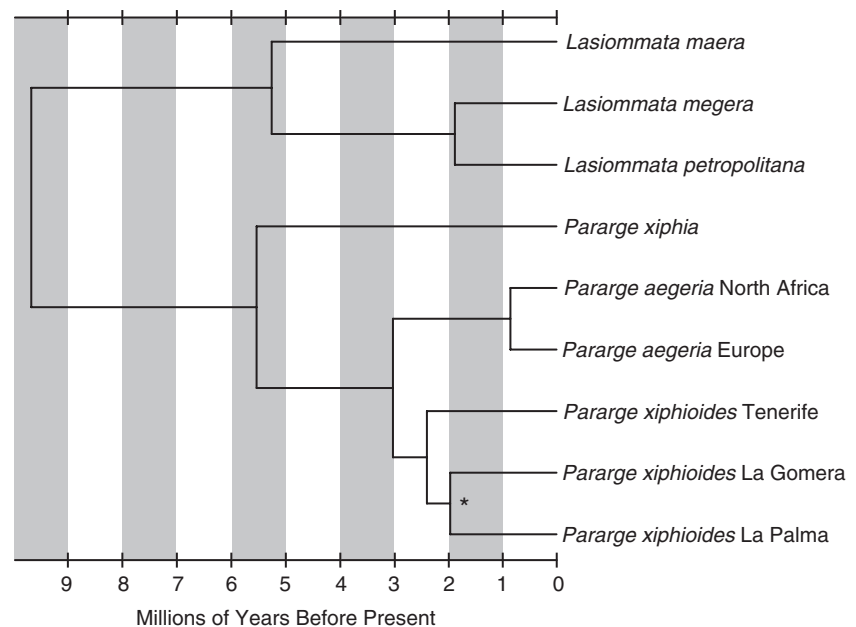
island to the African mainland which has *Pararge* on it) is about 190 km west of the African continent. The source of the ancestors of *P. xiphia* is not clear and may have been either Europe or North Africa.

Most likely, the Canary Islands were colonized from North Africa, from which the nearest island is only 95 km. Although we have yet to sample *P. xiphioides* from Gran Canaria, the pattern of colonization seems to be from older to younger islands, which corresponds to an east to west direction, with the population on Tenerife sister to the populations on La Gomera and La Palma. This result agrees with other studies on Canary Islands colonizations (Carranza *et al.*, 2000; Emerson *et al.*, 2000a; Rees *et al.*, 2001). With regard to butterflies, there is one study of the genus *Gonepteryx* Leach, 1815, in which the authors suggest that the colonization of the Canary Islands has occurred from Africa (Brunton & Hurst, 1998). However, it is crucial to include the population on Gran Canaria in order to draw any conclusions about the colonization pattern of *P. xiphioides*. The species has not been recorded on the two islands Lanzarote and Fuerteventura, the easternmost

islands of the archipelago, most probably due to the lack of suitable habitats on these islands.

One explanation for the short basal branch lengths for the *P. xiphioides* haplotype clade (see Fig. 3) could be a rapid diversification process on these islands. This would produce the observed basal branches with few character changes, but large genetic distances between islands, indicating genetic isolation between them (Table 1). High genetic divergences amongst the populations on different islands in the Canary Islands, compared with the mainland populations, are found also in psyllids (Percy, 2003). The estimated maximum age of *P. xiphioides* is 3.1 million years ( $\pm 0.46$ ) (Fig. 4). Thus, *P. xiphioides* is much younger than the formation of Tenerife (11.6 million years ago) and, once it colonized the Canary Islands, it was able to spread amongst the islands quickly.

Contrary to what might be expected from morphology, we have not discovered any indication that *P. a. aegeria* and *P. a. tircis* are separate evolutionary entities based on a mitochondrial gene and a nuclear gene. Instead, we found two separate clades within *P. aegeria* corresponding to the



**Fig. 4.** Chronogram for *Pararge*. The asterisk marks the node which was fixed at two million years.

European and African + Madeiran populations. This pattern is consistent in both genes. However, the morphological differences indicate that other genes are differentiated between *P. a. tircis* and southern European populations known as *P. a. aegeria*. The implication of this finding will affect comparative studies of *P. a. tircis* and *P. a. aegeria* in which Madeiran specimens have been used as representatives of the subspecies *P. a. aegeria* (e.g. Nylin *et al.*, 1994, 1995). It cannot be concluded that life history differences between the two subspecies are a consequence of recent adaptation to the habitat on Madeira (e.g. Nylin *et al.*, 1995). Instead, the Madeiran populations may carry with them adaptations to habitats in North Africa, which are beneficial also on Madeira. It is thus preliminary to conclude that over 100 generations is enough time to allow life history traits to evolve on Madeira (Nylin *et al.*, 1995), without a comparative study of populations from North Africa and Madeira.

It is of great interest to determine when the two mitochondrial clades of *P. aegeria* diverged, as this can help us to understand the process that might have promoted diversification. The results from dating should, however, be interpreted carefully. When using the age of islands to calibrate nodes, we obtain the maximum age of a clade if we assume that divergence has taken place on the island. Most likely, the ancestral butterflies colonized the islands later, maybe much later. However, the much used 2.3% pairwise sequence divergence per million years (Brower, 1994) gives a very similar age (1.4 million years) for the split between La Gomera and La Palma lineages as our assumed two million years (the age of the island of La Palma), lending some credence to the ages we discuss.

According to our dating, the split between the European *P. aegeria* and the North African populations occurred at about 0.87 million years ( $\pm 0.24$ ) ago (Table 2, Fig. 4). This falls within the Quaternary, which in Europe was dominated by repeated intervals of glaciations that affected the evolution of European species (Hewitt, 1996). During glacial periods, northern populations were extinguished or pushed further south. When the climate became warmer, the populations expanded their ranges northwards. The Mediterranean has been a strong barrier to migration (Taberlet *et al.*, 1998). During the Messinian salinity crisis (5.57–4.93 million years ago), when the Mediterranean was desiccated, Europe and Africa were joined into a single land mass (De Jong, 1998, and references cited therein), and this possibly enabled migration between the two continents.

The phylogenetic patterns we have uncovered present a difficult case for investigating the historical biogeography of the group. Three of the four major lineages show autapomorphic distributions (Madeira, Canary Islands and North Africa), whereas the one lineage sharing its distribution with the sister group (Europe) is in a highly derived position, suggesting convergence. Conventional methods would suggest that Europe is the ancestral range of the genus *Pararge*, and this is undoubtedly true. However, it does not shed light on the source from which the islands or North Africa were colonized.

The evolution of *Pararge* butterflies is thus an example of a seemingly simple case of speciation that, in fact, shows a rather complicated pattern. In order to understand this pattern, we must rely on the phylogeny. Five observations can be made: (1) the sister group to *Pararge* is (or appears to be) the Palaearctic genus *Lasionmata* (in fact, all potential sister groups to *Pararge* are Palaearctic; see Bozano, 1999); (2) the two island species of *Pararge* are not sister species, suggesting that Madeira and the Canary Islands were colonized independently; (3) the direction of colonization on the Canary Islands (from west to east) accords with other studies and suggests that the source of the colonists was North Africa; (4) the recent colonization of Madeira by *P. aegeria* took place from North Africa; and (5) the haplotypes of *P. aegeria* form a monophyletic group to the exclusion of the two other species and, importantly, the haplotypes in North Africa + Madeira and in Europe are reciprocally monophyletic.

The foregoing observations suggest strongly the following scenario to us. The ancestor of the genus *Pararge* colonized North Africa from the Palaearctic, perhaps during the Messinian salinity crisis (assuming that *P. xiphia* is at most five million years old). The ancestral populations in Europe subsequently went extinct. When this occurred, we cannot say, but at least prior to the recolonization of Europe by *P. aegeria* some four million years later (evidenced by no endemic European species or ancient haplotypes in European populations of *P. aegeria*). The ancestral species then colonized Madeira from North Africa soon after the island appeared above sea level, and speciated subsequently and evolved into what we know currently as *P. xiphia*. The ancestral populations in North Africa continued to evolve through time until a second colonization of the islands and speciation event took place, this time in the Canary Islands. The subsequent spread of *P. xiphoides* amongst the islands appears to have been rapid. The colonization of the Canary Islands apparently also was a single event, evidenced by the monophyly of current haplotypes of *P. xiphoides*.

After the divergence of *P. xiphoides* from ancestral *P. aegeria* populations, the North African populations continued to develop in isolation until perhaps 0.8 million years ago, when Europe was colonized. This colonization also appears to be a single event, evidenced by the deep divergence between, and reciprocal monophyly of, North African and European populations. Subsequent to the colonization of Europe, the species eventually spread to occupy its current range. It is quite likely that the European *P. aegeria* has spread and retracted its range several times, as there have been several glaciation periods during the past one million years. Recent observations in the U.K. have shown that *P. aegeria* has the ability to expand its range rapidly in response to climate changes (Hill *et al.*, 1999). Given the relatively deep divergence between the European and North African lineages, it is possible that the European *P. aegeria* survived glacial maxima in refugia away from the Iberian peninsula, such as the Balkan peninsula. Further evidence for the scenario described above comes from the



coloration of the wings. Four taxonomic entities retain the ancestral orange wing colour (which is also found in *Lasiommata*): *P. xiphia*, *P. xiphioides*, *P. a. aegeria* (North Africa) and *P. a. aegeria* (Europe). Our phylogenetic hypothesis suggests that the yellow colour of the northern *P. a. tircis* has evolved later, perhaps as an adaptation to colder climates.

An alternative biogeographical hypothesis, and one more parsimonious based on our phylogenetic hypothesis, would be that Madeira was colonized first from Europe by the ancestral *Pararge*. The Canary Islands were colonized subsequently from Madeira by the ancestor of *P. xiphioides* and *P. aegeria*, and then North Africa was colonized from the Canary Islands (before the differentiation of *P. xiphioides* on the islands). Finally, Europe was colonized from North Africa, completing a circle that took perhaps five million years. Both hypotheses require that the ancestral *Pararge* went extinct in Europe at some stage before the present. It is impossible to differentiate between these two hypotheses based on the evidence we have on hand. However, we believe that indirect evidence favours the first hypothesis over the second. Studies on other insects suggest that the island groups have been colonized from continental sources (Brunton & Hurst, 1998; Emerson *et al.*, 2000a, b; Rees *et al.*, 2001; Hundsdoerfer *et al.*, 2005). Although the colonization of Madeira by *P. aegeria* from North Africa cannot be seen as direct evidence of the same route by the ancestor of *P. xiphia*, in the absence of other evidence, it is suggestive. There is some evidence of inter-island group colonizations, but so far the direction of colonization has been inferred to be from the Canary Islands to Madeira (e.g. Emerson *et al.*, 2000b; Hundsdoerfer *et al.*, 2005).

Our results have important taxonomic implications for *Pararge*. First of all, *P. aegeria*, *P. xiphia* and *P. xiphioides* clearly form three good species that produce monophyletic clades with both mitochondrial and nuclear genes. This implies that the three species have had a relatively long period of independent evolution, an implication which is corroborated by our analysis of the ages of the clades. The second taxonomic implication is within the species *P. aegeria*, for which there is conflicting information regarding morphological and genetic data. On the basis of morphology, there are two clear subspecies found north and south of the Alps, respectively. Based on the sequences of two genes, there are two clear lineages which are highly diverged from each other, one in North Africa and the other in Europe. DNA taxonomy is enjoying great popularity at the moment (see, for example, Hebert *et al.*, 2003), although calls are being made not to discard traditional morphological data (see, for example, Wahlberg *et al.*, 2005). In this case, there are two COI haplotype lineages which have apparently not interbred for a long period of time (perhaps as long as 0.8 million years, although they are able to produce fertile offspring in the laboratory; Nylin *et al.*, 1994), and two morphological lineages that do interbreed but form a narrow hybrid zone and retain their morphological distinctiveness outside the hybrid zone. This would suggest that there are in fact three taxonomic

entities within *P. aegeria*, one in North Africa (and Madeira), one in Europe south of the Alps and one in Europe north of the Alps.

There are names available for each of the three entities: *meone* Stoll, 1780 (type locality: Algeria), *aegeria* Linnaeus, 1758 (type locality: South Europe and North Africa) and *tircis* Godart, 1821 (type locality: France). However, using these names is complicated by the fact that the type locality of *aegeria* is given as 'South Europe and North Africa' and no type material exists (see Honey & Scoble, 2001). Fixing the name to populations in southern Europe would require a neotype, and such an act is beyond the scope of this paper. On the other hand, the names *aegeria* and *tircis* have been used consistently for southern and northern European populations, respectively, and taking the name *meone* for the North African and Madeiran populations would not cause much taxonomic instability. We thus raise the name *meone* Stoll, 1780 from synonymy to subspecies status (*Pararge aegeria meone* **stat. rev.**) and restrict its use to populations in North Africa and Madeira. The name *Pararge aegeria aegeria* refers to populations in southern Europe with an orange background colour on the forewings, and the name *Pararge aegeria tircis* to populations in northern Europe with a light yellow background colour on the forewings.

Different genetic lineages in Europe and Africa have been found in several studies (Burban *et al.*, 1999; Percy, 2003; Hundsdoerfer *et al.*, 2005). Quite clearly, the Mediterranean remains a barrier for genetic exchange, even though the Iberian Peninsula and Morocco are separated by only 14 km at one point (Dobson & Wright, 2000). This does seem paradoxical, as *Pararge* clearly crossed large bodies of water to reach Madeira and the Canary Islands. However, a general pattern of intraspecific divergence between the two continents may exist, but studies, as yet, are few, especially in flying insects. A similar pattern is found in the satyrine *Lasiommata megera* (E. Weingartner, unpublished), as well as in the nymphalines *Melitaea cinxia* (Linnaeus, 1758) and *Melitaea phoebe* (Denis & Schiffermüller, 1775) (N. Wahlberg, unpublished). However, in the strong flyer *Polygonia c-album* (Linnaeus, 1758) (Nymphalidae), deep divergence between Europe and Morocco could not be found (E. Weingartner, unpublished). More taxa need to be studied in order to see if the pattern is consistent. If this pattern occurs in other taxa, it would suggest that there has been an event that has facilitated movement between North Africa and Europe at one point in the recent geological history of the region. What this event could have been remains to be discovered through comparative studies of other species with distributions in North Africa and Europe.

### Acknowledgements

We are grateful to Enrique Garcio-Barros, Karl Gotthard, Bengt Karlsson, Georg Nygren, Thomas Schmitt, Constanti Stefanescu, Michel Tarrier and Christer

Wiklund for providing specimens for this work. We thank Andrew Brower, Ian Kitching and an anonymous reviewer for constructive comments on a previous version of the manuscript.

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Accepted 26 September 2005

First published online 26 April 2006

#### Appendix 1. Collection localities, voucher numbers and GenBank accession numbers for the specimens included in this study.

Taxon	Voucher ID	Origin of sample	COI	<i>wingless</i>
Outgroup taxa				
<i>Kirinia roxelana</i>	25–26	Peloponnes, Greece	DQ176348	DQ176325
<i>Lasiommata maera</i>	30–5	Stockholm, Sweden	DQ176350	
<i>Lasiommata maera</i>	24–24	Ustou Valley, Ariège, France		DQ176328
<i>Lasiommata megera</i>	24–23	Ustou Valley, Ariège, France	DQ176351	DQ176326
<i>Lasiommata petropolitana</i>	24–21	Ustou Valley, Ariège, France	DQ176352	DQ176327
<i>Lopinga achine</i>	28–22	Gotland, Sweden	DQ176349	DQ176329
Ingroup taxa				
<i>Pararge aegeria aegeria</i>	1–1	Carcassonne, France	DQ176379	
<i>Pararge aegeria aegeria</i>	1–2	Carcassonne, Aude, France	DQ176394	
<i>Pararge aegeria aegeria</i>	1–3	Carcassonne, Aude, France	DQ176373	DQ176339
<i>Pararge aegeria aegeria</i>	1–4	Carcassonne, Aude, France	DQ176382	
<i>Pararge aegeria aegeria</i>	17–8	Carcassonne, Aude, France	DQ176384	
<i>Pararge aegeria aegeria</i>	17–9	Carcassonne, Aude, France	DQ176387	
<i>Pararge aegeria aegeria</i>	1–6	Bages, Aude, France	DQ176380	

## Appendix 1. Continued.

Taxon	Voucher ID	Origin of sample	COI	wingless
<i>Pararge aegeria aegeria</i>	1–8	Bages, Aude, France	DQ176386	
<i>Pararge aegeria aegeria</i>	24–18	Ustou Valley, Ariège, France	DQ176383	
<i>Pararge aegeria aegeria</i>	25–27	Peloponnes, Greece	DQ176375	DQ176344
<i>Pararge aegeria aegeria</i>	25–28	Peloponnes, Greece	DQ176388	
<i>Pararge aegeria aegeria</i>	30–7	Peloponnes, Greece	DQ176404	
<i>Pararge aegeria aegeria</i>	31–5	Madrid, Spain	DQ176403	
<i>Pararge aegeria aegeria</i>	31–6	Madrid, Spain	DQ176405	
<i>Pararge aegeria aegeria</i>	33–21	Algarve, Portugal	DQ176409	
<i>Pararge aegeria aegeria</i>	33–22	Algarve, Portugal	DQ176407	
<i>Pararge aegeria aegeria</i>	33–23	Algarve, Portugal	DQ176408	
<i>Pararge aegeria aegeria</i>	27–8	Anti-Atlas oriental, Morocco	DQ176377	DQ176346
<i>Pararge aegeria aegeria</i>	27–9	Rif occidental, Morocco	DQ176392	
<i>Pararge aegeria aegeria</i>	27–11	Env. Maaziz, Morocco	DQ176391	
<i>Pararge aegeria aegeria</i>	27–12	Haut Atlas central, Morocco	DQ176376	DQ176345
<i>Pararge aegeria aegeria</i>	27–13	Atlas Tellien, Morocco	DQ176389	
<i>Pararge aegeria aegeria</i>	27–14	Moyen Atlas méridional, Morocco	DQ176397	
<i>Pararge aegeria aegeria</i>	27–15	Moyen Atlas méridional, Morocco	DQ176390	
<i>Pararge aegeria aegeria</i>	6–4	Madeira, Portugal	DQ176374	DQ176340
<i>Pararge aegeria aegeria</i>	6–5	Madeira, Portugal	DQ176381	
<i>Pararge aegeria aegeria</i>	6–6	Madeira, Portugal	DQ176385	
<i>Pararge aegeria aegeria</i>	6–7	Madeira, Portugal	DQ176393	
<i>Pararge aegeria aegeria</i>	25–7	Madeira, Portugal	DQ176378	DQ176347
<i>Pararge aegeria aegeria</i>	25–8	Madeira, Portugal	DQ176406	
<i>Pararge aegeria aegeria</i>	25–9	Madeira, Portugal	DQ176398	
<i>Pararge aegeria aegeria</i>	25–10	Madeira, Portugal	DQ176399	
<i>Pararge aegeria aegeria</i>	26–4	Madeira, Portugal	DQ176395	
<i>Pararge aegeria aegeria</i>	26–5	Madeira, Portugal	DQ176396	
<i>Pararge aegeria aegeria</i>	29–19	Madeira, Portugal	DQ176400	
<i>Pararge aegeria aegeria</i>	29–20	Madeira, Portugal	DQ176401	
<i>Pararge aegeria aegeria</i>	29–21	Madeira, Portugal	DQ176402	
<i>Pararge aegeria tircis</i>	10–8	Meerdalwoud, Belgium	DQ176410	DQ176341
<i>Pararge aegeria tircis</i>	17–13	Stockholm, Sweden	DQ176412	DQ176343
<i>Pararge aegeria tircis</i>	17–14	Stockholm, Sweden	DQ176416	
<i>Pararge aegeria tircis</i>	18–1	Stockholm, Sweden	DQ176414	
<i>Pararge aegeria tircis</i>	18–2	Stockholm, Sweden	DQ176417	
<i>Pararge aegeria tircis</i>	18–3	Stockholm, Sweden	DQ176415	
<i>Pararge aegeria tircis</i>	16–1	Oxford, U.K.	DQ176413	
<i>Pararge aegeria tircis</i>	16–2	Oxford, U.K.	DQ176418	
<i>Pararge aegeria tircis</i>	16–3	Oxford, U.K.	DQ176411	DQ176342
<i>Pararge xiphia</i>	3–12	Madeira, Portugal	DQ176355	
<i>Pararge xiphia</i>	5–1	Madeira, Portugal	DQ176353	DQ176330
<i>Pararge xiphia</i>	5–2	Madeira, Portugal	DQ176356	
<i>Pararge xiphia</i>	24–27	Madeira, Portugal	DQ176358	
<i>Pararge xiphia</i>	24–28	Madeira, Portugal	DQ176361	
<i>Pararge xiphia</i>	24–29	Madeira, Portugal	DQ176354	DQ176331
<i>Pararge xiphia</i>	24–30	Madeira, Portugal	DQ176359	
<i>Pararge xiphia</i>	24–31	Madeira, Portugal	DQ176357	
<i>Pararge xiphia</i>	24–32	Madeira, Portugal	DQ176360	
<i>Pararge xiphioides</i>	27–1	La Gomera, Canary Islands, Spain	DQ176366	DQ176332
<i>Pararge xiphioides</i>	27–2	La Gomera, Canary Islands, Spain	DQ176367	DQ176333
<i>Pararge xiphioides</i>	27–4	La Palma, Canary Islands, Spain	DQ176362	
<i>Pararge xiphioides</i>	27–5	La Palma, Canary Islands, Spain	DQ176363	
<i>Pararge xiphioides</i>	27–6	La Palma, Canary Islands, Spain	DQ176369	DQ176335
<i>Pararge xiphioides</i>	27–7	La Palma, Canary Islands, Spain	DQ176364	
<i>Pararge xiphioides</i>	28–18	La Palma, Canary Islands, Spain	DQ176365	
<i>Pararge xiphioides</i>	28–19	La Palma, Canary Islands, Spain	DQ176371	DQ176337
<i>Pararge xiphioides</i>	3–10	Tenerife, Canary Islands, Spain	DQ176368	DQ176334
<i>Pararge xiphioides</i>	28–16	Tenerife, Canary Islands, Spain	DQ176370	DQ176336
<i>Pararge xiphioides</i>	28–17	Tenerife, Canary Islands, Spain	DQ176372	DQ176338