Istituto Comprensivo Materna Elementare Media Convenevole da Prato, Prato, Italy

Speciation in Mediterranean refugia and post-glacial expansion of *Zerynthia polyxena* (Lepidoptera, Papilionidae)

Leonardo Dapporto

Abstract

Migration of populations to and from glacial refugia is responsible for various cases of speciation and subspeciation in Europe. The pattern of distribution and the degree of diversification between lineages originated by isolation in different glacial refugia usually depends on ecological traits, especially to their dispersal ability. *Zerynthia polyxena* is a philopatric species, scattered in small populations and rarely colonizing mountain areas. These characteristics probably caused repeated isolation during the Quaternary and may have favoured diversification. Actually two studies, based on both morphological and genetic data, suggest the existence of two highly distinct lineages in Europe having in Northern Italy their contact zone. In this study, I applied geometric morphometrics to male genitalia and demonstrated that (i) two morphotypes exist in Europe approximately facing on the two sides of the Po River; (ii) the two lineages probably survived glaciations in Italy and the Balkan Peninsula, respectively; then the Balkans lineage expanded to Central and Eastern Europe; (iii) no hybrid populations seem to exist in the contact area and, in one locality at least, the two lineages live in sympatry without any evidence of intermediates. These results suggest that (i) two sister species of *Zerynthia* exist in Europe. Accordingly, *Papilio cassandra* Geyer, 1828 is reinstated, as *Zerynthia cassandra* stat. rev., as the species to which the *Zerynthia* from Italy South of the Po River belong. Male genitalia differences with *Zerynthia polyxena* are described.

Key words: Biogeography – butterflies – Europe – speciation – glacial refugia – colonization routes – *Zerynthia cassandra* – *Zerynthia polyxena*

Introduction

Due to its peculiar latitude and topography, Southern Europe represents a model area to study consequences of Quaternary climatic oscillations on living organisms' phylogeography (Taberlet et al. 1998; Hewitt 1999, 2000, 2004; Schmitt 2007). During glaciations, Central European climate was too cold and dry for most temperate species. Many taxa survived cold periods at lower latitudes and altitudes in the three large peninsulas of Southern Europe: Iberia, Italy and the Balkans. These areas were isolated from each other during glacial maxima because of the presence of mountain chains representing extensive and insurmountable ice areas (Taberlet et al. 1998; Hewitt 1999, 2000). As a consequence, Southern European populations have been repeatedly isolated in the three peninsulas during Quaternary glacial stages. During warm periods, species tended to expand northwards to Central and Northern Europe, but sometimes mountain chains still represented important barriers. Comparative phylogeography shows that each taxon largely represents a unique case with its own contraction⁄expansion history (Taberlet et al. 1998), nevertheless some common patterns, referred to as colonization paradigms have been described (Hewitt 1999, 2000). In particular, the generalized high genetic discrepancy between Italian lineages and those from the remaining of Europe suggests that the Alps represented the main barrier to northward post-glacial expansions (Taberlet et al. 1998). Consequently, Italy retains many endemic lineages evolved by isolation and successively trapped by the Alpine chain (Taberlet et al. 1998). To a lower extent, the Pyrenees were also an obstacle to the post-glacial spreading of several Iberian populations (Hewitt 1999, 2000). In contrast, Balkan lineages did not face important barriers to their expansion and their genomes predominate in most Central European taxa (Taberlet et al. 1998; Hewitt 1999, 2000, 2004; Schmitt 2007).

In several butterfly species, glacial and postglacial isolation produced diversification among lineages belonging to different refugia (Schmitt 2007). However, in most cases this diversification did not produce taxa differentiated at the specific level (Porter et al. 1997; Schmitt et al. 2005). This is probably due to the relatively brief isolation periods and to the high dispersal ability of butterflies. Actually, when different butterfly lineages met during their expansion, they formed hybrid zones (Schmitt 2007). In Europe, hybrid zones are clustered in 'suture zones' mostly located between the Alps and the Pyrenees (Schmitt 2007).

The differing rates of colonization through geographical barriers are responsible for most of the observed distributions (Taberlet et al. 1998). Dispersal ability depends on several species traits and divergence among lineages is expected to be higher in taxa showing low mobility, strict habitat requirements and inaptitude to live at high altitudes (Schmitt et al. 2003; Dapporto and Dennis 2009). Indeed, in these cases, gene flow across mountain chains is drastically reduced in glacial periods and it might still be impossible in warmer periods.

The papilionid butterfly *Zerynthia polyxena* (Denis and Schiffermüller, 1775) offers an opportunity to study the consequences of long-term lineage isolation in butterflies. Indeed, this species is strictly linked to micro-habitats where the larval food plant (*Aristolochia* spp.) grows and it is very unusual to find vagrant individuals far from such areas (Verity 1947). Furthermore, it is well know that *Z. polyxena* is a thermophilic species rarely found at altitudes higher than 900 m (Higgins and Riley 1983; Tolman and Lewington 1997). Finally, it has a unique annual generation flying for few weeks. All these factors suggest that *Z. polyxena* may have experienced long-isolation periods in its glacial refugia during the Quaternary.

Intriguingly, two papers independently suggested the existence in Europe of two highly differentiated lineages of *Zerynthia*. Coutsis (1989) described differences in male genitalia between specimens belonging to Sicily and Florence

Corresponding author: Leonardo Dapporto (e-mail: leondap@gmail.com)

(Tuscany) and specimens from France, Northern Italy and Balkans. Subsequently, Nazari and Sperling (2007) highlighted deep genetic divergences between two populations from Sicily and Imola (about 75 km from Florence) and some populations from Russia, Ukraine and the Balkans (Kosovo, Montenegro, Serbia and Greece). Nazari and Sperling (2007) concluded that such high divergence is quite suggestive of a potential speciation event, but they refrained from making taxonomic conclusions due to the absence of comprehensive morphological investigations. On the other hand, Coutsis (1989) argued that two morphotypes are present in Italy and concluded that it is desirable to investigate potential geographical overlaps of the two forms and the possibility that hybrid populations exist.

In this study, male genitalia morphology of a large sample of *Z. polyxena* from Europe has been examined, mainly focusing on the supposed contact area between Italy, France and the Balkans. The geometric morphometrics approach based on landmark and sliding semilandmarks was used (Bookstein 1997). This method represents a powerful tool to obtain numerical data about genitalia shape, which are independent of several biasing factors such as subjective evaluation and overall size of studied specimens (Mutanen 2005; Mutanen and Pretorius 2007; Dapporto 2008; Dapporto and Strumia 2008). The aim of the study is to verify (i) if two morphologically distinct lineages of *Zerynthia* exist in Europe, (ii) how their distribution could have originated on the basis of the established paradigm patterns of glacial refugia and post-glacial range expansion, (iii) which kind of contact exists between the two lineages (i.e. if they form hybrid populations), and (iv) if the two morphotypes are concordant with the hypothesis of Coutsis (1989) and Nazari and Sperling (2007) that two sister species of *Zerynthia* exist in Italy.

Material and Methods

Study species

The southern festoon *Z. polyxena* is a papilionid species distributed from Southern France through Italy and Central Europe to Balkans, Russia and Asia Minor. Adults are conspicuously coloured in yellow/white with many black, red and blue spots. Because of the very high variability of wing pattern, dozens subspecies and forms, even belonging to single-local populations, have been described. *Zerynthia polyxena* feeds only on a few species of *Aristolochia*. As this plant is locally distributed in several regions, this butterfly is usually scattered in small and vulnerable populations. For this reason, it is a species of community interest listed in the Habitat Directive 92/43 EEC under annex IV. The flight period is extremely short ranging from mid-March to mid-May depending on altitude and latitude (Verity 1947; Higgins and Riley 1983; Tolman and Lewington 1997).

Study sample and genitalia preparation

A total of 186 males belonging to private and museum collections were examined (author's and Gabriele Fiumi private collections, Roger Verity collection in the Museo di Storia Naturale Università di Firenze; Prola collection in the Museo Civico di Storia Naturale di Roma; collection Staatliche Naturwissenschaftliche Sammlungen Bayerns). The study area was divided into the following seven regions: (i) Southern France (Nîmes $n = 9$; Ardèche $n = 1$; Toulon $n = 8$; Saint Crépin $n = 4$; Villeneuve de Loubet $n = 5$); (ii) Northern Italy (as defined by Balletto and Cassulo 1995) (Torino $n = 13$; Salbertrand $n = 10$; Vigevano $n = 6$; Vercelli $n = 10$; Reggio Emilia $n = 3$; Modena $n = 6$; Bologna $n = 3$; Ventimiglia $n = 1$; Monte Beigua $n = 11$; Rapallo $n = 1$; La Spezia $n = 8$; Forlì $n = 1$; Ravenna $n = 2$; Belluno $n = 3$; Trieste $n = 1$; Udine $n = 1$); (iii) Southern Italy (Forte dei Marmi $n = 4$; Pisa $n = 5$; Pontremoli $n = 4$; Livorno $n = 3$; Prato $n = 9$; Firenze $n = 4$; Pari $n = 1$; Roma $n = 1$; Fano $n = 1$; Monticchio $n = 2$; Foggia $n = 1$); (iv) Elba Island, $n = 5$; (v) Sicily (Castelbuono $n = 1$; Etna env. $n = 7$); (vi) Balkans (Bosnia and Herzegovina, Brod $n = 1$; Croatia, Dalmatia $n = 1$, Zara $n = 2$, Pula $n = 2$, Potravlje $n = 2$, Zagreb $n = 3$; Macedonia, Skopje $n = 2$); and (vii) Central and Eastern Europe (Czech Republic Lednice $n = 3$, Harrachov $n = 1$; Slovak Republic $n = 1$; Austria, Wien $n = 2$, Gumpoldskirchen $n = 1$, Kitzeck $n = 2$, Burgenland $n = 1$; Hungary, Budapest $n = 3$; Ukraine, Kharkov $n = 4$) (Fig. 1). The categorization into seven geographical areas may appear subjective, but it has only been used to draw maps and charts since no analyses based on *a priori* classifications have been carried out.

Genitalia were dissected using standard procedures (Dapporto 2008). Abdomens were boiled in 10% caustic potash. Genitalia were cleaned and the left valva was mounted in Euparal between microscope slides and cover slips. Genitalia were photographed using a Nikon Coolpix 4500 camera (Nikon, Tokyo, Japan) mounted on a binocular microscope.

Fig. 1. Map of the study area: samples from France (black triangles); Central/Eastern Europe (black circles); Balkans (black squares); Northern Italy (grey circles); Southern Italy (white circles); Elba island (white triangle); Sicily (white squares)
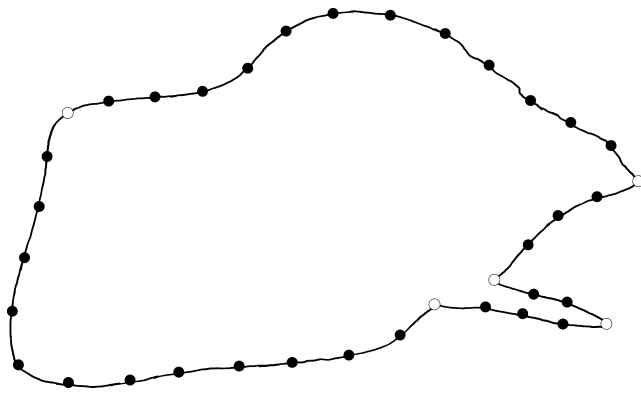
Fig. 2. Schematic representation of fixed landmarks (open circles) and sliding semi-landmarks (black circles) considered in geometrical morphometric analyses

Geometric morphometrics and statistical analyses

A combination of landmarks and sliding semi-landmarks was applied as in geometric morphometrics (Bookstein 1997). This method allows quantitative explorations and comparisons of shape. The thin-plate spline (TPS) series of programs was used for these analyses (Rohlf 2006a,b, 2007). The lateral sections of the valvae were examined (Fig. 2). Five points on the outline that could be precisely identified were considered as landmarks (type II and type III landmarks, Bookstein 1997), whereas the other points (sliding semi-landmarks) were allowed to slide along the outline trajectory to reduce uninformative variation (Bookstein 1997) (Fig. 2). Digital data for landmarks on genital photographs were carried out using TPSDIG 2.10 (Rohlf 2006a) and the definition of sliders using TPSUTIL 1.38 (Rohlf 2006b).

Generalized procrustes analysis was applied to the landmark data to remove non-shape variation in location, scale and orientation and to superimpose the objects in a common coordinate system (Bookstein 1997). Using the shape residuals from generalized procrustes analysis, partial warps were calculated, these are sets of variables containing shape information. Applying principal components analyses to partial warps, relative warps (RWs) were obtained. RWs can be used as variables in the following analyses. Furthermore, RWs can be visualized by thin-plate spline (TPS) deformation grids, which permit a visual comparison of shape differences. Generalized procrustes analysis, partial and relative, warp calculations and TPS visualization were carried out using TPSRELW 1.45 (Rohlf 2007).

To verify if the similarity pattern highlighted by geometric morphometrics has a statistical significance, I applied a K-Means clustering to the RW values. According to Nazari and Sperling (2007) and Coutsis (1989), two lineages seem to exist; for this reason, the formation of two clusters was imposed. To reduce the bias due to a high number of poorly informative variables, only the RWs explaining more than 1% of variance were included in K-Means (Dapporto 2008; Dapporto and Strumia 2008).

Results

After generalized procrustes analysis, 66 RWs were calculated. The RWs explaining more than 1% of variance were 11 (explaining a cumulative variance of 93.19%). A scatter plot of RW1 and RW2 (explaining respectively 37.01 and 19.63% of variance) revealed the presence of two discrete clusters of valva shape (Fig. 3). As shown by TPS deformation grids, both warps reflect extension and contraction of the distal tip of the valva forming a clear process in half of the specimens (left-up side of Fig. 3). Furthermore, RW1 also reflects differences in the length of the ventral valva process, while RW2 reflects its rotation respect to the tip of the valva. The shape variation showed by TPS is highly concordant with the description of Coutsis (1989). By maximizing and minimizing inter-cluster and intra-cluster differences respectively, K-Means identified two clusters. RW1, RW2 and RW8 revealed to be significantly different among the two clusters thus representing the variables responsible for the discrimination (Table 1). When compared with the scatter plot of Fig. 3, K-Means confirmed the validity of the two clusters. Indeed, all specimens in the left side of the dashed line of Fig. 3 are classified into cluster 1, while all the specimens on the right side are classified into cluster 2 (Fig. 3). Cluster 1 grouped all the specimens from Southern Italy, Sicily, Elba island and from several populations of Northern Italy. In particular, all specimens from Reggio Emilia, Modena, Bologna, Ventimiglia, Rapallo, La Spezia, Forlì, Ravenna and nine specimens from Monte Beigua are included in cluster 1. Cluster 2 grouped together specimens from France, Balkans, Central and Eastern Europe, all specimens from Northern Italy populations of Torino, Salbertrand, Vigevano, Vercelli, Belluno, Trieste, Udine and three specimens from Monte
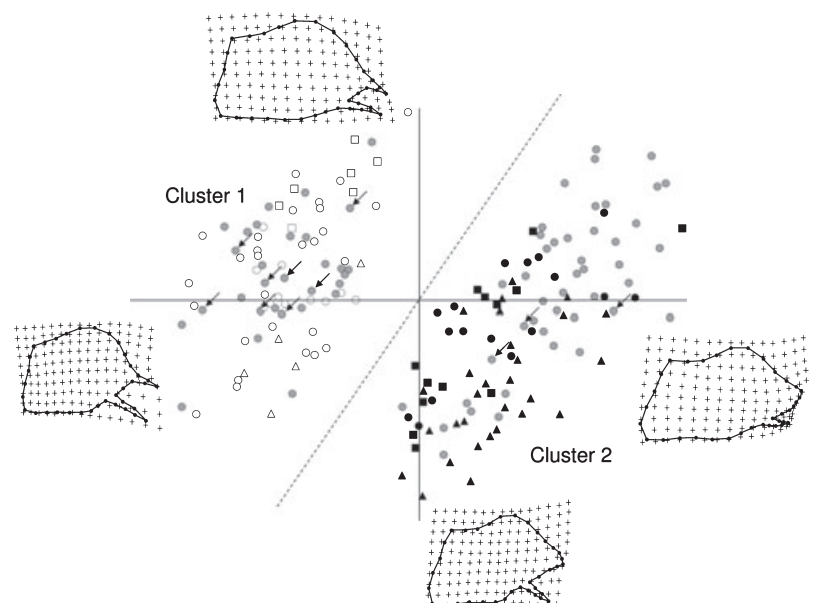
Fig. 3. Graphical representation of the first (*x*-axis) and of the second (*y*-axis) relative warps (RWs) of the valva analysis. Variations in shape along both axes are shown in thin-plate spline deformation grides. Dashed line indicates specimen separation into cluster 1 and cluster 2 obtained by K-Means. Arrows show specimens from Monte Beigua. Symbols for areas as in Fig. 1

Table 1. Anova table for K-Means clustering

	Anova	
Shape bariable	F	p-values
RW1	**575.584**	**0.000**
RW2	**22.628**	**0.000**
RW3	0.210	0.647
RW4	1.686	0.196
RW5	0.622	0.431
RW6	0.612	0.435
RW7	0.088	0.767
RW8	**7.110**	**0.008**
RW9	1.386	0.241
RW10	0.117	0.733
RW11	0.099	0.753

In bold variables showing significant differences between the two clusters.

Beigua (Fig. 4). There is thus evidence that each population hosts only one of the two lineages with the only exception of Monte Beigua where both morphs have been found (Fig. 4). However, also in this population there is no evidence of intermediate individuals and morphological differences among specimens belonging to the two lineages maintain the same order of magnitude (Fig. 3). The distribution of the two lineages shows a clear separation in Northern Italy with the Po River approximately representing the boundary line (Fig. 4).

Discussion

Zerynthia polyxena shows two clearly distinct morphotypes in Europe. Indeed, specimens can be separated into two clusters according to genitalia shape. The use of geometric morphometrics and K-Means avoided the use of any subjective shape evaluation and any *a priori* classifications of specimens, thus returning conservative results. Relative warps showing the highest significant values in K-Means also explain most shape variance, implying that most genitalia variation is involved in the differentiation between the two lineages. Separation of specimens into two clusters highlights a clear geographical pattern. Indeed, all specimens from Sicily, Elba and Southern Italy belong to the first cluster, while all specimens from Balkans, France and Central and Eastern Europe belong to the second cluster. All Italian specimens collected North to the Po River have been attributed to the second cluster, while specimens belonging to areas South to the Po River fall into the first cluster with the only exception of three specimens from Monte Beigua (Fig. 3). The observed postglacial distribution pattern of *Z. polyxena* in Europe (Fig. 5) highly resembles to the so-called 'grasshopper paradigm' (Hewitt 2000, 2004). *Chorthippus parallelus* (Zetterstedt, 1821), the common meadow grasshopper, is represented in Southern Europe by several lineages while in Central and Northern Europe, its haplotypes show low diversity and are similar to those of the Balkans (Hewitt 2000). The grasshopper paradigm is expected for species showing difficulties in crossing the mountain barriers represented by the Alps and Pyrenees. Indeed, propagulaes from the Balkans, not impeded by conspicuous mountain chains, easily expanded to Central Europe; populations from Italy and Iberia often remained trapped by Alps and Pyrenees. The main difference between the distribution pattern of *C. parallelus* and *Z. polyxena* seems to be the absence of an Iberian *Zerynthia* lineage. The Iberian *C. parallelus* are, however, strongly differentiated from the other lineages showing only limited hybridization in the Pyrenees, thus they are suggested to represent a sibling species (Butlin and Hewitt 1985). Intriguingly, the genus *Zerynthia* is represented in Iberia and the Maghreb by the closely related *Zerynthia rumina* (Linnaeus, 1758), which is found in exactly the same ecological niche than the other *Zerynthia* in the East. Therefore, *Zerynthia* genus seems to have many similarities with the *Melanargia galathea* (Linnaeus, 1758)/*Melanargia lachesis* (Hübner, 1790) and *Polyommatus coridon* (Poda, 1761)/*Polyommatus hispana* (Herrich-Schäffer, 1852) complexes (Schmitt 2007). Another difference between *C. parallelus* and *Z. polyxena* distributions emerges. The Southern Italian lineage

Fig. 4. K-Means attribution of specimens to the two lineages. Only the Monte Beigua sample (indicated by the arrow) shoes a mixed population. White circles, Southern Italian lineage, black circles Balkan lineage. White and black stars represent populations attributed to two different genetic lineages by Nazari and Sperling (2007)
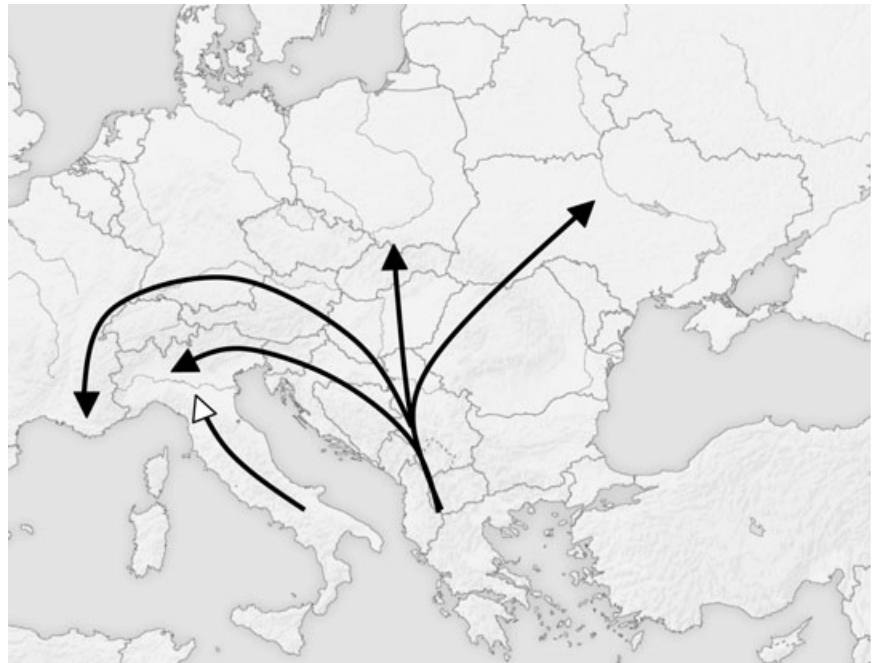
Fig. 5. Supposed post-glacial colonization routes for the two *Zerynthia* lineages

does not reach the southern slope of the Alps (except for the Mediterranean Ventimiglia population). There is no apparent barrier separating the two morphotypes as they approximately face on the two sides of the Po River. Despite the low mobility of *Z. polyxena*, the Po River cannot represent a barrier to its distribution because *Z. polyxena* is usually found in the swampy lowland areas of Italy (Verity 1947). Thus, the observed pattern is most likely the result of a large expansion of the Balkan lineage to Central Europe, France and Northern Italy, and a limited expansion of the Italian lineage along the Italian Peninsula. Most probably, during the last glacial maxima, the Italian lineage of *Z. polyxena* was restricted to the southernmost regions of the Italian Peninsula and Sicily.

In Northern Italy, where both lineages occur, there is no evidence for the occurrence of intermediate individuals. Indeed, Fig. 3 shows that Northern Italian specimens belonging to the two different lineages that are completely separated and shape differences are in the same order of magnitude as specimens from the other areas. Finally, on Monte Beigua, the two lineages are sympatric without any evidence of intermediate individuals (Figs 3, 4 and 7). In the sampling gap between the populations of both species in Northern Italy (Fig. 4), there might be other populations that might include both taxa flying together.

Mallet (2005) observed that about 12% of European butterfly species can hybridize, and actually, hybridization in post-glacial suture zones occurs in sister taxa recognized to be good species (e.g. *Melanargia galathea/Melanargia lachesis*, Habel et al. 2005 and literature therein). Hybridization is finally the rule between lineages diversified at subspecific or quasi-specific level. For example, *Pontia daplidice* (Linnaeus, 1758)/*Pontia edusa* (Fabricius, 1777), and different lineages of *Maniola jurtina* (Linnaeus, 1758) and *Erebia medusa* (Denis and Schiffermüller, 1775) show hybrid zones that can be recognized by transitional morphological and genetic traits (Porter et al. 1997; Schmitt et al. 2005; Schmitt and Müller 2007; Dapporto et al., in press). This seems not to be the case of *Zerynthia* in Italy where populations located only few

dozens of kilometres from each other show completely different genitalia morphology. Thin-plate spline configurations of RW1 and RW2 revealed that differences in valva shape between the two morphotypes are highly concordant with the description of Coutsis (1989), thus supporting his hypothesis of the presence of two *Zerynthia* species in Italy. Nazari and Sperling (2007) also supposed the existence of two *Zerynthia* species on the basis of genetic analyses. They observed a 2.4% sequence divergence between mtDNA of two Italian populations compared with those from Balkans and Eastern Europe. Despite the use of percent sequence divergence alone in defining species boundaries has been proven not adequate (Rubinoff and Holland 2005; Cognato 2006), a 2% divergence and even less is common between well-characterized sister species of Lepidoptera (Avise 1994; Hebert et al. 2003; Nazari and Sperling 2007). Nazari and Sperling also calculated the rate of mtDNA sequence divergence in *Zerynthia* to be between 2.3% and 3.1% per million years. Accordingly, the two lineages have diverged from a common ancestor about 1.0–0.8 MYA, approximately corresponding to the onset of the Günz glacial period of the Early Pleistocene (about 0.7 MYA).

It is inevitable to link the strong and ancient diversification of *Z. polyxena* to its ecological traits. Indeed, the degree of diversification among lineages is usually strictly linked to their dispersal and colonization capabilities (Taberlet et al. 1998). Schmitt et al. (2003) demonstrated that European populations of *Polyommatus icarus* (Rottemburg, 1775) do not show a regional split into genetically differentiated units. The authors suggested that *P. icarus* maintained an intact metapopulation structure in the Mediterranean area without strong diversification. Actually, *P. icarus* is a mobile butterfly, forming dense and continuous populations colonizing most landscapes from the sea level to 2900 m. Furthermore, it flies for about 7–9 months over several generations and the larva feeds on many plants (Tolman and Lewington 1997; Schmitt et al. 2003). *Zerynthia polyxena* represents an antipodal case as it is a sedentary butterfly showing a short-flight period, forming

Fig. 6. Males of *Zerynthia cassandra* (a–f) and *Zerynthia polyxena* (g–o). (a) neotype, Prato; (b) Etna, Sicily; (c,d) Monticchio; (e,f) Monte Beigua; (g) Potravlje; (h) Monte Beigua; (i) Villeneuve de Loubet; (j) Wien; (k) Vercelli; (l) Salbertrand; (m) Belluno; (n) Pula; (o) Lednice

small and scattered populations linked to a single plant genus and it is rarely found on mountain areas. Species having intermediate dispersal characteristics usually show different lineages in Europe with hybrid zones (Schmitt 2007). Future comparative studies will reveal possible correlation between species' ecological traits and their tendency to form and
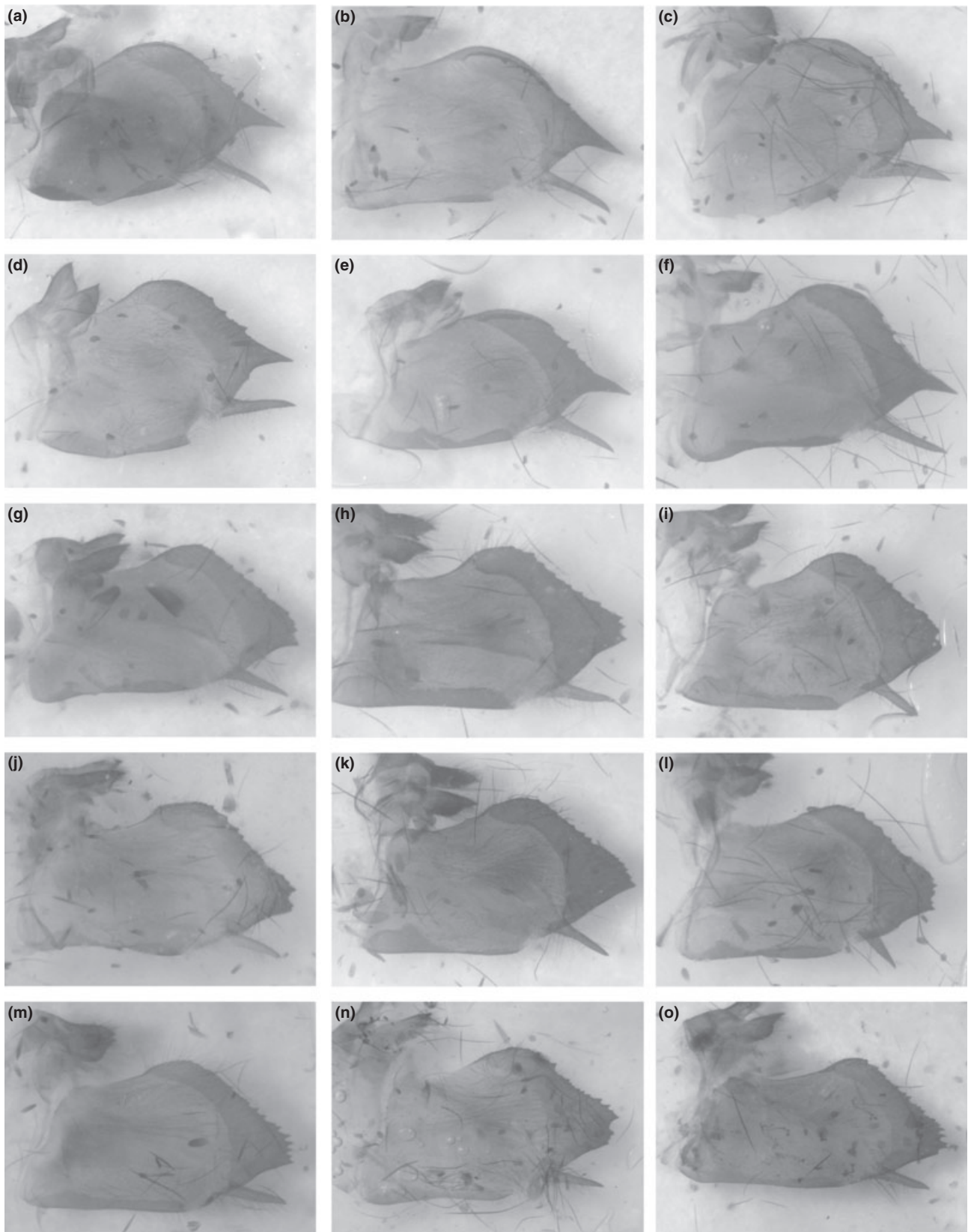
Fig. 7. Male genitalia of *Zerynthia cassandra*. (a–f) and *Zerynthia polyxena* (g–o). (a) neotype, Prato; (b) Etna, Sicily; (c,d) Monticchio; (e,f) Monte Beigua; (g) Potravlje; (h) Monte Beigua; (i) Villeneuve de Loubet; (j) Wien; (k) Vercelli; (l) Salbertrand; (m) Belluno; (n) Pula; (o) Lednice

maintain different lineages in Europe. In conclusion, there is genetic and morphometric evidence that there has not existed a gene flow between the two *Zerynthia* lineages since *ca.* 0.9 MYA (i.e. during many interglacial warm periods). The most obvious explanations can be the following: (i) they have not met in secondary contact until very recently; (ii) they met

in secondary contact, but they had already developed barriers to hybridization and gene flow; (iii) they met, limited gene flow existed, but hybrid populations have been later lost.

The high morphological and genetic divergence, the absence of intermediate populations and the existence of at least one area where the two lineages occur in sympatry, highly support the hypothesis that two *Zerynthia* sister species live in Europe, and it is thus necessary to revise the taxonomy of the two taxa.

Zerynthia cassandra (Geyer, 1828)

The type locality of *Z. polyxena* is Wien where the Balkan lineage occurs (Figs 3, 4 and 7). Many studies have been carried out describing several *Zerynthia* taxa in Italy on the basis of wing morphology. *Papilio cassandra* Geyer, 1828, and *Thais creusa* Meigen, 1829 are the oldest ones. These entities were described as distinct from *Z. polyxena* for the absence of a red spot on forewing apex and the presence of large black areas on both wings (Hemming 1934, 1937; Higgins and Riley 1983; Tolman and Lewington 1997). It has to be noted that to this subspecies were also referred France populations (Higgins and Riley 1983; Tolman and Lewington 1997) belonging to the Balkan lineage. Hemming (1934) revised the systematic position of these entities and indicated that these taxa should be considered as a distinct subspecies of *Z. polyxena*, and restricted the type locality of the two subspecies concluding that both *Papilio cassandra* and *Thais creusa* were based on specimens from Tuscany (Hemming 1934, 1937). In a first paper, Hemming (1934) also concluded that *T. creusa*, described in 1829 should be considered as the oldest name since he dated between 1827 and 1830 the Geyer table where *P. cassandra* was described. Successively, this hypothesis was revised by Hemming himself because new evidence belonging to a Geyer sale list of April 1828 proved that *P. cassandra* was described between July 1827 and April 1828 (Hemming 1937). Thus, *P. cassandra* should be considered as the older of the two names. Therefore, the peninsular Italian, Elba and Sicily populations are here considered to represent a distinct Italian endemic species which has to be named as *Zerynthia cassandra* (Geyer, 1828). The wing pattern is not a good discriminative trait because, as showed by Fig. 6, intraspecific differences are often larger than interspecific ones. Conversely, in *Z. cassandra* male genitalia have a prominent cylindrical and pointed process at the distal tip of the valva, always absent in *Z. polyxena*. The distal margin is less dentate and the ventral process of the valva (harpa) is usually longer and tighter than in *Z. polyxena* (Fig. 7). Aedeagus is fine and straight with clear basal enlargements.

So far as I have been able to establish, the type specimen or a series of *P. cassandra* are lost; there is no evidence of the existence of the Carl Geyer's butterfly collection in reference works concerned (Hagen 1862; Horn and Schenkling 1928–1929, Horn and Kahle 1935–1937, Horn et al. 1990). The specimens originally figured by Geyer (1828) were probably included in the collection of Jacob Hübner, destroyed by fire in Wien in 1848. Furthermore, although I found in Tuscany only *Z. cassandra* specimens, the existence of Tuscan localities inhabited by both *Z. cassandra* and *Z. polyxena* cannot be excluded. To secure the objective and unequivocal definition of the identity of *P. cassandra*, it is necessary to designate a neotype, following the Art. 75 of the Code. The neotype is a male labelled as follows: Italy: Prato: San Giorgio a Colonia: 20.V.2009, Leonardo Dapporto leg., deposited in the Museo di Storia Naturale dell'Università di Firenze 'La Specola', section Entomologia (Figs 6a and 7a).

Riassunto

Speciazione in differenti rifugi Mediterranei e espansione post-glaciale in Zerynthia polyxena (Lepidoptera, Papilionidae)

Le dinamiche glaciali e post-glaciali di contrazione ed espansione degli areali prodotto sono responsabili di buona parte dell'attuale differenziazione specifica e sottospecifica in Europa. I pattern di distribuzione e il livello di diversificazione fra linee genetiche originate dall'isolamento in diversi rifugi glaciali dipende in genere da caratteristiche ecologiche legate alle capacità dispersive delle singole specie. Infatti le capacità dispersive influenzano direttamente il flusso genico tra popolazioni. *Zerynthia polyxena* è una specie strettamente filopatrica, distribuita in piccole popolazioni isolate e raramente colonizza aree montuose. Queste caratteristiche possono aver favorito l'isolamento delle popolazioni durante i periodi freddi. Effettivamente due studi basati su evidenze genetiche e morfologiche hanno suggerito l'esistenza in Europa di due linee distinte che mostrano in Italia settentrionale la loro area di contatto. In questo studio ho applicato tecniche di morfometria geometrica ai genitali maschili di questa specie dimostrando che (i) due morfotipi sono effettivamente presenti in Europa e il fiume Po rappresenta approssimativamente l'area di contatto; (ii) i due morfotipi hanno probabilmente sopravvissuto all'ultima glaciazione in Italia e nella penisola balcanica. Successivamente le popolazioni balcaniche hanno occupato l'Europa centro-settentrionale, la Francia e parte dell'Italia settentrionale; (iii) le due linee non mostrano popolazioni con caratteristiche intermedie e, almeno in una località dell'Appennino ligure entrambi i morfotipi coesistono senza alcuna evidenza di ibridazione. Questi risultati sembrano quindi confermare l'ipotesi che (iv) due specie distinte di *Zerynthia* vivano in Italia. Di conseguenza, *Zerynthia cassandra* è elevata al rango di specie e ridescritta sulla base della morfologia dei genitali maschili.

References

Avise JC (1994) Molecular Markers, Natural History and Evolution. Chapman and Hall, New York.

Balletto E, Cassulo LA (1995) Lepidoptera Hesperioidea, Papilionidea. In: Minelli A, Ruffo S, La Posta S (eds), Checklist Delle Specie Della Fauna Italiana 89. Calderini, Bologna, pp 1–11.

Bookstein FL (1997) Landmark methods for forms without landmarks: localizing group differences in outline shape. Med Image Anal **1**:225–243.

Butlin RK, Hewitt GM (1985) A hybrid zone between *Chorthippus parallelus parallelus* and *Chorthippus parallelus erythropus* (Orthoptera: Acrididae): behavioural characters. Biol J Linn Soc **26**:287–299.

Cognato AI (2006) Standard percent DNA sequence divergence for insects does not predict species boundaries. J Econ Entomol **99**:1037–1045.

Coutsis JG (1989) Valval variation in *Zerynthia polyxena* (Denis and Schiffermüller) (Lepidoptera; Papilionidae). Ent Gaz **40:**281–282.

Dapporto L (2008) Geometric morphometrics reveal male genitalia differences in the *Lasiommata megera/*paramegaera complex (Lepidoptera, Nymphalidae) and the lack of a predicted hybridization area in the Tuscan Archipelago. J Zool Syst Evol Res **46:**224–230.

Dapporto L, Dennis RLH (2009) Conservation biogeography of large Mediterranean islands. Butterfly impoverishment, conservation priorities and inferences for an ecological "island paradigm". Ecography **32:**169–179.

Dapporto L, Strumia F (2008) The thorny subject of insular endemic taxonomy: morphometrics reveal no evidence of speciation between *Coenonympha corinna* and *Coenonympha elbana* butterflies (Lepidoptera: Nymphalidae). Zootaxa **1755:**47–56.

Dapporto L, Bruschini C, Baracchi D, Cini A, Gayubo SF, González JA, Dennis RLH. Phylogeography and counter-intuitive inferences in island biogeography: evidence from morphometric markers in the mobile butterfly *Maniola jurtina* (Linnaeus) (Lepidoptera, Nymphalidae). Biol J Linn Soc, in press.

Hagen HA (1862) Bibliotheca Entomologica. Die Literatur Über das Ganze Gebiet der Entomologie bis zum Jahre 1862. 1. Band A-M. Wilhelm Engelmann, Leipzig.

Hebert PND, Ratnasingham S, deWaard JR (2003) Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proc R Soc Lond B **270** (Suppl.)**:** S96–S99.

Hemming AF (1934) Revisional notes on certain species of Rhopalocera (Lepidoptera). Stylops **3:**193–200.

Hemming AF (1937) Hübner, a Bibliographical and Systematic Account of the Entomological Works of Jacob Hübner and of the Supplements Thereto by Carl Geyer, Gottfried Franz von Frölich and Gottlieb August Wilhelm Herrich-Schäffer, Vols 1& 2. Royal Entomological Society of London, London.

Hewitt GM (1999) Post-glacial re-colonization of European biota. Biol J Linn Soc **68:**87–112.

Hewitt GM (2000) The genetic legacy of the Quaternary ice ages. Nature **405:**907–913.

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. Phil Trans R Soc Lond B **359:**183–195.

Higgins LG, Riley ND (1983) A Field Guide to the Butterflies of Britain and Europe. Collins, London.

Horn W, Kahle I (1935-1937) Über Entomologische Sammlungen, Entomologen und Entomo-Museologie. Entomologische Beihefte. Deutsches Entomologisches Institut, Berlin.

Horn W, Schenkling S (1928-1929) Index Literaturae Entomologicae. Die Weltliteratur über die gesamte Entomologie bis inclusive 1863, 4 Bände. Berlin-Dahlem.

Horn W, Kahle I, Friese G, Gaedike R (1990) Collectiones entomologicae. Ein Kompendium über den Verbleib entomologischer Sammlungen der Welt bis 1960. Akad Landw Wiss DDR Teil I: A bis K.

Mallet J (2005) Hybridization as an invasion of the genome. Trends Ecol Evol **20:**229–237.

Mutanen M (2005) Delimitation difficulties in species splits: a morphometric case study on *Euxoa tritici* complex (Lepidoptera, Noctuidae). Syst Entomol **30:**632–643.

Mutanen M, Pretorius E (2007) Subjective visual evaluation vs. traditional and geometric morphometrics in species delimitation: a comparison of moth genitalia. Syst Entomol **32:**371–386.

Nazari V, Sperling FAH (2007) Mitochondrial DNA divergence and phylogeography in western Palaearctic Parnassiinae (Lepidoptera: Papilionidae): how many species are there? Insect Syst Evol **38:**121–138.

Porter AH, Wenger R, Geiger H, Scholl A, Shapiro AM (1997) The Pontia daplidice-edusa hybrid zone in northwestern Italy. Evolution **51:**1561–1573.

Rohlf FJ (2006a) TPSDIG, Version 2.10. Department of Ecology and Evolution, State University of New York at Stony Brook (http://life.bio.sunysb.edu/morph/).

Rohlf FJ (2006b) TPSUTIL, Version 1.38. Department of Ecology and Evolution, State University of New York at Stony Brook (http://life.bio.sunysb.edu/morph/).

Rohlf FJ (2007) TPSRELW, Version 1.45. Department of Ecology and Evolution, State University of New York at Stony Brook(http://life.bio.sunysb.edu/morph/).

Rubinoff D, Holland BS (2005) Between two extremes: mitochondrial DNA is neither the panacea nor the nemesis of phylogenetic and taxonomic inference. Syst Biol **54:**952–961.

Schmitt T (2007) Molecular biogeography of Europe: pleistocene cycles and postglacial trends. Front Zool **4:**11.

Schmitt T, Müller P (2007) Limited hybridization along a large contact zone between two genetic lineages of the butterfly *Erebia medusa* (Satyrinae, Lepidoptera) in Central Europe. J Zool Syst Evol Res **45:**39–46.

Schmitt T, Giessl A, Seitz A (2003) Did *Polyommatus icarus* (Lepidoptera: Lycaenidae) have distinct glacial refugia in southern Europe? Evidence from population genetics. Biol J Linn Soc **80:**529–538.

Schmitt T, Röber S, Seitz A (2005) Is the last glaciation the only relevant event for the present genetic population structure of the Meadow Brown butterfly *Maniola jurtina* (Lepidoptera: Nymphalidae)? Biol J Linn Soc **85:**419–431.

Taberlet P, Fumagalli L, Wust-Saucy AG, Cosson JF (1998) Comparative phylogeography and postglacial colonization routes in Europe. Mol Ecol **7:**453–464.

Tolman T, Lewington R (1997) Butterflies of Britain and Europe, Harper Collins, London.

Verity R (1947) Le Farfalle Diurne D'Italia 3. Divisione Papilonida. Famiglie Papilionidae e Pieridae. Marzocco, Firenze.