Rise and fall of island butterfly diversity: Understanding genetic differentiation and extinction in a highly diverse archipelago

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Dapporto L.[1,2]*, Cini A.[2]*, Menchetti M. [1,2]*, Vodă R.[3]*, Bonelli S.[3], Casacci L.P.[3], Dincă V.[1,4], Scalercio S.[5], Hinojosa J.C.[1], Biermann H.[6], Forbicioni L.[7], Mazzantini U.[8], Venturi L.[9], Zanichelli F.[10], Balletto E.[3], Shreeve T.G.[11], Dennis R.L.H.[11,12], Vila R.[1]

[1] Institut de Biologia Evolutiva (CSIC-Universitat Pompeu Fabra), Passeig Marítim de la Barceloneta 37, 08003, Barcelona, Spain

[2] Dipartimento di Biologia, Università degli Studi di Firenze, Via Madonna del Piano 6, 50109, Sesto Fiorentino, Florence, Italy

[3]Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università degli Studi di Torino, Via Accademia Albertina 13, 10123, Turin, Italy

[4] Centre for Biodiversity Genomics, Biodiversity Institute of Ontario, University of Guelph, Guelph, Ontario, N1G2W1, Canada

[5] Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Unità di Ricerca per la Selvicoltura in Ambiente Mediterraneo, Contrada Li Rocchi, 87036 Rende, Italy

[6] Markusstrasse, 17D-33014 Bad Driburg, Germany

[7] Via Roma 6, 57037, Portoferraio, Italy

[8] Legambiente Arcipelago Toscano, 57037, Portoferraio, Italy

[9] Parco Regionale della Maremma, via del Bersagliere 7/9, 58100, Alberese (GR), Italy

[10] Parco Nazionale Arcipelago Toscano, località Enfola, 57037, Portoferraio, Italy

[11] Centre for Ecology, Environment and Conservation, Department of Biological and Medical Sciences, Oxford Brookes University, Headington, Oxford OX3 0BP, UK

[12] Institute for Environment, Sustainability and Regeneration, Staffordshire University, College Road, Stoke-on-Trent ST4 2DE, UK

Dapporto L.: leondap@gmail.com; Cini A.: ciniales@gmail.com; Menchetti M.: mattiamen@gmail.com; Vodă R.: raluvoda@gmail.com; Bonelli S.: simona.bonelli@unito.it; Casacci L.P.: luca.casacci@unito.it; Dincă V.: vlad.e.dinca@gmail.com; Scalercio S.: stefano.scalercio@crea.gov.it; Hinojosa J.C.: sirsphingidae2@gmail.com; Biermann H.: biermannbd@gmx.de; Forbicioni L.: jjuniper8@gmail.com; Mazzantini U.: legambientearcipelago@gmail.com; Venturi L.: legambientearcipelago@gmail.com; Zanichelli F.: sagittarius.sagitta@libero.it; Balletto E.: emilio.balletto@unito.it; Shreeve T.G.: tgshreeve@brookes.ac.uk; Dennis R.L.H.: rlhdennis@aol.com; Vila R.: roger.vila@csic.es

* These authors contributed equally to this work

Corresponding author: Raluca Vodă, raluvoda@gmail.com

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ABSTRACT

Aim

We describe fine-scale diversity patterns of the entire butterfly fauna occurring on the Tuscan Archipelago. By assessing the traits associated with population diversification, haplotype uniqueness and extinction, we aim to identify the factors determining the origin and maintenance of genetic diversity, and population vulnerability to environmental changes.

Location

Tuscan Archipelago, Sardinia, Tuscany (Italy) and Corsica (France).

Methods

We built a mtDNA dataset (1303 COI sequences) for the 52 butterfly species reported in the Archipelago, also including specimens from neighbouring areas, and compiled data on 12 species traits and on the apparent extinction of species from the main islands. We calculated indices that measure genetic differentiation, and using phylogenetic regressions we evaluated the relationships between these indices and species traits. Finally, we inferred which traits are associated with disappearance of species on individual islands using phylogenetic regression.

Results

The overall spatial pattern of genetic diversity corresponded with the proximity of the areas, but strong contrasts were also identified between geographically close areas. Together with the island endemics, several common and widespread species had a high genetic diversification among islands and mainland. Phylogenetic regressions revealed that smaller-sized, more specialized species, with a preference for drier regions, displayed greater genetic structure and/or haplotype uniqueness. Species that disappeared from islands had a higher population diversification. Capraia has experienced a notable loss of diversity, which significantly affected species with shorter flight periods.

Main conclusions

Tuscan island butterflies are characterized by strong genetic contrasts and species differ in their contribution to the overall genetic diversity. By ranking the species for their contribution to genetic diversity and identifying the traits linked to the emergence and maintenance of diversity, we have developed a valuable tool for prioritizing populations as targets for monitoring and conservation action. The dataset constructed also represents a valuable resource for testing biogeographical hypotheses.

Keywords

Conservation biogeography, DNA barcoding, Lepidoptera, Phylogeography, Species traits, Tuscan Archipelago

INTRODUCTION

The worldwide biodiversity crisis calls for the identification, prioritization and protection of biodiversity hotspots, and understanding how biodiversity is generated and lost determines the success of this endeavour (Dirzo et al., 2014; Venter et al., 2014). The wealth of information now available in the "big data era" greatly facilitates these efforts, especially the ability to work at an unprecedented resolution (Hampton et al., 2013). For conservation biogeography this includes the increased availability of molecular, occurrence and trait data for various organisms (Ladle & Whittaker, 2011; Fernández-Palacios et al., 2015).

Islands host a disproportionate fraction of global biodiversity, often characterized by distinctive faunas (Whittaker & Fernández-Palacios, 2007), and therefore represent a model system to better understand general patterns in biogeography. Insular populations are typically exposed to high risks of extinction since restricted habitats can easily become unsuitable because of stochastic events or human disturbances acting at both local (habitat fragmentation, alteration, invasion by alien species) and global scales (e.g. climate change) (Fordman & Brook 2010). Understanding the factors that drive the emergence, maintenance and loss of island biodiversity is crucial for planning and implementing evidence-based conservation prioritization and protection measures (Ladle & Whittaker, 2011).

Remote oceanic islands experience infrequent colonization events followed by genetic drift and adaptive radiation (Rosindell & Phillimore, 2011). In contrast, biotas on less isolated islands are mostly assembled following frequent events of colonization and extinction, which produce nested communities of the source species, with few endemics (Whittaker & Fernández-Palacios, 2007). However, a pace of extinction and colonization on islands (turnover) slower than the dynamics occurring in the surrounding regions can generate intraspecific diversification and relictuality, which creates community distinctiveness among islands and from neighbouring mainland (Masini et al., 2008; Dapporto et al., 2012). Such diversity is usually represented by cryptic species or genetic lineages that can be detected only after in-depth molecular and/or morphologic analyses (Hernández-Roldán et al., 2016; Vodă et al., 2015a, 2016).

The occurrence of endemic and relict populations can be the result of deterministic processes, largely affected by species characteristics. According to a widely accepted paradigm of island conservation biogeography, genetic diversification and extinction probability of island populations are inversely related to their degree of mobility and generalism (Burney & Brumfield, 2009; Dennis et al., 2011; Salisbury et al., 2012; Dawson et al., 2014, but see also Kobayashi & Sota, 2016 for different patterns). This hypothesis has profound implications for conservation, because populations with unique genetic fingerprints are clearly irreplaceable, but potentially suffer from high extinction probability (Ricklefs, 2009). Nevertheless, this hypothesis has been rarely tested (Burney & Brumfield, 2009; Vodă et al., 2016), probably because of the absence of integrative datasets combining molecular and long-term occurrence data with species traits for entire species-rich taxonomic groups and from a large geographic area.

Here we tested if species having low dispersal capacities and restricted ecological requirements tend to colonize islands at a slower rate, experience reduced gene flow and thus have a higher diversification rates and a higher likelihood of local extinction compared with more mobile and generalist species. We designed an integrated framework (Fig. 1) based on a dataset comprising: i) a revised distribution list of the 52 butterfly species reported for the Tuscan Archipelago and the apparent disappearance of several species in the last 115 years, ii) 1303 cytochrome *c* oxidase subunit I (COI) sequences (658 bp) for all the species, including populations from the surrounding areas (Sardinia, Corsica and Tuscany), and iii) 12 species traits related to mobility, phenology, trophic generalism and climatic preferences. The analysis of this dataset allowed us to: i) describe the patterns of population diversification (genetic differentiation and haplotype uniqueness) among islands and neighbouring areas, ii) identify the functional traits that are correlated with the emergence of diversification and species disappearance on islands. Finally, iii) we ranked the species according to their contribution to the overall island diversity. Our integrated framework (Fig. 1) allowed us to provide evidence-based guidelines for butterfly conservation in a well-known Mediterranean diversity hotspot (Dennis et al., 2008).

METHODS

Study area and occurrence data

We carried out annual surveys of the butterflies of the Tuscan islands and the neighbouring areas of Sardinia, Corsica and Tuscany between 2000 and 2015 (Fig. 1a). We sampled the main biotopes from early spring to late autumn, with different tools (mostly Malaise traps and insect nets) and compared occurrence data from our surveys with published records dating back to 1900 (Appendix S1). Sampling was not done constantly throughout the 15 years but our extensive collections represent the most intensive sampling effort done on the butterflies of these islands, therefore it is reasonable to assume that a species reported in the past but not during the last decade has either become extinct or has significantly declined. We restricted our analysis of species disappearance to the three largest islands: Elba, Capraia and Giglio, for which sufficient historical data were available.

Genetic assessment

Using standard sequencing procedures (deWaard et al., 2008), we obtained 1303 COI sequences for specimens belonging to the 52 butterfly species reported for the Tuscan islands between 1900 and 2015, including specimens from Sardinia, Corsica, Argentario and four mainland areas (Fig. 1a). We also used 2940 sequences from other parts of Europe and Asia, mined from BOLD and GenBank. Information about specimens and sample size for each population is available in the "dataset.csv" file (Appendix S2). Sequences were aligned in Geneious 6.0.6 (www.geneious.com). All 4243 sequences used in this study are publicly available in “DDI – Tuscan Islands” (DS-DIDI, doi…) on BOLD at www.barcodinglife.org.

The butterfly species currently recognized by taxonomists, and for which trait data are available, show varying levels of intraspecific genetic divergence, including potential cases of cryptic taxa (Dincă et al., 2015). We considered as separate units most species recognized by the widely accepted checklist of the Fauna Europaea Project (Karsholt & Van Nieukerken, 2013; www.faunaeur.org) and also considered as single units taxa displaying a minimum COI p-distance lower than 3%. In butterflies, this threshold separates more than 90% of the recognized species (Hebert et al., 2003) and a recent study on Sicilian islands confirmed this observation (Vodă et al., 2016). We also repeated all the analyses without setting any distance threshold, and considered as single units only the species recognized by Fauna Europaea.

We calculated the genetic uncorrected p-distances among all sequenced specimens for each species by using the function “dist.dna” of the “ape” R package. We preferred p-distance to tree-based genetic distances because, at the intraspecific level, coalescence has not taken place and distances calculated on branch lengths of bifurcating phylogenetic trees do not properly reflect the reticulated evolutionary processes (e.g. Posada et al., 2001). Moreover, recent reviews indicate that p-distances are the best option in the analysis of COI data compared to other indices (Srivathsan & Meier, 2012). Based on p-distances, we obtained two measures for population differentiation: Dst and Gst (Nei, 1987). We also calculated the pairwise Gst among all pairs of populations for each species (see Appendix S1 for a description of the indices).

Based on the Gst pairwise matrices we produced for each species the mean Gst matrix, representing the degree of differentiation among areas based on all species. A Principal Coordinate Analysis (PCoA) was applied to this matrix to obtain a two dimensional representation of the overall diversity pattern among areas. Subsequently, we aligned this configuration with the geographic location of the areas by using the "procrustes" analysis from the "vegan" R package and tested the correlation between the PCoA configuration of Gst and the spatial location by using the vegan function “protest”. To visualize the pattern of similarity among islands in the geographic space, we projected the PCoA configuration in the RGB space using the R package “recluster” (Dapporto et al., 2014). The colour resemblance in the resulting configuration is directly proportional to the genetic similarity among the communities.

Four species (*Leptidea sinapis*, *Aglais urticae*, *Nymphalis polychloros*, *Argynnis paphia*) recorded for Tuscan islands in the last century but not during our surveys have also been analysed by computing the Dst and Gst between Tuscany, Sardinia and Corsica to estimate the fraction of diversity that has presumably disappeared.

Island haplotype uniqueness for each species was calculated as:

$$Uni = \sum_{h=1}^{n} \frac{\min(D_{h,m})}{occ}$$

where h are the haplotypes found on the Tuscan islands and not recorded on mainland areas of Europe and Asia, $\min(D_{h,m})$ is the p-distance between the h^{th} haplotype and the genetically closest haplotype from mainland, and occ is the number of islands (Tuscan islands, Sardinia and Corsica) where the h^{th} haplotype has been found. Uniqueness for a given species is higher when: 1) there are many insular endemic haplotypes, and/or 2) they show high divergence with respect to the closest mainland haplotype and/or 3) they are found in fewer islands. Uniqueness values (Uni) were calculated for each species occurring on any island except for the island endemics that do not have mainland populations (*Hipparchia aristaeus* and *H. neomiris*).

To assess the importance of each species for the diversity of the Tuscan Archipelago we ranked species according to Gst, Dst and Uni and subsequently summed the ranks. The patterns of genetic variation were also analysed by inferring maximum parsimony haplotype networks using the program TCS 1.21, with a 95% connection limit for all species except *H. neomiris* (94% connection limit) and *Coenonympha corinna* for which we used a fixed connection limit of 23 steps (Clement et al., 2000).

Species traits and phylogenetic regressions

Review studies suggest that only morphological, physiological or phenological features that can be measured on individual organisms and without reference to the environment or any other level of organization should be considered as functional traits (Violle et al., 2007; Moretti et al., 2016). Moretti et al. (2016) identified a series of 29 functional traits to cover the primary functions of invertebrates, divided into five major groups: morphology, feeding, life history, physiology and behaviour. Measuring the 29 traits on individuals for all the studied species would have entailed a major long-term effort that was beyond the scope of this study. Thus, based on literature data and personal observations, we assessed 12 species traits representing four of the five groups (excluding behaviour) identified by Moretti et al.

(2016): but encompassing morphology, feeding, life history and physiology. For each trait we formulated functional hypotheses (Table 1): a) Trophic generalism (feeding), was identified as i) the number of host plant genera reported in the literature; b) Mobility was assessed with morphological traits represented by ii) wingspan and obtained as the average between minimum and maximum size reported in literature; c) Phenology (life history traits) was identified as iii) the length of the flight period, iv) the first month when adults emerge, v) the last month when adults fly, and vi) voltinism. Finally, d) Climatic preference and tolerance (physiology) were assessed by proxy variables for eco-physiological responses to environmental conditions. These variables have been calculated by Schweiger et al. (2014) by modelling species distribution in Europe based on occurrence data, and then by averaging temperature and precipitation among the spatial cells where each species is predicted to occur. Although these indices cannot be considered as strict functional traits since they are obtained from the geographic distribution of the species (Violle et al., 2007), they are widely recognized as proxies for the traits responsible for eco-physiological responses to climate (e.g. Devictor et al., 2012). The variables we included are: vii) mean annual temperature viii) and precipitation, ix) standard deviations of the temperature mean and x) and precipitation, xi) upper 95% confidence limit of temperature mean, and xii) lower 95% confidence limit of precipitation mean.

Butterfly traits are usually highly inter-correlated but they can be conveniently reduced to factors by using ordination methods (Carnicer et al., 2013; Dapporto & Dennis, 2013). For morphology, life history and physiology traits we applied a Principal Component Analysis (PCA), using the R function “rda” and the components with eigenvalues higher than one have been used as variables for successive analyses. Some literature sources did not report the wingspan for all the studied species and we imputed the missing values by using the “mice” function of the “mice” R package (see Appendix S2). The algorithm imputes an incomplete variable by generating plausible values based on other variables in the data by Multivariate Imputations by Chained Equations (MICE) (Van Buuren & Groothuis-Oudshoorn, 2011).

The existence of a phylogenetic signal for the variables of each trait following the PCA ordination and for Dst, Gst and Uni was tested with Pagel’s lambda index by applying the “phylosig” R function of the "phytools" package. We used a Maximum Likelihood (ML) phylogenetic tree based on COI sequences for all the western Mediterranean butterflies – freely available in the package “recluster” (Dapporto et al., 2013)– as reference phylogeny. The tree was inferred with topological constraints at family and subfamily levels (see Appendix S1 for details). ML analyses were performed using RAxML BlackBox (Stamatakis et al. 2008). A GTR+Gamma+I model was selected and node supports were assessed through 100 rapid bootstrap replicates. Gst, Dst and Uni have been mapped onto the phylogenetic tree by using the “contMap” function of the “phytools” package. Character mapping was accomplished by estimating states at internal nodes using maximum likelihood and then by interpolating the states along each edge (Revell, 2013). The relationships between the variables and Dst, Gst and Uni have been assessed using phylogenetic stepwise regressions. We also employed Pagel's lambda as a model for the phylogenetic covariance of residuals and applied a two-way selection of variables based on the Akaike Information Criterion (AIC) as implemented in the function “phylostep” of the package “phylolm”. From the phylogenetic regressions we removed the species not recorded for the Tuscan Archipelago during the study period since no DNA sequences were available. We square-root transformed Dst and Uni to improve their normality and standardized the values of the traits with zeta-scores to provide a balanced contribution to the phylogenetic regression.

The importance of traits in explaining possible extinctions of butterflies on two islands (Elba and Capraia) has been assessed with a logistic phylogenetic Generalized Linear Model using the function “phyloglm” of the package “phylolm”, in which species found during our surveys and species that have not been confirmed, represented the binary response variable, and the trait variables the predictors. Logistic GLM was not performed for Giglio as only three species disappeared on this island. Effect size for models has been evaluated by plots of observed vs fitted values associated with Spearman rho correlation. “Phylolm” function was used to assess if species disappeared in at least one island showed higher values of Gst, Dst and Uni.

RESULTS

Based on the taxonomy proposed by Fauna Europaea and by applying a 3% threshold of COI divergence, we identified 52 units among the taxa reported in literature for the Tuscan Islands (hereafter ‘species’, Table 2). During our surveys on these islands we recorded a total of 46 species. The comparison between observations during the last decade and literature data from 1900 for Elba, Giglio and Capraia, identifies those species (see Table 2) that probably became extinct or strongly declined on these islands (respectively six, three and seven on Elba, Giglio and Capraia).

Dst was correlated with both Gst (Spearman rank test: rho 0.836, $P<0.001$) and Uni (rho 0.460, $P<0.001$), while Gst was not correlated to Uni (rho 0.192, $P=0.213$). Gst values showed an almost bimodal distribution (14 species with $Gst<0.25$ and 10 species with $Gst>0.75$, Table 2). Twenty-four species had haplotypes not recorded on the mainland and most of these species did not belong to endemic taxa (Table 2). Gst and Uni did not have a significant phylogenetic signal (lambda 0.218; $P=0.314$ and lambda <0.001; $P=1.000$, Fig. 2 and S51-

S52), while Dst had a significant effect (lambda 0.334; P=0.034; Fig. 2). Ordering species by the sum of ranks of the three indices (Dst, Gst and Uni) showed that, together with endemics, several common and widespread species provided a large contribution to diversity. The first quartile of the top ranking species comprised four species/groups with endemic elements in the Tuscan islands (*C. corinna*, *Lasiommata megera/paramegaera*, *H. neomiris*, *Aglais urticae/ichnusa*) and ten widespread species, most of them ubiquitous in Europe and not included in any protection list (Table 2).

The wingspan measures reported in the four literature sources used were highly correlated (Pearson R>0.9 for all pairs) and the PCA identified only one component with an eigenvalue higher than one (Table 1, Figure S46). For life history and physiologic traits two components were considered (Table 1, Figure S47). The first component of phenology was mainly linked to the length of the flight period (voltinism, number of months when adults occur), while the second was mainly linked to seasonality (first and last month of emergence). The first component for physiologic traits ordered species from those experiencing high temperatures and low precipitation to those living in colder and wetter areas, while the second component ordered species mostly according to their precipitation tolerance (Table 1, Figure S48). The six resulting variables showed a lower correlation among each other with Pearson correlation values always lower than 0.400 (Table S1). Among the six resulting variables, the number of host plants, phenology PC1, physiology PC1 and PC2 did not show a phylogenetic signal (lambda 0.282; P=0.228; lambda<0.001; P=1.000; lambda 0.062; P=0.711; lambda<0.001; P=1.000, respectively), while wingspan PC1 and phenology PC2 showed a significant effect (lambda 1.187; P<0.001; lambda 1.083; P<0.001, respectively).

The overall spatial pattern of genetic variation based on Gst corresponded to the proximity of the areas but with a rather low level of correlation (protest correlation 0.592, P=0.015, Fig. 3a,b).

The AIC procedure for the stepwise phylogenetic regression for Gst selected a model with four variables, but only mobility and trophic generalism had a significant effect (Table 3), meaning that smaller-sized and more generalist species had a higher Gst. For Dst, four variables entered the model - three were significant and showed that smaller-sized, more generalist species and those experiencing less annual precipitation had a higher variation (Table 3). Two variables entered the Uni model showing that species living in drier areas significantly had higher haplotype uniqueness (Table 3), while trophic generalism entered the model but without significant effect. Plots for observed vs fitted values of the three models (Fig. S53) showed large residuals indicating that Gst, Dst and Uni are only weakly explained by the measured traits (Spearman rho: Gst 0.537, Dst 0.430, Uni 0.368).

Species that had disappeared from at least one island showed significantly higher values of Dst, while no differences in Gst and Uni were found (Table 3). According to the logistic phylogenetic GLM, species that disappeared from Capraia had lower values in PC1 for phenology, corresponding to shorter flight periods (Table 4). A plot for observed vs fitted values (Fig. S54) revealed a good fit for this analysis (Spearman rho 0.798). For Elba we found no significant effect explaining the disappearance of the six species (Table 4).

The analyses in which we used only the taxonomy from Fauna Europaea returned very similar results to the ones in which we used a 3% threshold for species identification (see Table S2-S5 and Figure S55 in Appendix S1).

DISCUSSION

The integration of an updated taxonomic list, species occurrence spanning across 115 years (1900-2015), mitochondrial DNA sequences and species traits, allowed us to characterize the butterfly diversity in the Tuscan Archipelago at an unprecedented resolution and to infer which species traits explain the rise and decline of butterflies in this archipelago.

An area of biogeographic contrasts

The Tuscan Archipelago is an insular hotspot for butterfly diversity and stands out among European islands for hosting far more endemics than would be expected based on their geography (e.g. area and isolation) (Dennis et al., 2008), resulting in the highest priority for butterfly conservation among the circum-Italian islands (Dapporto & Dennis, 2008). Its unexpected level of endemicity and richness is a consequence of its intermediate location between Tuscany and the Sardo-Corsican region, generating a double filtering effect (Dapporto & Cini, 2007; Fattorini, 2009).

We found that a main determinant for the genetic make-up of populations is island location (see the protest analysis), the same as for the community composition at species level (Dapporto & Cini, 2007). Nevertheless, a model based only on a double filtering effect is not sufficient to explain the observed degree of genetic diversity. In fact, we identified strong contrasts between geographically close areas, such as between Montecristo (very similar to the Sardo-Corsican region) and Pianosa (more similar to Elba and the Italian Peninsula), which are separated by 30 km; between Capraia and Elba (same pattern as before, distance 33 km) and between Giglio and Argentario, separated by only 14 km (Fig. 1 and 3). Striking divergence among populations from nearby areas is unexpected in butterflies characterized by a high mobility resulting in a high capacity to track suitable environments (Wilson et al., 2010; Waters, 2011; Devictor et al., 2012). However, in the western Mediterranean (Tuscan islands included), chequered distributions of sister species and genetic lineages are a common phenomenon, probably due to the combination of several historical determinants and contemporary ecological forces (e.g., the connection between land masses during the Last Glacial Maximum, density-dependent phenomena, differences in climatic and environmental tolerances, Vodă et al., 2015a,b, 2016). Accordingly, more than 30% of the examined species, comprising both endemic and widespread taxa, had a Gst value higher than 0.5.

Endemic taxa also have unexpected patterns of intraspecific genetic diversity among islands. *Coenonympha corinna/elbana* is highly divergent among the three clades they form in: i) Tuscany, Elba and Giannutri; ii) Capraia and Corsica; and iii) Sardinia. This pattern only partially supports the current taxonomic separation into two species or subspecies (*C. corinna*: Sardinia, Corsica and Capraia; *C. elbana*: Elba, Tuscany and Giannutri) (Fig. 3c). *Hipparchia neomiris*, an endemic species from Sardinia, Corsica and Elba, also displayed notable intraspecific divergence. COI sequences from Elba and Corsica are differentiated by at least 2% compared to conspecific individuals from Sardinia. By contrast, no genetic diversification was detected in the *Plebejus idas* group, since the endemic *P. bellieri* from Sardinia and Corsica (recognized as a good species in Fauna Europaea) shared COI barcodes with the population from Elba, treated as a species in some works, and with the mainland populations, elsewhere reported as *P. idas* or *P. abetonicus* (Balletto et al., 2015).

Phylogenetic regressions suggest that the species adapted to a dry climate, the small-sized ones and the host plant specialists have a higher degree of island uniqueness and of population diversification among islands. According to our hypotheses, these species traits can facilitate the emergence and maintenance of these genetic contrasts since typical Mediterranean species thrive on islands, while species with reduced dispersal and poor colonization capabilities probably experience reduced gene flow.

Several species previously recorded on three Tuscan islands were not observed in the last 10 years, suggesting that they became extinct or declined considerably (Table 2). On Giglio only three species have not been recorded during the last decade, but they include *C. corinna,* the most emblematic species for the archipelago, as well as *A. agestis* and *Polyommatus icarus*, both showing high levels of population diversification (Table 2).

Elba has apparently lost six species. In this case as well, some of the taxa have diversified populations in the study area (*Aglais urticae/ichnusa*, *Leptidea sinapis* and *Nymphalis polychloros* showed a Gst higher than 0.4). For this island we found no species traits correlated with disappearance and the fraction of likely extinct species (11.5%) was lower than on Capraia where 30% of the species reported in the past have disappeared.

Two species that disappeared from Capraia were insular endemics (*H. neomiris* and *H. aristaeus*) and others showed divergent populations in the study area (*P. cecilia*, *L. phlaeas*, *M. jurtina*). The species that went extinct represented a fraction of the fauna with a short flight period. As the length of the flight period is correlated with inter-island dispersal in the Tuscan Archipelago (Dapporto et al., 2012), species with a short flight period have a lower probability of re-colonizing from surrounding areas. Interestingly, there are no typical springtime species on Capraia and taxa with short flight periods are typically monovoltine, with adults emerging at the beginning of summer, aestivating during the hottest weeks and laying eggs in September/October. Aestivation in the Mediterranean region is known for *M. jurtina* (Scali, 1971) and *Hipparchia semele* (García-Barros, 1988), a species closely related to *H. aristaeus*. Both of them have disappeared from Capraia, together with two other Satyrinae (*P. cecilia* and *H. neomiris*), which tend to be frequent in woodlands and scrub/maquis during the hottest and driest months. Because there are barely any remaining woods on Capraia, aestivation may represent an important stress period with current temperature increases (Shreeve et al., 2009; Cerrato et al., 2016). Compared to Elba and Giglio, Capraia is more isolated and this could have hampered a rescue effect for many species. Moreover, Elba has the highest mountain peak among the small Italian islands (Monte Capanne, 1019m) and woodlands are common on both Giglio and Elba. The higher environmental heterogeneity of these two islands could have provided a wider range of suitable areas for many species under environmental stress and climatic oscillations.

Extinction events over long periods are expected on islands based on the equilibrium theory (MacArthur & Wilson, 1967), but they should be paralleled by colonization events, which was not the case for the Tuscan islands. On Elba only *Anthocharis cardamines* has been discovered after 1950; on Capraia only *P. aegeria* and *A. agestis* have been found after intensive field research between 1970-1980, when almost all the seven extinct species were present; only *C. rubi* has been recently discovered on Giglio.

Guidelines for the conservation of the Tuscan Archipelago butterflies

Ranking species according to their contribution to genetic diversity (Table 2) shows that butterfly diversity in the Tuscan Archipelago is encompassed both by insular endemic taxa as well as by widespread species (*A. agestis, C. pamphilus, C. alceae, Melitaea nevadensis, M. jurtina, P. aegeria* and *Zerynthia cassandra*). While most of these latter species are currently treated as being of ‘Least Concern’ in the European and Italian Red Lists (Van Swaay et al., 2010; Balletto et al., 2015), some of them disappeared from several islands causing loss of faunistic and genetic diversity. Some of the populations that have apparently disappeared in the last 10 years may be still rediscovered following dedicated field researches, as occurred for *Z. cassandra*, discovered on Elba in 1932 but lost before our intensive collection effort (Appendix S1). Species that disappeared from at least one island showed a higher overall population diversification (Dst) compared to persisting species. In fact, our analyses revealed that species accumulated genetic diversification because of their reduced migration and colonization capabilities; but these characteristics can also produce higher extinction risk due to reduced gene flow and rescue effect. We showed that one of the main peculiarities of Tuscan islands is the occurrence of strong genetic contrasts among nearby areas. If an insular relict or a genetically endemic population goes extinct, it is likely that it would be replaced by conspecific propagules from the nearest source, thus lowering the ancestral genetic diversification. There is also evidence for Mediterranean butterflies that the presence of endemic and relict island populations limits the colonization by mainland populations probably due to density-dependent phenomena (Dapporto et al. 2012, Vodă et al. 2015b). The establishment of the Tuscan Archipelago National Park in 1996 represented a fundamental step for the broad-scale protection of island communities. Nevertheless, specific conservation strategies tailored on particularly valuable species are still lacking because evidence-based information is missing. Our integrated approach, by prioritizing species according to their contribution to genetic diversity and by identifying the impact of ecological drivers on the emergence and extinction of differentiated populations, allows conservation priorities to be established with a necessity for regular monitoring schemes. Such schemes should evaluate population consistence and health, particularly focusing on population size and trends, genetic load and persistence of habitat suitability.

This study also suggests that the reduction of shady areas (woodlands and shrublands) might have been a driver of species loss in the Tuscan Archipelago, mostly on Capraia and Montecristo. It has been already reported that deforestation can be a main factor for butterfly extinction in Mediterranean islands. For example, the strong reduction of shady areas has been linked with post-glacial loss of butterflies on Malta with the recent extinction of most relict elements (Vodă et al., 2016). We thus advocate, as a specific conservation action, adopting environmental management procedures aimed at preserving and favouring environmental heterogeneity, thereby increasing resources availability and suitable habitats for a larger number of butterfly species (Dennis, 2010). The increase of temperature predicted by recent climate change scenarios suggests that environmental heterogeneity will play a pivotal role in buffering increasing thermal and drought stress.

In conclusion, this study shows that the integration of molecular and trait data with long-term occurrence records allows the identification of the eco-evolutionary processes underlying the high butterfly diversity in the Tuscan Archipelago, a Mediterranean diversity hotspot (Dennis et al., 2008). Evidence-based priorities for future conservation actions have been provided following the theory of conservation biogeography (Ladle & Whittaker, 2011). Public institutions, such as the NGO Legambiente and the Tuscan Archipelago National Park that have collaborated in this project, have already used some of these results to raise awareness for island diversity protection and to implement key conservation measures for butterflies.

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Data accessibility. The complete dataset, together with R scripts to replicate the analyses published in the paper, are available in the Supporting Information. All the COI sequences are available in "DDI – Tuscan Islands" (DS-DIDI, doi…) on BOLD at www.barcodinglife.org.

Biosketch

Members of the research team are actively engaged in insect biogeography, systematics and conservation, with specific interest in unravelling the historical and present-day factors responsible for species distributions across mainland Europe and Mediterranean islands.

LD, RaV, AC, MM and RoV conceived the idea; LD, RaV, AC, MM, RoV, VD, LF, HB, LPC, SS, FZ, UM and LV collected the data and the specimens in the study area; LD, RaV, VD and RoV assessed the taxonomy of the study species; LD, MM, AC and SS compiled the trait data; LD, VD, JCH, RaV, MM, AC, RoV, FZ, UM and LV managed and obtained the COI sequences; LD, HB and EB gathered the occurrence data; LD, RaV, MM, JCH and AC performed the data analysis; all the authors discussed the results and participated in writing the paper.

Table 1 Species traits used in the study with the description of the type of trait (sensu Moretti et al., 2016), in bold and the relative functional hypothesis in italics; the trait(s) measured; a description of the trait(s), the literature sources and the weights obtained by each trait in the first two Principal Components (in the cases when the analysis has been carried out) (PC1 and PC2). PCA on the single variable of host plants has not been carried out and for wingspan only the first component returned an eigenvalue higher than 1.

Type of trait	Functional hypothesis	Trait measured and description	Sources	PC1	PC2
Feeding	Species feeding on a large number of plants have a wider niche, thus a higher potential to colonize islands, compared to species feeding on fewer plant species (Dennis et al., 2012)	**Number of host plant genera** used by larvae as reported in two literature sources	Lafranchis (2007)	-	-
			Tolman & Lewington (2008)		
Morphology	Large-sized species are characterized by high mobility (Sekar, 2012) which increases the probability of crossing sea barriers (Dennis et al., 2012)	**Wingspan,** calculated as the mean between minimum and maximum wing size reported in four main sources for European butterflies. Tshikolovets (2011) reported size for both males and females	Higgins & Riley (1970)	0.448	-
			Lafranchis (2000)	0.448	-
			Pamperis (2009)	0.446	-
			Tshikolovets (2011) males	0.448	-
			Tshikolovets (2011) females	0.448	-
Life history	Phenological attributes characterize the period of the year and the duration of the most mobile life stage in butterflies, i.e. the winged	**Month of adult first emergence,** ranging from the coldest, January (1), to the warmest, August (8). No butterfly species has a later first emergence in the study area	Authors' collection data	-0.293	-0.822

	adults. These characteristics can affect the probability of crossing sea barriers (Dapporto et al., 2012; Dennis et al., 2012) and can interact with climatic changes in determining extinction probabilities	**Last month when adults fly**, ranging from January (1) to December (12)	Authors' collection data	0.533	-0.482
		Length of the flight period: number of months when the adults occur in the study area	Authors' collection data	0.587	-0.189
		Voltinism: number of generations/year in the study area	Authors' collection data and Tolman & Lewington (2008)	0.535	0.237
Physiology	Mean climatic conditions of the areas inhabited by a species are considered as good proxies for their ecophysiological response to climate (Devictor et al., 2012). They can affect the probability of species' persistence in the warm and dry Mediterranean climate that characterize the Tuscan islands	**Mean temperature** occurring in the 50×50 km spatial cells where the species has been modelled to occur	Schweiger et al. (2014)	0.334	-0.387
		Mean precipitation in the same spatial cells as above	Schweiger et al. (2014)	-0.292	-0.607
		Maximum temperature tolerance: upper 95% confidence interval for temperature mean	Schweiger et al. (2014)	0.334	-0.387
		Minimum precipitation tolerance: lower 95% confidence interval for precipitation mean	Schweiger et al. (2014)	-0.332	-0.413
		Overall temperature tolerance: standard deviation for temperature mean	Schweiger et al. (2014)	-0.332	0.313
		Overall precipitation tolerance: standard deviation for precipitation mean	Schweiger et al. (2014)	-0.337	-0.056

Table 2 The studied species ordered according to the cumulative ranks calculated for Gst, Dst and Uni, thus representing their rank for the contribution to genetic diversification. Dis, species that disappeared from Elba (E), Giglio (G) or Capraia (C). The last seven columns represent the uniqueness values scored by the haplotypes of each species on each island (Gor, Gorgona; Cap, Capraia; Elb, Elba; Pia, Pianosa; Mon, Montecristo; Gig, Giglio; Gia, Giannutri). "-" indicates that the species has not been reported on that particular island, "NA" indicates that the species has been reported but that the population was not included in this study because of lack of genetic data, “End” denotes the species that do not have mainland populations and for which it was not possible to calculate haplotype uniqueness, and “*” marks an old record of *Pontia sp.* on Capraia, which could not be correctly assigned to either *P. edusa* or *P. daplidice*. Taxa endemic to the insular region (Sardinia, Corsica and Tuscan islands) are written in bold, while the six species that were not recorded on islands during our surveys in the last 10 years are highlighted in grey.

Species	Gst	Dst	Uni	Dis	Gor	Cap	Elb	Pia	Mon	Gig	Gia
Coenonympha corinna	0.943	2.194	0.190	G	-	0.456	0.101	-	-	NA	0
Hipparchia neomiris	0.871	0.968	End	C	-	NA	End	-	-	-	-
Lasiommata megera/paramegaera	0.966	0.615	0.370		0	0.798	0.076	0	0.152	0	-
Coenonympha pamphilus	0.864	0.860	0.152		-	-	0.152	-	-	-	-
Aricia agestis/cramera	0.738	0.984	0.094	G	-	0.152	0.095	0.046	-	NA	NA
Pararge aegeria	0.880	0.636	0.076		NA	0.076	NA	0.076	NA	NA	NA
Melitaea nevadensis	0.455	0.492	0.988		-	-	0.988	-	-	-	-
Carcharodus alceae	0.779	0.258	0.095		0	NA	0	0.456	NA	0.152	-
Maniola jurtina	0.603	0.298	0.085	C	-	NA	0.092	0.076	-	0	-
Lycaena phlaeas	0.762	0.098	0.051	C	-	NA	0	0	0.152	0	-
Plebejus bellieri/idas	0.616	0.134	0.051		-	-	0.152	-	-	-	-
Pyronia cecilia	0.260	0.224	0.091	C	-	NA	0	0.152	-	0.076	-
Aglais urticae/ichnusa	0.937	0.449	NA	E	-	-	NA	-	-	-	-
Zerynthia cassandra	0.293	0.053	0.152		-	-	0.228	-	-	-	-
Callophrys rubi	0.846	0.279	0		-	-	0	-	-	0	-
Argynnis pandora	0.171	0.150	0.076		-	-	0.076	-	-	0.114	-
Hipparchia aristaeus	0.119	0.019	End	C	NA	NA	End	-	-	End	-
Favonius quercus	0.393	0.053	0.051		-	-	0.152	-	-	-	-
Melitaea cinxia	0.379	0.540	NA	E	-	-	NA	-	-	-	-
Leptidea sinapis	0.501	0.081	0	E	-	-	NA	-	-	-	-
Iphiclides podalirius	0.701	0.063	0		-	-	0	-	-	-	NA
Pieris napi	0.432	0.133	0		NA	-	0	-	-	-	0
Polyommatus icarus	0.216	0.018	0.076	G	-	0.076	0	0	-	NA	-
Gonepteryx rhamni	0.323	0.237	NA	E	-	-	NA	-	-	-	-
Thymelicus acteon	0.432	0.067	0		-	-	0	-	-	-	-
Pieris rapae	0.118	0.050	0.054		0.157	0.051	0	0.038	NA	0	0
Melitaea didyma	0.344	0.070	0		-	-	0	-	-	-	-
Celastrina argiolus	0.114	0.011	0.076		0	0	0.051	0	0	0.076	-
Nymphalis polychloros	0.415	0.043	NA	E	-	-	NA	-	-	-	-
Vanessa atalanta	0.127	0.009	0.051		NA	0.051	0	NA	0	0	NA
Hipparchia statilinus	0.281	0.053	0		-	-	0	-	-	-	-
Pontia edusa	0.079	0.015	0.051	C	-	*	0.076	NA	-	0	NA
Leptotes pirithous	0.298	0.048	0		-	0	0	NA	0	0	0
Issoria lathonia	0.431	0.014	0		-	-	0	-	-	0	-
Spialia sertorius	0.417	0.009	0		-	-	0	-	-	-	-
Pyronia tithonus	0.260	0.015	0		-	-	0	-	-	-	-
Limenitis reducta	0.185	0.015	0		-	-	0	-	-	-	-
Gonepteryx cleopatra	0.385	0.004	0		0	0	0	0	-	0	0
Vanessa cardui	0.032	0.007	0.038		0	0	0.076	0	NA	0	0.076
Lampides boeticus	0.044	0.005	0.038		0	0.152	0	0	0	0	NA
Pieris brassicae	0	0	0.076		NA	NA	0	0	NA	0.101	NA
Anthocharis cardamines	0	0	0.051		NA	NA	0.051	NA	NA	NA	NA
Papilio machaon	0	0	0.038		0	NA	0	0	-	0.152	-
Argynnis paphia	NA	0	0	E	-	-	NA	-	-	-	-
Colias croceus	NA	0	0		0	0	0	0	NA	0	NA

Euchloe ausonia	NA	0	0		-	-	0	-	-	-	-
Gegenes pumilio	NA	0	0		-	-	0	-	-	0	-
Pyrgus armoricanus	NA	0	0		-	-	0	-	-	-	-
Satyrium ilicis	NA	0	0		-	-	0	-	-	-	-
Charaxes jasius	0	0	0		NA	0	0	NA	-	0	0
Glaucopsyche alexis	0	0	0		-	-	0	-	-	-	-
Pieris mannii	0	0	0		-	-	0	-	-	-	-

Table 3 Trait variables selected by the AIC phylogenetic regressions for Gst, Dst and Uniqueness (Uni), and the differences in Gst, Dst and Uni between species that have disappeared from at least one island, compared to species that have not disappeared.

	Trait variables	Estimate	StdErr	t.value	p.value
Gst	Host Plants	-0.100	0.045	-2.226	0.032
	Mobility PC1	-0.141	0.046	-3.031	0.004
	Phenol PC1	0.058	0.043	1.363	0.181
	Ecophy PC2	0.068	0.040	1.713	0.095
Dst	Host Plants	-0.110	0.043	-2.550	0.015
	Mobility PC1	-0.126	0.047	-2.660	0.011
	Phenol PC1	0.060	0.042	1.454	0.153
	Ecophy PC2	0.119	0.039	3.042	0.004
Uni	Host Plants	-0.045	0.028	-1.643	0.108
	Ecophy PC2	0.102	0.033	3.112	0.003
Gst	Disappearance	0.111	0.093	1.201	0.236
Dst	Disappearance	0.213	0.087	2.452	0.018
Uni	Disappearance	0.014	0.063	0.215	0.831

Table 4 The effects of trait variables in the logistic phylogenetic regressions for species disappearance in Elba and Capraia.

Island	PC1/PC2	Estimate	StdErr	z.value	p.value
Elba	Host Plants	-0.380	0.469	-0.810	0.418
	Mobility PC1	0.412	0.482	0.855	0.393
	Phenol PC1	-0.382	0.544	-0.702	0.483
	Phenol PC2	-0.522	0.437	-1.194	0.233
	Ecophy PC1	-0.813	0.584	-1.393	0.164
	Ecophy PC2	0.766	0.561	1.367	0.172
Capraia	Host Plants	-0.969	0.972	-0.997	0.319
	Mobility PC1	1.605	1.206	1.331	0.183
	Phenol PC1	-6.375	3.002	-2.124	0.034
	Phenol PC2	2.835	1.869	1.517	0.129
	Ecophy PC1	-1.820	1.196	-1.522	0.128
	Ecophy PC2	2.753	1.783	1.544	0.123

Supporting Information

Additional Supporting Information is available in the online version of this study:

Appendix S1 {Supplementary methods and results containing the taxonomic assessment for each species, the occurrence data on Tuscan islands from 1900 to 2015 and the COI assessment for all the species. Supplementary results for PCA and Phylogenetic Models are also provided}

Appendix S2 {The dataset in the form of a fasta file (sequences_TA.fas), the information about the specimens used in the study (dataset.txt), the table containing the ecological traits for the 52 species (selected.traits.txt) and the R scripts used to carry out the analyses (Dapporto_et_al_script.R)}

Conflict of Interest: The authors declare no conflicts of interest