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Testing Classical Species Properties with Contemporary Data: How "Bad Species" in the Brassy Ringlets (*Erebia tyndarus* complex, Lepidoptera) Turned Good

PAOLO GRATTON[1,2,*], EMILIANO TRUCCHI[3,4], ALESSANDRA TRASATTI[1], GIORGIO RICCARDUCCI[1], SILVIO MARTA[1], GIULIANA ALLEGRUCCI[1], DONATELLA CESARONI[1], AND VALERIO SBORDONI[1]

[1] *Dipartimento di Biologia, Università di Roma "Tor Vergata", Via della Ricerca Scientifica, 00133 Roma Italy;* [2] *Department of Primatology, Max Planck Institute for Evolutionary Anthropology, Deutscher Platz, 6, 04103 Leipzig Germany;* [3] *Department of Biosciences, Centre for Ecological and Evolutionary Synthesis, University of Oslo, PO Box 1066, Blindern, Oslo 0316 Norway; and* [4] *Department of Botany and Biodiversity Research, University of Vienna, Rennweg 14, 1030 Vienna, Austria*

[*] *Correspondence to be sent to: Department of Primatology, Max Planck Institute for Evolutionary Anthropology, Deutscher Platz, 6, 04103 Leipzig, Germany. Email: paolo_gratton@eva.mpg.de*

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Abstract.—Alls peciesc onceptsa rer ootedi nr eproductive,a ndu ltimatelyg enealogical,r elations.G eneticd ataa ret hus the most important source of information for species delimitation. Current ease of access to genomic data and recent computational advances are blooming a plethora of coalescent-based species delimitation methods. Despite their utility as objective approaches to identify species boundaries, coalescent-based methods (1) rely on simplified demographic models that may fail to capture some attributes of biological species, (2) do not make explicit use of the geographic information contained in the data, and (3) are often computationally intensive. In this article, we present a case of species delimitation in the *Erebia tyndarus* species complex, a taxon regarded as a classic example of problematic taxonomic resolution. Our approach to species delimitation used genomic data to test predictions rooted in the biological species concept and in the criterion of coexistence in sympatry. We (1) obtained restriction-site associated DNA (RAD) sequencing data from a carefully designed sample, (2) applied two genotype clustering algorithms to identify genetic clusters, and (3) performed within-clusters and between-clusters analyses of isolation by distance as a test for intrinsic reproductive barriers. Comparison of our results with those from a Bayes factor delimitation coalescent-based analysis, showed that coalescent-based approaches may lead to overconfident splitting of allopatric populations, and indicated that incorrect species delimitation is likely to be inferred when an incomplete geographic sample is analyzed. While we acknowledge the theoretical justification and practical usefulness of coalescent-based species delimitation methods, our results stress that, even in the phylogenomic era, the toolkit for species delimitation should not dismiss more traditional, biologically grounded, approaches coupling genomic data with geographic information. [Species delimitation; RAD sequencing; isolation by distance; next-generation sequencing; genotype clustering; parapatric species; alpine butterflies, *Erebia*.]

Despite the classic debate concerning species concepts and species delimitation (see, e.g., Mallet 2013; Heller et al. 2014 and references therein), a large majority of biologists agree that species are one of the fundamental units in the living world, both as subjects of the evolutionary process and as targets of conservation policies. However, species are dynamic entities, which can be regarded as transient segments of relative stability throughout the evolutionary process, connected by "gray zones" where their delimitation will remain inherently ambiguous (Pigliucci2 003; de Queiroz 2007). Biological entities that currently exist in these "gray zones" do not show all of the diagnosable properties that characterize "good" species, and have sometimes been humorously referred to as "bad" species (Descimon and Mallet 2009).R egardlesso ft heird ifferences,a ll species concepts share the idea that members of the same species are linked by tighter genealogical relations to each other than to members of different species (de Queiroz 2007).I nt hisp erspective,s pecies delimitation may correspond to the identification of gaps in the network of genealogical relations, and, in most cases, genetic markers represent the single most important tool for species delimitation (e.g., Fujita et al. 2012).I ndeed,t her ecenta vailabilityo f plentiful genetic markers provided by next-generation sequencing techniques is greatly increasing our ability to resolve species gaps, especially for tangled situations stemming from recent speciation/radiation events (e.g., Hipp et al. 2014). Recent computational approaches combine coalescent theory with Bayesian or likelihood methods to provide objective, genealogy-based, and statistically grounded tests of alternative hypotheses of species delimitation (e.g., Yang and Rannala 2010; Ence and Carstens 2011).H owever,a lthought echniques allowing application of such tests to genome-wide data are rapidly developing (Leachée ta l.2 014), they remain computationally very intensive. Moreover, coalescent-based species delimitation approaches are centered on statistical comparison of two extreme models of population structure (complete panmixia vs. complete reproductive isolation) that do not necessarily capture the expected attributes of biological species. Lastly, coalescent-based species delimitation approaches are not geographically explicit, and do not allow researchers to exploit the information conveyed by the spatial distribution of genetic diversity.

Indeed, one of the most universally recognized properties of "good" biological species is that populations of different species should be able to coexist in sympatry while maintaining their distinctiveness, thanks to mechanisms of reproductive isolation (Mayr 1942).E xtensivec oexistencei ns ympatryo f reproductively isolated populations can be maintained only if the two species attained a sufficient degree of niche segregation (Levine and HilleRisLambers 2009).

Therefore, "good" species whose niches strongly overlap may still meet in contact zones, which will not evolve into wide, clinal, genomic-scale hybrid zones (e.g., Dufresnes et al. 2014; Poelstra et al. 2014) if reproductive isolation is effective. On the other hand, allopatric populations whose present demographic isolation merely results from the onset of geographic barriers to dispersal may maintain traces of past isolation by distance (IBD) well after such barriers have arisen (e.g., Good and Wake 1992). Therefore, different hypotheses about evolutionary processes (reproductive isolation accompanied by ecological divergence, secondary contact with varying degrees of intrinsic reproductive isolation, purely geographic allopatric divergence) generate testable predictions about the geographic distribution of genetic markers. An approach to species delimitation based on such predictions draws from a well-established tradition (Good and Wake 1992; Sites Jr and Marshall 2003),b uti ss omewhatn eglectedi nr ecent literature (but see, for an analysis of this kind, Streicher et al. 2014).

The *Erebia tyndarus* species complex (brassy ringlets, Lepidoptera: Nymphalidae: Satyrinae) is an assemblage of closely related alpine species, ranging from the Iberian Peninsula to western North America. The case of the brassy ringlets represents an intriguing example of how, by considering different diagnosable properties of species, researchers gradually recognized a growing number of taxa, leading Descimon and Mallet (2009) to list this group among examples of "bad" species. Members of the *E. tyndarus* complex have been characterized by (1) subtle morphological differences (Warren1 936);(2)k aryotypes(e.g., de Lesse 1953); (3) cross-breeding experiments (Lorkovi´d 958), and (4) molecular markers, such as allozymes (Lattes et al. 1994; Martin et al. 2002) and mitochondrial DNA (Martine ta l.2 002; Albre et al. 2008).T hese data allowed the identification of two main clades: the "*ottomana*" clade, including lower-elevation taxa from southwestern Asia and southeastern Europe, and the "*tyndarus*" clade, whose representatives occupy high-elevation grasslands in the mountains of southern Europe, in the Altai and the Rocky Mts. The "*tyndarus*" clade comprises a so-called "terminal" group (Albre et al. 2008) including the four species occurring in the Alps (Fig. 1a,c). Interestingly, the taxonomy and genetic relationships within the "terminal" clade could not be satisfactorily resolved. Since the works of de Lesse (1953, 1955, 1956) and Lorković (1958), four species are most usually recognized in the "terminal" clade (*E. tyndarus*, *E. cassioides*, *E. nivalis*, *E. calcaria*). These species display complex, often discontinuous and mostly parapatric geographic distributions (Fig. 1c), with narrow contact zones (typically less than 1 km, see Sonderegger 2005). Appreciable areas of sympatry only occur between *E. nivalis* and *E. cassioides*, in the Eastern Alps and in a small area of the western Alps (Fig. 1b,c). In areas of sympatry, *E. cassioides* and *E. nivalis* tend to displace each other along an altitudinal gradient, where *E. nivalis* occupies higher elevations than *E. cassioides* (Lorković 1958).I nf act,w hilea llo thers peciesi n the group are univoltine, *E. nivalis* is reported to be a semivoltine butterfly (Sonderegger 2005), a life history trait whose fitness effect clearly depends on temperature, and hence elevation. Rare instances of natural hybridization have been reported (Descimon and Mallet 2009)a mongp arapatrica nds ympatric species of the "*tyndarus*" group (Fig. 1b). Lorković (1958) performed cross-breeding experiments, which indicated a gradient of "sexual affinity" (i.e., postzygotic and prezygotic reproductive isolation) from allopatric (weak) to parapatric (intermediate) and sympatric (very strong isolation) species pairs in the *E. tyndarus* group (Fig. 1b). On the other hand, differences in the number of chromosomes did not seem to have a major effect on reproductive isolation (a pattern consistent, e.g., with recent findings on *Leptidea* butterflies by Lukhtanov et al. 2011).T heseo bservationss uggestt hat the degree of reproductive isolation was shaped by natural selection in the presence of gene flow, perhaps through reinforcement, rather than in allopatry.

Despite the system having been relatively well studied, all analyses of genetic variation in the "terminal" clade failed to find support for the "traditional" four-species taxonomy. Lattes et al. (1994), based on allozyme data focusing on *E. cassioides*, suggested that populations of this taxon from Western Alps, Apennines, and Pyrenees should be moved to a separate species (*E.c armenta*). Martin et al. (2002) did not find evidence of reciprocal monophyly across the four putative species at both allozyme and mtDNA markers, the latter result being confirmed by Albre et al. (2008) with an expanded mtDNA data set. These observations raise the hypothesis that the "terminal" clade of the *E. tyndarus* group represents an instance of very recent divergence, whose diagnosable morphological and (in the case of *E. nivalis*)e cologicald ifferences,a ndd ifferentd egrees of reproductive isolation may depend on the fixation of a small number of genes in an otherwise homogeneous genomic background (Feder and Nosil 2010; Poelstra et al. 2014).

In the present study, we analyzed nucleotide sequence variation at genome-wide markers (RAD sequencing, Baird et al. 2008)i n4 6i ndividualss ampleda crossm osto f the geographic distribution of the *E. tyndarus* "terminal" clade (*sensu* Albre et al. 2008). We employed multivariate analysis and model-based Bayesian clustering to identify genetic clusters within the *E. tyndarus* "terminal" clade and tested for IBD within and among genetic clusters. Under our operational criterion, a reliable species boundary is identified between two well-resolved genotype clusters if (1) a pattern of within-clusters IBD is detected, and (2) genetic differentiation between pairs of individuals belonging to different clusters shows no clear dependence on their geographic distance (i.e., individuals sampled in, or near to, contact zones do not tend to be genetically intermediate). We then analyzed morphometric variation at traits commonly used for diagnosis of butterflies of the *E. tyndarus* group in the field to assess the level of consistency between

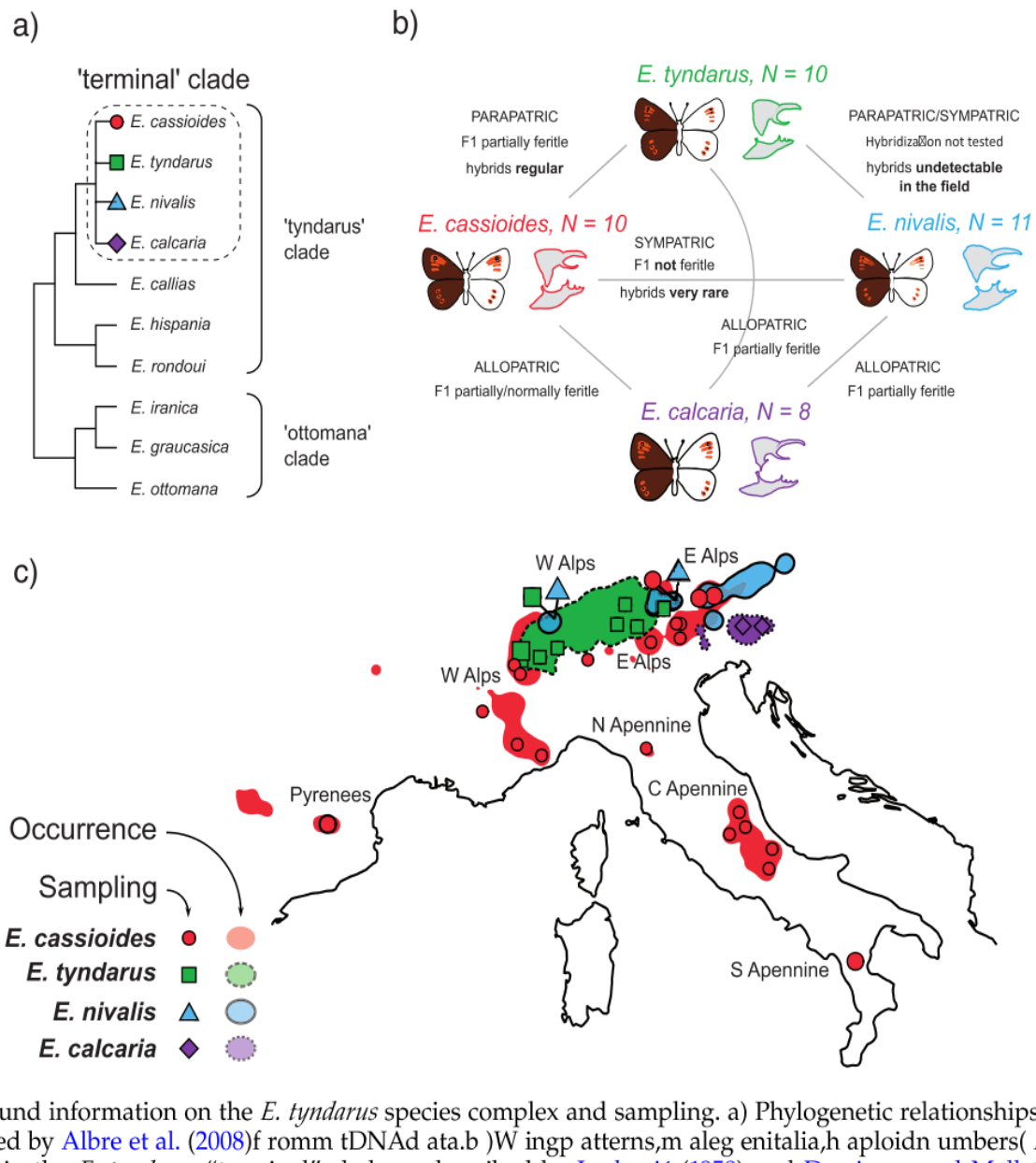

FIGURE 1. Background information on the *E. tyndarus* species complex and sampling. a) Phylogenetic relationships within the *E. tyndarus* species complex inferred by Albre et al. (2008)f romm tDNAd ata.b)W ingp atterns,m aleg enitalia,h aploidn umbers(*N*), biogeographic, and reproductive relations in the *E. tyndarus* "terminal" clade as described by Lorković (1958)a nd Descimon and Mallet (2009).c)G eographic distribution of the four traditionally recognized species in the *E. tyndarus* "terminal" clade and location of samples employed in this study. Each species is represented by a different color/shade and symbol. Ranges of occurrence for each species (distinct by color/shade and outline style) are thresholded two-dimensional kernel density estimated from 855 occurrence records compiled from various sources. Area of symbols that represent sampling sites is proportional to sample size, with the smallest circles corresponding to $N=1$.

our species delimitation and traditionally recognized taxa.

Lastly, we performed a coalescent-based Bayes factor delimitation (BFD*; Leaché et al. 2014) analysis to statistically evaluate two competing hypotheses consistent with our clustering of RAD genotypes. Results from this method strongly supported the splitting of genotype clusters separated by distributional gaps into separate species, regardless of the amount of differentiation, the morphological similarities, and the fact that their genetic divergence was consistent with a simple IBD model.

Our study represents the first genetic analysis based on a consistent set of markers across most of the range of the whole *E. tyndarus* "terminal" clade, a group that has so far resisted genetic-based species delimitation and is regarded as a classic example of problematic taxonomic resolution. We demonstrate that relatively simple hypothesis-driven tests based on the geographic distribution of genetic diversity allow resolution of the systematics of the "bad" species in this brassy ringlets complex. At the same time, our results indicate that coalescent-based species delimitation methods might lead to overconfident or even incorrect conclusions when dealing with allopatric populations or incomplete geographical sampling.

METHODS

Sampling

Samples of adult *E tyndarus E. cassioides*, *E. nivalis*, and *E. calcaria* for genetic analyses were collected by the authors and collaborators in July–August 2012 at 41 sites in the Alps, Apennine, and Pyrenees (Fig. 1c and Supplementary Appendix 1 available on Dryad at http://dx.doi.org/10.5061/dryad.3n5c9).B utterflies were netted and immediately placed in 95% ethanol, after removing the wings for morphometric analyses. Sampling was representative of the whole distribution range of the taxa included in the *E. tyndarus* "terminal clade" (*sensu* Albre et al. 2008),w itht heo nlyi mportant exception of the small pockets of the *E. cassioides* range in the Balkan peninsula, and of the Italian population

of *E. calcaria* (Fig. 1c). All samples were provisionally assigned to one of the four "traditional" species based on the geographic location and/or elevation of sampling sites.

Molecular Methods

Genomic DNA was extracted from the thorax of 46 individuals (26 *E. cassioides*, 1 1*E. tyndarus*, 6 *E. nivalis*, and 3 *E. calcaria*)u singa C TABp rotocol (Doyle and Doyle 1987) with RNAse. RAD libraries were prepared according to Etter et al. (2011) with a few modifications. High-fidelity SbfI restriction enzyme (New England Bioloabs) was used to digest 300 ng genomic DNA from each individual, and digested DNA was ligated to 150 nmol barcoded Illumina P1 adaptor (Microsynth), with all 6 bp barcodes differing by at least two bases. DNA was then pooled into three libraries, containing 10–18 individuals each, and sheared in a Bioruptor™ sonicator (Diagenode), using 8× 30 s on-and-off cycles. Sheared DNA was concentrated using MinElute™ Columns (QIAGEN), migrated on agarose gel, size-selected at 300–500 bp and extracted from agarose using a QIAquick™ Gel Extraction Kit (QIAGEN). Blunt ends were repaired using a Quick Blunting™ Kit (New England Biolabs) and DNA was again purified with MinElute™ Columns before and after 3′-dA overhang addition with Klenow Fragment $3' \rightarrow 5'$ (New England Biolabs). Illumina P2 adaptors were then added and 22 cycles of final amplification were performed on 15μL of each of the three libraries using 50μL Phusion High-fidelity Master Mix (New England Biolabs) in a total volume of 100μL. The resulting RAD libraries were finally combined and sequenced on a single lane of an Illumina 2500 HiSeq platform at the Norwegian Sequencing Centre (Oslo, Norway).

Reads without the complete SbfI recognition site were discarded from further analysis, as were reads containing one or more bases with a Phred quality score below 10, or >5% of the positions below 30. Libraries were demultiplexed using the process_radtags program from the *STACKS* pipeline (Catchen et al. 2011), which automatically corrects for single-bp errrors within the barcode. A *de novo* assembly of the final, quality-filtered and demultiplexed data set was performed in ustacks (*STACKS* pipeline, Catchen et al. 2011) to produce a draft catalog of putative loci. In the terminology of the *STACKS* pipeline, a "stack" is a set of identical sequences, and several stacks can be merged to form a putative locus. We set the minimum number of sequences to form a stack at 15 (-m parameter) and the maximum distance between stacks at the same locus (-M parameter) at 3, so that stacks could be merged to form a locus if they differed by three nucleotides or less. Note that, since this threshold applies to pairwise comparisons, the total number of nucleotide differences at a locus may be significantly higher. At any rate, putative loci containing more than 10 variable sites were discarded as possible paralogues, as were putative loci with unusually high coverage and putative loci that were not sequenced in at least three individuals. For our final analyses, we also excluded from the filtered catalog all loci that were not sequenced in at least one-third of individuals in each of the prior geographic "populations", to obtain a data set with a small and balanced amount of missing data and reduce the number of loci affected by polymorphism at restriction sites (Arnold et al. 2013, Davey et al. 2013).T hefi lteredd atas etw asfi nally compared to the NCBI GenBank nucleotide collection using the megablast algorithm, in order to identify and remove exogenous sequences. All downstream analyses employed a stringently filtered data set of polymorphic putative RAD loci.

Genotype Clustering and Identification of Introgressed Individuals

We explored the variation of our sample at the retained putative RAD loci by performing a principal components analysis (PCA) using the *dudi.pca* R function ("ade4" package, Dray and Dufour 2007).T hei nput data consisted of the frequencies (0 = absent, 0.5 = heterozygote, 1 = homozygote) of each haplotype at a given locus (allele) in each sampled individual. Missing data were replaced by the mean frequency of the haplotype in the sample. We then performed a cluster analysis of genotypes on PCA-transformed data using the *find.clusters* R function ("adegenet" package, Jombart 2008), which is based on performing successive *k*-means with an increasing number of clusters (k). Bayesian information criterion (BIC) was applied to compare clustering models with k from 1 to 20, and retaining the first 40 principal components.

In order to trace evidence of recent introgression, we performed a Bayesian model-based clustering accounting for potential admixed origin of individuals using *Structure v2.3.3* (Pritchard et al. 2000).F ort his analysis, we again used genotypic data (where each unique haplotype at a given locus was considered as an allele). We applied the "admixture" model with uncorrelated allelic frequencies among populations to obtain estimates of the proportion of ancestry (q) of each individual genotype in each of the K clusters. *Structure* MCMCs were run for 500,000 generations after a 100,000 generations burn-in. We ran *Structure* for values of K from 2 to 10, with 10 replicates for each value of K, and examined the values of $ln\ \Pr(D|K)$ (logarithm of the posterior probability of the data given the number of clusters) to determine the most likely number of clusters. Runs with extremely low values of $ln\ \Pr(D|K)$ (i.e., more than one order of magnitude lower than the median $ln\ \Pr(D|K)$ for the same K) were removed as outliers. In order to visualize the genetic relationships among clusters at the most probable value of K, we computed a neighbor-joining (NJ) tree based on the net-nucleotide distance among clusters.

Isolation by Distance

We tested for IBD both within and between species by comparing matrices of genetic distances D

(Cavalli-Sforza and Edwards 1967)a nd *log*-transformed least-cost path (LCP) geographic distances among pairs of samples. LCP distances were computed by aggregating a digital elevation model in 10×10 km cells, and setting the conductance of all cells with maximum elevation >1000 m.a.s.l. to 1 and of all other cells to 0 (simple Euclidean distances were also tested and provided essentially identical results). To assess statistical correlation among matrices we applied Mantel tests (1000 randomizations) to distance matrices.

Wing Morphometry

In order to evaluate morphological differentiation among genetic clusters, we measured three characters of the forewing that were proposed to possess some diagnostic value: wing size, extension of eyespots, and shape of the wing margin (Sonderegger 2005). Only males were considered for these analyses, because of the small number of females in our sample and clear sexual dimorphism apparent in preliminary analyses (data not shown). In order to provide a larger sample for the exploration of morphological variation, we also considered 29 additional individuals (Supplementary Appendix 1), either sampled for this study or retrieved from the Lepidoptera collection of Valerio Sbordoni (GRBio code VSRM, www.grbio.org).

Details about morphometric measures are given in the Supplementary Appendix 2. We obtained a synthetic description of morphological differences among genetically determined species by performing a linear discriminant analysis (LDA) with wing size, eyespots extension, and the four most important PCs of wing margin shape variation (see Supplementary Appendix 2) as the predictors. The LDA training sample included those males with available genetic data ($N=36$), and the grouping factor was based on results from previous genetic clustering and IBD analyses (Supplementary Appendix 1). As our sample included only one genetically determined *E. calcaria* male, we assigned *E. calcaria* as the putative species to six additional individuals from Slovenia, based on the assignment of the three Slovenian samples with genetic data (i.e., the single male and two females, see Supplementary Appendix 1). The remaining 23 individuals were used to explore the variation at morphological traits but were not included in the LDA training sample. The R function LDA (package "MASS"; Venables and Ripley 2002)w asu sedt ofi tt heL DA.

Coalescent-based species delimitation.—Weu sedt he method of BFD* with genomic data described by Leaché et al. (2014) to statistically evaluate two competing hypotheses that were consistent with the previous genotype clustering analyses and especially relevant for our study. In particular, we compared two species delimitation hypotheses: (H1) clusters of individuals provisionally assigned to the same species, and showing a signal of IBD in between-clusters comparisons, were lumped; (H2) each cluster resulting from the *k*-means model with the lowest BIC (see above) was considered as a separate species.

The BFD* approach uses path sampling to estimate the marginal likelihood (ML) of a population divergence model directly from SNP data (without integrating over gene trees) and has been shown to be robust to a relatively large amount of missing data (Leaché et al. 2014), being especially suited for RAD sequencing data. We performed the BFD* analysis using SNAPP (Bryant et al. 2012), implemented as a plugin in BEAST 2.3.0 (Bouckaert et al. 2014), and analyzing a data matrix constructed by selecting a single SNP at random from each RAD locus (as in Leaché et al. 2014). We estimated ML of each model by running path sampling with 24 steps (50,000 MCMC steps, 10,000 pre-burnin steps). The Bayes Factor (BF) test statistic (2*ln(BF)) was then used to compare the strength of support according to the framework of Kass and Raftery (1995). In the SNAPP analysis, we set a prior for $\theta = 4\mu N_e$ as a gamma distribution with $\alpha = 2.15$ and $\beta = 2600$ (mean $= 8 \times 10^{-4}$). Assuming a mutation rate $\mu = 10^{-8}$ substitutions $\times$ site^{-1} $\times$ generation^{-1} (e.g., Kondrashov and Kondrashov 2010; Lynch 2010),o ur prior corresponds to a reasonable range of effective population sizes (mean $N_e = 25000$, 99% HPD 170–66 500), similar in magnitude to the genetically estimated effective sizes of other regional populations of *Erebia* butterflies (Hammoutie ta l.2 010). However, though estimates of effective population sizes and divergence times obtained by SNAPP depend strongly on the choice of priors, BFD* analyses have been shown to be robust to prior misspecification (Leachée ta l.2 014).

RESULTS

Genotyping by RAD Sequencing

The final quality-filtered and demultiplexed data set contains 172 million reads, each 84 bp long. Raw sequence data have been submitted to the NCBI Sequence Read Archive (SRA, accession SRP065834). The first draft RAD catalog contains 24,547 putative loci. However, a large number of these putative loci were sequenced only in a small number of samples (median number of sequenced individuals per locus = 6). One individual (ATSAJ3.25, *E. cassioides* E Alps, Supplementary Appendix 1) was genotyped at just 486 putative loci due to inefficient sequencing, and was removed from further analyses. The remaining 45 samples were sequenced at 3913–7809 putative loci. The number of loci shared between pairs of individuals is consistent with our provisional species assignment and geographic origin (Supplementary Fig. S1): individuals from the same geographic populations (as identified by major distributional gaps, see Fig. 1c and Supplementary Fig. S1) share on average 49.4% (±6.7% SD) of loci, while individuals from different populations of the same species share 41.2% (±5.1% SD) of loci and individuals from different provisional species share only 22.2%

(±4.7% SD) of loci. The latter result clearly shows that polymorphism at restriction sites accounts for a significant fraction of the missing data, and is itself a clear indication of the strong genetic differentiation among species (see Lexer et al. 2013; Hipp et al. 2014). However, since samples from the same population do not share more than 50–70% of loci, it is likely that ~30% of the putative loci in the draft catalog are exogenous (due to environmental and laboratory contamination) or incorrectly assembled. Therefore, we estimate that each individual genome contains ~3000–4000 genuine RAD loci, which is consistent with an a priori estimate based on a typical lepidopteran genome size of ~500 Mb and GC content about 37–40% (Goldsmith and Marec 2010). Filtering out putative loci with too many missing data (see "Methods" section) and loci with a very high number of polymorphisms (>10) restricted our data set to 400 polymorphic putative loci with 11.4% (±6.5% SD among individuals) missing data. A BLAST search for highly similar matches in the GenBank nucleotide collection revealed that two sequences out of 400 are 100% or 99% identical to segments of published *Wolbachia* genomes (*wPip_Mol* strain, GB accession HG428761.1 from pos. 804,578 to pos. 804,495 and *wHa* strain GB accession CP003884.1 from pos. 240,874 to pos. 240,957, respectively). These results indicate that this endosymbiont is present in all of the analyzed populations or that it was present in some ancestral population (as the retrieved *Wolbachia*-likef ragments might represent insertions into the host's genomes, see, e.g., Koutsovoulos et al. 2014). These two sequences were excluded from further analyses. The final data set consisted of the remaining 398 putative polymorphic loci, containing 2039 SNPs (SNPs per putative locus: median 5, mean 5.11, SD 2.65).

Genotype Clustering and Identification of Introgressed Individuals

Principal components analysis (PCA) clearly shows that individual genotypes form four clusters in the space of the first three principal components (Fig. 2a). Accordingly, BIC of *k*-means clustering decreases steeply until *k* (number of clusters) = 4, meets a minimum at $k=6$ and increases sharply afterwards (Fig. 2b). The clustering for $k=4$ corresponds to the four "traditional" species as identified by morphological and geo-altitudinal criteria (Fig. 2a). At $k=6$, the *cassioides* sample is further segmented into three clusters that group together samples from adjacent geographic areas: (1) Eastern Alps, also including the Orobian Alps sample; (2) Southern and Central Apennines; and (3) Western Alps, Pyrenees, and Northern Apennines. Running the *find.clusters* function on the *cassioides* sample alone confirms the same clustering (Fig. 2c), with $k=3$ corresponding to the lowest BIC (data not shown). The PCA plot for the *cassioides* samples shows how, within this species, the space of genetic variation largely corresponds to the geographic space (Fig. 2c, d).

The posterior probability of the *Structure* model has a clear maximum at *K* (number of clusters) = 6 (Fig. 3b). The resulting clustering (Fig. 3a) almost perfectly matches the results from the PCA and *k*-means analysis (Fig. 2a,c), with the exception that the individual from Northern Apennines is now explicitly identified as admixed between the Western Alps + Pyrenees cluster and the Central + Southern Apennines cluster. The NJ tree calculated on net-nucleotide distances between pairs of clusters clearly shows that the three clusters including *cassioides* samples are much closer to each other than to the remaining clusters (Fig. 3c). All *calcaria* and *tyndarus* samples are unambiguously assigned to a species-specific cluster with $q>0.999$. Conversely, *Structure* identifies one *nivalis* individual from the Western Alps sample (a location where *nivalis* and *tyndarus* are sympatric), as having mixed ancestry, with $q=0.082$ (90% CI 0.050–0.119) to the *tyndarus* cluster (Fig. 3a).

Isolation by Distance

Mantel tests show that genetic distances are significantly dependent on least-cost path geographic distances for within-species tests in *E. cassioides* (Pearson's $r=0.646$, $P=0.001$) and *E. tyndarus* ($r=0.370$, $P=0.005$) (Fig. 4). *Erebia calcaria* and *E. nivalis* also show a very strong positive correlation between genetic and geographic distances ($r=0.996$ and $r=0.844$, respectively), but we did not perform Mantel tests within these species owing to small sample size. Conversely, all between-species comparisons yield negative and non-significant correlations, with the exception of distances between *E. tyndarus*–*E. nivalis* pairs, which returned a positive and marginally significant correlation ($r=0.357$, $P=0.048$).

Wing morphometry.—Our morphometric analysis (Fig. 5) shows that the four detected species differ from each other in the space of wing morphology traits (size, extension of eyespots, shape of forewing margin; Fig. 5a), although they are not completely separable (Fig. 5b). Moreover, the morphometric characteristics of each cluster appear to match the reported features of traditionally recognized species *E. tyndarus*, *E. cassiodes*, *E. nivalis*,a nd *E. calcaria*. *Erebia cassioides* is the most distinctive species, being largely separated from the others on the first LD, which accounted for over 83% of the inter-species differentiation. Our *E. cassioides* samples are characterized by larger size, stronger development of eyespots and pointed shape of wing margin (Fig. 5b), consistent with the diagnostic traits used for field identification (Sonderegger 2005). According to our discriminant analysis (which removed the effect of elevation on wing size, see Supplementary Appendix 2), size does not play any role in separating *E. calcaria*, *E. tyndarus*,a nd *E. nivalis* from each other (Fig. 5b). Indeed, these three species, though occupying slightly overlapping morphological spaces,

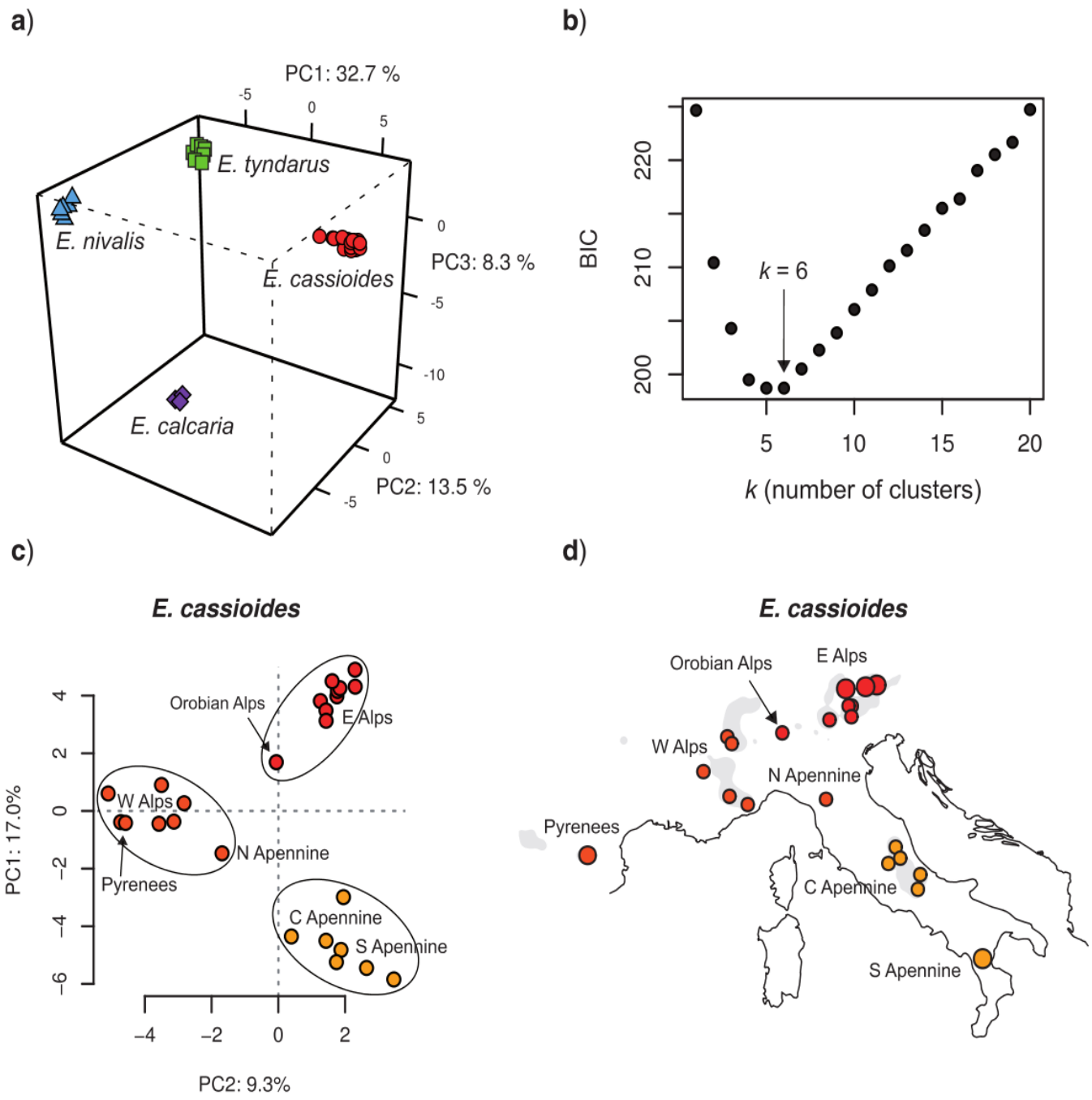

FIGURE 2. Principal components analyses (PCA) and *k*-means cluster analyses. a) Three-dimensional scatterplot for the first three principal components of a PCA performed on all samples. Individuals provisionally assigned to each of the four "traditional" species are shown with different colors and symbols (diamond: *E. calcaria*;t riangle: *E. nivalis*;s quare: *E. tyndarus*;c ircle: *E. cassioides*).b)B ayesiani nformationc riterion scores as a function of the number of clusters (*k*) for successive *k*-means clusterings based on the PCA performed on all samples. c) Scatterplot for the first two principal components of a PCA performed on samples of *E. cassioides*.E llipsesa ndd ifferents hadeso fr ed/orangeh ighlight the results of *k*-means clustering. Labels indicate the geographic origin of genotyped samples. d) Geographic origin of genotyped samples of *E. cassioides*.C olorsa ndl abelsa si n(c).G rays hadingi ndicatest her angeo f *E. cassiodes*.

are characterized by subtle characteristics of the wing margin (mostly PC4 in Fig. 5b) and, marginally, by the size of eyespots, with the *E. calcaria* cluster sporting the least developed spots, and *E. nivalis* the relatively more developed spots. These results are again consistent with the reported morphological features separating the traditionally recognized species (Sonderegger2 005; Tolman and Lewington 2008).

Coalescent-based species delimitation.—Weu sedt heB FD* method to statistically evaluate two species delimitation hypotheses: (H1) four species, corresponding to the "traditional" taxa (*E. tyndarus*, *E. cassioides*, *E. nivalis*, *E. calcaria*);(H2)s ixs pecies,c orrespondingt ot hes ix clusters identified by the PCA+k_{means} approach with the lowest BIC. The two models thus differed only for the status attributed to the three clusters in *E. cassioides* (eastern Alps, western Alps + Pyrenees + N Apennine, C Apennine + S Apennine), which were recognized as a single species in H1 and as three separate species in H2. The estimated (*ln*) marginal likelihoods are −5027.21 and −4787.96 for H1 and H2, respectively, corresponding to $2*\ln(BF_{H1-H2}) = -478.5$, thus decisively supporting H2 over H1. The estimation of marginal likelihoods required ∼40 days/CPU per model on a Xeon 2.6 GHz processor.

DISCUSSION

"Bad Species" Turn Good

Our analyses of RAD sequencing data, combining different approaches to genotype clustering with simple tests of IBD, unambiguously shows, for the first time,

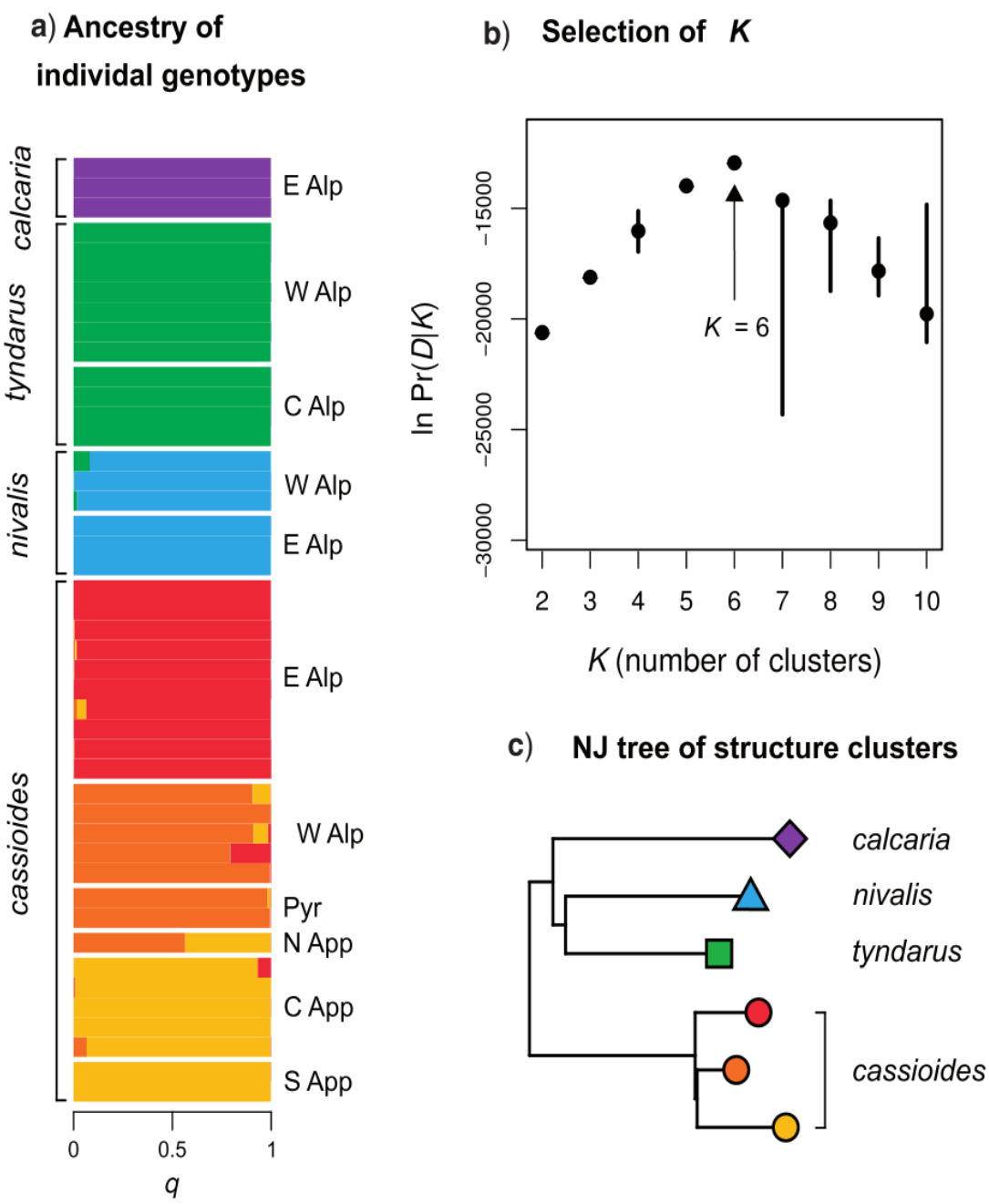

FIGURE 3. Model-based Bayesian clustering. a) Barplot showing the inferred ancestry of 45 individual genotypic profiles in each inferred cluster for $K = 6$. Each horizontal bar represents one individual, with colors indicating the proportion of ancestry (q) attributed to each cluster. Italicized names on the left indicate the provisional identification of samples in each of the four traditionally recognized species. b) Natural logarithm of the posterior probability of the clustering model (ln Pr($D|K$)) as a function of the number of clusters (K). Dots indicate mean values over 10 replicates, and error bars indicate extreme values after removal of outliers (see "Methods" section for details). c) Neighbor-joining (NJ) tree based on the net nucleotide distance among the six inferred clusters. Color-coding as in (a), symbols correspond to the provisional identification of all members of each cluster in each of the four traditionally recognized species (diamond: *E. calcaria*;t riangle: *E. nivalis*;s quare: *E. tyndarus*;c ircle: *E. cassioides*).

that the *E. tyndarus* "terminal" clade contains four highly differentiated genetic clusters (Figs. 2a and 3a,c). These clusters satisfy the expected properties of biological species concerning the geographic distribution of genetic diversity (i.e., clear signals of IBD within species, and no general intermediate genetic composition of individuals from contact zones). Remarkably, the species delimitation obtained by genetic analyses matches the "traditional" four-species taxonomy recognized, e.g., by Lorković (1958) and based on a diverse set of data, ranging from morphology to karyotypes and cross-breeding experiments. Indeed, the four clusters correspond to the available descriptions of geographic distributions and morphological traits of *E. tyndarus*, *E. cassioides*, *E. nivalis*, and *E. calcaria* (e.g., Sonderegger 2005; Albre et al. 2008). Lower level, geographic population structure is apparent in the most widely distributed species, *E. cassioides* (Figs. 2c and 3a). However, our analyses show that, contrary to the between-species comparisons, genetic differentiation among individuals of *E. cassioides* is largely accounted for by an IBD model (Figs. 2c,d and 4). Therefore, although our data partly confirm the genetic differentiation between Eastern and Western populations of *E. cassioides* reported by Lattes et al. (1994), the observed pattern of IBD argues against the recognition of Western populations of *E. cassioides* as a separate species (*E.c armentas ensu* Lattes et al. 1994).

Our data also demonstrate that genetic introgression among the four species is, at most, very limited. In particular, there is no indication that individuals of *E. cassioides* and *E. tyndarus* sampled near or within contact zones possess alleles typical of the other species (Figs. 2a and 3a), and no positive dependence of genetic

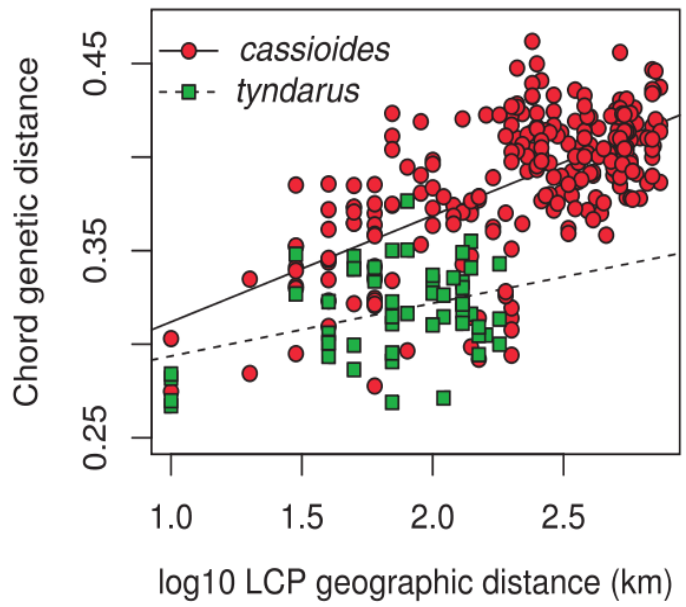

FIGURE 4. Isolation by distance. Chord genetic distances among individuals of *E. tyndarus* (green squares) and among individuals of *E. cassioides* (red circles) as a function of $\log_{10}$ pairwise least-cost path (LCP) geographic distance. Lines represent least-squares regressions (dashed for *E. tyndarus*,c ontinuousf or *E. cassioides*).

distance on geographic distance in between-species comparisons. A similar pattern is also observed between *E. cassioides* and *E. nivalis* (Figs. 2a and 3a). Moreover, a generalized introgression between *E. tyndarus* and *E. nivalis* can be excluded, since most individuals from the *E. nivalis* to *E. tyndarus* contact zone in Switzerland (Fig. 1c) are unambiguously assigned to either one or the other species (Figs. 2a and 3a). However, at least one individual is admixed (Fig. 3a), with an estimated 0.05–0.12 of *E. tyndarus* genes, and thus very unlikely to be an F1 hybrid. This observation is especially interesting since the *E. nivalis* × *E. tyndarus* cross-breeding was never tested by Lorković (1958), and morphological identification of hybrids of these two species in the field is considered practically impossible (Descimon and Mallet 2009).M oreover,a ndc onsistently,s omed egree of IBD is detected when *E. nivalis–E. tyndarus* pairs are compared. Our sample is too limited to draw proper inferences about the rate of introgression among the different species. However, it is sufficient to conclude that (1) hybridization between *E. nivalis* and *E. tyndarus* did occur in recent generations, but (2) the genetic distinctiveness of each species is not disrupted by the occurrence of some, probably fertile, hybrids, thus arguing for the status of "good" species under a slightly relaxed biological species concept (Coynea ndO rr2 004).

By showing a clear genetic differentiation among the four species within the "terminal" clade of the *E. tyndarus* species complex, our results may appear at odds with previous studies, which reported much more ambiguous patterns (Lattese ta l.1 994; Martin et al. 2002; Albre et al. 2008). A possible explanation for this discrepancy might be found in the much higher number of genetic markers employed in this study (∼400 RAD loci vs. ∼15 allozyme loci or a single mtDNA locus). However, we repeated our PCA+k_{means} clustering (see the "Methods" section) on random samples of 5, 10, 20, 40, 80, and 160 RAD loci, and we found that the "correct" four-species clustering was obtained in over 80% of the samples already with as few as 20 loci (Supplementary Fig. S2). Moreover, the "correct" clustering was obtained in over 95% of the samples when NJ trees were built on distance matrices based on 20 loci (Supplementary Fig. S2). Therefore, it seems unlikely that such a difference might be solely due to the higher resolution provided by our next-generation sequencing approach. As for the two studies employing allozyme markers, it is possible that non-neutral evolution of metabolically important enzymes represent a partial explanation. A hint in this direction may be provided by the fact that, in both Martin et al. (2002) and Lattes et al. (1994), samples of *E. nivalis* appear as the most genetically differentiated, consistent with the stronger ecological divergence of this species. Moreover, both cited studies relied on pairwise genetic distances among populations, rather than analyzing individual genotypes, so that strong genetic drift in a few populations might have increased measures of genetic divergence, and/or misidentification of some individuals might have led to biased results. On the other hand, the observed lack of reciprocal monophyly in mtDNA trees (Martin et al. 2002; Albre et al. 2008)m ight, in principle, result from incomplete lineage sorting. However, our RAD sequencing data show that a large fraction of the putative loci is fixed for alternative alleles between species samples. For example, *E. tyndarus* and *E. cassioides*,w hichw erer epresentedb yt hel argest samples in our analyses, are fixed for alternative alleles at 112/398 loci, with evidence for incomplete lineage sorting (i.e., SNPs where both alleles were observed in both species) at only 25/398 loci. These figures suggest that incomplete lineage sorting at mtDNA is quite unlikely. An intriguing hypothesis is raised by the finding of sequences from the *Wolbachia* genome in our raw RAD sequencing data set. *Wolbachia* endosymbionts are known to facilitate introgression of mtDNA across species, by generating a fitness advantage for infected females compared to non-infected females (Bachtrog et al. 2006). Therefore, it is reasonable to hypothesize that a *Wolbachia* infection occurring after the initial divergence of *E. cassiodes* may have led to the fixation of a single, recent, *Wolbachia*-associatedm itochondrial lineage across all four species through rare events of hybridization and subsequent selective introgression. We aim at testing this hypothesis by analyzing an mtDNA data set larger than those already published and simulating mtDNA genealogies under a divergence scenario estimated from RAD sequencing data.

Remarks on Species Delimitation

Our analyses show that unambiguous delimitation of species in the *E. tyndarus* "terminal" clade can be accomplished by genomic analyses of a small number of individuals, employing a relatively large set of genetic markers, coupled with a carefully planned sampling and an analysis of the geographic distribution of genetic diversity. Indeed, our results suggest that the previous

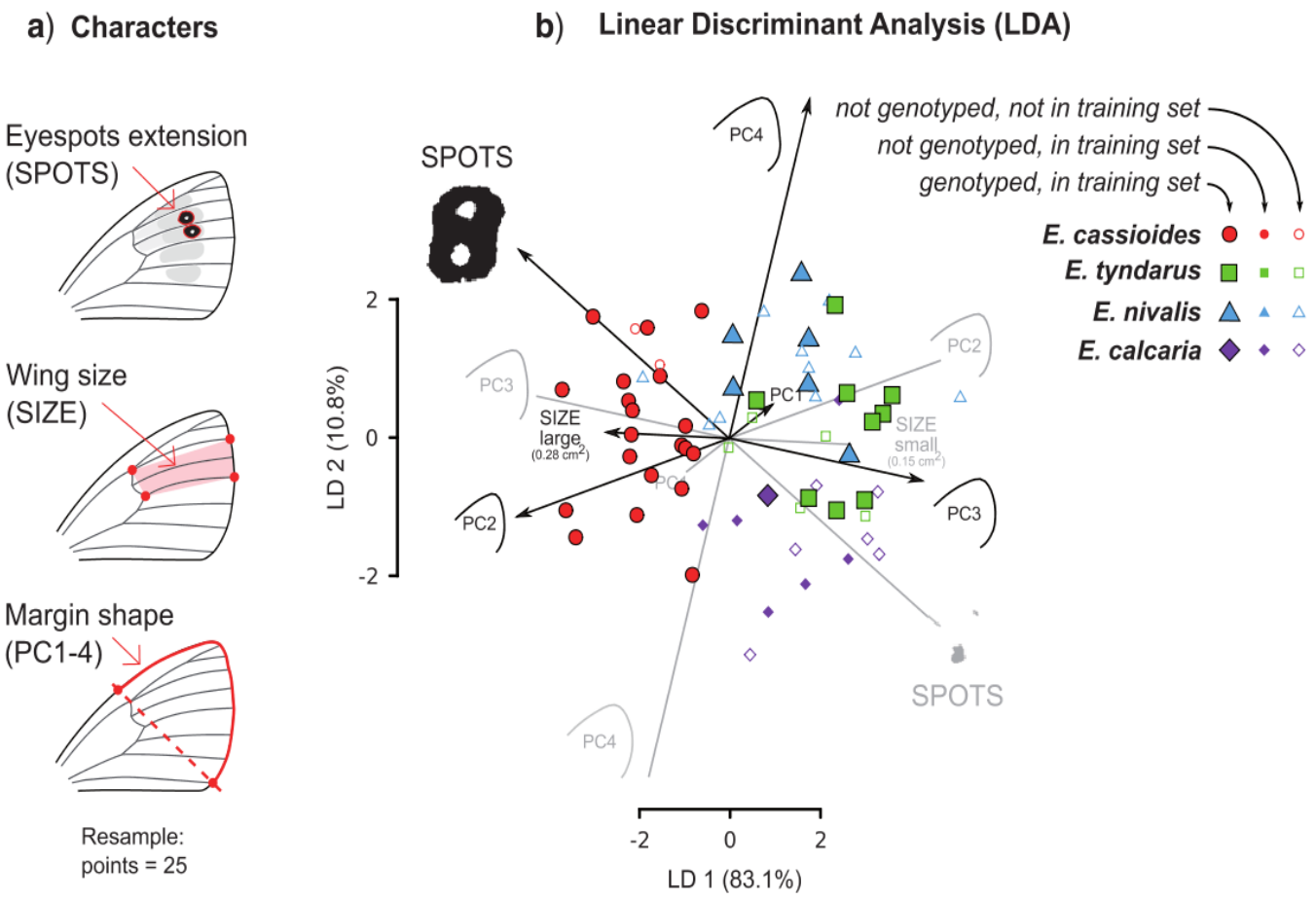

FIGURE 5. Analysis of forewing morphometric characters. a) Measured characters (see Supplementary Appendix 2 for details). b) Scatterplot of the first two discriminant functions (LD1 and LD2) from a LDA. Large, full symbols represent individuals sequenced at RAD loci; small, full symbols, represent individuals not genetically analyzed which were included in the LDA training sample; small, empty dots represent individuals that were employed in the analysis of morphological variation, but were not included in the LDA training sample. Colors and symbols indicate provisional species assignment. Vectors are proportional to the coefficients of SPOTS, SIZE, and the first four principal coordinates (PCs) of the wing margin's shape in LD1 and LD2. Features of extreme individuals are represented at each end of the vectors, except for PC1, which has very little diagnostic significance.

lack of a genetically supported delimitation of species in this group was mostly due to the lack of "good" data (adequate sampling, individual-based analyses, and multiple genetic markers) than to the intrinsic absence of clear genealogical gaps among "bad" species. Indeed, RAD data allow us to clearly identify these gaps between species, and have more than enough power even to discriminate among geographically separated populations of *E. cassiodes* (Fig. 2c,d). Importantly, the coalescent-based BFD* decisively supports the modeling of three regional assemblages of *E. cassiodes* as separate species (H2) rather than as a single one (H1). Since these three population assemblages are separated by obvious geographic gaps in the species range (Fig. 1c), and given that the formal model tested in the BFD* concerns one panmictic populations (H1) vs. three completely isolated populations (H2), it is hardly surprising that H2 is a better fit to the data. However, our analyses show that genetic divergence among all populations of *E. cassiodes* fits an IBD model, just as if no geographic gaps existed. These results highlight that, if geography is not carefully accounted for, the application of coalescent-based species delimitation methods (especially when used with powerful genomic data) may be misleading. Indeed, it may result in (1) strong statistical support for the split of geographically separated populations into different species, even when genetic differentiation is a simple function of geographic distance and/or (2) incorrect identification of multiple species when an incomplete sample of a continuously distributed species is analyzed.

Importantly, our geography-based approach to species delimitation is rooted in expectations directly deriving from the biological species concept (i.e., coexistence in sympatry, e.g., Mayr 1942),a ndi sa ne xample of how non-genetic information can, and whenever possible should, guide the interpretation of results from genetic species delimitation. Indeed, we propose that analyses of the kind proposed in this study should be carefully considered before taking on more sophisticated (and computationally intensive) methods, and that, more generally, the geographic component of genetic differentiation should always be accounted for. Our point here is not to question the theoretical validity or practical usefulness of coalescent-based species delimitation methods. We rather argue that, even in the phylogenomics age, the toolkit for species delimitation should not dismiss more traditional, biologically grounded approaches that allow combination of genetic data with other sources of information.

Lastly, it is worth stressing that our phylogenomic clustering fully matches our a priori species identification, which is based on morphological traits, distribution, and ecology, but which ultimately relies on the work of a past generation of researchers that also examined karyological traits (deL esse1 953;

Lorković 1958). Therefore, while properly designed phylogenomic analyses can provide reliable tests for species delimitation, we stress that the recognition of a set of populations as a "good" species should never neglect a broader, multidimensional examination of their biological attributes. In particular, if "gray zones" are inherent to the speciation process and/or to the philosophical attributes of the species concept (Pigliucci 2003),s peciesd elimitationw illn evera mount to a straightforward analytical pipeline returning a dichotomous response, but will always consist of a multidimensional exploration of how any particular set of populations meet a given body of properties at the morphological, behavioral, ecological, and genetic level (e.g., Mayr 1957; Sbordoni 1993, Edwards and Knowles 2014).

Supplementary Data

Data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.3n5c9.

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