Integrative analysis of DNA phylogeography and morphology of the European rose chafer (Cetonia aurata) to infer species taxonomy and patterns of postglacial colonisation in Europe

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Abstract

Integrative taxonomy has been proposed as a framework to unify new conceptual and methodological developments in quantitative assessment of trait variation used in species delimitation, but empirical studies in this young branch of systematics are rare. Here we use standard phylogenetic and parsimony network analyses on nuclear and mitochondrial DNA (Cox1, ITS1) of 230 individuals from 65 European sampling sites in order to deduce population structure of Cetonia beetles from geno- and haplotypes. Statistical measures of population differentiation are inferred on genealogical and geographical scales to test hypotheses about species limits and population history. By combining results of phylogenetic structure with features of morphology, including genital shape morphometrics and discrete external body characters, as well as with measures of population genetics, we attempt to integrate the results as a test of the validity of species limits, in particular of currently recognised subspecies. Despite high Cox1 divergence between some haplotype lineages, even some sympatric lineages (9%, e.g. N2 vs. N4), nDNA and morphology, as well as pattern of geographical and genealogical divergence measured by AMOVA analysis did not support the hypothesis of separate species. Highest divergence in nuclear markers was found among Italian populations of C. aurata pisana and C. a. sicula, and moderately high fixation indices along measurable morphological divergence suggest the correctness of their status as ‘subspecies’. Divergence time estimates of the lineages suggest a glacial divergence in different refugia between the major haplogroups, while population differentiation in mtDNA among these was primarily attributable to restricted gene flow caused by geographic isolation.

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1. Introduction

The renewed interest in understanding and preserving biodiversity on Earth has resulted in radical new developments in the fields of taxonomy and systematics. However, in many groups of organisms, the number of extant species, as well as exact species limits remains poorly known, due in part to difficulties in using traditional species descriptions for identification. This severely limits our ability to study many groups of organisms in a meaningful way, and precludes proper assessment of, for example, their conservation status, their relevance to ecosystem function, or their role in agriculture and forestry. Large scale DNA sequencing (‘barcoding’) has been proposed to overcome this ‘taxonomic impediment’ as it could greatly speed up species identification and perhaps could contribute to the delimitation and description of the 90% or so species on Earth which still have not been formally named (Tautz et al., 2002; Hebert et al., 2003a). Early results were promising (Hebert et al., 2003b) and developments in the field rapid (Vogler and Monaghan, 2007). Moreover, DNA sequences provide a very powerful tool to recognise structure within a species complex (e.g. Gómez-Zurita et al., 2000). This is essential for reconstruction of range evolution (phylogeography) of a taxon and also to define conservation units (Vogler and deSalle, 1994a), both evolutionary significant units and manageable units (Moritz, 1994; Paetkau, 1999). Due to shortcomings of mtDNA-based species delineation and its frequent incompatibility with morphology and traditional taxonomy, integrative taxonomy has been proposed as a framework to unify new conceptual and methodological developments for quantitative assessment of lines of evidence used in species delimitation (e.g. Schlick-Steiner et al., 2010; Palad et al., 2010). However, few empirical studies have been completed. Recognition of conservation units seems
particularly important for ‘flagship species’. These species are used by conservation advocacy groups to represent entire guilds of species as indicators of optimal habitat conditions (e.g. Habitats Directive, formally known as Council Directive 92/43/EEC on the Conservation of natural habitats and of wild fauna and flora) or, due to their attractiveness, they may be used as outstanding examples for hundreds of species with similar life styles by political entities in order to make the public aware of environmental problems (Kuratorium Insect of the year, 2000). For flagship species, such as the European rose chafer Cetonia aurata (Linné, 1761), facts concerning taxonomy, distribution and ecology are generally better known than they are for other less charismatic species.

This comparatively large scarab beetle (Coleoptera: Scarabaeidae: Cetoniinae) is, with its greenish-coppery integument, one of the most attractive beetles in central Europe. Twenty-eight species are united in the Palearctic genus Cetonia (Krajčík, 1998). While adults of Cetonia aurata feed on pollen and are frequent visitors of many types of wild and cultivated flowers, its larvae feed on decaying wood and organic matter. During the past few decades, this species has become a constant inhabitant of composts in gardens and nurseries and may cause damage in rose plantations. Cetonia aurata is widely distributed from the Pyrenees to Siberia and has been subdivided into five subspecies (Smetana, 2006). Subsequent attempts to justify these divisions have used very subtle differences in male genital characters (e.g. Aliquo, 1983; Baraud, 1992; Lisa, 1999, 2001). Recently, a sixth subspecies was described from central Asia (Rataj, 2000). In central Europe the prevailing colour is green, while in southern European populations there is remarkable variation in colour, which has spawned some 82 taxon names (Krajčík, 1998), some are valid species, others synonyms (e.g. Mulsant, 1842; Nedelkov, 1909; see Smetana, 2006), and others as unavailable infrasubspecific names (by rules of zoological nomenclature: e.g. Heller, 1900; Bourgin, 1943, 1945; Dellacasa, 1973; Leplat, 1996; Caubet, 1999). Interestingly, the same colour forms do occur in different subspecies (Lisa, 1999, 2001).

In the present study, we used a representative sample of a wide range of European Cetonia aurata populations and applied standard phylogenetic and parsimony network analyses to nuclear and mitochondrial DNA data (ITS1 and Cox1) in order to deduce population structure from geno- and haplotypes. Statistical measures of population differentiations at genealogical and geographical scales were inferred to test hypotheses about species limits and population history. By combining phylogenetic structure with features of external and genital morphology, including shape morphometrics and discrete body characters, as well as with measures of population genetics, we attempted to integrate the results for a test of the validity of species limits. Comparing genetic diversity among populations and identifying evolutionary units (Avise, 2000; Crandall et al., 2000) in Cetonia populations may assist decision makers in answering questions regarding conservation management and recovery strategies for these beetles.

2. Materials and methods

2.1. Taxon sampling, DNA extraction, and DNA sequencing

We extracted DNA from 230 individuals of Cetonia aurata from 65 localities across Europe (Supplement Tables 1 and 11), including the populations assigned separate subspecific rank (Cetonia aurata aurata (Linnaeus, 1761), C. aurata pisani Heer, 1841, C. aurata sicula Aliquo, 1983, C. aurata pallida Drury, 1773), as well as three other closely related Cetonia species that were available to us (C. delgrangei, C. carchumii and C. aurataeformis). Morphospecies identifications were based on the keys of Baraud (1992) and Lisa (1999). The outgroup comprised species of Potosia spp., which are the closest relatives to Cetonia available in Europe (Mico et al., 2008). DNA was extracted from thoracic leg muscle tissue using Promega WizardSV extraction plates. Following DNA extraction, beetles and male genitalia were dry mounted. Vouchers are deposited at the Zoological Research Museum A. Koening, Bonn (ZFMK).

Gene regions used here included the mitochondrial cytochrome oxidase subunit 1 (Cox1) and the internal transcribed spacer (ITS1) fragment. PCR and sequencing was performed using primers CI-1-2183 (Jerry) and TL2-N-3014 (Pat) for Cox1 (Simon et al., 1994) and ITS1 F/ITS1 R (Vogler and DeSalle, 1994). Sequencing was performed on both strands using BigDye v. 2.1 and an ABI3730 automated sequencer. Sequences were edited manually using Sequencher v. 4.8 (Gene Codes Corp., Ann Arbor, MI, USA). All sequences used in the present study have been submitted to GenBank (Supplement Table 1).

2.2. Phylogenetic analysis

Progressive alignment procedures of sequences were conducted in ClustalX 2.0 (Thompson et al., 1997) under default gap opening penalties (GO) of 15 and extension penalties (GE) of 6.66. In order to obtain lines of evidence for species evolution from mitochondrial and nuclear sequences, Cox1 and ITS1 sequences were analysed separately. Model-based phylogenetic analyses under Maximum Likelihood (ML) were performed in PhyML (Gouin and Gascuel, 2003) using a GTR model (as selected by Modeltest using AIC; Posada and Crandall, 1998; Akaike, 1974) with all parameters estimated from the data and four substitution rate categories. Split networks as implemented in SplitsTree4 v4.12.3 (Huelsen and Bryant, 2006) were used to represent incompatible and ambiguous signals in the Cox1 data set. In such a network, parallel edges, rather than single branches, are used to represent the splits computed from the data. In order to accommodate incompatible splits, a split network may, and often does, contain nodes that do not represent ancestral species. Because of this, the representation of evolutionary history provided by the network is only “implicit” (Huelsen and Bryant, 2006).

2.3. Analysis of molecular variation

DNA character variation was analysed using statistical parsimony analysis (Templeton, 2001) at a connection limit of 95%. This procedure partitions the data into networks, i.e. subgroups, of closely related haplotypes connected by branches with a <95% probability to be non-homoplastic. For mtDNA of insects, these networks usually group haplotypes above the species-level (e.g. Templeton, 2001; Wiens and Penkrot, 2002; Cardoso and Vogler, 2005; Pons et al., 2006; Ahrens et al., 2007; Astrin et al., 2012). Statistical parsimony networks were determined using TCS v.1.3 (Clement et al., 2000). Separate analyses were conducted on Cox1 and ITS1. Hypotheses of population separation to define potentially diagnosable groups a priori were based either on occurrence at different geographic localities or on morphological traits. We measured divergence (uncorrected p-distance) within and among networks using Cox1. A spatial analysis of molecular variance algorithm (SAMOVA; Dupanloup et al., 2002) was used to explore the population configuration. Given the number of groups (K), the population configuration with the highest differentiation among groups (Fst) was estimated using a simulated annealing procedure by SAMOVA 1.0. The analysis was run for k = 2–20 and significance of fixation indices was tested with 1000 permutations. The optimum number of population groups for a set of sample populations is expected to show the highest index Fst for the different values of k. To test whether genetic differentiation among ‘populations’ was significant, we used analysis of molecular variance (AMOVA; Excoffier et al., 1992) implemented in ARLEQUIN v. 3.5 (Excoffier
et al., 2005). This approach was applied on the 95% cut-off networks from TCS analysis, on twelve arbitrarily and 'a priori' defined geographical regions, and was implemented in SAMOVA for the different grouping simulations. As a complimentary investigation, we tested for geographical associations among haplotypes and clades based on the most parsimonious haplotype network, followed by a nested cladistic analysis (NCPA) (Templeton et al., 1995; Templeton, 1998, 2001) as implemented in the program ANeCA v1.2 (Panchal and Beaumont, 2008). This was performed separately on the two markers in order to explore what factors shaped the species' history. Despite NCPA's past popularity (Templeton, 2008), there are several drawbacks and limitations associated with the method e.g. a high false positive rate, the lack of measure of confidence in its support for estimates and the inability to test specific alternative hypotheses following the rejection of a simple null hypothesis by a permutation test (for more details see Knowles and Maddison, 2002; Petit, 2008; Beamont et al., 2010). However, it must be regarded as an important and useful exploratory tool (Templeton, 2009a,b; Beamont et al., 2010).

Evolutionary analyses, including calibration of the nodes, were conducted in BEAST 1.6.1. As a topology constraint for the BEAST analysis we used a newly estimated phylogenetic tree (in PhyML) based on the Cox1 data, with only a single terminal for each of the [95% cut-off] parsimony networks obtained from TCS analysis. We estimated the timing of divergence of the seven major haplotype lineages using a distance-based and a Bayesian approach as implemented in MEGA5 (Tamura et al., 2007) and BEAST 1.5.2 (Drummond and Rambaut, 2007), respectively. Due to lack of suitable fossil records, we applied the two values of molecular rate of evolution of 2.3% and 3.54% sequence divergence per Million years [Myr] proposed as standard divergence of insect Cox1 DNA by Brower (1994) and Papadopoulou et al. (2010), respectively, in order to calibrate the tree. The substitution model was set to GTR-I as suggested by the Akaike information criterion implemented in jModelTest (Posada, 2008). Using BEAST, two independent runs (10 million generation each) were performed on the dataset. Chains were sampled every 1000 generations, with the initial 10% of the samples removed during burn-in. A newly estimated Maximum Likelihood (ML) tree (PhyML) using Cox1 data with only a single terminal from each of the 95% cut-off parsimony networks (TCS analysis) was used as the topology constraint (MEGA) or starting tree (BEAST), respectively. The results of the independent

**Fig. 1.** PhyML tree (left) and neighbour-joining network (right) of the Cox1 sequences of *Cetonia aurata* and related species with the extension of the parsimony networks (N 1–7) mapped on the tree with coloured bars. Bootstrap values above 50% are shown in grey close to the tree branches. The Sicilian specimens of N5 are marked in the tree with a black dot while in the NJ network being grey shaded. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
BEAST runs were combined in the program LogCombiner 1.5.2 (http://beast.bio.ed.ac.uk/LogCombiner) to check for convergence and mixing of independent chains. Trees resulting from the three independent runs were summarised in a consensus tree using TreeAnnotator 1.5.2 (http://beast.bio.ed.ac.uk/TreeAnnotator).

We used the General Mixed Yule Coalescent model (GMYC) of Pons et al. (2006) to infer species boundaries by delimiting mtDNA clusters based on the transition from slow to faster apparent branching rates on the gene tree expected at the species boundary (Acinas et al., 2004). This method optimises a threshold age such that nodes before the threshold are considered to be diversification events (i.e. reflect cladogenesis generating the isolated species) and nodes subsequent to the threshold reflect coalescence occurring within each species. For this approach, we pruned all identical haplotypes from our Cox1 data set and recalculated the tree (PhyML), which was subsequently made ultrametric in Pathd8 (Britton et al., 2007) with the root of an arbitrary age of one. GMYC modelling was performed using the GMYC-script, available as a tool within the splits package for R (Ezard et al., 2009).

2.4. Morphology

In order to infer a morphological differentiation between the populations and to explore congruence with DNA-based clusters, each specimen included in the molecular analyses was scored for nine discrete morphological characters, and an outline analysis was performed on the apex of genitalia in male specimens. The partial outline of the male’s right paramere (part of the intromittent genital organs, in dorsal view) was digitized from images captured on a digital microscope. The partial outline was extracted using TPSDig2.1 (Rohlf, 2006), and the curves subsequently converted into a set of 100 semi-landmarks. These curves produce a measure that is independent of size in this sample. Using the Eigenshape program as implemented in Morpho-tools (available on www.morpho-tools.net) (Krieger, 2006), the set of outline coordinate points was converted from Cartesian (x,y) form to the \( \phi \) form of the Zahn and Roskies (1972) shape function. The resultant shape functions were expressed as raw net angular deviation (\( \phi \), mean centred, no standardization). Eigenshape analysis (MacLeod and Rose, 1993; MacLeod, 1999) was performed in Eigenshape v.2.6 as implemented in Morpho-tools using the covariance for calculation of the similarity matrix and \( \phi \). Of the 40 eigenaxes produced, further analysis was performed on 12 eigenaxes, which explained 95% of the variation in the samples. Based on the informative eigenaxes, we performed a Canonical Variates Analysis (CVA), with the samples grouped according to the mitochondrial DNA networks and the recognised subspecific units, C. a. pisana and C. a. sicula.

3. Results

3.1. Molecular trees and parsimony networks

We obtained 826 base pairs [bp] of the 3’ portion of Cox1 from 147 adults and larvae and a fragment of the nuclear ITS1 (963 aligned positions) was isolated from 192 adults and larvae. The latter varied little in length among the ingroup (from 922 to 947 bp). In total we identified 94 and 25 different haplotypes for Cox1 and ITS1, respectively.

![Fig. 2. The geographical structure of the Cetonia species as represented by the Cox1 networks from 95% connection limit (N 1–7, coloured as in Fig. 1) of C. aurata shown for the major geographical regions (a – Iberian peninsula, b – France, c – UK, d – central Europe, e – northern Europe, f – NW Balkan, g – Corsica, h – Sardinia, i – central and southern Italy, j – Sicily, k – southern Balkan, l – Lebanon). Small circles symbolise geographical regions sampled with less than 5 specimens. The numbered dots represent the collecting sites for the sampling of this study (see Supplement Table 11).](image-url)
The Maximum Likelihood tree (Likelihood: $\log L = -4279.98$) based on the Cox1 sequences revealed, in addition to *C. delagrangei*, *C. carthami* and *C. aurataeformis*, two principal clades (Fig. 1). One clade comprised *C. aurata aurata*, the other contained populations of *C. aurata pisana* and *C. aurata sicula* (Baraud, 1992; Lisa, 1999). Specimens of the latter were not monophyletic (Fig. 1, tree terminals marked with black dots). The *C. aurata aurata* clade is composed of two major clades with broadly overlapping geographical ranges (Fig. 2). The tree topology is generally consistent with the neighbour-joining network based on the Cox1 data (Fig. 1, right side) and the combined data (Supplement Fig. 1). The latter clearly shows the same divergence between the *C. aurata aurata* clade and the *C. a. pisana/ sicula* clade, but also shows divergence between the two clades of *C. aurata aurata*.

The ML tree of ITS1 sequences (not shown) did not reveal single species monophyletic clades (except for *C. delagrangei*). One large clade was composed of the representatives of *C. aurata*, *C. carthami* and *C. aurataeformis*. Interestingly, the latter formed a monophyletic clade within the specimens of *C. a. pisana*. Specimens of *C. aurata aurata* showed much less variation than did specimens of *C. aurata pisana*.

### 3.2. Analysis of molecular variation

Statistical parsimony analysis with TCS v.1.3 (Clement et al., 2000) on the Cox1 sequences (Templeton et al., 1992) resulted in seven independent networks (mapped on the ML tree and the neighbour-joining network in Fig. 1). The number of steps by which two haplotypes have a 95% statistical probability of being linked without homoplasy was calculated to be 12. These separate parsimony networks broadly matched the major clades of the Cox1 ML tree. The *C. aurata aurata* clade splits into three separate networks (Fig. 1), one of these included only a single specimen from the Aegean Islands (network 3). On the other hand, only a single network (network 5, Fig. 1) resulted for the specimens of *C. aurata pisana*, which also included the specimens of *C. aurata sicula* from Sicily. Among networks 2, 4 and 5 we found extensive differentiation. Their detailed interior relationships are depicted in Fig. 3. In particular, network 2 had high amount of divergence among the Balkan populations with six subgroups separated by three steps (Fig. 3). The southeastern populations of the Balkans were separated into subclades, which clustered geographically to some extent, but their genetic diversity was unevenly distributed.

Fig. 3. Details of parsimony networks based on Cox1 sequences of *C. aurata* specimens belonging to separate network 2, 4 and 5 showing major features of geography of haplotypes and nested clades with divergence inferred by the NCPA shown as broken contour lines: red – restricted gene flow with isolation by distance; blue – allopatric fragmentation; green – contiguous range expansion (nested clades with divergence not inferred by NCPA not shown, see Supplement Table 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
On the other hand, network 2 contained a few very widespread haplotypes (Fig. 3, haplotype 3). In contrast to network 2, network 4 did not show much variation across the Balkans, resulting in only two three-step subclades (Fig. 3). Network 5 contains four subclades separated by eight steps (Fig. 3), showing little geographical clustering, except for the separate populations of *C. a. sicula* from Sicily (highlighted yellow, Fig. 3). The Calabrian populations of network 5 (haplotype 78, Fig. 3) nest together with central Italian specimens (haplotype 44, Fig. 3), and they do not show an intermediate position relative to the island population of Sicily.

Statistical parsimony analysis on the ITS1 sequences of *Cetonia* resulted in two independent networks (Fig. 4), one comprising *C. delagrangei*, the second composed of all the other specimens, including the three species *C. aurataeformis*, *C. aurata*, and *C. carthami*. In the latter network, most of the variation occurred among specimens of *C. aurata pisana* (corresponding *Cox1* network 5), while the specimens of *C. aurata aurata* were poorly differentiated and were represented by only three genotypes. A few genotypes of ITS1 (Fig. 4) shared *Cox1* haplotypes that were part of different *Cox1* networks (network 2, 4) with one (1.62) being very widespread.

Our GMYC analysis resulted in a vast over-splitting for the four species of *Cetonia* included in this study. The analysis resulted in 15 GMYC units, with a confidence interval of 12–18 entities. The likelihood ratio between GMYC and the null model was 20.45 (with significance in the LR test $p = 0.0001$; $\text{LnLH}(_{\text{GMYC}}) = 630.34$; $\text{LnLH}_{0} = 620.11$). The *C. aurata* lineage separated into 12 GMYC clusters, in particular network 2 was subdivided into three GMYC units while network 4 was congruent with a single GMYC unit. The largest over splitting was found in N5 where the Sicilian populations (*C. aurata sicula*) resulted in four separate GMYC units.

### 3.3. Spatial analysis of genetic population differentiation

NCPA was separately performed on *Cox1* and ITS1 data. The topology of their networks (Figs. 3 and 4) was the basis for establishing the hierarchical nesting design for subsequent statistical tests of geographical association of haplotypes. Inferences on historical or population structure were made using the automatic tool ANeCA v1.2 (Panchal and Beaumont, 2008), which is based on the inference key of Templeton et al. (1995). These analyses suggest ‘restricted gene flow with isolation by distance’ as the predominant

<table>
<thead>
<tr>
<th>Clade</th>
<th>Chain of inference</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cox1</td>
<td>1-2 1-19-2-11-12 NO</td>
<td>Contiguous range expansion</td>
</tr>
<tr>
<td></td>
<td>1-5 1-19 NO</td>
<td>Allopatric fragmentation</td>
</tr>
<tr>
<td></td>
<td>1-29 1-2-11-17-4 NO</td>
<td>Restricted gene flow with isolation by distance*</td>
</tr>
<tr>
<td></td>
<td>2-15 1-2-11-17-4 NO</td>
<td>Restricted gene flow with isolation by distance*</td>
</tr>
<tr>
<td></td>
<td>2-47 1-19 NO</td>
<td>Allopatric fragmentation</td>
</tr>
<tr>
<td></td>
<td>3-9 1-2-11-17-4 NO</td>
<td>Restricted gene flow with isolation by distance*</td>
</tr>
<tr>
<td></td>
<td>4-8 1-2-11-17-4 NO</td>
<td>Restricted gene flow with isolation by distance*</td>
</tr>
<tr>
<td></td>
<td>4-10 1-2-3-4 NO</td>
<td>Restricted gene flow with isolation by distance*</td>
</tr>
<tr>
<td>ITS1</td>
<td>1-8 1-19-2-11-12 NO</td>
<td>Contiguous range expansion</td>
</tr>
<tr>
<td></td>
<td>2-2 1-19 NO</td>
<td>Allopatric fragmentation</td>
</tr>
<tr>
<td></td>
<td>2-6 1-2-11-12 NO</td>
<td>Contiguous range expansion</td>
</tr>
<tr>
<td></td>
<td>3-2 1-2-11-12 NO</td>
<td>Contiguous range expansion</td>
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<tr>
<td></td>
<td>3-3 1-19 NO</td>
<td>Allopatric fragmentation</td>
</tr>
<tr>
<td></td>
<td>4-1 1-2-11-12-13-14 NO</td>
<td>Long-distance colonisation and/or past fragmentation</td>
</tr>
</tbody>
</table>

(4 (three-step) subclades on Peleponneses). On the other hand, network 2 contained a few very widespread haplotypes (Fig. 3, haplotype 3). In contrast to network 2, network 4 did not show much variation across the Balkans, resulting in only two three-step subclades (Fig. 3). Network 5 contains four subclades separated by eight steps (Fig. 3), showing little geographical clustering, except for the separate populations of *C. a. sicula* from Sicily (highlighted yellow, Fig. 3). The Calabrian populations of network 5 (haplotype 78,
process to explain patterns of Cox1 haplotype distribution, while ‘contiguous range expansion’ was the principle process suggested by ITS1 (Table 1). Significant inferences (Table 1) were depicted in networks 2, 4 and 5 and represented in Figs. 3 and 4 (for Cox1 and ITS1 sequences, respectively) (for detailed nesting structure see Supplement Tables 2 and 3). However, for the most of the nested clades (Cox1: 86%, ITS1: 67%), either the ‘null’ hypothesis could not be rejected, or outcomes were inconclusive. In fact, most of the nesting units overlap geographically and most of the major clades had inconclusive outcomes, probably due to poor geographic sampling. Inference of some geographically well-defined clades (e.g. nested clade 3–5 and 2–6 for Cox1 and ITS1, respectively, for the Sicilian network 5 populations, see Fig. 1) were not recognised as significant during automated NCPA inference. We have therefore not illustrating the complete geographical nesting structure.

Sampling sites failed to cluster for either of the markers (in Cox1, data were available for 44 sites, in ITS1, 56) during SAMOVA analysis. Fixation indices for the various simulations with $n_0$ ($n = 1–20$) were continuously decreased for ITS1 but increased for Cox1 (Supplement Fig. 2), in such way that no maximal $F_{ct}$ could be identified (for detailed results see Supplement Table 4).

We therefore used separate networks as identified with statistical parsimony analysis and 12 predefined geographical regions (see Fig. 2) to explore genetic differentiation through AMOVA. The differentiation between the separate parsimony networks was moderate ($F_{st} = 0.08$) but significant (Tables 1 and 2) as revealed by AMOVAF-Statistics (Excoffier et al., 1992). The exception to this was apparent for the comparisons with C. delagrangei due to the limited number of specimens available for this analysis. The corrected pairwise differences between the separate networks were fairly high, ranging from 3% to 11% (Supplement Table 5), and were higher between network 2 and network 4 than between network 2 and network 5. A multidimensional plot of the corrected pairwise differences reveal that mtDNA differentiation was most similar between C. aurata aurata (networks 2–4), C. a. pisana (network 5) (including C. a. sicula) and the other Cetonia species (C. delagrangei and C. aurataeformis) – all of these groups clearly separating into well-defined clusters (Supplement Fig. 3). Among arbitrarily predefined geographical regions, differentiation for Cox1 was high ($F_{st} = 0.71$). Significant pairwise fixation indices ($F_{st}$) ranged between 0.09 (populations of northwestern and southeastern Balkans) and 0.93 (Iberian and Lebanon populations, i.e. C. aurataeformis vs. C. delagrangei) (Supplement Table 6). While populations with recognised species-status had consistently high fixation indices, that was not the case for the various C. aurata aurata populations. Regions (Italy, Sicily) with specimens belonging to the separate Cox1 network 5 (ssp. pisana and ssp. sicula) had similarly high fixation indices, however, the differentiation between Sicilian populations and those of the Italian mainland was lower ($F_{st} = 0.39$).

The pattern of variation between/among ‘populations’ within separate parsimony networks and within predefined geographical regions was remarkable. Greatest variation was seen between populations within the networks (91.9%), while much less (29.1%) was apparent between populations and geographical regions.

In a Mantel test performed on the pairwise $F_{st}$ of ITS1 and Cox1 data grouped according to separate Cox1 networks and predefined geographical regions, we found a remarkable and significant

![Fig. 5](image)

**Fig. 5.** Plots of pairwise $F_{st}$’s for ITS1 and Cox1 data between (A) the specimens of each separate Cox1 network, and (B) the various predefined geographical regions.
correlation among $F_{st}$ values for both markers among geographical regions ($r = 0.57$, $p < 0.0001$), while $F_{st}$ values for differentiation among the separate Cox1 networks were not significantly correlated ($r = 0.39$, $p = 0.099$). To compare the pairwise $F_{st}$ of both markers for geographical regions and separate networks, we plotted both values against each other (Fig. 5). While incongruent divergence between the markers became evident among the separate networks (Fig. 5A), we recognised three mostly distinct clusters of $F_{st}$ plots with low, medium and high differentiation among geographical regions. Pairwise comparisons with low divergence were observed between Balkan (including Croatian) and central, western and northern European sites, while medium divergence was found mainly between the Italian populations and the latter European sites. The transition to highly differentiated region pairs was smooth, with gaps not as wide as between the low and medium values. The cluster with high $F_{st}$ values mainly comprised regions containing separate species ($C. carthami$, $C. aurataeformis$, $C. delagrangei$), but also contained geographically distant populations (Sicily vs. northern Europe).

3.4. External morphology and genital shape

Of the nine discrete external morphological characters (Supplement Tables 7 and 8) scored for each specimen, four were among
those which traditional taxonomy (Baraud, 1992; Lisa, 1999) regards as most important for distinguishing between the three defined subspecies of *Cetonia aurata*. Only two of the nine characters (Fig. 6B and D), i.e. the punctuation and surface constitution of elytra, were found to be consistent with ML tree topology and with geographic clustering, while all others were encountered in the various character states among all lineages (Fig. 6A and C). Weak to moderately strong elytral punctuation and silky dorsal shine of elytra was associated only with specimens in network 5 from the Italian mainland, i.e. excluding Sicilian populations (which are assigned to a separate subspecies, *C. aurata sicula*). Interestingly, Italian specimens in network 4 always shared these character states with those of network 5, while all other specimens of network 4 had robust elytral punctures and metallic dorsal shine. Intermediate states (i.e. moderately strong punctures) occurred in Italy as well as in other European populations.

The morphometric approach revealed that 12 eigenaxes represent 95% of the cumulative variation of shape of the tip of the paramere for the analysed dataset (not including outgroups) (Supplement Table 9). Eigenaxes one, two and three shared alone 42.38%, 18.03%, and 12.05% of variation, respectively. As for genetic variation, we checked the separate Cox1 networks and predefined
geographical regions for their divergence of variation, i.e. respective groups were plotted on the morphospace obtained from the CVA (Fig. 7) based on the 12 eigenaxes (see Supplement Table 10). Cetonia carthami and the specimen of C. aurata in the separate Cox1 network 3 had to be excluded since we had only single male specimens available. Plots revealed an almost complete overlap between specimens of separate Cox1 networks 2 and 4 (Fig. 7A), indicating no significant differentiation in male genital shape between these two major haplotype clades. The same was valid for the specimens of haplotype network 5, except the Sicilian populations showed a wider overlap with network 4 rather than with network 2. In contrast to that, Sicilian specimens of network 5 representing C. aurata sicula were distinctly differentiated from those of Italian mainland, as well as from those of network 2 and network 4, although showing limited overlap in morphospace with specimens of network 2 and network 4 (Fig. 7A, CVA axes 1–2). Cetonia aurataeformis clustered distinctly distant from all C. aurata specimens. Overlap between geographical regions, particularly among the specimens formerly assigned to C. aurata, Cox1 clustered distinctly distant from all network 4, although showing limited overlap in morphospace with those of Italian mainland, as well as from those of network 2 and network 4, although showing limited overlap in morphospace with specimens of network 2 and network 4 (Fig. 7A, CVA axes 1–2). Cetonia aurataeformis clustered distinctly distant from all C. aurata specimens. Overlap between geographical regions, particularly among the specimens formerly assigned to Cox1 networks 2 and 4, was significantly reduced (Fig. 7B), consequently we concluded that most of the observed variation was related to geographical isolation and not to the mtDNA divergence apparent from the Cox1 data. A MANOVA on Eigenaxes 1–12, however, failed for most of the group pairs due to insufficient sampling of male specimens (for this reason, a few areas had to be excluded from the MANOVA analysis: Sweden, UK, Sardinia, Corsica).

Based on standard rates of sequence divergence of the Cox1 data (2.3% and 3.5%) (Brower, 1994; Papadopoulou et al., 2010), we explored divergence times between the different genetic clusters. Age estimates performed on the Cox1 tree in BEAST and in MEGA were quite similar (calibrated trees not shown). The split between network 2 and networks 3/4 apparently occurred from late in the last glacial maximum to the early Holocene (Allen et al., 1999). Node ages ranged from 4.5 (3.5%) to 15 kyr (2.3%) (BEAST) and from 9 (3.5%) to 14 kyr (2.3%) (MEGA) while the split between networks 2–4 and network 5 was estimated around 12.3 (3.5%) to 49.8 kyr (2.3%) (BEAST) and 30 (3.5%) to 46 kyr (2.3%) (MEGA). The “split” between the populations from Italian mainland and Sicily (network 5) had about the same age as the splits between network 2 and networks 3/4.

4. Discussion

Analysis of genetic data, and in particular of mtDNA, holds the promise for the discovery of morphologically cryptic taxa (e.g. Hebert et al., 2004; Blaxter, 2004) and to establish a universal system for taxonomic reference (e.g. Tautz et al., 2002). Due to known pitfalls in barcoding (e.g. Moritz and Cicero, 2004), an integrated approach that uses different lines of evidence to infer taxonomy has been advocated (e.g. Will et al., 2005). Padial et al. (2010) presented protocols that integrate various lines of evidence, but many primary choices remain subjective and the method chosen to test for congruence of evidence may influence the outcome of the analysis (see Schlick-Steiner et al., 2010).

In the current analysis, we explored variation of different traits (mtDNA, nDNA, morphology, shape) using a variety of approaches to detect statistically definable groups that might fit at least one available species concept. We alternatively compared the hypothesis of genetic divergence with that of spatial divergence applying diverse methods of population genetics.

The phylogeography of Cetonia aurata elaborated with nuclear and mitochondrial DNA resulted in a widely incongruent pattern between the two data sets. Variation and divergence of nDNA and mtDNA markers were in part uncorrelated, as shown by Fig. 5A and by a Mantel test of fixation indices. Since our numerous techniques revealed few significant results (NCPA) and failed to elucidate species boundaries (GMYC) and geographical structuring (SAMOVA) (probably due at least in part to sampling issues with regard to the number of populations per group; Fitzpatrick, 2009), we based the major part of our investigation on predefined groups that were evident from the Cox1 statistical parsimony analysis (separate networks) and from geographical origin of our specimens.

The divergence of network 5 and networks 2–4 (Figs. 1 and 2) shows characteristics of a parapatric separation (Balkan vs. Appenine refugium; Schmitt, 2007). Automated inference of NCMA (Supplement Tables 2 and 3) failed, however, to recognise this pattern, likely due to subsequent admixture of haplotypes from northern or eastern Balkan populations of C. aurata aurata with those from the Appenine peninsula. The situation between networks 2, 3 and 4 is much more complex. In all three mtDNA networks, most genetic diversity is distributed in the Balkans, with little geographic structure apparent from current distributions of haplotypes. We propose two plausible explanations for this pattern: the strong dispersal capacity of the species, or human related (accidental or purposeful) translocations of living individuals (see Lisa, 1999). Whether one or two of the three haplotype lineages (networks 2–4) might represent the fourth subspecies of C. aurata in Europe [C. a. pallida occurring mainly in Turkey, but also recorded from the Adriatic coast and the southern Balkans (Lisa, 1999)] remains uncertain. We found no morphological evidence supposedly characteristic of this subspecies (Baraud, 1992; Lisa, 1999). The timing of splits between the various haplotype lineages in Cetonia aurata with mtDNA remains problematic due to the lack of suitable fossils for tree calibration. In addition we have an external source of data that would allow us to determine the evolutionary rate of mtDNA. The widely used standard divergence (Brower, 1994) was shown to be not valid in many cases (Papadopoulou et al., 2010) and the application of various “standards” of molecular rates does not necessarily sharpen the estimates. However, if we assume the dating of the networks 4–2–3 split correctly to be around the late Pleistocene (ca. 15 thousand years ago [kyr]), further differentiation within the separate networks must have occurred during the re-colonisation process of Europe, or later in the Holocene. However, mitochondrial divergence during the last glacial maximum (LGM) appears to be delayed since lineage sorting does not correspond directly with either the onset or centre of the LGM. Nevertheless, divergence estimates among major lineages of Cetonia, in particular the split between the haplotype networks 2 and 3 + 4, seem to fall within widely separated southern European populations (i.e. refugia of Appenine peninsula vs. Balkans) as is also evident from fossil pollen records of deciduous Quercus (Brewer et al., 2002), which presently show a distribution similar to that of Cetonia in Europe.

Combining the ITS1 parsimony network topology with mtDNA haplotypes (Fig. 4) suggested that hybridisation has occurred between C. aurata aurata and C. aurata pisana [network 4 haplotypes sharing the genotype (15.15) with network 5 haplotypes in Corsica and central Italy, which is nested among the other genotypes of network 5 (Fig. 4)]. Added to this, specimens from Corsica share morphological key traits with the Italian population (Fig. 6B and D). The fact that genotype 1.62 includes haplotypes from three different networks (Fig. 4) might be an indicator of the origin of the C. aurata aurata clade (Fig. 4, nested clade 3–2).

The study of body colouration did not reveal a link between colour and genetic structure (Fig. 6C), although some of this inconsistency may be explained by poorly sampled lineages, e.g. C. carthami. Thus, it must be assumed that colouration diversity is due to polymorphism. This generally agrees with Lisa (2001), who stated that after rearing numerous specimens of different colour forms, that colour seemed not to be a character genetically
transferred, but rather was influenced by climatic or ecological factors of the habitat. Henrrotte et al. (1984) argued that the chemical composition of breeding substrate of rose chafers like Cetonia can influence the concentration of magnesium within the specimens (although it may differ significantly between different genera) and cause cuticular modifications (i.e. its thickness, supposedly a genetically induced polymorphism) that might play a role in determination of colour (e.g. Kurachi et al., 2002; Davis et al., 2008; Akamine et al., 2011).

The underlying processes responsible for the differentiation of mtDNA in the C. aurata aurata (mtDNA networks 2–4) appeared to be caused primarily by restricted gene flow due to geographic isolation (network 2, Table 1 and Fig. 3), and in a few instances, by allopatric fragmentation (network 4, Fig. 3). At present, we have no explanation for the major split between networks 2 and 4.

The most significant question is: What is the taxonomic status of the separate Cox1 networks representing the generally accepted subspecies of Cetonia aurata, i.e. aurata, pisana, and sicula (Baraud, 1992; Lisa, 1999)? The Cox1 distances between aurata and pisana are almost as great as it is between them and each of the other Cetonia species analysed here (C. carthami, C. australisformis, C. delagraenei) (Supplement Fig. 3). However, the record of hybridisation between C. a. aurata and C. a. pisana, as well as the weaker fixation indices (Fig. 5) of both molecular markers would suggest subspecies status rather than that of separate species. The morphometric results of genital shape (parameres) indicate almost no differentiation between the C. a. pisana and C. a. aurata, in contrast to the high differentiation between subspecies C. a. sicula and C. a. pisana (mtDNA network 5), which showed only limited mtDNA divergence.

The complex phylogeographical pattern in European Cetonia aurata is only partly congruent between mtDNA and nDNA, as well as with other traits, particularly with morphology. This seems to call for an integrated taxonomic approach (e.g. Padial et al., 2009) that uses various lines of evidence for taxon delimitation. As shown in our example of Cetonia aurata, barcoding of Cox1 alone may not resolve problems of species taxonomy, but may just sort out unusual species clusters through preliminary screening (Padial et al., 2010). Although we have a set of novel methodologies at hand to estimate species and population divergence directly from measurable traits (Duncanlop et al., 2002; Pons et al., 2006; Vogler and Monaghan, 2007), these may fail when sampling is not adequate. Nevertheless, using population divergence statistics, such as AMOVA, and multivariate analysis may help to assess congruence of lines of evidence for species borders, as they did during the present study, and thus to choose between alternative hypotheses. As Lim et al. (2012) and Lohse (2009) recognised, sampling is often a crucial issue dictated by the biological systems themselves.

Bergsten et al. (2012) illustrated that increased sampling and extended geographical scale may increase the number of cases of “strange taxonomy” (e.g. non-monophyletic [morpho]species) greatly (up to 80%). Before we can have confidence in the use of these DNA-based approaches for rapid biodiversity assessment (e.g. environmental sequencing), we need more empirical studies to test the robustness and limits of our methods, and to further investigate various techniques for the integration of independent lines of evidence (Schlick-Steiner et al., 2010; Padial et al., 2010).

Acknowledgments

We are grateful to A.P. Vogler and his labgroup for helpful discussions and to Giuseppe Carpaneto, Eckehard Rössner, Arno Thomaes, Jason Maté, Bruno Massa, Roberto Lisa, Guido Sabatinielli, Denis Keith, Eric Jiroux, Ignazio Sparaccio, Olaf Jäger, and Conrad Gillet for collecting specimens. We thank the two anonymous referees who helped to improve the final version of the manuscript.

Dirk Ahrens was supported by the German Science Association (DFG/1H175/2); Petr Šipek would like to acknowledge the institutional resources of Ministry of Education, Youth and Sports of the Czech Republic for the support of science and research.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.05.016.

References


