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When morphological identification meets genetic data: the case of *Lucanus cervus* and *L. tetraodon* (Coleoptera, Lucanidae)

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Abstract

The European stag beetle *Lucanus cervus*, widely distributed across Europe and in some Near East countries, is one of the best known coleopteran species listed in the European Habitats Directive 92/43/EEC and it is considered a flagship species for conservation of saproxylic fauna. *Lucanus tetraodon* is a closely related species whose geographical distribution is still poorly known and debated. The two species have a sympatric occurrence in central Italy, and in some localities of these areas, many individuals show a mosaic of morphological traits that makes species assignment nearly impossible. We used both mitochondrial and nuclear markers to analyse these specimens. The mitochondrial results evidenced that the two species represent well-defined genetic entities with mitochondrial DNA introgression. This pattern could be the result of either hybridization or of a convergence of morphological characters under local selective pressures in areas of sympatric occurrence. The nuclear marker was polymorphic across the two species and therefore did not reveal hybridization, even if many are the supports to this phenomenon. The most plausible explanation for this genetic pattern is a very recent divergence of the two species which share a common origin and thus a common *wg* genotype.

Key words: mtDNA – hybridization – Habitats Directive – stag beetles – Wingless

Introduction

The European stag beetle *Lucanus cervus* (Linnaeus 1758) is one of the best known coleopteran species listed in the European Habitats Directive 92/43/EEC (Annex II and subsequent modifications and additions), and it is considered a flagship species for conservation of saproxylic fauna (Thomaes et al. 2008). *L. cervus* is widely distributed across Europe and strays into some Near East countries (Franciscolo 1997; Bartolozzi and Sprecher-Uebersax 2006; Harvey et al. 2011). It occurs in the northern and central regions of Italy, as far as Latium and Umbria, but is apparently absent in Sardinia and the southern regions (Franciscolo 1997; Bartolozzi and Maggini 2007). There are old records from Sardinia (Bargagli 1872) and Campania (Luigioni 1929), but they have never been confirmed by further findings. There is also a congeneric species in Italy, *Lucanus tetraodon* Thunberg, 1806 (considered in the past a subspecies of *L. cervus*: Paulian 1941), but its geographical distribution is still poorly known and debated. This species was first described from Italy (*terra typica*: 'Italia' without further indications) and subsequently recorded in Albania (Kraatz 1860), Greece (Van Roon 1910), southern France (Colas 1949), Corsica (Baraud 1993), Algeria (Séguy 1955) and northern Spain (Murria Beltrán and Murria Beltrán 2009). Therefore, *L. tetraodon* can be considered a Mediterranean species with a relict distribution pattern. Nevertheless, the extent of its occurrence should be critically revised because the data from Maghreb and Balkan countries are old and vague, often indirectly reported by catalogues (Winkler 1929;

Benesh 1960; Maes 1992; Baraud 1993; Bartolozzi and Sprecher-Uebersax 2006) but not confirmed by recent observations. Most records of this species refer to the Italian peninsula, where it is traditionally considered to inhabit the centre and south, including Sicily (Mikšic 1959a,b, 1961; Bartolozzi 1986; Franciscolo 1997; Lapiana and Sparacio 2006; Santoro et al. 2009; Trizzino et al. 2013), usually associated with meso-xerophilous oaks and other broadleaf trees. Recent reports of populations in Emilia Romagna (Fabbri 2010), Liguria and Lombardy (Varese Province) have extended the Italian distribution range northward (Zilioli and Pittino 2004; Trizzino et al. 2013; Audisio et al. 2014). The Lombardy record represents the northernmost limit of *L. tetraodon,* and this population could be a relict population favored by local macroclimatic conditions. As observed for other taxa, the Insubria area (a portion of pre-Alpine western Lombardy) is often considered a xerothermic oasis with southern elements in its faunal community (Bhagwat and Willis 2008). The two species have a well-documented area of overlap in central Italy (Liguria, Emilia-Romagna, Tuscany, Marche, Umbria and Latium) where they have been found in syntopy (Bartolozzi 1986; Santoro et al. 2009; Trizzino et al. 2013; Cortellessa et al. 2014; Fig. 1a).

Lucanus cervus is a highly variable species, especially the males which show great variation in body size, mandibular length, size and position of the mandibular median tooth, and number of antennomeres of the terminal antennal club (Harvey et al. 2011). Allometric studies showed continuous variation between two morphological types of males, the so-called major (=hyperthelic) and minor (=hypothelic), with a relation between mandibular development and body size (Kawano 2000; Knell et al. 2004; Harvey and Gange 2006; Hardersen et al. 2011). These morphological differences are influenced by the quantity/quality of food during the larval stages and by other seasonal constraints (Paulian and Baraud 1982; Paulian 1988; Baraud 1993; Harvey and Gange 2006; Hardersen et al. 2011; Gotoh et al. 2014). This extreme variation is not present in *L. tetraodon*, which is usually characterized by a more homogeneous aspect,

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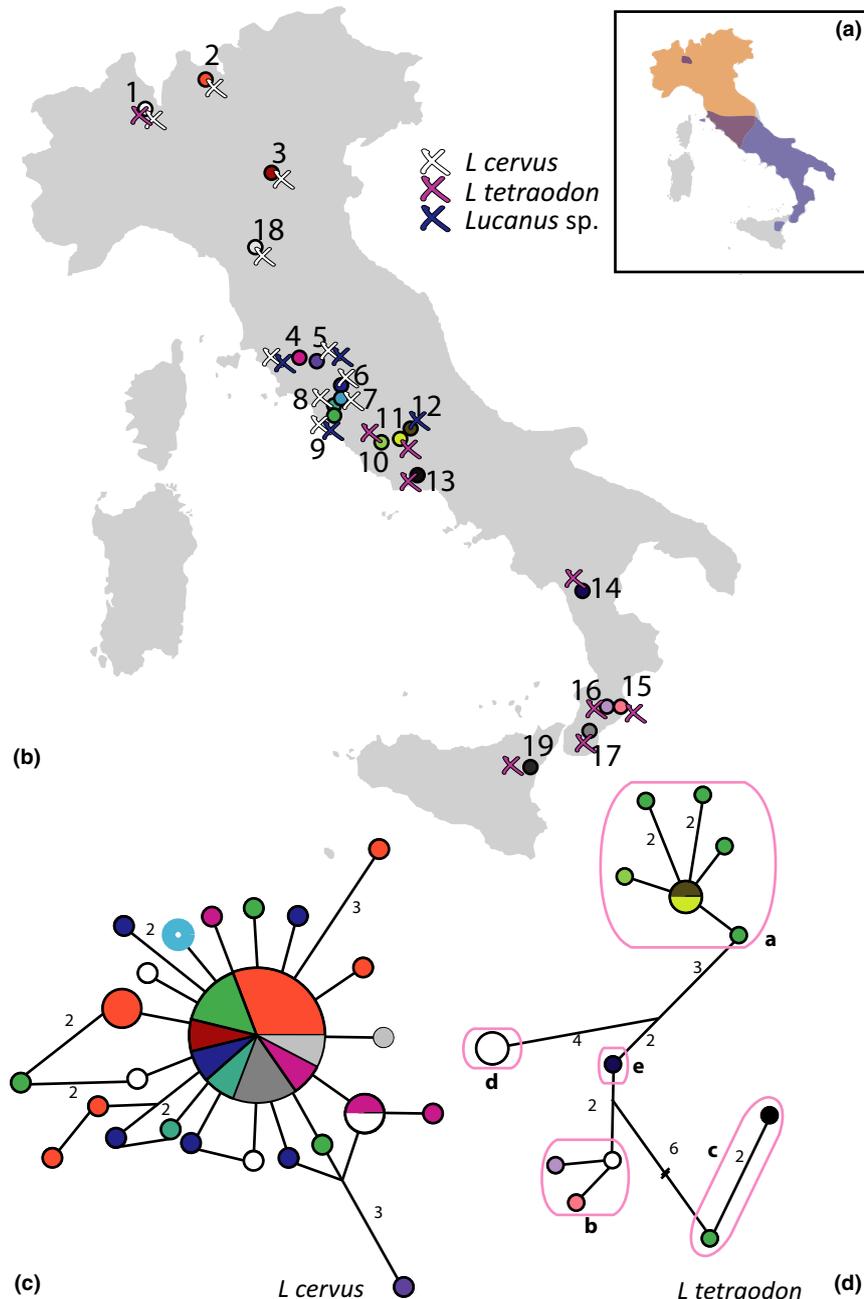
Fig. 1. (a) The Italian distribution of *Lucanus cervus* (light mask) and *L. tetraodon* (dark mask), following the CK Map distribution (Bartolozzi and Maggini 2007). (b) Map of the 19 sampled localities (numbers and circles). The name of each locality is reported in Table 1. For each locality, it is also reported the species assignment for the individual sampled, *L. cervus* (white cross), *L. tetraodon* (light cross) and *Lucanus* sp. (dark cross). On the bottom, parsimony networks performed on the two molecular clades, *L. cervus* (c) and *L. tetraodon* (d). The haplotypes are coloured following the colour of the localities in (b). The pink frames indicate the haplogroups (a–e) of the *L. tetraodon* clade.

similar to the smaller *L. cervus* specimens. Female morphology does not vary as much, making in some cases difficult to easily distinguish the two species based exclusively on female individuals. Males of both species use their antler-like mouthparts and the wrestling style of combat in the competition for a mate (Eberhard and Gutierrez 1991; Harvey and Gange 2006).

Franciscolo (1997) and Ballerio et al. (2010) provided a list of diagnostic characters discriminating *L. cervus* and *L. tetraodon* in Italy. The identification keys are based on characters useful to identify individuals from areas of allopatric occurrence. These characters, shown in Figs S1 and S2, include (1) the number of articles in the antennal club, (2) the position of the median tooth

in male mandibles and (3) the fore tibia ratio (protibia length/ protibia width). However, these characters not always allow a clear distinction in sympatric populations of the two species. In the areas of syntopic occurrence, many individuals show a mosaic of morphological traits that makes species assignment difficult.

This issue represents a taxonomic challenge but also has important implications in conservation. Indeed, *L. cervus* is a species of conservation concern in the EU legislation, while *L. tetraodon* is not, even though both are saproxylic species associated with ancient woods. Hence, correct identification of specimens is critical and can affect legal constraints and conservation measures to

Table 1. List of analysed specimens.

Species	N Ind	Code	N loc	Loc	Region	Map
L. cervus	(3)	lc5/lc20/*lc21*	9	Varese	Lombardy	1
L. tetraodon	(6)	lt7/lt8/*lt9*/lt10/*lt11*/lt12				
L. cervus	(10)	*lc22/lc23/lc24/lc25/lc26/lc27/lc28/lc29/lc30/lc31*	10	Sondrio	Lombardy	2
L. cervus	(2)	*lc14*/**lc41**	2	Mantua	Lombardy	3
L. cervus	(2)	lc17/*lc18*	4	Roccalbegna	Tuscany	4
Lucanus sp.	(2)	lsp6/lsp7				
Lucanus sp	(3)	*lsp1*/lsp2/**lsp8**	11	Acquapendente	Latium	5
L. cervus	(8)	lc1/*lc2/lc3/lc4*/**lc37/lc38/lc39/lc40**				
L. cervus	(1)	lc32	1	Viterbo	Latium	6
L. cervus	(1)	lc19	1	Ronciglione	Latium	7
L. cervus	(2)	lc15/*lc16*	2	Manziana	Latium	8
L. cervus	(8)	*lc6*/lc7/lc8/*lc9*/lc10/lc11/*lc12/lc13*	10	Castelgiuliano	Latium	9
Lucanus sp	(2)	lsp3/lsp4				
L. tetraodon	(1)	lt2	1	Frascati	Latium	10
L. tetraodon	(1)	lt3	1	Genazzano	Latium	11
Lucanus sp.	(1)	lsp5	1	Jenne	Latium	12
L. tetraodon	(2)	lt1/**lt13**	2	Ausoni Mt.	Latium	13
L. tetraodon	(2)	lt5/**lt14**	2	Mormanno	Calabria	14
L. tetraodon	(1)	lt6	1	Stilo	Calabria	15
L. tetraodon	(1)	lt4	1	Mongiana	Calabria	16
L. tetraodon	(1)	**lt15**	1	Aspromonte Mt.	Calabria	17
L. cervus	(4)	*lc33/lc34/lc35/lc36*	4	Orrido di Botri	Tuscany	18
L. tetraodon	(1)	**lt16**	1	Etna Mt.	Sicily	19

Species assignment (*Species*), number of individuals analysed for each species at each locality (*N.ind*), code of each individual (*Code*), total number of individuals per locality (*N.loc*), name of locality (*Loc*), region and map reference number (*Map*). "Code" character in regular typing for individuals for which both *coI* and *wg* sequences are available; bold typing available *wg* only, no *coI*; italic typing available *coI* only, no *wg* (see Table S1 for further details on the sample).

be implemented in the field. The reliable identification of individuals and populations is a fundamental issue in conservation biology for the purpose of avoiding erroneous decisions and efficiently targeting conservation efforts and resources (Frankham 2010; Zachos et al. 2013).

It is important to plan conservation strategies for specialized taxa with a wide distribution that can suffer local extinction (Kotze and O'Hara 2003) and thus to adapt conservation measures to local populations. Yet, some local populations previously considered valid species and endangered because of their restricted distribution range have turned out to be part of a widespread related species and require a conservation strategy based on ESUs (Evolutionarily Significant Units) (Moritz 1994; Solano et al. 2013).

This study represents one of the few molecular approach to lucanid phylogeny (Lin et al. 2011; Cox et al. 2013; Kim and Farrell 2015) and aims to describe, by means of two molecular genetic markers, the genetic differentiation of *Lucanus* in Italy. Besides, we hypothesize the evolutionary meaning of the mosaic of morphological characters in areas of sympatric occurrence. In fact, the mixed morphological traits found in sympatric populations may hide processes of hybridization and introgression or may derive from the convergence of morphological traits under natural selection. The latter phenomenon has been defined as 'convergent character displacement' (Grant 1972). The character states of sympatric species may be selected to converge, even though few cases have been described thus far in vertebrates and invertebrates (Vadas 1990; Scott and Foster 2000; Leary 2001; Hassall 2014) with respect to the more common 'divergent character displacement' (Brown and Wilson 1956; Grant 1972; Butlin 1989; Howard 1993; Hostert 1997; Kawano 2002).

Materials and Methods

Sample

The diagnostic morphological characters proposed by Franciscolo (1997) and Ballerio et al. (2010) were used to identify the specimens using comparative material and available identification keys (Baraud 1993; Franciscolo 1997; Sabatinelli 2009–2012; Ballerio et al. 2010).

The complete data set consisted of 65 Italian *Lucanus* samples coming from specialist's and museum's collections captured by traps in the field between 2008 and 2013 (Table S1), at the end of the reproductive season and preserved in 95% ethanol: 41 *L. cervus*, 16 *L. tetraodon* and eight individuals (indicated as *Lucanus* sp.) for which identification was not possible because of mixed diagnostic characters (see Table 1 and Figs S1 and S2). In total, 19 localities were sampled in Latium (nine localities; *L. cervus* $N = 21$, *L. tetraodon* $N = 4$ and *Lucanus* sp. $N = 6$), Lombardy (three localities; *L. cervus* $N = 15$, *L. tetraodon* $N = 6$), Tuscany (two localities; *L. cervus* $N = 6$ and *Lucanus* sp. $N = 2$), Calabria (four localities; *L. tetraodon* $N = 5$) and Sicily (one locality; *L. tetraodon* $N = 1$). For details of the sampling localities and their distribution, see Fig. 1b and Table 1.

Molecular genetic markers: Cytochrome oxidase 1 and Wingless

DNA was extracted from dissected metafemoral muscles of 66 individuals following the salting out procedure described by Aljanabi and Martinez (1997). Two markers were amplified: a fragment of the mitochondrial *cytochrome C oxidase subunit I* (*coI*) gene and the nuclear Wingless gene (*wg*). For both genes, PCR amplifications were carried out in a 25 μl volume containing: 1.25 U of Taq DNA polymerase (Bioline, London, UK), 0.8 pmol of each primer, 1 mM each dNTP (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, US) and 5 μl of 10X buffer + 1.5 mM MgCl$_2$. The MJ MINI Personal Thermal Cycler (BIO-RAD Laboratories Hercules, California, USA) was used for the PCR amplifications. PCR products were purified with the Charge Switch® PCR Clean-Up Kit (Invitrogen) and sent to an external sequencing service (BMR – Genomics, Padua, Italy).

An 831 bp fragment at the 3' terminus of the mtDNA *coI* gene was amplified and sequenced for 55 of the *Lucanus* individuals (36 *L. cervus*, 12 *L. tetraodon* and 7 *Lucanus* sp.; see Table 1) and for one individual of *Dorcus parallelipipedus* used as outgroup. The universal primers C1-J-2183 [5'- CAACATTTATTTTGATTTTTTGG-3'] and TL2-N-3014 [5'-TCCAATGCACTAATCTGCCATATTA-3'] were used (Simon et al. 1994). The PCR amplification conditions were as follows: 94°C denaturation (30 s), 50°C annealing (1 min) and 72°C extension (30 s) for 33 cycles, followed by a 7-min elongation step at 72°C. The same forward and reverse primers were used to sequence the fragment in double strand.

The resulting sequences were edited and aligned using Geneious v. 4.8.3 (Biomatters Ltd Auckland, 1010 New Zealand). Sequences have been deposited in GenBank under accession nos. KU139606 – KU139695 (Table S1). Phylogenetic trees were constructed using the Bayesian Inference (BY) approach. A generalized time-reversible model with a proportion of invariable sites and heterogeneous substitution rates following a gamma distribution (GTR + I + G, Rodríguez et al. 1990) was selected by jmodeltest (Nylander et al. 2004) using the AICc criterion. BY phylogenetic reconstructions were performed under the selected substitution model. BY inference was carried out with mrbayes v. 3.2.1 (Huelsenbeck and Ronquist 2001) by running 1 000 000 generations, with Markov chains sampled every 1000 generations. A burn-in of 10% was applied, and the remaining trees were used to compute a 50% majority rule consensus tree and posterior probabilities. The *coI* sequence of *L. c. cervus* (GenBank accession no. FJ606555) from Lin et al. (2011) was added to the analysis. *Lucanus hermani*, *L. formosanus*, *L. planeti*, *Neolucanus doro doro* and *Prosopocoilus astacoides blanchardi* (GenBank accession no. FJ606552, FJ606620, FJ606553, FJ606550 and FJ606548, respectively) were used as outgroup.

The mean pairwise *p* distances of *coI* sequences between and within the clades indicated by the phylogenetic analyses was estimated using mega v. 5.05 (Tamura et al. 2011). We reconstructed parsimony networks of *coI* haplotypes with tcs v. 1.21 (Clement et al. 2000), in default settings, calculating connection limits at 95%, to describe the relationships within the clades.

To investigate possible events of hybridization between the two species in the areas of sympatric occurrence, we amplified a partial fragment of the nuclear *wg* gene in a subset of 34 individuals (14 *L. cervus*, 13 *L. tetraodon* and 7 *Lucanus* sp.; see Table 1) using the two primers described in Brower and De Salle (1998) *LepWg1a* [5'-GARTGYAART-GYCAYGGYATGTCTGG-3'] and *LepWg2a* [5'-ACTICGCARCAC-CARTGGAATGTRCA-3']. This gene was selected because it has proved effective in discriminating among closely related *Lucanus* species (Lin et al. 2011). The thermal cycling parameters used in these amplifications were as follows: 95°C for 5 min, followed by 33 cycles of 94°C for 1 min, 50°C for 30 s and 72°C for 1 min, with the final elongation step extended to 7 min. The resulting PCR products were checked in electrophoresis agarose gel and yielded two bands. We isolated and purified the target gene band (490 bp) by the pre-cast gel system method described in Gibson et al. (2010), using the Lonza FlashGel® System according to the manufacturer's instructions. This method allows isolation of the exact band without any further purification procedure, producing DNA suitable for sequencing without cloning procedures. The sequences were used to design specific *Lucanus* primers for subsequent double strand sequencing, *Wing-F* [5'-CCGCCTCAAGGACCGCTTCG-3'] and *Wing-R* [5'-AATTGCACCTTTCGACGATGGCG-3']. *Wing-F* bind at the position 73 of reference sequences and the *Wing-R* bind at the position 475. The resulting sequences were inspected for double peaks, edited and aligned using geneious v. 4.8.3 (Biomatters). Because of the presence of multisite heterozygotes (see results) in the *wg* alignment, the gametic phase of each haplotype was resolved using phase (Garrick et al. 2010) implemented in dnasp v. 5. The two most common alleles (highest probabilities) for heterozygous individuals were selected. For the resulting genotypes, we used mega v. 5.05 to calculate the mean pairwise *p* distances within and between the clades indicated by the *coI* phylogenetic analyses.

Results

The *coI* fragments resulted in an alignment of 56 specimens and 721 bp. The Bayesian consensus tree topology is reported in Fig. 2 (only posterior probability values exceeding 70% are shown). Two main clades are highly supported (p.p. = 1), mostly corresponding to the two species. The specimens with the mosaic of morphological characters fall into both clades. Most of the *Lucanus* sp. specimens (5 of 7) are in the *L. cervus* clade [Lsp1 and Lsp2 from Acquapendente (locality 5); Lsp3 from Castelgiuliano (locality 9); Lsp6 and Lsp7 from Roccalbegna (locality 4)] while the other two [Lsp5 from Jenne (locality 12) and Lsp4 from Castelgiuliano (locality 9)] cluster in the *L. tetraodon* clade.

Finally, two individuals from Varese (locality 1, Lt7 and Lt12) and identified as *L. tetraodon* cluster in the *L. cervus* clade, whereas four individuals from Castelgiuliano (locality 9, Lc7, Lc9, Lc10 and Lc11) and identified as *L. cervus* cluster in the *L. tetraodon* clade. There is low variability of *coI* within the two clades, but mainly within *L. cervus*, in which the Bayesian approach revealed no kind of structuring. A certain amount of structure is present in *L. tetraodon*. Accordingly, the mean pairwise genetic distance between the two clades is high (10.9%), while the mean distance within clades is 0.3% in *L. cervus* and 1.2% in *L. tetraodon*.

The parsimony networks performed separately on the two clades are reported in Fig. 1. The *L. cervus* clade shows a star-like pattern with no internal structuring (Fig. 1c). The main haplogroup contains specimens from eight of the nine localities where the species was captured. Conversely, five distinct haplogroups are detectable in the *L. tetraodon* clade (Fig. 1d). We defined the haplogroups on the basis of parsimony steps (>3). Haplogroup A includes seven individuals from the area of sympatric occurrence in central Italy [Castelgiuliano (locality 9) $N = 4$; Frascati (locality 10) $N = 1$; Genazzano (locality 11) $N = 1$; Jenne (locality 12) $N = 1$]. Directly related to this group are haplogroup D from the site of sympatric occurrence in northern Italy [Varese (locality 1) $N = 2$], haplogroup B from northern and southern Italy [Varese (locality 1) $N = 1$; Calabria (localities 15 and 16) $N = 2$] and haplogroup E from southern Italy [Calabria (locality 14) $N = 1$]. Finally, haplogroup C is the most divergent one, including two individuals from central Italy [Castelgiuliano (locality 9) $N = 1$; Ausoni Mt (locality 13) $N = 1$].

Sequencing of the nuclear *wg* fragment resulted in an alignment of 34 specimens and 375 bp. The sequences are invariant except for 10 positions characterized by double peaks (Fig. 3). The alignment (Fig. 3) revealed four types of homozygous sequences, distributed over 13 individuals and eight localities (three of which are sites of sympatric occurrence: localities 1, 9 and 5, Fig. 1b and Table 1), and 22 multisite heterozygotes, 19 of which at multiple sites, distributed over 12 localities including the four sites of sympatric occurrence (locality 4 in addition to the previous ones). The homozygous and heterozygous sequence typologies are randomly distributed among *L. cervus*, *L. tetraodon* and *Lucanus* sp. The mean pairwise genetic distances between (1.1%) and within (0.9% in *L. cervus*, 0.7% in *L. tetraodon*) the two clades identified by *coI* are low and similar.

Discussion

In Italy, *L. cervus* and *L. tetraodon* display strong morphological diversity in allopatry. However, in areas of sympatric occurrence they show a mosaic of the external morphological traits traditionally used to distinguish the two species (Franciscolo 1997). Whereas in allopatry the same morphological diagnostic characters show congruence, in sympatry the presence of mixed combinations of these traits often makes correct species attribution impossible, except in cases of markedly hyperthelic *L. cervus* individuals.

The *coI* tree (Fig. 2) shows marked genetic differentiation between *L. cervus* (Lc) and *L. tetraodon* (Lt), with the presence of two clades with maximum support (p.p. = 1). These two clades are separated by a high genetic distance (11%), which can be considered very high for Coleoptera (Hebert et al. 2003). In contrast, there is a low genetic distance within the two clades (0.3% and 1.2% for *L. cervus* and *L. tetraodon* respectively). Hence, the *coI* marker is highly efficient for distinction of the two species in Italy. This divergence was not as clear cut when
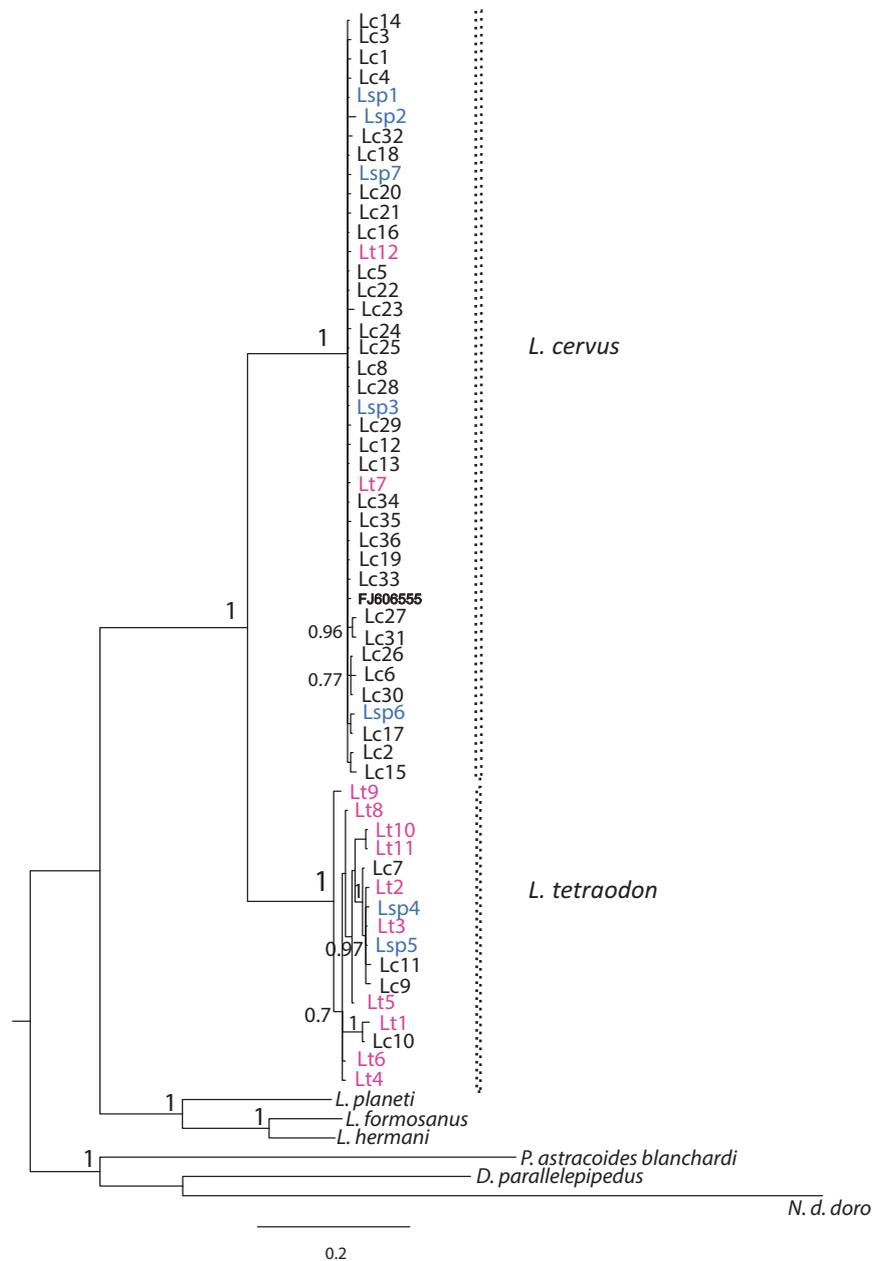
Fig. 2. Bayesian tree topology. Posterior probability major of 70% is reported at nodes. The specimens are indicated following the morphological species assignment, *Lucanus cervus* (Lc), *L. tetraodon* (Lt) and *Lucanus* sp. (Lsp). The two major clades correspond to the two species.

the same marker was used in the Palearctic sample of Cox et al. (2013). The probable reasons are that the taxonomic status of *L. cervus*, and its several purported subspecies is largely unresolved and some of them, occurring in Balkan and Near East countries, probably represent distinct species more closely related to *L. tetraodon* than to *L. cervus*. Moreover, the speciation events involving both species in peninsular Italy are probably sufficiently recent to be detected with this marker in contrast to the events involving *L. cervus* over all its distribution area.

The individuals with mixed characters (Lsp) fall within either *L. cervus* or *L. tetraodon*. More interestingly, some specimens from overlapping areas attributed to *L. tetraodon* are genetically *L. cervus* (two individuals) and vice versa (four individuals). While the percentage of assignment by traditional morphological characters in allopatry is 100%, it decreases to 82% for *L. cervus* and to 62.5% for *L. tetraodon* in the areas of sympatric occur-

rence. In these areas, the percentage of identification estimated over our sample, is about 17.5% for *L. cervus* (with 5% misidentification) and 37.5% for *L. tetraodon* (with 25% misidentification). The disproportion in assignment is certainly due to the occurrence of more easily identifiable hyperthelic ('major') *L. cervus* individuals.

The presence of individuals morphologically attributed to *L. cervus* but mitochondrially *L. tetraodon* and vice versa could be due to (1) introgression of mitochondrial DNA and thus hybridization between the two species or (2) the convergence of morphological traits in sympatry.

In the similar case study of *Onthophagus* beetles (Pizzo et al. 2006 and references therein), when two species were sympatric and syntopic (sharing the same environmental influences), the morphological similarity and/or intermediate morphological traits were interpreted in two main ways: (1) speciation
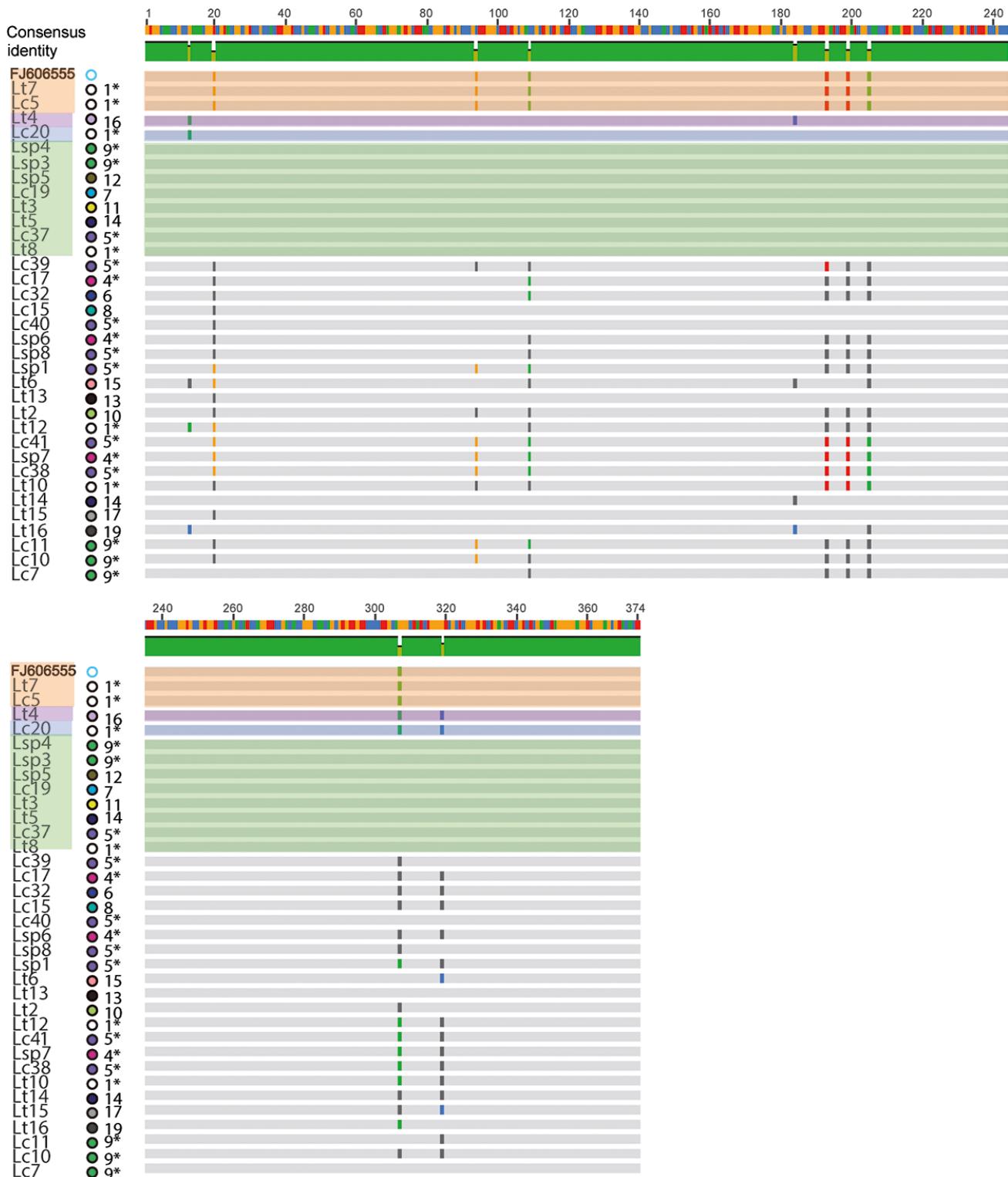
Fig. 3. Alignment of nuclear *wg* sequences of 34 specimens. The 'identity' bar reports the 10 polymorphic positions (see text for details). The first 13 highlighted specimens represent the four types of homozygous sequences. The remaining grey sequences are the 22 individuals with heterozygous positions. Homozygous position: yellow (G), blue (C), green (T) red (A). Heterozygous position: dark grey. The circles and the numbers represent the sampling localities for each specimen (see Fig. 1 and Table 1 for the numbers correspondence). The asterisks indicate the localities of sympatric occurrence of the two species (see text and the Table 1 for details).

occurred in sympatry and the morphological homogeneity depended on convergence due to ecological adaptation to the local environment and sexual selection (through assortative mating and sexual conflict), (2) the genetic divergence occurred in allopatry and was followed by the (re-)establishment of sympatry.

In the latter case, hybridization could occur if the speciation was not complete.

Consequently, in our case, the hybridization may have lead to the mosaic of morphological traits between the two species in sympatry. A detailed quantitative analysis of genital features is

highly desirable because, if two closely related species differ markedly in genital features, mating isolation is undoubted. If the genitalia are similar, the two species may copulate and mating isolation cannot be confirmed without mating experiments because it could act through different mechanisms including species-specific recognition (production of pheromones, emission of sounds, courtship behaviour, etc.).

Our target nuclear gene analysis was intended to find evidence of hybridization. The *wg* sequence alignment (Fig. 3) revealed four types of homozygous haplotypes and 22 heterozygous individuals with heterozygosis in 10 positions. The distribution of this polymorphism does not agree with either the species assignation or the geographical distribution. The analysis of genetic divergence within and between the *wg* phased genotypes of the two clades showed similar values (0.9%, 0.7% and 1.1% respectively) and, most importantly, values comparable with the within species genetic divergence calculated in *L. formosanus* (Lin et al. 2011). The mean value of between species divergence calculated by Lin et al. (2011) was 2.7%.

Therefore, the *wg* marker does not follow the pattern expected for hybridization between the two species in the area of sympatric occurrence. In this case, we expected to have heterozygous individuals in sympatric populations, likely associated with the undefined Lsp morphology, and to have corresponding homozygous individuals in the allopatric ones. We also expected to have genetic divergence between the two species close to the value obtained between congeneric species for the same marker. Yet, the *wg* gene shows a spread polymorphism across the two species, apparently in contrast to the strong species structure of the mitochondrial marker. The most plausible explanation is very recent divergence of the two species which share a common origin and thus a common *wg* genotype. The nuclear marker, although it worked efficiently in the discrimination of *Lucanus* sister species (Lin et al. 2011) in Eastern stag beetles, resulted not suitable for the discrimination of Italian species and to detect hybridization in this study.

Lucanus cervus and *L. tetraodon* are traditionally considered sister species. Nevertheless, Cox et al. (2013) showed that the taxonomic assessment of *L. cervus* in its distribution range is largely unresolved. Morphological studies have revealed at least seven subspecies of *L. cervus*, which in some cases may correspond to different species. The presence of 4–7 species of the genus *Lucanus* with a partially overlapping distribution (Fujita 2010; Cox et al. 2013 and references therein) makes the situation more difficult to unravel. This is reflected by the paper of Cox et al. (2013), where *L. cervus* was found to be not monophyletic, based on partial COI sequences, and the specimens of *L. tetraodon* were included among other species.

In Italy, the two species display marked mitochondrial diversity, but there is a high degree of genetic homogeneity within both clades. The *coI* parsimony network provides hints on the origin and evolutionary dynamics of the speciation processes. As expected from the tree and the genetic distances calculated within the clade (0.3%), the *L. cervus* parsimony network (Fig. 1c) has a perfect star-like pattern, showing extreme genetic homogeneity of the Italian populations of this species. The *L. tetraodon* network (Fig. 1b) shows less genetic homogeneity (genetic distance within the clade 1.2%). This is probably due to the different geological events involving the two parts of the Italian peninsula which produced strong genetic isolation of populations in the south and less isolation in the north. These data, associated with a very high degree of genetic divergence between species, are common in many other beetles and have helped to pinpoint their origin during the Pleistocene climatic oscillations (Hewitt 1999). The presence of distinct haplogroups with no evident geographical

structure could be the result of recent recolonization events and random extinction of haplotypes, as seen for other beetle species (Solano et al. 2013).

In the second possible scenario, there is no hybridization between the two taxa. This implies that the morphological mosaic depends on convergence due to ecological adaptation to the local environment or sexual selection. 'Convergent character displacement' (Grant 1972) has been hypothesized when the character states of sympatric species may be selected to converge. This phenomenon, the opposite of the more common and well-documented 'divergent character displacement' (Brown and Wilson 1956; Grant 1972; Waage 1979; Butlin 1989; Howard 1993; Hostert 1997; Kawano 2002), has been described in some vertebrates and invertebrates (Vadas 1990; Scott and Foster 2000; Leary 2001; Hassall 2014). A reproductive advantage of hypothelic males with respect to hyperthelic ones has been suggested for *L. cervus* (Harvey and Gange 2006), and a morphological convergence of *L. cervus* and *L. tetraodon* in areas of syntopic occurrence may reflect a similar advantage. A future study of the hypothelic:hyperthelic ratio in *L. cervus* in sympatric vs. allopatric conditions would be helpful in this regard. Yet, the adaptive value of the characters used for taxonomic identification is unknown because no studies have been conducted on the involvement of these structures in intraspecific and interspecific interactions (e.g. fightings before copula) and in feeding behaviour or on the environmental constraints acting on them. Therefore, it is impossible to hypothesize possible selective pressures that might lead these characters to converge in the same locality and environment.

The aim of our study was to provide scenarios of the genetic diversity and evolution of Italian stag beetles. Considering the mosaic of morphological characters (and referring to similar data on other beetles, i.e. Pizzo et al. 2006), the hybridization hypothesis indicates the need of a more extensive assessment of genetic and genomic variability of these species and a more thorough search for possible hybrids in sympatric populations. On the other hand, if the interspecific competition has led to a temporal shift on the reproductive activity, supporting the absence of hybridization, an extended morphological revision to redefine the diagnostic characters of *L. cervus* and *L. tetraodon* will be needed. Furtermore, the identification of additional diagnostic characters for specimens from areas of sympatric occurrence is recomended. Our study confirms the finding by many other authors that resolving taxonomic uncertainties is crucial to set conservation priorities and plan conservation actions. A thorough taxonomic revision of Italian stag beetles is needed to clarify the phylogenetic position and conservation status of *L. tetraodon*. Although this species is not protected like *L. cervus*, it deserves to be taken into account if there is evidence for the hybridization hypothesis. The most appropriate definitions for conservation purposes are based on ESUs and gene flow (Frankham 2010). The sympatric populations of the two species seem to share gene flow and they probably mate but we do not know if their progeny are sterile. The lack of a universally recognized definition of species creates enormous difficulties in conservation biology.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Mandible shape in male specimens of *Lucanus cervus* and *L. tetraodon*

Figure S2. Right antennal club of *Lucanus cervus* and *L. tetraodon*

Table S1. Table of the samples used in this study.