Different behaviour of mitochondrial and nuclear markers: introgression and the evolutionary history of *Chrysocarabus* (Coleoptera: Carabidae)

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Phylogenetic analyses of *Chrysocarabus* taxa using different markers result in different phylogenetic trees. In particular, the mitochondrial gene tree contradicts the results of morphological and inbreeding studies. Two very different haplotypes of *Carabus splendens* Olivier, 1790 do not form a clade within this phylogenetic tree. We have earlier proposed that contradictory results are due to introgression. To verify our hypothesis, we analysed the internal transcribed spacer 2. No substitutions were observed in these nuclear sequences between the individuals of *Carabus splendens,* which contain the different mitochondrial haplotypes in question. The differences in the gene trees based on mitochondrial and nuclear sequences can be explained with at least two introgression events.

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1. Introduction

The taxon *Chrysocarabus*, a subgenus of *Carabus*, includes several taxa that seem to be very closely related, because of the occurrence of hybrids in the field (Puisségur 1964, Mossakowski *et al.* 1986, 1990). The number of species varies between seven and nine depending on the author. Further phylogenetic analyses of this subgenus based on (i) different morphological character complexes (Malausa *et al.* 1983, Mossakowski 1979, Marciniak 1995), (ii) cross breeding studies (Allemand & Malausa 1984), (iii) allozyme variability (Braun 1988) and (iv) mitochondrial DNA sequences (Prüser 1996, Düring *et al.* 2001) resulted in different phylogenetic trees.

Contradictory results between trees inferred from different character sets are commonly encountered and may depend on different processes (see e.g., Maddison 1997). Incongruity between morphological taxonomy and results based on mitochondrial DNA data were described for the first time in the genus *Carabus* by Prüser (1996) for European species and by Su *et al.* (1996) for closely related species of the Japanese subgenus *Ohomopterus*. Su *et al.* (1996) interpreted their results by the assumption of parallel evolution, which resulted in so called "type switching". Sota and Vogler (2001) rejected the type switching hypothesis and demonstrated that the mechanism of incongruity was multiple introgression in their comprehensive molecular study.

In *Chrysocarabus*, Prüser (1996) and Düring *et al.* (2001) found two very different mitochon-

drial haplotypes (ht) in *Carabus splendens*, which do not form a clade in the phylogenetic trees. One of these haplotypes is very similar to one found in *C. punctatoauratus* while the other is nearly identical to those found in *C. rutilans*. Both form a clade with these two species in the mitochondrial gene tree. The geographical distribution of both haplotypes was investigated by Düring *et al.* (2000). Ht1 is spread over the whole distribution area of the species while ht2 is only present in some northern, isolated populations and in the western part, where it occurs in some populations together with ht1. As the most likely explanation of these results, an introgression event has been proposed, which involves the transmission of a complete mitochondrial genome by horizontal gene transfer (Düring *et al.* 2000). In this case, the maternal inheritance of the mitochondrial genome leads to misinterpretations, because the phylogeny reflects the history of the genes and not the history of the species. In contrast to the mitochondrial genome, recombination dilutes simultaneously introgressed nuclear DNA in the population by backcrossing. If our introgression hypothesis is correct, gene trees based on mitochondrial and nuclear DNA sequences should be different. In order to verify the proposed introgression hypothesis we analysed DNA sequences of the fast evolving nuclear internal transcribed spacer 2 (ITS 2). Possible incongruity between the placements of the taxa should allow conclusions about the underlying reticulate evolutionary processes.

2. Material and methods

DNA sequences of the ITS 2, including parts of the 2.0S rRNA, 5.8S rRNA and 28S rRNA, were determined via PCR and direct sequencing from the following *Chrysocarabus* species*: Carabus splendens* Olivier, 1790, *C. lineatus* Chevrolat, 1837, *C. lateralis* Chevrolat, 1840, *C. auronitens* Fabricius, 1792, *C. punctatoauratus* Germar, 1824, *C. rutilans* Dejean, 1826 and *C. hispanus* Fabricius, 1787. *Carabus* (*Macrothorax*) *morbillosus* DeLapouge, 1899 was included as the outgroup. The analysis included three specimens of *Carabus splendens*, which carry the three different mitochondrial haplotypes ht1, ht1a, and ht2. DNA sequences were deposited in the GenBank database (Acc. Numbers DQ683169-DQ683178).

DNA was extracted with the commercial QIAamp tissue kit (QIAGEN). PCR amplification and primer sequences (ITS3 and ITS4) were specified in White et al. (1990). Direct sequencing was done on an ABI 373A sequencer using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequence alignment was done with the program ClustalX (Thompson et al. 1997). The full data set consisted of 664 sites (52 variable, 21 parsimony informative, outgroup included). After gap sites and parts of uncertain homology were removed, the data set finally consisted of 636 characters (v: 49, p. i.: 18). For data analysis, we applied the parsimony method (MP) using PAUP* 4.0b10 (Swofford 1998) to allow a strict comparison with the published CO2 data. Additionally, calculations with the maximum likelihood method (ML, PHYLIP 3.6, with empirical base frequencies, transition/transversions = 1:2, and global rearrangements; Felsenstein 2004) were performed. The support of the branches was checked by bootstrap analysis (1,000 pseudoreplicates) and decay index (MP only). Both data sets (with and without gaps) were analysed by ML.

3. Results

The analysed data set without alignment gaps contains 26 variable and 17 phylogenetically informative positions within the ingroup. Sequence divergences (p-distance) are shown in Table 1. No substitutions were observed in the ITS 2 sequences (p-distance = zero) between the individuals of *Carabus splendens*, which contain the three different mitochondrial haplotypes (ht1, ht1a, ht2).

Phylogenetic analysis using parsimony of the ITS 2 DNA sequences resulted in two most parsimonious trees (54 steps). The strict consensus tree is shown in Fig. 1. All three specimens of *C. splendens* containing the mitochondrial haplotypes ht1, ht1a, and ht2 cluster on a single branch. They form a clade with C. *lineatus/C. lateralis,* which is supported by a high bootstrap value of 96%. The *Chrysotribax* species (*C. rutilans* and

Table 1. Sequence divergences in *Chrysocarabus* taxa. Nucleotide differences are given as percentage of divergence (lower left part based on nuclear sequences of ITS 2, upper right part from Düring *et al.* (2001) based on mitochondrial CO2 DNA sequences).

	C. splendens									
	ht1	ht1a	ht2	*lin*	*lat*	*auro*	*punc*	*rutil*	*hisp*	*mor*
splendens ht1	–	0.58	5.53	5.39	5.53	5.82	1.16	5.53	3.78	8.44
splendens ht1a	0.00	–	5.39	5.39	5.53	5.97	1.16	5.39	4.08	9.02
splendens ht2	0.00	0.00	–	0.87	1.16	6.70	5.82	0.15	7.42	9.61
lineatus	0.63	0.63	0.63	–	0.58	6.49	5.82	1.02	7.57	9.17
lateralis	0.63	0.63	0.63	0.00	–	6.70	5.97	1.31	7.57	9.46
auronitens	1.73	1.73	1.73	2.04	2.04	–	6.26	6.84	6.55	9.17
punctatoauratus	1.73	1.73	1.73	2.04	2.04	0.31	–	5.82	3.78	9.02
rutilans	2.67	2.67	2.67	2.99	2.99	1.89	1.89	–	7.42	9.75
hispanus	2.36	2.36	2.36	2.67	2.67	1.26	1.26	1.26	–	9.32
morbillosus	5.03	5.03	5.03	5.35	5.35	4.87	4.87	5.35	5.35	–

C. hispanus) are grouped together with a moderate bootstrap value (85%) and form a clade with *C. auronitens* and *C. punctatoauratus* (75% bootstrap value).

The ML method resulted in a similar tree (Fig. 2). The three *C. splendens* haplotyes form a separate clade. *C. rutilans* builds a branch with *C. hispanus* and *C. punctatoauratus* with *C. auronitens*, respectively. These clades are supported by high bootstrap values. The results of the data set which includes gaps are very similar. The boot-

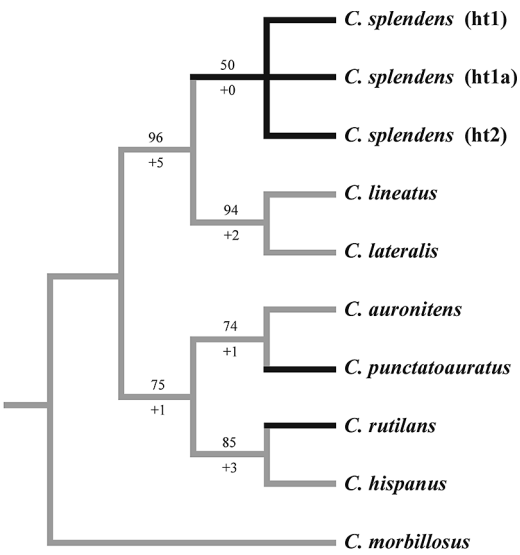

Fig. 1. Strict consensus tree of two MP trees (54 steps) based on nuclear ITS 2 DNA sequences of *Chrysocarabus* species. Bootstrap values (above line) and decay indices (below) indicate support of the nodes. Black bars mark the taxa mentioned in the introgression hypothesis.

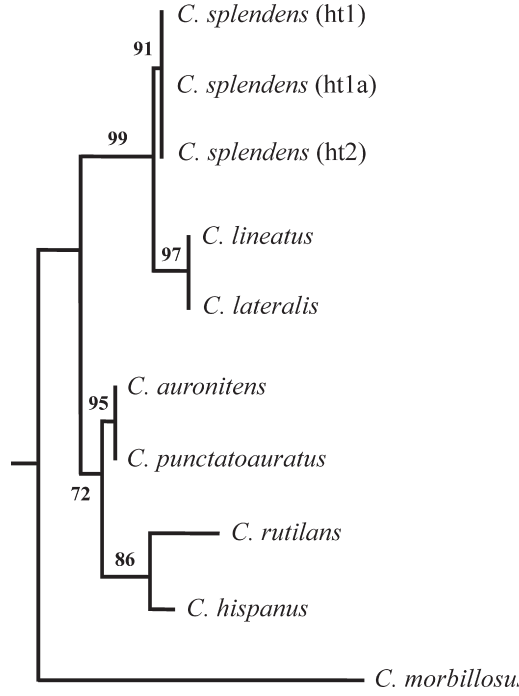

Fig. 2. Maximum likelihood tree based on ITS2 sequences of *Chrysocarabus* species. Numbers indicate branch support by bootstrap values (1,000 pseudoreplicates). Ln likelihood: –1204.27981.

strap support of both analyses do not differ significantly with the exception of the branch which connects *C. auronitens/punctatoauratus* and *C. hispanus/rutilans*. This is about 10 percent points higher (results not shown).

4. Discussion

4.1. Comparison of nuclear and mitochondrial gene trees

The phylogenetic analysis of the ITS 2 DNA sequences by parsimony results in moderate to well supported branches (Fig. 1). Several of these branches contrast with the results of former phylogenetic analyses based on mitochondrial DNA sequences of the CO2 gene, although the DNA was isolated from the same individuals (Düring *et al.* 2001; see Fig. 3). The maximum likelihood analysis (Fig. 2) of the ITS 2 data resulted in a tree similar to that of Figure 1, the branches of which are supported by high bootstrap values. In particular, the branch of most interest (*C. splendens*, *lineatus* and *lateralis*) is well supported.

The lack of base substitutions in the nuclear DNA sequences between the individuals of *C. splendens*, which carry the mitochondrial ht1, ht1a, and ht2, is remarkable because it clearly demonstrates the monophyly of this species. *C. splendens* seems to be the sister taxon to *C. lineatus*/*C. lateralis* in the ITS 2 gene tree as well as in morphological studies (e.g., Mossakowski 1979). This is in contrast to the genetic distances of approximately 5% in the mitochondrial CO2 gene (Table 1) where the mitochondrial haplotypes of *C. splendens* are spread over the phylogenetic tree. In the mitochondrial gene tree ht1 and ht1a form a clade with *C. punctatoauratus* while ht2 is grouped together with *C. rutilans*.

Additionally, in the nuclear gene tree, *C. rutilans* and *C. hispanus* form a sister group. This is in accordance with the classical morphological taxonomy where both taxa are described as *Chrysotribax*. However, this well established group is not found in the mitochondrial gene tree. The species *C. auronitens* and *C. punctatoauratus,* which were sometimes considered to be

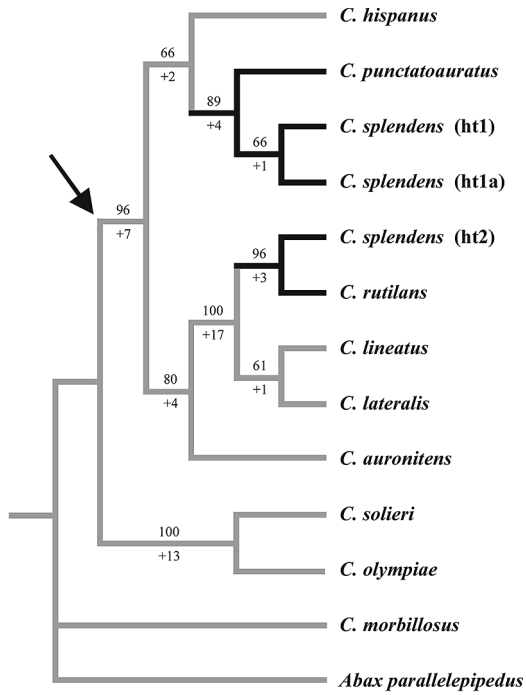

Fig. 3. Phylogenetic tree based on mitochondrial CO2 DNA sequences of *Chrysocarabus* species (after Düring *et al.* 2001). Bootstrap values (above line) and decay indices (below) indicate support of the nodes. Black bars mark the taxa mentioned in the introgression hypothesis. Arrow: see Discussion, section 4.2.

subspecies, also form a clade in the ITS 2 gene tree (p-distance 0.31), although this grouping is not present in the CO2 gene tree. Only the sister group relationship of *C. lineatus* and *C. lateralis* is present in both the nuclear and the mitochondrial gene tree. However, these species are sometimes discussed as being conspecific (Allemand & Malausa 1984).

The nuclear gene tree corresponds with morphological results and cross breeding studies, thus this tree may be interpreted as the organismal tree. The terminal branches of our tree have also been found by Sota and Ishikawa (2004) who studied the phylogenetic relationships within *Carabus* by means of two nuclear genes. Six of seven species of the inner group of *Chrysocarabus* (Fig. 1, without *C. lateralis*) resulted in the same pairs of sister species. But no resolution was found between these pairs. Additionally, the positioning of the mitochondrial haplotypes of *C.*

splendens conflicts with the interpretation of the mitochondrial gene tree as the species tree (Düring *et al.* 2001).

4.2. Introgression or ancestral polymorphism?

Different phylogenetic trees inferred from independently inherited genetic markers, such as nuclear and mitochondrial DNA sequences, can be the result of convergence, pseudogenes (mitochondrial genes transferred into the nuclear genome), gene duplication, ancestral polymorphism or introgression (Maddison 1997). However, high homoplasy content was not found in the mitochondrial and nuclear DNA data sets of the *Chrysocarabus* subgenus. In addition, no evidence for pseudogenes, e.g., differences in the length of the PCR amplificates, unreadable base positions, indels, or stop codons (Zhang & Hewitt 1996), was found in the mitochondrial DNA sequences.

If the existence of ancestral polymorphism (or gene duplication) is proposed to explain the differences between the mitochondrial and the nuclear gene tree, the two haplotypes (ht1/ht1a and ht2) of *C. splendens* must have been present in their last common ancestor in the mitochondrial tree (see the arrow in Fig. 3). This split can be dated using reference values of evolutionary rates of the mitochondrial genome. However, for a valid determination, only the genetic divergence of a taxon pair can be used which splits at the same time as the haplotypes of *C. splendens*. Two taxa, viz. *C. hispanus* and *C. lineatus* (or *C. lateralis*), fit this condition in both the mitochondrial and the ITS 2 gene tree. The sequence divergence in the mitochondrial data set is 7.6%. Assuming a divergence rate of 2% per million years in the mitochondrial genome (Brown et al. 1982), this split is approximately 3.8 myrs old. Lower divergence rates have been proposed for carabids by Prüser & Mossakowski (1998), Su *et al.* (1998), Brückner (2002) and Düring (2004), and for cicindelids by Barraclough and Vogler (2002). Therefore, this split may be slightly older, but probably not more recent.

Consequently, the ancestral polymorphism of the last common ancestor (see arrow in Fig. 3) should also be at least 3.8 myrs old. However, the sequence divergence is 0.15% between *C. splendens* ht2 and *C. rutilans* and 1.16% between *C. splendens* ht1/1a and *C. punctatoauratus*. These genetic distances are too low to support this hypothesis. The events which lead to the different gene trees must have occurred much more recently. Therefore, ancestral polymorphism and gene duplication are unlikely explanations for the different gene trees.

4.3. Introgression in *Chrysocarabus*

According to the arguments mentioned above, the differences in the results of the phylogenetic analyses of mitochondrial and nuclear gene trees are most likely the result of introgression. The following scenario with at least two introgression events explains the major differences between the mitochondrial gene tree and the organismal tree:

(1) *C. splendens* (ht2) transferred its mitochondrial genome to *C. rutilans* by introgression, leading to their closer relationship in the mitochondrial CO2 gene tree. Consequently, the CO2 data set contains no non-introgressed (= no original) mitochondrial genome of *C. rutilans*.
(2) The mitochondrial genome of *C. splendens* (ht1) was acquired by introgression from *C. punctatoauratus*, followed by diversification into two subtypes (ht1 and ht1a).

However, the different position of *C. auronitens* in mitochondrial and nuclear gene trees suggests further potential introgression events which cannot be resolved by the available data sets.

The introgression of mitochondrial genomes by hybridisation requires a sympatric existence of the species involved. This was recently the case in *C. splendens* (ht1) and *C. punctatoauratus* (Fig. 4), but not in *C. splendens* (ht2) and *C. rutilans*. In accordance with the current distribution areas, the transfer of the mitochondrial genome from *C. punctatoauratus* to *C. splendens* seems a more recent event than the corresponding transfer from *C. splendens* to *C. rutilans*. However, this hypothesis conflicts with the genetic distances in the mitochondrial data set in which the genetic dis-

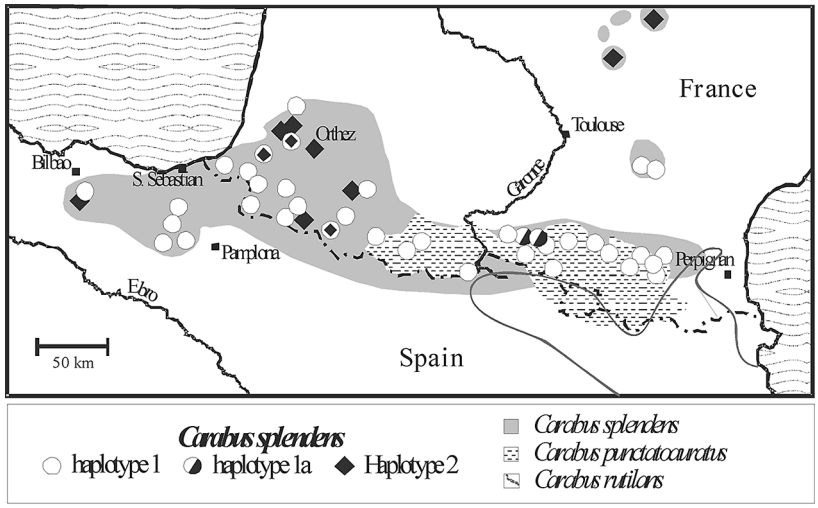

Figure 4. Distribution area and haplotypes of *Carabus splendens*. Modified after Düring *et al.* (2000). Ht1 is spread over the whole area while ht2 is only present in some northern isolated populations and in the western part where it occurs in some populations together with ht1.

tance between *C. splendens* (ht1) and *C. punctatoauratus* is higher (1.16%) than between *C. splendens* (ht2) and *C. rutilans* (0.15%). Nonetheless, the distribution areas of both taxa have certainly changed after the last introgression event due to the quaternary climate fluctuations. The conflict between the genetic and distribution data is probably the expression of these area dynamics in *Chrysocarabus*.

In conclusion, our analyses point out a serious problem that may occur when gene trees are uncritically interpreted as species trees. Recently separated taxa with incomplete genetic isolation are potentially able to transfer genetic material if they experience a secondary contact. In regard of the maternal inheritance of the mitochondrial genome, these gene transfers will lead to misinterpretations, as we have demonstrated in the subgenus *Chrysocarabus*. Therefore, the analyses of different character complexes such as mitochondrial and nuclear genes or molecular and morphological characters, is a suitable way to obtain well established organismal trees and to reveal the underlying molecular evolutionary processes.

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