Introgression or low molecular differentiation?  
The case of Carabus maacki

Abstract

Background: Carabus (Limnocarabus) maacki aquatilis and C. (Euleptocarabus) porrecticollis of the Japanese Island Honshu reveal a remarkably low sequence difference in the mitochondrial ND5 gene, similar to that existing between the subspecies of C. porrecticollis although the morphology is quite different. On the other hand C. maacki aquatilis from Japan is quite distinct to European C. clatratus in ND5 sequences and that was the reason to established C. maacki as a separate species, formerly a subspecies of C. clatratus. My hypothesis is that data on these taxa are better interpreted by assuming that C. maacki is in fact a C. clatratus but suffered in its Japanese form aquatilis an introgression from C. porrecticollis.

Material and Methods: Twenty-one specimens were sequenced and seven sequences were retrieved from GenBank. They comprise C. porrecticollis and C. maacki aquatilis form Japan, C. maacki maacki from the Asian mainland, and C. clatratus from far East and Europe. The data were analysed using the maximum likelihood method and were presented as a tree and as visualisation of sequences differences.

Results: The hypothesis that C. maacki is a C. clatratus but suffered an introgression by C. porrecticollis is supported by the results because (i) C. maacki maacki from Russian Primorje clustered with Far East and European C. clatratus in mitochondrial ND5 sequences, and (ii) Japanese C. maacki aquatilis clustered with C. clatratus in the nuclear gene data of wingless, but both differ in the same way to C. porrecticollis.

Conclusions: The presented data corroborate the hypothesis of an introgression from C. porrecticollis to the population of C. maacki aquatilis on Honshu, Japan. The taxon maacki cannot maintain species status. Its two subspecies should be considered as subspecies of C. clatratus (C. clatratus maacki from the Asian mainland, C. clatratus aquatilis from Japan).

INTRODUCTION

In their study on Carabus porrecticollis, Kim et al. (1) used sequences of the mitochondrial gene ND5 as a molecular marker for populations all over its distribution area on Honshu, Japan. Taking the Japanese C. maacki aquatilis as an outgroup, the authors obtained the surprising result that C. maacki aquatilis differed from C. porrecticollis only in the same extent as the C. porrecticollis subspecies do among themselves: the maximum divergence in ND5 sequences between the three lineages of C. porrecticollis was 3.1%, identical to the difference between C. porrecticollis and C. maacki aquatilis. They suggested a split of C. porrecticollis into several species instead of subspecies (1, p. 444).
This interpretation will be questioned in the present paper, because hybridisation between species is known very well in Carabus from nature and laboratory experiments (e.g. 2, 3). Even Jeannel (4) described a new species that turned out to be a hybrid. Molecular markers in various Carabus species and subgenera documented evolution of separate taxa by introgressive hybridisation: e.g. Chrysocarabus solieri (5), Ohomopterus (6, 7, 8, 9, 10), Chrysocarabus splendens – rutilans (11, 12), Chrysocarabus splendens – punctataauratus (13), Leptocarabus (14) and Mesocarabus (15).

The C. clatratus from the Russian province of Primorje was described as subspecies C. maacki Morawitz, 1862 and the Japanese form as C. aquatilis Bates, 1883. Imura et al. (16) ranked C. maacki as a separate species, based on the large differences between C. clatratus from Europe and C. maacki aquatilis from Japan (Kimura-2-Parameter (17) of 5.4 – 5.3 % (18); threshold values of K2P are usually greater than 3 between closely related species). Deuve (19, 20) followed this interpretation and promoted C. maacki from subspecies to species. This is the status in ‘The Carabus of Japan’ (21) (Imura & Mizusawa 2013), as well as in Schütze & Kleinfeld (22).

On the other hand, Löl & Smetana (23) and Lorenz (24) placed C. maacki as a subspecies of C. clatratus. Osawa et al. (25) did not use the name maacki; their Limnocarabus of Japan was ranked as a subspecies and named C. clatratus aquatilis. For practical reasons, I use the classification of Imura et al. (16) (species status of C. maacki with C. maacki aquatilis and the nominate form C. maacki maacki as subspecies) in order to use unambiguous allocation of names.

Only a few species exist within the genus Carabus that occur over a wide range in the Palaearctic region, one of which is Carabus (Limnocarabus) clatratus L. with a distribution area ranging from Ireland to the Far East. Carabus (Euleptocarabus) porrecticollis is endemic to the Japanese island Honshu. The subgenera Limnocarabus and Euleptocarabus comprise a monophyletic clade without doubt (1, 16), based on morphological characters (i.e. elytra sculpture, spine at the internal sac) and molecular data. Carabus maacki is morphologically closer to C. clatratus than to C. porrecticollis: e.g. in the shape of the body, the structure of the elytra (Fig. 1) and in characters of the endophallus, the spine of which is long in C. clatratus and C. maacki but short in C. porrecticollis (Fig. 2). (See also 16, 25, and 28).

Hypothesis

An introgression event will be tested as an alternative hypothesis.

The high molecular similarity of C. maacki aquatilis and C. porrecticollis exists because C. maacki suffered an introgression from C. porrecticollis in former times. The large difference between C. clatratus and C. maacki aquatilis is a result of this introgression event. In consequence, C. maacki should be downgraded to the rank of subspecies.

Because the C. maacki data are only from Japanese specimens and only from a mitochondrial gene (which is
only propagated maternally), three comparisons can generate this result:

- Specimens from the Asian mainland (C. maacki maacki) have a different pattern of the ND5 sequence than the Japanese ones (C. maacki aquatilis).
- The ND5 differences between both C. clatratus and C. maacki maacki from the Asian mainland compared to C. porrecticollis are similar, but much greater than those between C. porrecticollis and C. maacki aquatilis from Japan.
- Nuclear sequence data will display minor differences between C. clatratus and C. maacki from Japan, as well as from the Asian mainland but greater to C. porrecticollis.

**MATERIAL AND METHODS**

The specimens used for the analyses are listed in Table 1. They include C. porrecticollis, C. maacki from Japan (C. m. aquatilis) and the Asian mainland (C. m. maacki), and C. clatratus from Europe and Far East (Jakutsk).

**Molecular methods**

The NADH-Dehydrogenase subunit 5, including a part of tRNA_Phe (ND5; 1083 bp), was analysed as a mitochondrial gene. The primer-pair His and Phe for ND5 (29) was used to perform PCR and sequencing. In some cases, intermediate primers were used for ND5 (e.g. 270 and 270r, 850 and 850r) (30). As a nuclear gene, we analysed the protein coding wingless (wg, 440 bp) using the primers CARWL-1 und CARWL-2 (6). Most sequences used here were generated by Michael A. Miller (www.kmbioservices.de).

Sequences were aligned with CLUSTALX (31), distances calculated using MEGA6 (32) and phylogenetic analyses were performed using the Maximum Likelihood Method (DNAML in PHYLIP 3.6) (33) using speedier but rougher analysis, empirical base frequencies, a transition/transversion ratio of 2.0, global rearrangements, and bootstrap procedure with 1000 pseudo-replicates. Additionally, a visualisation is given of sequence differences position per position.

**RESULTS**

The mitochondrial ND5 and the nuclear wingless gene of twenty-one specimens were sequenced. The suitable data included sixteen new ND5 sequences and ten of wingless. In addition, seven sequences were revealed from GenBank, four of ND5 and three of the wingless gene (Tab. 1). An additional four wingless sequences from other locations were excluded because they differed only in singletons.

**Mitochondrial gene**

**Distances ND5**

The Japanese C. maacki aquatilis exhibited minor differences in comparison to C. porrecticollis and major differences in comparison to C. clatratus but also high values in comparison to C. maacki maacki from the Asian mainland (Primorje) (Table 2). On the other hand, C. maacki maacki from the Asian mainland was very similar to C. clatratus (framed numbers in Tab. 2). Such low distances were also found within the C. maacki aquatilis taxon.
Table 1 List of Carabus specimens used for this study.

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* #157 = #N241 of EU798736.1 ND5 Wg

Table 2 Pair-wise distances of mitochondrial ND5 sequences (p %) of representative specimens of Carabus clatratus, C. porrecticollis and C. maacki from different regions.

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Carabus maacki: Introgression or low molecular differentiation?

D. Mossakowski

Maximum likelihood tree: ND5

The Japanese Carabus maacki aquatilis clustered with C. porrecticollis in the maximum likelihood tree of ND5 data, while the Carabus maacki from the Asian mainland was put into the clatratus group that includes specimens from different regions of Russia and Europe (Fig. 3). This cluster was supported by a high bootstrap value.

Visualisation of sequence differences: ND5

A lot of positions with exclusive patterns were marked in dark green in Figure 4. The Japanese Carabus maacki aquatilis shared at least 13 and an additional 7 tentative nucleotide positions with C. porrecticollis but none was exclusive with C. maacki maacki from the Asian mainland. In addition, at ten positions C. maacki aquatilis differed from all other specimens involved. In contrast, C. maacki from the Asian mainland shared the same bases with C. clatratus from Europe and the Far East in the aforementioned character blocks.

Nuclear protein-coding gene

Maximum likelihood tree: wingless

The tree constructed by use of a nuclear gene, wingless (Fig. 5), resulted in one well-supported cluster of European C. clatratus and C. maacki aquatilis. Carabus maacki maacki could not be included because fresh material was not available.

Visualisation of sequence differences: wingless

The data for wingless gene demonstrated in detail, that the pattern of nucleotides was identical for C. clatratus and C. maacki aquatilis in at least nine exclusive positions. Only one, or at most two, positions displayed a contradictory pattern (highlighted in blue or grey in Table 4).

DISCUSSION

The introgression hypothesis was supported by the presented data in all three criteria examined:

(i) A specimen from the Asian mainland (C. maacki maacki) displayed a similar pattern to that of C. clatratus from various regions in the mitochondrial ND5 sequence but a pattern quite different to the Japanese ones (C. maacki aquatilis); (ii) The same result was also apparent in the nucleotide differences as shown by abstract values (% similarity) and by visualisation of the base patterns. (iii) The nuclear sequence data of wingless displayed minor differences between C. clatratus and C. maacki aquatilis from Japan but greater differences with C. porrecticollis.

Figure 3 Majority rule consensus tree of ND5 sequences constructed using DNAML. Bootstrap values >50% are shown.

Figure 4 ND5 base positions of C. clatratus, C. maacki maacki (Primorje), C. m. aquatilis (Japan), and C. porrecticollis that differ in more than one specimen (= singletons omitted).
These findings are in agreement with the molecular data of Kim et al. (1). They analysed a mitochondrial gene in their study of the *C. porrecticollis* populations of Honshu and included the Japanese *C. maacki aquatilis* as an outgroup. Therefore, they could not detect hybridisation events and they stated for *C. porrecticollis* (p. 444): “...there may be several phylogenetic species here instead of just one species with several populations”.

Kim et al. (1) found three groups in their thorough investigations of *C. porrecticollis*. Two of these match the described subspecies, the nominate form in the north and *C. kanaiensis* in central Honshu (Kinki district); the third, from southwestern Honshu (Chugoku district), did not cluster unambiguously and its status remained open. A third subspecies, *C. porrecticollis pacificus*, was described later from specimens collected from between the Pacific coast and the Fuji Mountain (38).

Considering the morphology, Imura et al. (16) have stated that the molecular differences were small between *Carabus maacki aquatilis* and *C. porrecticollis*, although these taxa were quite different morphologically. Also Kleinfeld (39) drew attention to this remarkable phenomenon.

The present status of Japanese *C. maacki maacki* may be interpreted by an introgressive hybridisation a long time ago for two reasons. The ‘*porrecticollis*’ mitochondrial of *C. maacki maacki*, which differs clearly from that of the *C. porrecticollis* species, and the morphological similarity between *C. clatratus* and both *C. maacki* forms, in contrast to *C. porrecticollis*.

Both facts indicate that there must have been a lot of backcrosses of the hybrid with *C. maacki aquatilis* despite its partial sympatry with *C. porrecticollis* in the northernmost part of Honshu.

All data conform with the hypothesis of an introgression from *C. porrecticollis* to *C. maacki aquatilis*. This interpretation conforms with the well-known hybridisation tendency of *Carabus* species from nature (2, 3) and laboratory experiments (5-15).

An alternative interpretation of the data is theoretically conceivable under the assumption of incomplete lineage sorting. A calculation was not done because some sequences don’t have the full length, the mitochondrial and the nuclear sequences are partly from different specimens or locations, due to the difficulty in obtaining fresh specimens from many regions, and the missing knowledge of some parameters, such as population size, that are necessary for the procedure. Additionally, the data gave a

![Figure 5](image_url)

**Figure 5.** Majority rule consensus tree calculated from sequences of a nuclear gene (wingless) of *Carabus clatratus*, *C. maacki aquatilis* and *C. porrecticollis*. Bootstrap values >50 are shown. The * indicates the branch with *C. clatratus* from Europe.

![Figure 6](image_url)

**Figure 6.** Base positions of a nuclear gene (wingless) of *C. clatratus*, *C. maacki aquatilis* and *C. porrecticollis* that differ in more than one specimen (= singletons omitted).
clear indication, in the nuclear sequences only one or at most two base positions displayed a contradictory pattern (Fig. 6), that may be interpreted by incomplete lineage sorting.

In consequence, the rank of *C. maacki* must be downgraded to subspecies rank; following priority rules, it should be considered that *C. maacki maacki* and *C. maacki aquatilis* are two subspecies of *C. clatratus*. *Carabus clatratus maacki* from the Asian mainland and *C. clatratus aquatilis* from Honshu, Japan. To retain two subspecies is in agreement with the differences in ND5 sequences depending on the long separation of Japan from the Asian mainland. The subspecies of *P. porrecticollis* maintain their status.

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