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Introgression or low molecular differentiation? The case of *Carabus maacki*

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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).

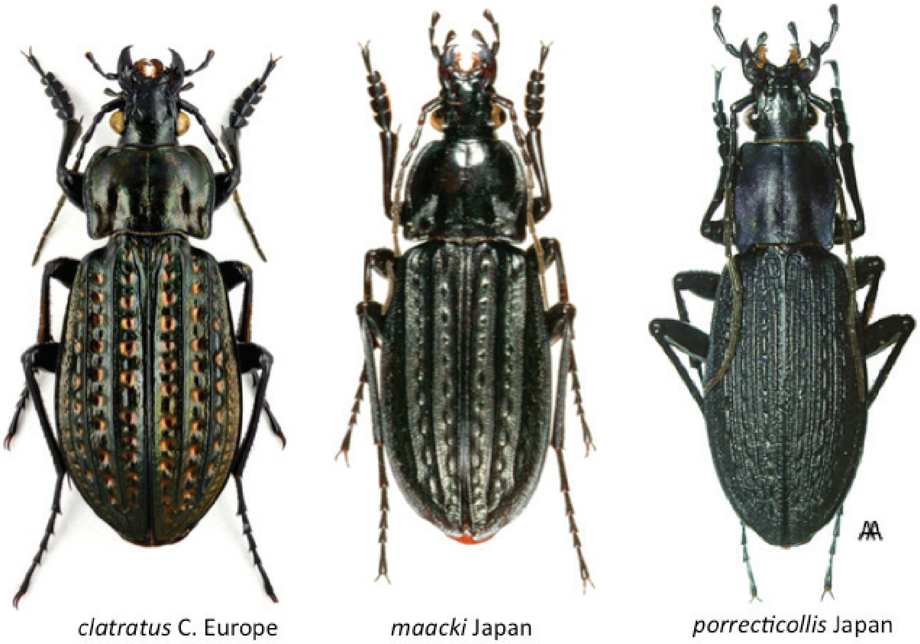


Figure 1 *Habitus of male* Carabus (Limnocarabus) clatratus[1] *and* C. maacki aquatilis[2] *and* C. (Euleptocarabus) porrecticollis[3]. *By courtesy of O. Bleich[1] (26); Y. Katsuro[2] (in 27), and A. Anitchenko[3] (27)*

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 – punctatoauratus (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, Löbl & 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

clatratus Ural *maacki* Vladivostok *porrecticollis* Japan

Figure 2 *Aedeagus with inflated internal sac of* C. clatratus[1], C. maacki maacki[1], *and* C. porrecticollis[2]. *By courtesy of O. Berlov[1] (28) and Y. Imura & K. Mizusawa[2] (21).*

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.*

Carabus species	Sub-species	Sample #	Country Province	Location	Collector/ Donator	Collect. date	GenBank #
porrecticollis	kansaiensis	196	JP Osaka	Mt. Kongo	T. Sota	2010	KX377443
porrecticollis	kansaiensis	196	JP Osaka	Mt. Kongo	T. Sota	2010	KX380583
porrecticollis	kansaiensis	S19	JP Shiga	Yogo	T. Sota	2010	KX380579
porrecticollis	porrecticollis		JP Nagano	Matsumoto	(34) Sota & Ishikawa		AY183585
maacki	aquatilis	195	JP Aomori	Tsugaru	Y. Okuzaki/T. Sota	2010	KX377447
maacki	aquatilis	195	JP Aomori	Tsugaru	Y. Okuzaki/T. Sota	2010	KX380581
maacki	aquatilis		JP Aomori		(34) Sota & Ishikawa		AY183586
maacki	aquatilis	75	JP Yamagata	Oishida-machi, Takanosu	Y. Nagahata/K. Kubota	12.06.2006	KX380580
maacki	aquatilis	S17	JP Aomori	Tsugaru	Y. Okuzaki/T. Sota	2010	KX380584
maacki	aquatilis	S18	JP Aomori	Tsugaru	Y. Okuzaki/T. Sota	2010	KX380585
maacki	aquatilis		JP		(29) Su et al.		D50358
maacki	maacki	162	RU Primorskiy krai	Lazo	D. Vasiliev/A. Matalin	1989	KX380588
clatratus	jansoni	190	GB Scotland	Isle of Harris	M. Briggs	16.06.2012	KX377440
clatratus	jansoni	190	GB Scotland	Isle of Harris	M. Briggs	16.06.2012	KX380589
clatratus	jansoni	202	IE Galway	Connemara	G. Kaule	2007	KX377444
clatratus	jansoni	202	IE Galway	Connemara	G. Kaule	2007	KX380582
clatratus	jansoni	191	IE Galway	Connemara	G. Kaule	2007	KX377442
clatratus	jansoni	192	GB Scotland	Dundonnell	G. Pozsgai, D.M.	03.06.2012	KX377442
clatratus	jansoni	157*	IE Galway	Kilmacduagh, Turlough	R.Anderson	24.05.2003	EU798736.2
clatratus	clatratus		NL Nord Brabant	De Kempen	(35) Deuve et al.		JQ646802
clatratus	clatratus	156	DE Lower Saxony	Pevestorf	D.M.	20.01.2003	KX377435
clatratus	clatratus	128	RU Jakutsk	Nerh-an	Museum Naturk. Berlin	29.04.1904	KX380587
clatratus	clatratus	138	RU Jakutsk	Nizhniy Bestyakh	S. Kurbatov/A. Matalin	July 2001	KX377446
clatratus	clatratus	138	RU Jakutsk	Nizhniy Bestyakh	S. Kurbatov/A. Matalin	July 2001	KX380586
clatratus	auraniensis	159	AT Lower Austria	March, Drösing	W. Paill	27.11.2002	KX377437
clatratus	arelatensis	56	FR Bouche-du-Rhone	Camargue	D.M.	23.09.1980	KX380590
clatratus	antonelli	39	IT Ravenna	Palude di S. Vitale	B. Lassalle	5.1970	KX380593
clatratus	antonelli	40	IT Ravenna	Palude di S. Vitale	B. Lassalle	5.1970	KX380592
clatratus	antonelli	85	IT Roma		Senckenberg Museum Frankfurt		KX380591
clatratus	antonelli	203	IT Siena	Montepulciano	A.Petrioli/A. Casale	18.01.2005	KX377445
Apotomopterus	iris		CN Sichuan	Daliangshan	(36) Kim et al.		AB031504
Apotomopterus	sauteri		CN Jiangxi	Jinggangshan	(36) Kim et al.		AB031488
Hygrocarabus	variolosus		DE Bavaria	Murnauer Moos	(37) Arndt et al.		AF231700
		* #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.*

			clatratus		maacki				porrecticollis	
			Far East		Prim	Japan				
	Taxon	Region	1	2	3	4	5	6	7	8
1	clatratus	RU Jakutsk 128	–	0.6	0.6	5.2	6.0	5.6	4.8	5.2
2	clatratus	RU Jakutsk 138	0.6	–	0.4	5.4	5.8	5.4	5.0	5.4
3	maa. maacki	RU Primorje 162	0.6	0.4	–	5.4	6.3	5.8	5.0	5.4
4	maa. aquatilis	JP Yamagata 75	5.2	5.4	5.4	–	0.8	0.4	3.3	3.3
5	maa. aquatilis	JP GenBank	6.0	5.8	6.3	0.8	–	0.4	4.2	4.2
6	maa. aquatilis	JP Aomori S17	5.6	5.4	5.8	0.4	0.4	–	3.7	3.7
7	porr. kansaiensis	JP Osaka 196	4.8	5.0	5.0	3.3	4.2	3.7	–	0.8
8	porr. kansaiensis	JP Shiga S19	5.2	5.4	5.4	3.3	4.2	3.7	0.8	–

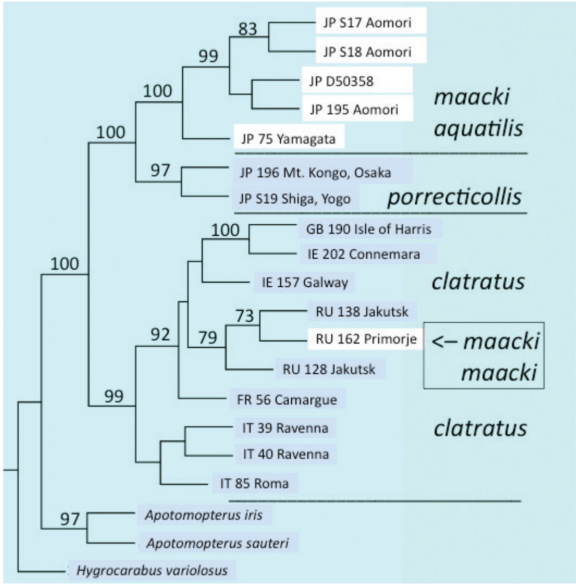


Figure 3 *Majority rule consensus tree of ND5 sequences constructed using DNAML.*
Bootstrap values >50% are shown.

Maximum likelihood tree: *ND5*

The Japanese *C. maacki aquatilis* clustered with *C. porrecticollis* in the maximum likelihood tree of ND5 data, while the *C. maacki 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 *C. 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 the other specimens involved. In contrast, *C. maacki 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.

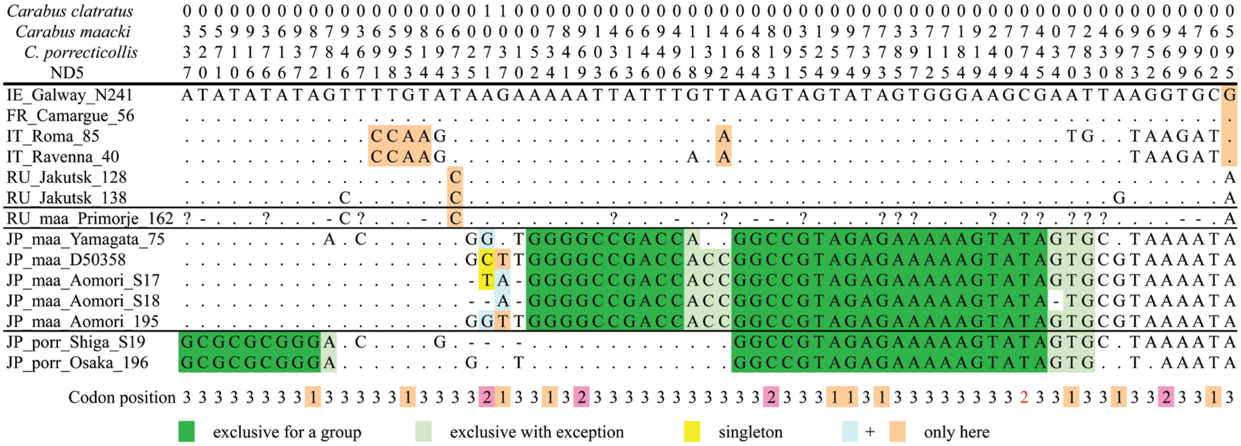
Visualisation of sequence differences: *wingless*

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*.



Carabus clatratus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Carabus maacki	3	5	5	9	9	3	6	9	8	7	9	3	6	5	9	8	6	6	0	0	0	0	0	7	8	9	1	4	6	6	9	4	1	1	4	6	4	8	0	3	1	9	9	7	7	3	3	7	7	1	9	2	7	7	4	0	7	2	4	6	9	6	9	4	7	6	5
C. porrecticollis	3	2	7	1	1	7	1	3	7	8	4	6	9	9	5	1	9	7	2	7	3	1	5	3	4	6	0	3	1	4	4	9	1	3	1	6	8	1	9	5	2	5	7	3	7	8	9	1	1	8	1	4	0	7	4	3	7	8	3	9	7	5	6	9	9	0	9
ND5	7	0	1	0	6	6	6	7	2	1	6	7	1	8	3	4	4	3	5	1	7	0	2	4	1	9	3	6	3	6	0	6	8	9	2	4	4	3	1	5	4	9	4	9	3	5	7	2	5	4	9	4	9	4	5	4	0	3	0	8	3	2	6	9	6	2	5
IE_Galway_N241	A	T	A	T	A	T	A	T	A	G	T	T	T	T	G	T	A	T	A	A	G	A	A	A	A	A	T	T	A	T	T	T	G	T	T	A	A	G	T	A	G	T	A	T	A	G	T	G	G	G	A	A	G	C	G	A	A	T	T	A	A	G	G	T	G	C	G
FR_Camargue_56	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
IT_Roma_85	.	.	.	.	.	.	.	.	.	.	.	.	C	C	A	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	T	A	A	G	A	T	.
IT_Ravenna_40	.	.	.	.	.	.	.	.	.	.	.	.	C	C	A	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A	G	A	T	.
RU_Jakutsk_128	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
RU_Jakutsk_138	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A
RU_maa_Primorje_162	?	-	.	.	.	?	.	.	.	-	C	?	.	.	.	-	.	C	.	.	.	.	.	.	.	.	.	?	.	.	.	-	.	.	?	.	-	-	.	?	.	.	.	.	?	?	?	.	.	.	.	?	.	?	?	.	?	?	?	.	.	.	.	-	-	.	A
JP_maa_Yamagata_75	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.	G	G	.	T	G	G	G	G	C	C	G	A	C	C	A	.	.	G	G	C	C	G	T	A	G	A	G	A	A	A	A	A	G	T	A	T	A	G	T	G	C	.	T	A	A	A	A	T	A
JP_maa_D50358	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	T	T	G	G	G	G	C	C	G	A	C	C	A	C	C	G	G	C	C	G	T	A	G	A	G	A	A	A	A	A	G	T	A	T	A	G	T	G	C	G	T	A	A	A	A	T	A
JP_maa_Aomori_S17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	T	A	-	G	G	G	G	C	C	G	A	C	C	A	C	C	G	G	C	C	G	T	A	G	A	G	A	A	A	A	A	G	T	A	T	A	G	T	G	C	G	T	A	A	A	A	T	A
JP_maa_Aomori_S18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	A	-	G	G	G	G	C	C	G	A	C	C	A	C	C	G	G	C	C	G	T	A	G	A	G	A	A	A	A	A	G	T	A	T	A	-	T	G	C	G	T	A	A	A	A	T	A
JP_maa_Aomori_195	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	T	T	G	G	G	G	C	C	G	A	C	C	A	C	C	G	G	C	C	G	T	A	G	A	G	A	A	A	A	A	G	T	A	T	A	G	T	G	C	G	T	A	A	A	A	T	A
JP_porr_Shiga_S19	G	C	G	C	G	C	G	G	G	A	.	C	.	.	.	.	G	.	-	-	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	C	C	G	T	A	G	A	G	A	A	A	A	A	G	T	A	T	A	G	T	G	C	.	T	A	A	A	A	T	A
JP_porr_Osaka_196	G	C	G	C	G	C	G	G	G	A	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	C	C	G	T	A	G	A	G	A	A	A	A	A	G	T	A	T	A	G	T	G	.	.	T	.	A	A	A	T	A
Codon position	3	3	3	3	3	3	3	3	1	3	3	3	3	3	1	3	3	3	3	2	1	3	3	1	3	2	3	3	3	3	3	3	3	3	3	3	3	2	3	3	3	1	1	3	1	3	3	3	3	3	3	3	3	2	3	3	1	3	3	1	3	3	2	3	3	1	3

🟩 exclusive for a group
🟩 exclusive with exception
🟨 singleton
🟦 + 🟧 only here

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).*

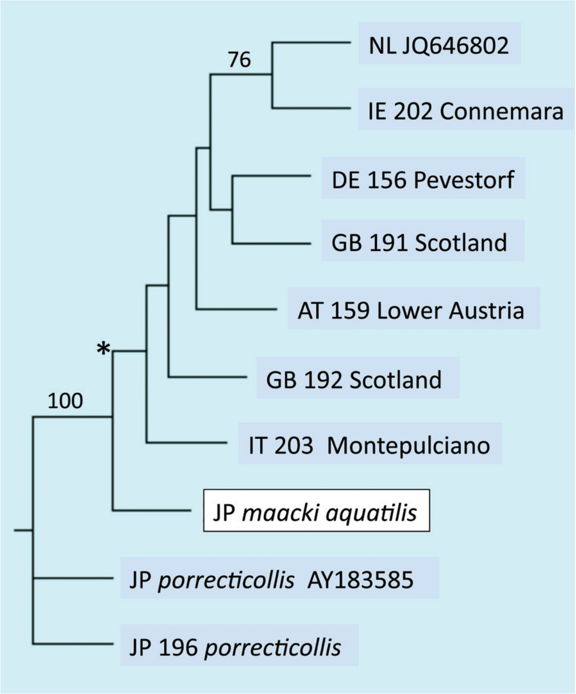


Figure 5 *Majority rule consensus tree calculated from sequences of a nuclear gene (wingless) of* Carabus clatratus, C. maacki aquatilis *and* C. porrecticollis.

*Bootstrap values >50 are shown. The * indicates the branch with* C. clatratus *from Europe.*

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)*.

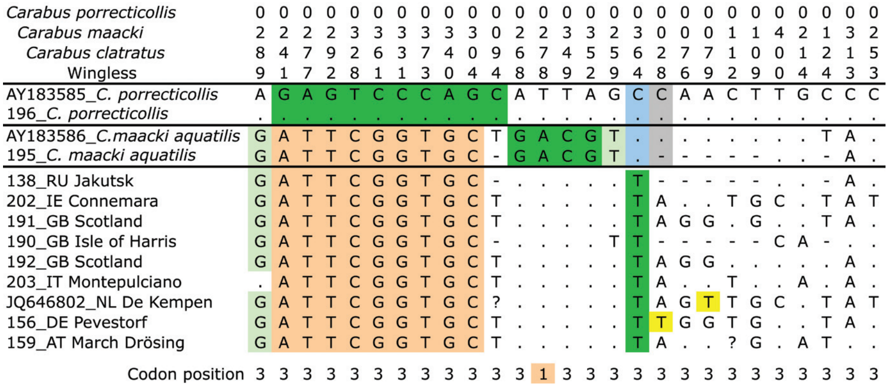
Considering the morphology, Imura et al. *(16)* have stated that the molecular differences were small between *Carabus maacki aquatilis* and *C. prorrecticollis*, although these taxa were quite different morphologically. Also Kleinfeld *(39)* drew attention to this remarkable phenomenon.

The present status of Japanese *C. maacki maacki* may be interpreted by an introgressive hybridisation a long time ago for two reasons. The '*porrecticollis*' mitochondrion 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



Carabus porrecticollis *Carabus maacki* *Carabus clatratus* Wingless	0289	0241	0277	0292	0328	0361	0331	0373	0340	0304	0094	0268	0278	0349	0352	0259	0364	0028	0076	0079	0112	0109	0400	0214	0124	0313	0253
AY183585_*C. porrecticollis*	A	G	A	G	T	C	C	C	A	G	C	A	T	T	A	G	C	C	A	A	C	T	T	G	C	C	C
196_*C. porrecticollis*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AY183586_*C.maacki aquatilis*	G	A	T	T	C	G	G	T	G	C	T	G	A	C	G	T	.	.	.	.	.	.	.	.	T	A	.
195_*C. maacki aquatilis*	G	A	T	T	C	G	G	T	G	C	-	G	A	C	G	T	.	-	-	-	-	-	.	.	-	A	.
138_RU Jakutsk	G	A	T	T	C	G	G	T	G	C	-	.	.	.	.	.	T	-	-	-	-	-	.	.	-	A	.
202_IE Connemara	G	A	T	T	C	G	G	T	G	C	T	.	.	.	.	.	T	A	.	.	T	G	C	.	T	A	T
191_GB Scotland	G	A	T	T	C	G	G	T	G	C	T	.	.	.	.	.	T	A	G	G	.	G	.	.	T	A	.
190_GB Isle of Harris	G	A	T	T	C	G	G	T	G	C	-	.	.	.	.	T	T	-	-	-	-	-	C	A	-	.	.
192_GB Scotland	G	A	T	T	C	G	G	T	G	C	T	.	.	.	.	.	T	A	G	G	.	.	.	.	.	A	.
203_IT Montepulciano	.	A	T	T	C	G	G	T	G	C	T	.	.	.	.	.	T	A	.	.	T	.	.	A	.	A	.
JQ646802_NL De Kempen	G	A	T	T	C	G	G	T	G	C	?	.	.	.	.	.	T	A	G	T	T	G	C	.	T	A	T
156_DE Pevestorf	G	A	T	T	C	G	G	T	G	C	T	.	.	.	.	.	T	T	G	G	T	G	.	.	T	A	.
159_AT March Drösing	G	A	T	T	C	G	G	T	G	C	T	.	.	.	.	.	T	A	.	.	?	G	.	A	T	.	.
Codon position	3	3	3	3	3	3	3	3	3	3	3	3	1	3	3	3	3	3	3	3	3	3	3	3	3	3	3

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 *C. porrecticollis* maintain their status.

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