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Craspedacusta sowerbii, Lankester 1880 – population dispersal analysis using COI and ITS sequences

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ABSTRACT

Craspedacusta sowerbii *(Hydrozoa, Limnomedusae, Olindiidae) is a freshwater jellyfish, which was discovered in England in 1880. Although thought to originate in South America, it became obvious that the species is native to the Yangtze River system in China. It has spread from China into lakes all over the world. Many different species, variations and sub-species have been described based on morphological characters. Specimens discovered in North America were described as separate species, as morphological differences appeared to be significant compared to European specimens. Even within Europe, differences were assumed to be obvious. Up to this point, three valid species are published; others are considered by various scientists to be true species as well, but mostly are recognized as variations. To obtain further insight into population dynamics of* C. sowerbii *as well as molecular information on the species itself, sequences of internal transcribed spacers (ITS) and cytochrome oxidase subunit I (COI) have been used to analyze specimens collected in Germany and Austria. These sequences have been compared to sequences published of different Chinese* Craspedacusta *species and variations. In addition, morphological descriptions were compared. For the COI sequences, we found uniformity throughout the complete set of samples. However, no comparisons could be made, as no data had been published on COI of Chinese specimens. ITS1, 5.8S and ITS2, including partial 18S and 28S, sequences, were almost uniform within the German populations, showing only minor base pair exchanges. However, comparisons to Chinese organisms indicated, that the investigated sequences of* C. sowerbii *from Germany and Austria are similar, although not identical in morphology, to* Craspedacusta sowerbii *var*. kiatingi *from China. Overall our data support the assumption that there are three valid species, with the possibility of* C. ziguiensis *being a fourth one, and several, morphological quite different sub-species or variations of the freshwater jellyfish* C. sowerbii.

Key words: Cnidaria, cytochrome oxidase, Hydrozoa, internal transcribed spacers, Limnomedusa

1. INTRODUCTION

Craspedacusta sowerbii, Lankester 1880 is a freshwater jellyfish within the family Olindiidae (Hydrozoa, Limnomedusae). Originating from the Yangtze River area in China (Kramp 1950, 1961), it has been introduced to many freshwater habitats worldwide (Acker & Muscat 1976; Boothroyd *et al*. 2002; Pennak 1956; Silva & Roche 2007). Although several freshwater species within the genus *Craspedacusta* exist, only *C. sowerbii* exhibits such a worldwide distribution. Means of distribution are less likely the short-lived medusa stage but more likely the minute polyp or any of the vegetative reproductive phases, such as frustules (Dejdar 1934; Reisinger 1957). After its discovery and subsequent first scientific description in England in 1880 (Lankester 1880), many reports of its occurrence were published within the following decades. However, it was impossible to reconstruct how the jellyfish was distributed and if there have been singular or multiple introduction events. Within recent years large numbers of new findings have been collected. Overall, *C. sowerbii* occurs in nearly all regions of Germany and new locations can be added on a regular basis (Fritz *et al*. 2007).

Several *Craspedacusta* species, subspecies and variations have been described from China and one species from Japan. Although the systematics and phylogeny are not clear on these descriptions, it seems that up to this point there are three valid *Craspedacusta* species, i.e., *C. sowerbii* (Lankester 1880), *C. iseanum* (Oka & Hara 1922), and *C. sinensis* (Gaw & Kung 1939). Others, described as new species, are *C. brevenima* (He & Xu 2002), *C. chuxiongensis* (He *et al*. 2000), *C. sichuanensis* (He & Kou 1984), and *C. ziguiensis* (He & Xu 1985). One species, *C. vovasi*, has been placed in a different genus by the authors themselves (Naumov & Stepanjants 1971; Jankowki *et al*. 2001). In addition, several other subspecies and variations have been reported, although they have been subsequently described in other publications as species (He 2003).

Molecular markers have become a valuable tool to determine different level relationships amongst the Cnidaria including higher level taxa analyses as well as genera, species and population level studies (Collins *et al*. 2006; France & Hoover 2002; Jankowski 2001; Jankowski *et al*. 2008; Parker *et al*. 1998). Although many observations on *C. sowerbii* have been reported over the years, nothing is known on the population dynamics and potential temporal or spatial dynamics of introduction events.

Tab. 1. Sampling locations of freshwater jellyfish medusae in quarry lakes in Germany and Austria including accession numbers for COI/ITS.

Lake	Location	Collection date	Sequence analysys	Accession number COI/ITS
Flückiger See	Freiburg, Baden-Wuerttemberg	Oct. 2006	COI, ITS	FJ423614/FJ423625
Diezsee	Limburg, Rheinland-Pfalz	Sept. 2006	COI, ITS	FJ423623/FJ423620
Canyon Süplingen	Süplingen-Haldensleben, Saxony-Anhalt	Aug. 2006	COI, ITS	FJ423618FJ423628
Schönbach	Herborn, Hessen	Aug. 2006	COI, ITS	FJ423613/FJ423624
Klingenberg	Langenfeld, Northrhine-Westphalia	Aug. 2006	COI, ITS	FJ423617/FJ423627
Hohwiesensee	Ketsch, Baden-Wuerttemberg	Aug.2006	COI, ITS	FJ423619/FJ423629
Matschelsee	Kürzell, Baden-Wuerttemberg	Oct. 2006	COI, ITS	FJ423615/FJ423626
Löbejun	Löbejun, Saxony Anhalt	Aug. 2006	COI, ITS	FJ423616/FJ423633
Streitköpfle	Linkenheim-Hochstetten, Baden-Wuerttemberg	Aug.2007	ITS	-/FJ423631
Lußhardtsee	Kronau, Baden-Wuerttemberg	Aug. 2007	ITS	-/FJ423622
Gänsedrecksee	Speyer, Rheinland-Pfalz	Aug. 2007	ITS	-/FJ423621
Willersinn II	Karlsruhe, Baden-Wuerttemberg	Aug. 2007	ITS	-/FJ423630
Alte Donau/ Danube	Vienna, Austria	unknown	ITS	-/FJ423632

Therefore, this study was conducted to obtain molecular information on *C. sowerbii* populations within Germany and Austria (for easy reading called "German" from now on) using internal transcribed spacers (ITS) and cytochrome oxidase subunit I (COI). The sequence information was used to obtain more insight on the usefulness of COI for species barcoding (Hebert & Gregory 2005; Hebert *et al*. 2003) as well as potential information on population dispersal and introduction events of *C. sowerbii*. We also gained a deeper insight into the systematic and phylogenetic positioning of the German freshwater jellyfish population within the *Craspedacusta* group.

2. METHODS

2.1. Specimen collection and preservation

Jellyfish medusae were collected from eleven different lakes within Germany as well as one water body in Austria (Tab. 1). The specimens were collected by SCUBA divers, who participated in a project using laymen to obtain scientific data on the distribution of aquatic neobiota. Identification was reliable as there are no other hydrozoans with free swimming medusae stages in Europe. Immediately after collection, the specimens were preserved in 99% Ethanol molecular biology grade (Roth, Karlsruhe, Germany) and stored at 4 °C. Prior to DNA extraction ethanol was removed by placing specimens in a dilution series (75%, 50%, 30%, 10% ethanol in water and pure water) for 10 min at each step and two times 30 min in pure water.

2.2. DNA extraction and PCR

DNA was obtained by using a DNA preparation kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. One complete medusa was used for each extraction. DNA concentration was measured using a NanoDrop® Spectrophotometer ND-1000 (Peqlab, Erlangen, Germany).

An approximately 600 base pair (bp) partial sequence of the cytochrome oxidase subunit I (COI) was amplified using the forward primer primers 5'-GGTCAACAACAAATCATAAAGATATTGG-3' and the reverse primer 5'-TAAACTTCAGGGTGACCAAA-AAATCA-3' (Folmer *et al*. 1994). Amplification was performed in 20 µL total reaction volume with 200 ng template DNA, 1 U Taq polymerase (TaKaRa BIO INC., Shiga, Japan), 3.9 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTP, and 2 mmol L^{-1} of each oligonucleotide primer. The COI sequence was amplified with an initial denaturation step of 5 min at 94 °C, followed by five cycles of 1 min at 94 °C, 1.5 min at 45 °C and 1.5 min at 72 °C; 35 cycles of 1 min 94 °C, 1.5 min at 50 °C, 1 min at 72 °C and a final extension of 5 min at 72 °C. An 800 bp partial sequence of the ribosomal DNA was amplified using the primers forward primer 5'-CCCTTTGTACACACCGCCCGTCGCT-3' and the reverse primer 5'-CTTTGGGCTGCAGTCCCAAGCA-ACCCGACTC-3' (Odorico & Miller 1997). This partial sequence included parts of the 18S rDNA and 28S rDNA, the complete ITS1 and ITS2 regions as well as the 5.8S rDNA region. The PCR reaction mixture was equivalent to the mixture for the amplification of the COI sequence (see above) in a final volume of 20 µL. An initial denaturation at 96 °C for 3 min was followed by 30 cycles (95 °C for 1 min, 52 °C for 30 s, 72 °C for 1 min) and a final extension at 72 °C for 7 min.

2.3. Cloning and DNA sequencing

PCR-products were cleaned using Nucleo-Spin® Extract II kit (Macherey-Nagel) following the manufacturer's instructions. Subsequently they were ligated into pCR®4-TOPO vector (Invitrogen, Carlsbad, USA) and introduced into competent *Escherichia coli* One Shot®TOP10 cells (Invitrogen) following the manufacturer's instructions. Plasmid DNA was subsequently isolated from cultures with the NucleoSpin® Plasmid Kit (Macherey-Nagel). Insert sizes were verified performing an EcoR1 digestion and subsequent agarose gel electrophoresis.

Sequences of interest (800 bp) were sequenced with M13forward as well as an M13reverse primer by

Tab. 2. Distance matrix of the given 800 bp including partial 18S, ITS1, 5.8S, ITS2 and partial 28S regions of available *Craspedacusta* species. Identical sequences of German and Austrian samples (distance = 0-0.1%) have been combined to *C. sowerbii*. Ranges are given for multiple entries per species. Numbers in brackets indicate number of different sequences used, no number indicates single sample.

	C. sinensis (7)	*C. brevinema*	*C. sowerbii* (Germany) (11)	*C. sichuanensis*	*C. kiatingi* (5)	*C. sowerbii*, Streitkoepfle	*C. sowerbii*, Diez	*C. ziguiensis*	*C. sowerbyi* (11)	*C. xinyangensis* (2)
C. sinensis (7)	0.1-1.6	0.1-1.4	9.1-10.2	8.9-10	8.9-10.2	8.9-10	9-10	10-11.1	9.9-11.9	9.9-11.4
C. brevinema	0.1-1.4	-	9.1-9.3	9.1	8.9-9.1	9.1	9.1	10.2	10.9-11.3	10.9
C. sowerbii (11)	9.1-10.2	9.1-9.3	0-0.1	0-0.1	0-0.4	0	0-0.1	3.1-3.2	8.4-8.7	8.4-8.7
C. sichuanensis	8.9-10	9.1	0-0.1	-	0-0.3	0	0	3.1	8.2-8.7	8.4-8.5
C. kiatingi (5)	8.9-10.2	8.9-9.1	0-0.4	0-0.3	0-0.3	0-0.3	0-0.3	3.1-3.3	8.4-8.8	8.2-8.5
C. sowerbii Streitköpfle	8.9-10	9.1	0	0	0-0.3	-	0	3	8.4-8.7	8.4-8.5
C. sowerbii Diez	9-10	9.1	0-0.1	0	0-0.3	0	-	3.1	8.2-8.7	8.4-8.6
C. ziguiensis	10-11.1	10.2	3.1-3.2	3.1	3.1-3.3	3	3.1	-	9.5-10	9.7
C. sowerbyi (11)	9.9-11.9	10.9-11.3	8.4-8.7	8.2-8.7	8.4-8.8	8.4-8.7	8.2-8.7	9.5-10	0-0.8	0-0.9
C. xinyangensis (2)	9.9-11.4	10.9	8.4-8.7	8.4-8.5	8.2-8.5	8.4-8.5	8.4-8.6	9.7	0-0.9	0.4

Macrogen (Seoul, Korea). Three clones of each individual specimen were sequenced with forward and reverse primers resulting in a total of six sequences.

2.4. Sequence Analysis

Sequences were edited and aligned manually as well as subsequently analyzed using the ARB (version 06.11.28) software package (Ludwig *et al*. 2004). Of each specimen three clones were sequenced with forward and reverse primers. For each specimen all six resulting sequences were aligned and resulted in one consensus sequence representing the majority decision where needed. The resulting consensus sequences together with available sequences from the databases (AY513614 - AY513641 and AY730675 - AY730678) were analyzed applying the Neighbor Joining, Maximum Likelihood and Maximum Parsimony algorhythms. Of these three analyses a strict consensus tree was constructed (Ludwig *et al*. 1998).

2.5. Database deposition

The resulting nucleotide sequence data reported in this paper have been deposited in the GenBank nucleotide sequence database with accession numbers FJ423613 through FJ423633 (Tab. 1).

3. RESULTS

3.1. Cytochrome oxidase I (COI)

All resulting COI sequences (FJ423613 through FJ423620) from all tested specimens were identical. No comparisons with *Craspedacusta sowerbii* from other countries or other *Craspedacusta* species were possible, as no fresh material was available and no gene data were available in online data bases such as NCBI or Genbank.

3.2. Internal transcribed spacers (ITS)

The ITS region included parts of the 18S and 28S regions, the 5.8S and the two ITS regions I and II. The alignment used for analysis and sequence comparison included 1,091 positions. Amongst the German specimens, minor differences were found. *C. sowerbii* from Löbejün showed a 9 bp insertion, which can be found identically in *C. ziguiensis* from China, at position 299-307. None of the other analyzed sequences exhibited this insertion. As this insertion can be suspected to be a single event, we did treat it as a single position in further analysis to avoid overestimation of this event. Single nucleotide insertions at several positions result in the slightly elevated similarity of the samples from Diezsee, Klingenberg and Streitköpfle to the Chinese *C. kiatingi*, as compared to the rest of the German populations. Compared with sequences from Chinese specimens obtained from the NCBI database more differences could be found and are represented in the resulting distance matrix shown in table 2. The corresponding strict consensus representation of Neighbour Joining, Maximum Likelihood and Maximum Parsimony analyses is shown in figure 1. Three clusters of *Craspedacusta* species are found within the sequences from the Yangtze population, with the German population being part of the "*kiatingi*" cluster.

3.3. Morphology

Data have been compiled from different sources to compare morphological features of the observed species and subspecies (Lankester 1880; Dejdar 1934; Kramp

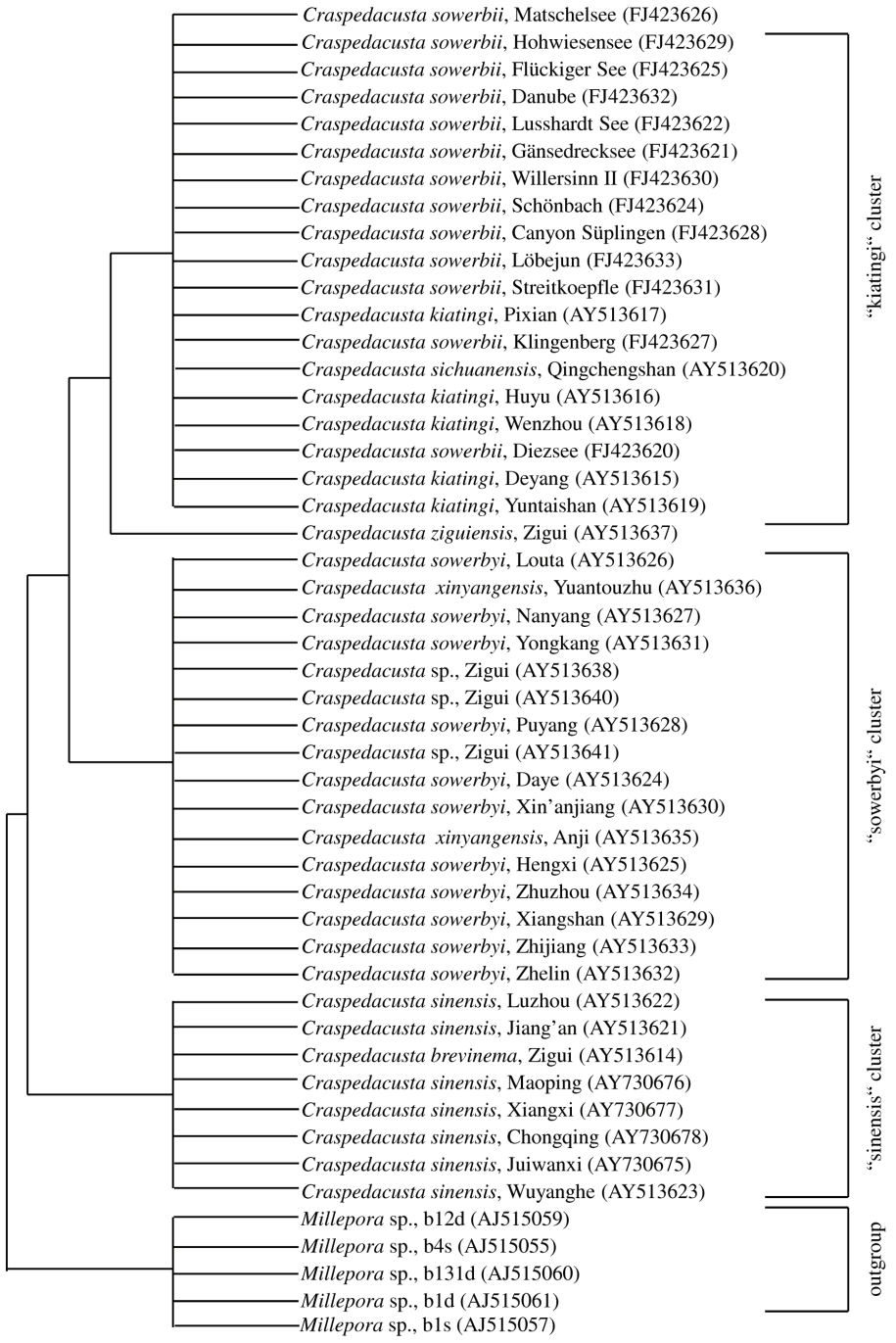

Fig. 1. A strict consensus representation of the Neighbor Joining, Maximum Likelihood and Maximum Parsimony analyses of the given 800 bp including partial 18S, ITS1, 5.8S, ITS2 and partial 28S regions of available *Craspedacusta* including *Millepora* sp. as next available relatives (outgroup).

1950, 1961; He & Xu 1985; 2002, 2003; He *et al*. 2000; Jankowski 2001). Although *C. sowerbii* described in Germany and other European countries look totally different from *C. kiatingi*, newly budded *C. sowerbii* look exactly as the described *C. sowerbyi* var. *kiatingi*. (Chinese specimens found in GenBank have been written with *-yi*. For differentiation purposes between Chinese and German specimens we kept the *-yi* spelling for the Chinese, and the *-ii* for the German specimens.) The umbrella is not flattened, but hemispherical and the gonads are small and pocket-like. On the other hand all mature German and Austrian specimens used for this study, as well as most of the worldwide described specimens look with some variations similar to *C. sowerbyi* specimens.

4. DISCUSSION

Although, described and discovered outside of China for the first time in 1880 (Lankester 1880), it is not known when the freshwater jellyfish was actually introduced into worldwide aquatic systems. The rapidly incoming reports after the first description indicate that the jellyfish may have been already present for quite some time, or at least since exotic ornamental aquatic plants, its potential source of transportation, were used in aquaria and parks. Still, new occurrences of the jellyfish are discovered within in Germany (Fritz *et al*. 2007) and worldwide (Arbaèiauskas & Lesutienë 2005, Pérez-Bote et al. 2006, Saadalla 2006). It is difficult to determine whether these are new introductions, local dispersal or just new observations. The increasing number could be due to several, not necessarily independent, factors i) The distribution is in fact increasing, ii) the medusae only occur for a short period of time during summer and need to be looked for or are found accidentally, iii) the polyp stage is minute and usually not detected but might have existed within a habitat long before the conditions were optimal for medusa formation and iv) increasing, recreational, aquatic activities such as SCUBA diving lead to an increase in observations. After the description of multiple sub-species and species, it seemed to be interesting to look into the location of origin, compare described species and elucidate the implications for introduction events.

Comparing morphological features, *C. sowerbii* found in Germany looks very different to *C. sowerbii* var. *kiatingi*, which is named as species by He (He 2003) and was described as variation at first description (Gaw & Kung 1939). The very small gonads, like in *C. ziguiensis*, make it difficult to see the similarity to *C. sowerbii*, let alone the differences in nematocyst arrangement. However, due to its similarity within the ITS region and morphological comparisons and discussions (Kramp 1950), the data support the status of *C. s.* var. *kiatingi* as a subspecies and indicate the origin of freshwater jellyfish found within Germany to be located in the Kiating area. The sub-species *Craspedacusta sowerbyi xinyangensis*, (He 1980) is indeed a form of *C. sowerbii*. On the other hand, the molecular data indicate that *C. brevinema* is a variation of *C. sinensis*, which also supports the fact that *C. sinensis* is a separate species. In addition, *C. ziguiensis* may be a separate species as indicated by a difference of approximately 3% in the distance matrix. This is also supported by mt16S and SSU analyses (Collins *et al*. 2008).

C. sichuanensis has morphological features similar to *C. sinensis*, however, some like *C. iseanum* (Jankowski 2001). The ITS sequences suggest genetic similarity with *C. s.* var *kiatingi* (Bouillon & Boero 2000; Jankowski 2001). This is supported by our data, however, a distance of 3.1% and hence, 5.3% to the Chinese *C. sowerbyi* might indicate a separate variation or subspecies. Sequences of the newly described species *C. chuxiongensis* (He 2003), have not been made publicly available yet and could not be compared. However, based on the data shown above, it is likely to be yet another variation of *C. sowerbii*. As mentioned by other scientists (Kramp 1950; Bouillon & Boero 2000; Jankowski 2001), our data support the fact that all Chinese "species", with the exception of *C. sinensis*, seem to be variations of either the latter or *C. sowerbii*. Hence at least three clusters or genetically separate groups of *Craspedacusta* seem to exist in the Yangtze River system. The "*kiatingi* cluster", the "*sinensis*" cluster and a third cluster comprising *C. sowerbyi* species. Regarding the ITS sequences, the German populations show a high genetic similarity to the "*kiatingi*" cluster, and hence most probably originated from *C. kiatingi*.

Craspedacusta exhibit a highly variable morphology, which has been already shown, when specimens were described as new species wherever *C. sowerbii* was observed for the first time (Browne 1906; Potts 1906; Roch 1924). Even specimens occupied one large, but interconnected river system show a huge variability as is seen in the populations observed and described from the Yangtze River system. Not only geography seems to play an important role but also, of course, the conditions of abiotic factors during the development of medusae (Kramp 1950). In addition, as so often, specimens are not described when newly collected and unpreserved. Handling and fixation methods also might lead to different conclusions as specimens are, for example, contracted when preserved and, subsequently, exhibited supposedly obvious differences (Dejdar 1934; Kramp 1950). One important feature, the shape of nematocysts as well as their arrangement in warts, is given a lot of attention although some investigations show difficulties in actually finding these differences when comparing more than one specimen and using fresh material (Dejdar 1934). Nematocysts indeed have taxonomic value at species level, however, it has to be considered that size, types and location can differ within a specimen depending on its stage of metagenesis (Östman 2000). As a result from these observations, many morphological features, which lead to the differentiation between different species, are questionable going conform with other authors (Dejdar 1934; Kramp 1950). And hence the molecular data not necessarily contradict morphological information, but rather ask for re-evaluation of some morphological investigations.

5. CONCLUSIONS

In general, the COI sequence is informative for barcoding (Hebert *et al*. 2003; Hebert & Gregory 2005) and therefore is a useful tool for some groups. However, earlier studies have shown that it is not useful for all taxonomic groups where it works for some species and does not for others (Heim *et al*. 2007a, Heim *et al*. 2007b). Even within the Cnidaria, COI evolved differently, leading to differences between the Scyphozoa,

Anthozoa and Hydrozoa (Huang *et al*. 2008), with more variation in the first group. It would be useful at this point to investigate the COI sequences of different species of *Craspedacusta* as well as comparing the sequences of different variations. Among the specimens found in Germany, which presumably belong to the very same species, no genetic variations were found. Hence the investigated region of the COI gene, bears no information on the population level whatsoever, but it is consistent (identical) within the German population of *C. sowerbii*. Still the German lakes could be regarded as hosting just one, genetically connected population of *C. sowerbii*. At least the German population only consists of one single species. Further investigation including COI information from more populations and species will elucidate the phylogenetic information content of COI sequences within freshwater medusae.

The ITS sequences again were identical throughout the samples from German lakes analyzed in this investigation. However they were slightly different from those *C. sowerbyi* sequences originating from China. These differences lay well within the ones observed between different Chinese populations of *C. sowerbyi* and *C. kiatingi*. This observation not only leads to the conclusion that *C. sowerbii* and *C. kiatingi* are in fact the same species, but also shows, that the sequences ITS1, 5,8S rDNA and ITS2 together allow the discrimination between genetically separated populations. We hence conclude, that the German population of *C. sowerbii* can be regarded as one population, presumably with some extent of genetic exchange or just too young to undergo diversification. It originated from the Chinese population of *C. kiatingi* and was shortly after invasion genetically separated from the original population in China. We furthermore suppose *C. kiatingi* to be renamed as *C. sowerbii* var. *kiating* due to sequence identity or similarity. However, the investigation and analyses of additional molecular markers and the re-evaluation of the morphological details applying similar preservation and sampling techniques and comparing similar stages of metagenesis are necessary to verify the status of a species, as well as obtaining data on the populations' diversity. For further investigations on *Craspedacusta* species, we suggest to include morphological information on all stages of metagenesis, at least ITS sequences and additional molecular markers like preferably COI and 18S rDNA.

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