

# Hidden diversity under morphology-based identifications of widespread invasive species: the case of the 'well-known' hydromedusa *Craspedacusta sowerbii* Lankester 1880

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## Abstract

*Hidden diversity under morphology-based identifications of widespread invasive species: the case of the 'well-known' hydromedusa *Craspedacusta sowerbii* Lankester 1880.* A relatively scarce number of morphological features available for delimiting closely related species and an increasingly worrisome scenario on Global Climate Change causing the rapid dispersion of invasive alien species can lead to the rapid spread of reports of a given species around the world. *Craspedacusta sowerbii* Lankester, 1880 is considered the most widespread freshwater jellyfish species and has been reported in numerous locations on all continents except Antarctica. Recently, a few medusae attributed to *C. sowerbii* were collected from a water reservoir (Bin El Ouidan) in Morocco, this being the first confirmed record of the species from North Africa. The morphology of these newly collected specimens agrees well with previous descriptions, but mitochondrial (*Cox1* and *16S*) and nuclear *ITS* (*ITS1–5,8S–ITS2*) molecular data lead to a discussion of a more complex general view concerning the number of species, synonyms and nomenclatural problems hidden behind the reports of *Craspedacusta sowerbii*.

Key words: *Craspedacusta*, Cryptic species, Medusa, Invasive species, NIS, *Cox1*, *16S*, *ITS*, Morocco

## Resumen

*La diversidad oculta en las identificaciones basadas en la morfología de especies invasoras de amplia distribución: el caso de la "bien conocida" hidromedusa *Craspedacusta sowerbii* Lankester 1880.* El número relativamente escaso de características morfológicas utilizadas para delimitar especies estrechamente relacionadas y el panorama cada vez más preocupante en el que el cambio climático global provoca la rápida dispersión de especies exóticas invasoras pueden conducir a la difusión precipitada por todo el mundo de informes sobre una especie determinada. *Craspedacusta sowerbii* Lankester, 1880, que se considera la especie de medusa de agua dulce más extendida, ha sido observada en numerosos lugares en todos los continentes, excepto en la Antártida. Recientemente, se recogieron algunas medusas atribuidas a *C. sowerbii* en un embalse artificial (Bin El Ouidan) en Marruecos, que representaron el primer registro confirmado de la especie en el norte de África. La morfología de estos especímenes recién recolectados concuerda con las descripciones anteriores, pero los datos moleculares mitocondriales (*Cox1* y *16S*) y nucleares *ITS* (*ITS1–5,8S–ITS2*) suscitan un debate general más complejo con respecto al número de especies, las sinonimias y los problemas nomenclaturales ocultos tras los informes de *Craspedacusta sowerbii*.

Palabras clave: *Craspedacusta*, Especies crípticas, Medusa, Especies invasoras, NIS, *Cox1*, *16S*, *ITS*, Marruecos

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## Introduction

Among the recommendations suggested by a wide panel of specialists to ensure progress in the management of aquatic NIS (non-indigenous species), the first was the availability of taxonomic expertise (see Ojaveer et al., 2014). Taxonomists produce the basic knowledge for understanding biodiversity (Agnarsson and Kuntner, 2007; Linse, 2017). The second was the need to use molecular tools, as classical taxonomy often requires additional sources of information for species description and identification (Goldstein and DeSalle, 2011), leading to an integrative taxonomy (DeSalle et al., 2005; Rubinoff et al., 2006a, 2006b; Pires and Marinoni, 2010; Chen et al., 2011).

Despite this, reliable identification of organisms to species level is one of the greatest constraints. The lack of specialists and the inaccuracy of species identifications often result in an erroneous interpretation of the actual biodiversity and inadequate conservation policies, from local to global levels. This is the so-called 'taxonomic impediment' (Hoagland, 1996; Giangrande, 2003; Dar et al., 2012). Avoiding this problem by limiting OTUs data matrices to higher taxonomic levels or considering functional biodiversity (Cernansky, 2017) is not a viable solution, especially when working on NIS, whose influence at different levels on native ecosystems is well documented (e.g. Bax et al., 2003; Wallentinus and Nyberg, 2007; Walther et al., 2009; Poulin et al., 2011; González-Duarte et al., 2016; among others).

The freshwater genus *Craspedacusta* includes a still uncertain number of hydromedusan species (Bouillon et al., 2006; Fritz et al., 2009; Jankowski et al., 2008). The most reported species around the world in this genus is *Craspedacusta sowerbii* Lankester, 1880, which can be found in all continents and subcontinents except Antarctica (Dumont, 1994). This species is native to the Yangtze River basin in China (Kramp, 1961) and is considered a cosmopolitan invasive species. *C. sowerbii* colonizes all types of freshwater habitats, i.e. streams, freshwater lakes, ponds, reservoirs and rivers (Raposeiro et al., 2011; Karaouzas et al., 2015). *C. sowerbii* was first described from specimens found in a water-lily tank in Regent's Park, London, England in 1880 (Lankester, 1880a). Later, the species was reported from many different localities: United States (Garman, 1916), Hawaii, South Australia (Thomas, 1950), New Zealand, the Philippines, China, Japan (Acker, 1976), France, Sweden, Portugal (Ferreira, 1985), Canada (McAlpine et al., 2002), Spain (Pérez-Bote et al., 2006; Medina-Gavilán and González-Duarte, 2018), Mexico (Moreno-Leon and Ortega-Rubio, 2009), Brazil (Silva and Roche, 2007), Uruguay (Mañé-Garzón and Carbonell, 1971), India (Riyas and Kumar, 2017), Italy (Schifani et al., 2018), Chile (Fraire-Pacheco et al., 2017; Fuentes et al., 2019), Turkey (Balik et al., 2001; Bekleyen et al., 2011), Israel (Gasith et al., 2011) and Greece (Karaouzas et al., 2015). From the African continent, it has been recorded with certainty only from South Africa (Rayner, 1988; Rayner and Appleton, 1989, 1992). The recent record of a *Craspedacusta* species in Lake Manzala (Delta

Nile), reported by Gasith et al. (2011: 147 and SM1), is based on a series of doubtful identifications (initially ascribed to the genus *Limnognathia*) and comments on a brief mass occurrence of medusae (see Elster et al., 1960; Elster and Vollenweider, 1961; Dumont and Verheye, 1984: 315; Dumont, 1994, 2009: 496 for additional information on that bloom event).

The life cycle of *Craspedacusta sowerbii* includes both polyp (assuming asexual reproduction) and free-swimming stages (involved in sexual reproduction) (Bekleyen et al., 2011; Gasith et al., 2011). The appearance of the active medusa stage is related to an increase in water temperature (Bekleyen et al., 2011). Occurrences of this pelagic stage are sporadic, lasting only a few weeks, usually in the late summer and autumn (Minchin et al., 2016). The polyp stage is often overlooked because of its small size, having a wide capacity to tolerate different temperature and light conditions (see Payne, 1924; Boulenger and Flower, 1928; Acker, 1976; Acker and Muscat, 1976). The polyp and medusa stages are rarely reported together (see Failla-Siquier et al., 2017). Duggan and Eastwood (2012) established a protocol to find polyp stages that would be usable even in water reservoirs where the medusa stage had not been previously observed. These authors reached the conclusion that *C. sowerbii* is more common and widespread than is apparent from observations of medusae. Estimating the timing of introduction of this species in a given region is therefore difficult if it is only carried out after jellyfish findings have been recorded.

A few individuals of a hydromedusa species were recently detected in a Moroccan reservoir. These specimens were initially identified (based on morphological characters) as the well-known alien widespread species *Craspedacusta sowerbii*. This record is the first confirmed finding of this species in North Africa. However, a molecular study of this material and its comparison to previously available information revealed a more complex scenario, with nomenclatural and biogeographic implications. As this hydromedusa species is often reported in lists of alien species, the correct specific identification of the different *Craspedacusta* lineages becomes an urgent challenge to correctly understand how many invasion events and species could be involved.

The present paper aims to stress the risks linked to the current trend of exponentially increasing numbers of morphology-based reports of invasive species. An integrative view, including both morphology and molecular information, should be applied as a rule for checking the current identity of these 'well known' species as there are several examples of cryptic species that are difficult or impossible to delimit due to overlapping morphological characters.

## Material and methods

### Sample collection

Bin El Ouidan reservoir is located in Azilal province (coordinates: 6° 27' 50" W; 32° 6' 24" N), at 810 m a.s.l.

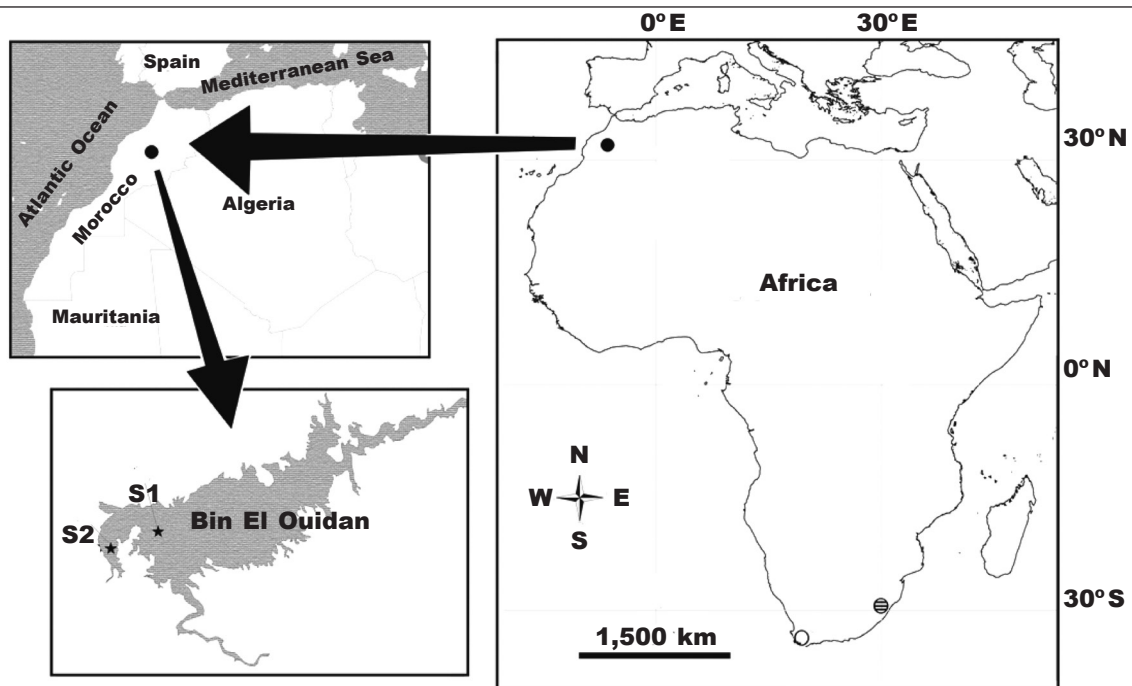


Fig. 1. Localisation of Bin El Ouidan reservoir in Morocco: S1, site 1; S2, site 2; open circle, Rayner and Appleton (1992); striped circle, Rayner (1988) and Rayner and Appleton (1989); solid circle, present record.

Fig. 1. Localización del embalse Bin El Ouidan en Marruecos: S1, punto 1; S2, punto 2; círculo blanco, Rayner and Appleton (1992); círculo rayado, Rayner (1988) y Rayner and Appleton (1989); círculo negro, registro presente.

(fig. 1). It covers 3,740 ha and has a maximum depth of nearly 100 m. The total volume reaches 1,384 million m<sup>3</sup>. The reservoir was built between 1949 and 1953.

In December 2015 we surveyed two sites (fig. 1), and found medusa stages attributable to the genus *Craspedacusta* at site S1 (6° 26' 13" W; 32° 05' 33" N). In total, four specimens were collected by scuba divers from the water-column between 1 and 5 m of depth. The water temperature, as indicated by the dive computer, was 20°C and the visibility was 3 m. Two specimens were fixed in absolute ethanol for the molecular study, while the other two were fixed in formalin 4% for morphological observations.

#### Nomenclatural remarks

Despite the precise nomenclatural comments by Fritz et al. (2007: 54) about the discovery, first descriptions of this jellyfish species (see also Allman, 1880; Lankester, 1880a, 1880b), and ICZN decision (see also Allen, 1910; Stiles, 1910), several subsequent authors still reported the species with the specific epithet 'sowerbyi'. In the original description, Lankester (1880a: 148) used the spelling *Craspedacusta sowerbii* in honour of Mr. Sowerby, understanding the genitive singular of the complete latinization of Sowerby to Sowerbius. The use of the form 'sowerbyi' must be considered an

erroneous spelling (Zarazaga, pers. comm.). In this case, Article 33.3.1 of the ICZN (1999) about the predominant use of erroneous spellings cannot be applied (indeed, according to Fritz et al. (2009) it is about 40% of all references). Thus, in order to avoid the use of 'sowerbyi', all references to the species of Lankester in this paper will be made with the original spelling.

#### Morphological observations and measurements

Observation and photography of different parts of the medusae were performed with a camera (ToupCam™) attached to light microscopy (Olympus CX41). A Panasonic Lumix FZ28 camera was used for macroscopic photography. Measurements of bell and gametogenic tissues and tentacle length were performed using ImageJ 1.46r software (NIH, Bethesda, MD, USA).

#### Phylogenetic analysis

Total genomic DNA was extracted from two EtOH-preserved specimens using the E.Z.N.A. DNA kit (OmegaBiotech) following the manufacturer's instructions. The *Cox1* and *16S* mitochondrial regions as well as the nuclear *ITS* region (*ITS1–5,8S–ITS2*) were sequenced as proposed by Fritz et al. (2009) and Karaouzaz et al. (2015) for comparative purposes.

The *Cox1* region was amplified using the primers dgLCO1490 5'-GGT CAA CAA ATC ATA AAG AYA TYG G-3' and dgHCO2198 5'-TAA ACT TCA GGG TGA CCA AAR AAY CA-3' (Meyer et al., 2005), the *16S* region was amplified using the primers *16S*. Cunningham.F.1mod 5'-ACG GAA TGA ACT CAA ATC ATG TAA G-3' and *16S*. Cunningham.R.2 5'-TCG ACT GTT TAC CAA AAA CAT A-3' (Bridge et al., 1995). An 800 bp partial sequence of the nuclear DNA was amplified using the forward primer 5'-CCCTTTGTACACACCGCCCGTCGCT-3' and the reverse primer 5'-CTTTGGGCTGCAGTCCCA-AGCAACCCGACTC-3' (Odorico and Miller, 1997). This last 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 (Odorico and Miller, 1997; Fritz et al., 2009). Each PCR used 1 U of MyTaq Red DNA Polymerase (Bioline), 10 µM of each primer, approximately 30 ng of genomic DNA, and was brought to a final volume of 25 µL with H<sub>2</sub>O. *Cox1* PCR was carried out using the following cycle profile: initial denaturation at 95°C for 1 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 42°C for 15 s, and extension at 72°C for 10 s, and a final extension at 72°C for 5 min. The *16S* and *ITS* PCRs used the same cycle profile, but 58°C and 60°C as annealing temperatures respectively. PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFisher Scientific) following the manufacturer's instructions. Purified products were electrophoresed on an ABI PRISM® 3730xl Genetic Analyzer and sequence traces were edited using Sequencher™ v4.0. The obtained sequences were compared with homologous sequences from EMBL-Bank of other Olinidiidae species. Available sequences of campanulariid *Obelia* species were used as out-group in the implemented phylogenetic analyses. The alignments of the different sets of sequences were carried out using MUSCLE, as implemented in MEGA6 (Tamura et al., 2013). After alignment, the best nucleotide substitution model was selected using Modeltest as implemented in MEGA6, according to the Akaike Information Criterion (AIC) and hierarchical likelihood ratio test (hLRT). The *16S* dataset (40 olinidiid + 1 campanulariid sequences) had 554 positions, with a total of 276 variable and 232 parsimony-informative sites. The *Cox1* dataset (64 olinidiid + 1 campanulariid sequences) had 680 positions, with a total of 270 variable and 232 parsimony-informative sites. The *ITS* dataset sequences (55 *Craspedacusta* + 1 campanulariid sequences) had 933 positions, with a total of 378 variable and 209 parsimony-informative sites. The phylogenetic reconstructions were obtained applying Maximum Likelihood (ML) and Bayesian inference methods. ML method was carried out in MEGA6, and based on the T92 + G model (*16S*), GTR + I (*Cox1*), and K2 + G model (*ITS*) (Kimura, 1980; Tamura, 1992; Nei and Kumar, 2000) using the NNI heuristic method (Nearest Neighbor Interchange) and 1000 bootstraps replications (Felsenstein 1985). The Bayesian Inference was carried out in MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), using the model GTR + G

(Iset nst = 6 rates = gamma), 10<sup>7</sup> generations and discarding 25% initial trees.

The material studied here has been deposited in the Museu de Ciències Naturals in Barcelona (MZB), the collection of the first author (JAO-UIZ) at the University Ibn Zohr of Agadir, Morocco, and the collection of the research team Biodiversidad y Ecología Acuática in the University of Seville, Spain (BECA).

## Results

### Systematics

Phylum Cnidaria  
 Class Hydrozoa  
 Subclass Trachylinae  
 Order Limnomedusae  
 Family Olinidiidae Haeckel, 1879  
 Genus *Craspedacusta* Lankester 1880

*Craspedacusta sowerbii* Lankester 1880

See Lewis et al. (2012) and Jankowski (2001) for a complete list of synonyms.

### Material examined

MZB 2018-0758 one specimen formalin fixed.  
 MZB 2018-0757 one specimen fixed in absolute ethanol. JAO-UIZ(H1) one specimen formalin fixed. BECA(H1) one specimen fixed in absolute ethanol. Moreover, total DNA extraction from the specimen in MZB 2018-0757 [in BECA as BECA(H2)] and from BECA(H1) are kept within the molecular DNA collection of BECA. All specimens with the same sampling data as above described in the section Material and methods.

### Morphological remarks

The medusa was the only stage recovered (fig. 2A). No polyps were found, and all specimens are female. The average bell/umbrella diameter is 20 ± 1 mm (19–21), flattened form. The mouth has four slightly folded lips overpassing the umbrella margin. Four gametogenic tissues pouch-like structures (7–11 mm length) are hanging from the radial canals. They are opaque in the basal fold-like part and translucent and voluminous in the apical part, giving a triangular shape (fig. 2B). The tentacles have no organs of adhesion and are connected to the marginal end of the umbrella on the ring canal (fig. 2E). Four long perradial tentacles (7–9 mm in length) emerging from the end of the four radial canals at the umbrella margin. About 60 medium tentacles (2.0–4.5 mm in length) arising from the pole of the bell were counted between the four long tentacles. Approximately 420 shorter tentacles (0.5–1.5 mm in length) extend around the bell edge. The three different sizes of tentacles are organized in a regular distribution along the umbrella edge. Along the tentacles, nematocysts are grouped in patches that



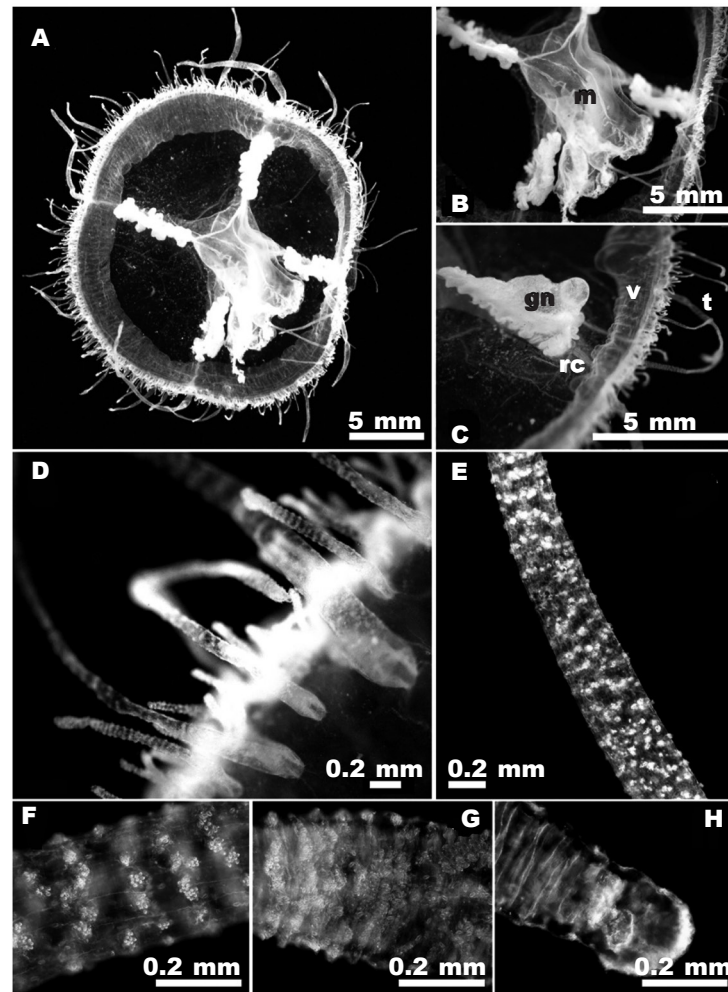


Fig. 2. *Craspedacusta sowerbii* Lankester, 1880: A, subumberllar surface. B, m, manubrium; C, gn, gametes; rc, radial canal; t, tentacles; v, velum. D, dispersion of tentacles. E, arrangement of nematocysts in per-radial tentacle. F, arrangement of nematocysts in the middle part of tentacle. G, arrangement of nematocysts in the apical part of tentacle. .

Fig. 2. *Craspedacusta sowerbii* Lankester, 1880: A, superficie subumberllar. B, m, manubrio. C, gn, gametos; rc, canal radial; t, tentáculos; v, velo. D, disposición de los tentáculos. E, ordenación de nematocistos en tentáculos perradiales. F, ordenación de nematocistos en parte media de un tentáculo. G, ordenación de nematocistos en el extremo apical del tentáculo. H, extremo apical del tentáculo.

are arranged in spaced parallel rings (fig. 2E, 2B), distances between consecutive rings are distinctly reduced distally (fig. 2G).

#### Phylogenetic analyses

*Cox1* analyses (fig. 3) placed the sequences obtained in this work for the two Moroccan specimens in a well-supported clade (Bootstarp [Bts.] 99, posterior probability [PP.] 0.99) with the German sequences and a Chinese (Sichuan province) sequence constituted, with an internal *p*-distances (German–Moroccan to Chinese sequence) of 0.3%. This last German–Moroccan–Chinese clade is the sister group of a relatively

poorly supported clade with two well defined groups, a Switzerland sequence and a well-supported clade (Bts. 100, PP. 1) including a conglomerate of Chilean–Italian–Indian–Grecian–Chinese sequences (all of which were also attributed to *C. sowerbii*), average uncorrected *p*-distance between these last two clades 17.5%. Average uncorrected *p*-distance between German–Moroccan–Chinese clade and Switzerland sequence 13.6%. Average uncorrected *p*-distance between German–Moroccan–Chinese clade and Chilean–Italian–Indian–Grecian–Chinese clade was 16.3%. Phylogenetic hypotheses based in *Cox1* suggest that there are at least three *Craspedacusta* species in Europe.

The 16S analyses (fig. 4) indicate that the analyzed Moroccan individuals merge well among other *Craspedacusta* sequences. There is very little previous 16S information on *Craspedacusta sowerbii*, just a sequence from Lake Huato (USA), another from Uruguay, two sequences from Switzerland, and a sequence of unknown locality (KY077294). Moroccan sequences form a well-supported clade (Bts. 99, PP. 1) with the sequence KY077294 and the two Switzerland sequences. Uncorrected *p*-distance between Morocco–unknown locality and Switzerland sequences is 0.2%. This last European clade (having in mind the unknown origin of one of the sequences) is the sister group of the clade formed by both American sequences (USA + Uruguay), which are identical. All these mentioned sequences, all identified as *C. sowerbii*, are related to another two *Craspedacusta* species (*C. sinensis* and *C. ziguiensis*) in a relatively well-supported clade (Bts. 82, PP. 0.95). The clade grouping all *Craspedacusta* species is the sister group of *Limnocyclus tanguiniae*, with high support (Bst. 90, PP. 0.99). Mean uncorrected *p*-distance between American populations attributed to *C. sowerbii* (USA and Uruguay) and the Moroccan–Switzerland–unknown–origin sequences is  $4.3\% \pm 0.1$  (range 4.2–4.5%). The genetic distance between the other two *Craspedacusta* species (*C. ziguiensis* and *C. sinensis*) is 6.2%, and the distance between the Moroccan specimens and the latter species is 6.8% and 8.3%, respectively. In general, all Olindiidae genera are well supported in this 16S phylogenetic hypothesis. Inter-genera genetic distances (uncorrected *p*-distances) in 16S seem to be around 13–30%. According to 16S' knowledge, a single species occurs in Morocco and Switzerland that is different from that present in America (USA + Uruguay).

The ITS phylogenetic analyses (fig. 5) benefits from a higher number of sequences; thus, the analyses is here focused on the genus *Craspedacusta* instead of the whole available olindiid taxa. *Craspedacusta* sequences are mainly obtained from central Europe and China, although recent sequences from Chile and Sicily (Italy) have been published. Three main clades can be detected, all of them including Chinese specimens. Clade I (Bts. 99, PP. 1), includes Chinese sequences attributed to *C. kiatingi* and *C. sichuanensis*, as well as all German sequences attributed to *C. sowerbii* and the sequences obtained in this study from Moroccan specimens. The sister group of Clade I is composed of a single sequence of *C. ziguiensis* from China [support between both sister groups (Bts. 99, PP. 1)]. On the other hand, Clade II (Bts. 99, PP. 1), includes Chinese sequences attributed to *C. sinensis* and *C. brevinema*, while Clade III (Bts. 90, PP. 0.56) includes Chinese sequences attributed to *C. sowerbii* and *C. xinyangensis*, as well as sequences from Italy and Chile.

Sequences from Clade I (where the Moroccan specimens are included) have genetic distances (uncorrected *p*-distances) between 0.0 and 0.9% (mean and SD  $0.1\% \pm 0.2$ ), while this Clade I is  $3.8\% \pm 0.1$  (range 3.6–4.2%) distant from its sister group (*C. ziguiensis*). Uncorrected *p*-distances between Clade I and Clade II are  $19.9\% \pm 0.4$  (range 18.9–20.6%),

while distances between Clade I and Clade III are  $11.3\% \pm 0.31$  (range 10.0–12.4%), finally, distances between Clade II and Clade III are  $17.7\% \pm 0.37$  (range 15.8–18.5%).

According to our current ITS knowledge, four main lineages (species) can be detected, two of them (Clades I and III) including specimens identified as *C. sowerbii*. At least two species are present in Europe, while the known American sequences (Chile) and those from Central Europe and North Africa are definitively different lineages (Clades I and III, respectively).

Unfortunately, there is no homogenous knowledge of the three genetic markers here examined along the entire distributional area where specimens attributed to *C. sowerbii* have been reported. Figure 6 shows the worldwide distribution of the main clades detected in the separate analyses of the three markers (see also fig. 3, 4, and 5 for comparison).

## Discussion

### Morphological remarks

*Caraspedacusta sowerbii* has been recorded in several localities around the world. However, many identifications are not fully reliable since the records do not give detailed morphological characters (Moreno–Leon and Ortega–Rubio, 2009; Jakovčev–Todorović et al., 2010; Stefani et al., 2010; Gasith et al., 2011; Souza and Ladeira, 2011; Galarce et al., 2013; Gomes–Pereira and Dionísio, 2013; Fraire–Pacheco et al., 2017). Moreover, many hydromedusae species have several similar morphological characters especially within the genus *Craspedacusta* (Jankowski, 2001), and only a few records gave more detailed descriptions of specific morphological characters (Kramp, 1950; Jankowski, 2001; Lewis et al., 2012). Indeed, up to eleven *Craspedacusta* species have been described, mostly recorded from China only (Jankowski, 2001). However, according to Bouillon et al. (2006) and Jankowski et al. (2008), many species may not be valid and are likely to be just morphological variations of the same species. Jankowski (2001) studied all the species recorded within *Craspedacusta* in detail and found that only three should be considered valid (*C. sowerbii*, *C. iseanum* Oka and Hara, 1922 and *C. sinensis* Gaw and Kung, 1939), and two are uncertain (*C. sichuanensis* He and Kou, 1984 and *C. ziguiensis* He and Xu, 1985); the rest seem to be synonyms of *C. sowerbii*, keeping in mind that two other species were synonymised [the marine species *C. vovasi* Naumov and Stepanjants, 1971 and the brackish water one *C. marginata* Modeer, 1791 (see Hummelinck, 1938)]. In the present paper, the morphological characteristics of our specimens coincide with the typical characters of the medusae belonging to the genus *Craspedacusta* (Russell, 1953; Bouillon and Boero, 2000; Bouillon et al., 2004, 2006). They have, apart from a well-developed marginal nematocysts ring, four simple radial canals from which pouch-like gametogenic tissues are hanging, and centripetal vesicles embedded in the

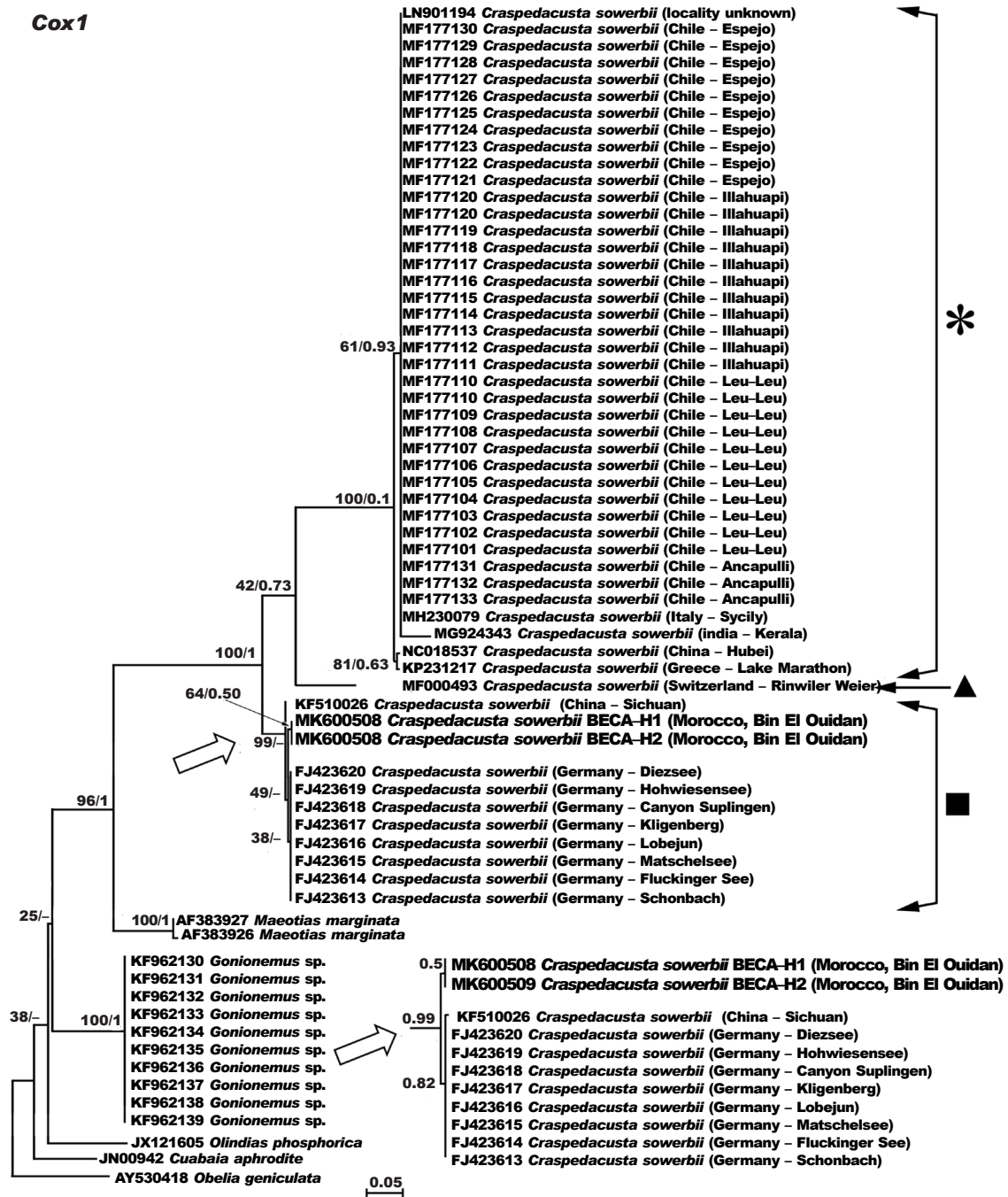


Fig. 3. Molecular analysis by the ML method. Relationship of olindiid species using *Obelia geniculata* as outgroup; the analysis is based on *Cox1*. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The white arrow indicates the clade where Moroccan sequences merge; note the slightly different arrangement of the sequences by Bayesian method (subtree at the bottom right of the figure). Symbols of groupings/clades correspond to the *Cox1* map in figure 6.

Fig. 3. Análisis molecular mediante el método de la máxima verosimilitud. Relación entre especies de olindidos utilizando *Obelia geniculata* como grupo externo; el análisis se basa en *Cox1*. El árbol está dibujado a escala y la longitud de las ramas indica el número de sustituciones por sitio. La flecha blanca indica el clado en el que se insertan las secuencias marroquíes; nótese que existe una ligera diferencia en la ordenación de las secuencias obtenidas mediante el método bayesiano (subárbol en la esquina inferior derecha de la imagen). Los símbolos de las agrupaciones o clados son los mismos que aparecen en el mapa *Cox1* de la figura 6.

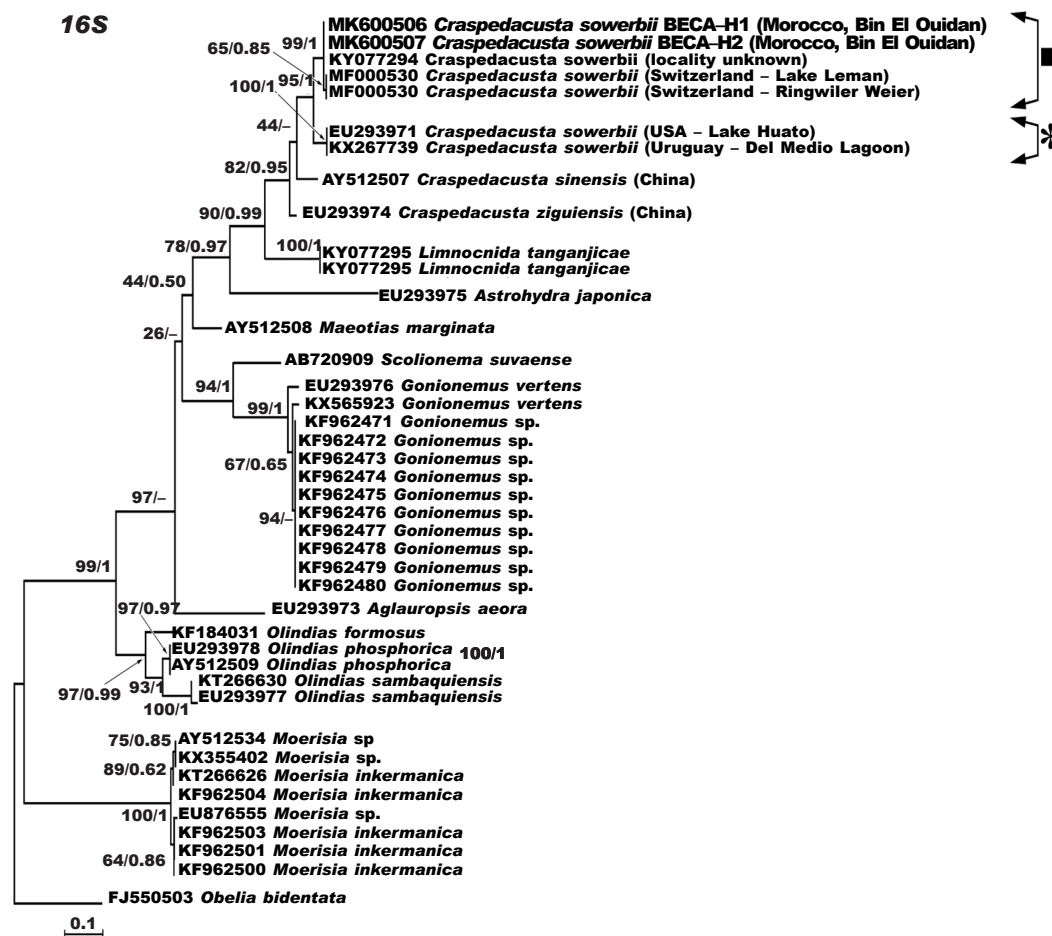


Fig. 4. Molecular analysis by the ML method. Relationship of olindiid species using *Obelia bidentata* as outgroup, the analysis is based on 16S. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Symbols of groupings/clades correspond to the 16S map in figure 6.

Fig. 4. Análisis molecular mediante el método de la máxima verosimilitud. Relación entre especies de olíndidos utilizando *Obelia bidentata* como grupo externo; el análisis se basa en 16S. El árbol está dibujado a escala y la longitud de las ramas indica el número de sustituciones por sitio. Los símbolos de las agrupaciones o clados son los mismos que aparecen en el mapa 16S de la figura 6.

velum as internal closed ecto-endodermal statocysts. Because these common similar characters within the *Craspedacusta* species lead to confusion and doubts when identifying a specimen and ascribing it to a determined species, the application of more specific characters is needed. The here-observed specimens identified as *C. sowerbii* have four prominent large per-radial tentacles, which are clearly shorter in *C. sinensis* (Kramp, 1950; Jankowski, 2001). This latter species, also found in China, is very similar to *C. sowerbii*. According to Kramp (1950), it differs from *C. sowerbii* also in having a markedly irregular distribution of the different tentacle sizes (which are evenly distributed in our medusae), as well as a characteristic nematocyst distribution on tentacles. In our specimens, transverse belts of clustered groups of two to 10 nematocysts cover the tentacles, while in *C. sinensis*, nematocysts

are located at the end of elongated cylindrical papillae that are not arranged in transverse rings on tentacles (Kramp, 1950; Jankowski, 2001). Moreover, in active swimming specimens, *C. sinensis* is easily recognizable by its remarkable changes in the umbrella diameter. This species actually varies from 0.48 cm at systole (contracted bell-shaped umbrella) to 1.8 cm during diastole (maximum dilated flattered umbrella) (Kramp, 1950). On the other hand, the observed extended tubular statocysts of different lengths and embedded in the velum of the here-studied specimens confirm our identification and discard the possibility of ascribing our medusae to *C. iseanum*. This species, found in Japan, is also very similar to *C. sowerbii*. According to Uchida (1955), the statocysts in *C. iseanum* are oval-shaped. Moreover, adult specimens in this species vary from five to 18 mm of umbrella diameter and have up to



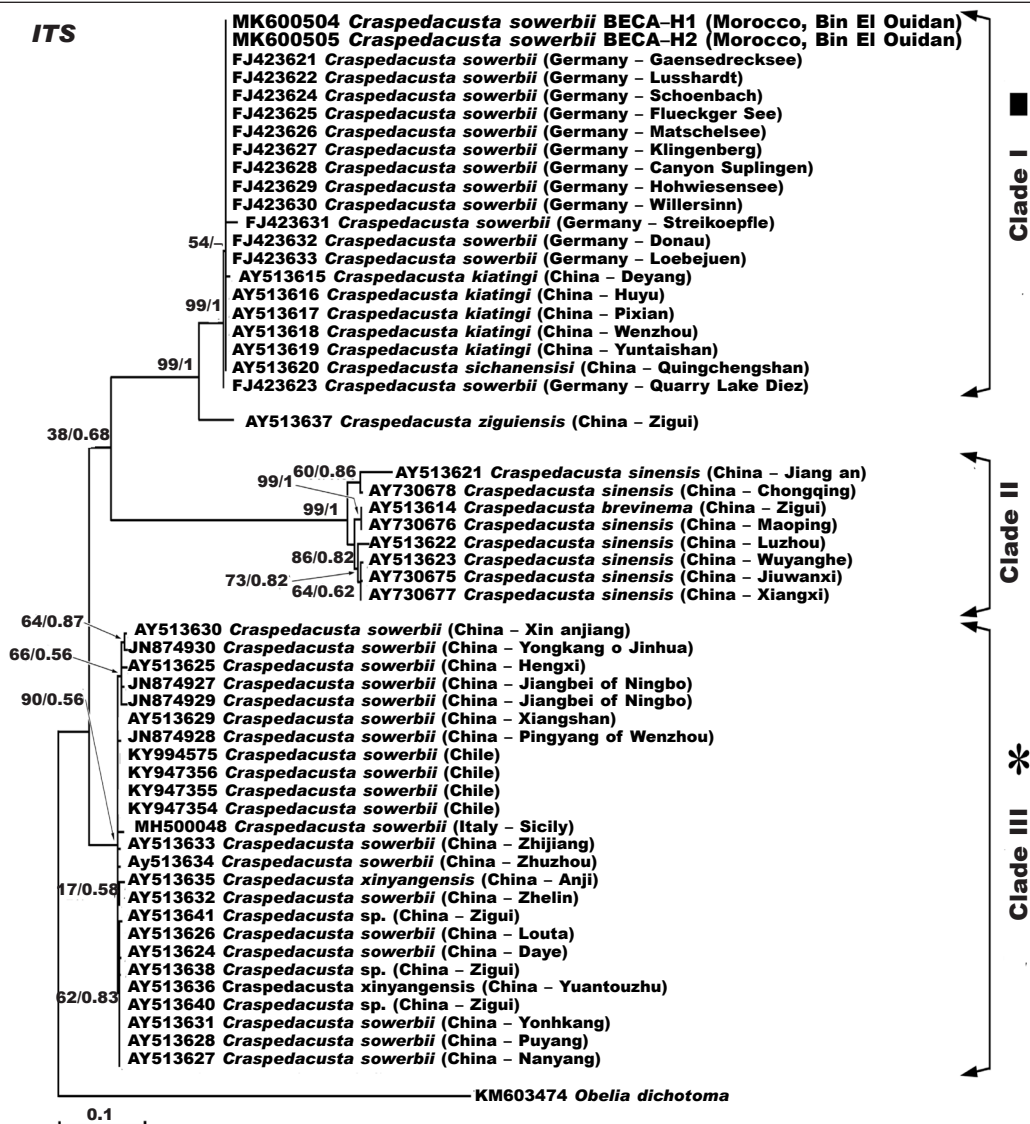


Fig. 5. Molecular analysis by the ML method. Relationship of *Craspedacusta* species using *Obelia dichotoma* as outgroup; the analysis is based on ITS. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Symbols of groupings/clades correspond to of the ITS map in figure 6.

Fig. 5. Análisis molecular mediante el método de la máxima verosimilitud. Relación entre especies de *Craspedacusta* utilizando *Obelia dichotoma* como grupo externo; el análisis se basa en ITS. El árbol está dibujado a escala y la longitud de las ramas indica el número de sustituciones por sitio. Los símbolos de las agrupaciones o clados son los mismos que aparecen en el mapa ITS de la figura 6.

128 tentacles (Lewis et al., 2012), while *C. sowerbii* adult specimens, like in the here-observed medusae, can reach up to 25 mm of umbrella diameter and have more than 400 tentacles (Russell, 1953; Jankowski, 2001). Concerning the nematocysts, *C. iseanum* have scattered, and not clustered, nematocysts on the tentacles. Nevertheless, all these specific characters may be trustworthy only when dealing with the identification of adult living specimens or at least adult well-preserved ones.

#### Phylogenetic analyses

Despite the abundant literature reporting the occurrence of *Craspedacusta sowerbii* around the world (see Dumont, 1994; Didžiulis and Žurek, 2013 for additional references), available molecular information is relatively scarce. Part of this information is published as a representation of the genus (or family) for general phylogenetic papers about different cnidarian taxa (e.g. Collins, 2002; Kayal et al., 2015; Grange

et al., 2017), and there are few specific papers on the phylogeny, molecular systematics, diversity and distribution of the genus *Craspedacusta* (Zou et al., 2012; Fritz et al., 2009; Zhang et al., 2009). A number of sequences can be obtained in databases such as GenBank, ca. 55 of them for the *ITS* region (for seven putative species), ca. 50 sequences are for the *Cox1* fragment (all of them attributed to *C. sowerbii*), and seven are for the *16S* (ascribed to three different species).

As previously commented, recent morphological (Jankowski 2001) or molecular (Fritz et al., 2009; Zhang et al., 2009) contributions drastically reduced the number of species to three (or maybe five). On one hand, for Jankowski (2001), only *C. sowerbii*, *C. iseanum*, and *C. sinensis* (and perhaps *C. sichuanensis* and *C. ziguiensis*) could be considered valid. On the other hand, the two simultaneous contributions by Fritz et al. (2009) and Zhang et al. (2009) pointed out the existence of different lineages (in the former) and species (in the later), thus stressing the lack of consensus on the real diversity and systematics of the genus.

Fritz et al. (2009) considered that the morphology of their German and Austrian samples agrees with *C. sowerbii*, and hence the Chinese *ITS* sequences (identical to their European material) attributed to *C. kiatingi* should be considered as *C. sowerbii* var. *kiatingi* (Gaw and Kung, 1939; Kramp, 1950). Fritz et al., (2009) identified three main clusters within their dataset: 'sinensis' [for *C. sinensis*, and *C. brevinema* (considered by these authors as a variety of the former)], 'sowerbyi' [sic, for Chinese sequences of *C. sowerbii* and *C. xinyangensis* (considered by these authors as a variety of the former)], and "kiatingi" (for the German and Austrian sequences of *C. sowerbii*, and the Chinese sequences of *C. sichuanensis* and *C. kiatingi* (considered by these authors as a variety of their European *C. sowerbii*)), remaining as doubtful the status of *C. ziguiensis*. In short, for Fritz et al. (2009) the "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*". Although the identification of *C. sinensis* and *C. ziguiensis* as different species seems to be clearly stated, the assignable different specific name to be used for the two other Clades ('kiatingi' and 'sowerbii') is not so clearly defined in this last paper.

Zhang et al. (2009) analysed eight putative *Craspedacusta* species using the nuclear marker *ITS*. Obviously, the trees obtained by these authors show similar conclusions, as both research groups shared a similar set of sequences: *C. xinyangensis* should be the synonym of *C. sowerbii*, *C. sichuanensis* the synonym of *C. kiatingi* and *C. brevinema* the synonym of *C. sinensis*, while the taxonomic status of *C. ziguiensis* is still uncertain. However, the main difference between the two contributions is the implications of those Austrian and German sequences, defining a clade *C. kiatingi*–*C. sowerbii*. The Chinese authors were probably unaware at that moment that a number of European sequences could be attributed to their *C. kiatingi*.

Recently, Schifani et al. (2018) and Fuentes et al. (2019) obtained sequences from specimens identified as *C. sowerbii* from Sicily (Italy) and Chile, respectively. In their *Cox1* analyses, Schifani et al. (2018) identified a Sicilian–Grecian–Chinese clade as *C. sowerbii*, a second German–Chinese clade as *C. kiatingi*, and a third clade of unknown origin as *Craspedacusta* sp. (actually, this last sequence was deposited by Dr. P. Schuchert from a polyp stage specimen collected in Ringwiler Weier (Canton Zurich, Switzerland) (see also features part of this sequence in GenBank Accession number MF000493). The *ITS* analyses of these authors also identify a Sicilian–Chinese *C. sowerbii* clade and a German–Chinese *C. kiatingi* clade (the origin of the sequence FJ423632 is indicated to be German Donau (Danube) in GenBank).

It is somehow ironic that the type locality of this considered-to-be invader alien species was a water-lily tank in the Botanical Garden in Regent's Park, London (Lankester, 1880a), while the origin of the species (and the diversity hot-spot of the genus *Craspedacusta*) is currently supposed to occur in the Yangtze River valley in China (e.g. Didžiulis and Žurek, 2013). Many changes have occurred since the late 19<sup>th</sup> century in the type locality, which finally disappeared in 1932 (C. Magdalena, pers. comm.). Anyway, the species was subsequently reported also from Southern England (Broom Water, Teddington), only a few kilometres away from the type locality (Green, 1998). Thus, it is plausible that future sequences obtained from a specimen collected in the London area could be considered a topotype (or a neotype could be established, since the original type material seems not to have been deposited in any institution), and could help to soundly and univocally define *C. sowerbii*. The nomenclatural consequences of the current absence of molecular data from the area of the type locality are in fact very important.

For the moment, according to the *ITS* phylogenetic hypothesis, mainland Europe and Morocco share the same haplotype, which is also shared with specimens identified as *C. kiatingi* and *C. sichuanensis* from China, the diversity hot-spot of this hydromedusa genus. Most Chinese specimens identified as *C. sowerbii* are in a well separated clade (see fig. 5). It is possible to speculate on two scenarios: 1) the South England sequences are identical (or similar) to those from mainland Europe and Morocco (Clade I); and 2) the South England sequences are different from those from mainland Europe and Morocco, but identical (or similar) to those identified as *C. sowerbii* by Zhang et al. (2009) and that from Sicily (Clade III). The direct consequence of the first scenario would be that *C. kiatingi* and *C. sichuanensis* sequences from Zhang et al. (2009) must instead be attributed to *C. sowerbii*, while another available name should be selected for those sequences identified as *C. sowerbii* by Zhang et al. (2009), the Clade 'sowerbyi' of Fritz et al. (2009), including the Sicilian and Chilean specimens (Clade III in this paper). Conversely, the consequences of the second scenario suggest that at least two species of *Craspedacusta* occur in Europe and North Africa, *C. sowerbii* in Southern England,

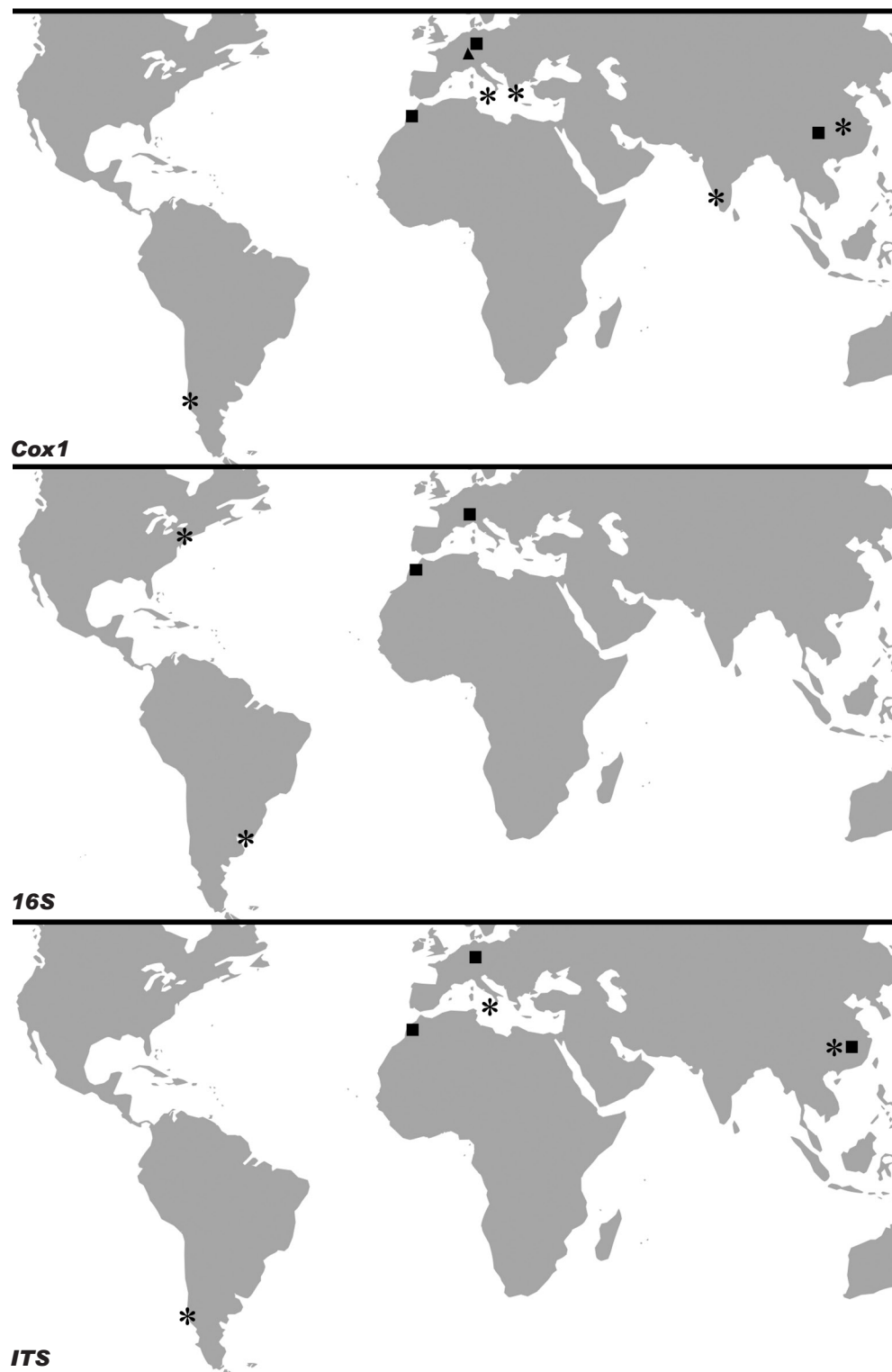


Fig. 6. Known worldwide distribution of the observed groupings (similar sequences, or clades) attributed to *Craspedacusta sowerbii* in the different phylogenetic analyses carried out in this paper (see comparatively fig. 3, 4, and 5).

Fig. 6. Distribución mundial conocida de las agrupaciones observadas (secuencias similares o clados) atribuidas a *Craspedacusta sowerbii* en los diferentes análisis filogenéticos llevados a cabo en este trabajo (compárense las fig. 3, 4 y 5).

and *C. kiatingi* [or another available name according to the ICZN (1999)] in the rest of this area, as well as in China. For the moment we have no *ITS* information from the specimens collected in Switzerland, Greece, or India.

The *Cox1* phylogenetic hypothesis clearly shows that Europe experienced the invasion of at least three *Craspedacusta* species, one of them in central Europe (Germany) and Morocco, the second one is currently present in Greece and Sicily, while the third one is found in Switzerland (see fig. 3 and 6). The two first invasions are connected (or simply share similar sequence) with their respective Chinese populations. This was already detected by Karaouzas et al. (2015), suggesting that the phylogeny of the genus is in need of further investigations, as genetic distances between the *C. sowerbii* clades are around 15%. In the available *Cox1* information for olindiid species, all genera except *Craspedacusta* are represented by a single species or haplotype, making difficult to discuss about the expected range of genetic distances at species level. In our phylogenetic hypothesis uncorrected *p*-distances between olindiid genera varies between 15 and 26%. As in the previous discussion, the name to be used for each *Craspedacusta* species will depend on the knowledge of a (still unknown) *Cox1* sequence from a putative Southern England population, possibly after the establishment of a neotype.

Information based on our *16S* phylogenetic hypothesis about *Craspedacusta* species delimitation is scarce, but it is well defined that a single species that can currently be identified in America (Lake Huato, USA and Uruguay) is different from the one present in North Africa (Morocco), Switzerland, and an unknown locality (sequence KY077294, see Grange et al., 2017). The same problem already discussed in assigning the name of *C. sowerbii* to one or another clade is present here. For this marker it is possible to discuss about the relative genetic distances (uncorrected *p*-distances) that are recognized between species of another olindiid genus, the genus *Olindias* (see Bouillon et al., 2004: 206, 2006: 435). Genetic distances between the three species of *Olindias*, from which *16S* sequences are available, vary from 5.5–5.7% (*O. phosphorica* to *O. sambaquiensis*) to 9.8–10.0% (*O. formosus* to *O. sambaquiensis*) (see also Collins et al., 2005, 2008). The genetic distance observed between North African–Switzerland and American sequences identified as *C. sowerbii* is 4.2–4.5%, between North African–Switzerland and *C. ziguiensis* and *C. sinensis* it is 7.8–8.9% and 6.8–7.4%, respectively; and between the latter two species it is 6.2%. This suggests that American specimens attributed to *C. sowerbii* should perhaps be considered a different species from the specimens analysed here from North Africa, as well as from those from Switzerland.

#### Final remarks

The described scenario could be much more complicated when considering that in the type locality of *C. sowerbii*, the aquatic plants of the water-lily tank in

Regent's Park (i.e. the potential dispersal vector of this hydromedusa species) were imported from Brazil, and not from China (C. Magdalena, pers. comm.). At present, it is difficult to know when the dispersion of *Craspedacusta* species from Easter Asia began, and most of the proposed vectors are in part speculative. Perhaps there was a combination of initial introduction by trade of aquatic plants and a subsequent natural dispersion by aquatic animals (e.g. birds, insects). For this reason, to solve this unstable nomenclatural and biodiversity problem, it is highly desirable to start with an important (or at least representative) field and molecular sampling programme in Southern England, in order to decide which haplotype (or set of related haplotypes) could be considered the true *Craspedacusta sowerbii*.

At this moment, for the present contribution, the most parsimonious solution would be the existence of a single species (clade) in England and the relatively close Central Europe (also shared by the Moroccan examined specimens). If this is correct, this clade should retain the specific epithet *sowerbii*, and then, as in the same group of sequences there are some attributed to *C. kiatingi* by Zhang et al. (2009), these sequences and individuals should also be assigned to *C. sowerbii*. According to these considerations, all materials included in the 'sowerbyi' clade of Fritz et al. (2009) and the clade that included all sequences attributed to *C. sowerbii* by Zhang et al. (2009) (including the Chilean and Sicilian sequences) should be assigned to a different species, which should be selected among the available names after a complete bibliographical and morphological review. It has also become clear that according to *Cox1*, at least three *Craspedacusta* species are present in Europe (see fig. 6, *Cox1*): one in central Europe (and Morocco), one in Greece and Sicily (for the moment *ITS* sequences from the Greek specimens are not available), and one in Switzerland (for the moment *ITS* information is not available). Unfortunately, no geographical information is currently available for a *16S* sequence (KY077294, see Grange et al., 2017) and a *Cox1* sequence (LN901194, see Kayal et al., 2015). In the future, knowing the origin of these and other additional sequences would provide important information on invasion events concerning this intriguing hydromedusa species.

The possible introduction vectors of *Craspedacusta sowerbii* in the recorded new sites generally remain unidentified. Accordingly, several hypotheses about the possible introduction paths have been mentioned in different reports and works on this species (Dumont, 1994; Angradi, 1998; Karaouzas et al., 2015). They can mainly be resumed in two possible vectors: 1) vectors facilitated by human activities, and 2) natural vectors. With regard to the former, the most likely dispersal vector may be the transfer of the species polyp stage, or the result of a resistance structure in aquaria or exhibition tanks, or an association with commercial ornamental aquatic plants or animals (Oscoz et al., 2010; Gasith et al., 2011; Gomes-Pereira and Dionisio, 2013; Minchin et al., 2016). The minuscule and hardly recognizable resting forms of the species make its unintentional human-mediated



dispersal likely. Some authors reported observations of *C. sowerbii* (medusa or polyp stage) coinciding with the introduction of stocked fish or aquatic plants (Parent, 1982). As for the second possible vector, desiccated podocysts attached to body parts of aquatic invertebrates and vertebrates (including birds) could have allowed this species to colonize near freshwater reservoirs (Jankowski, 2001; Jankowski et al., 2008). The cysts are able to survive for about 40 years while being completely desiccated (Bouillon and Boero, 2000; Bouillon et al., 2006; Lewis et al., 2012). These resting bodies may accidentally be transferred to new sites on bird's feet or plumage. Then, in favorable conditions, cysts turn into medusae and podocysts become polyps that can lead to more budding. This makes the aerial passive dispersal a possible introduction path for *C. sowerbii* (Parent 1982; Dumont 1994; Oscoz et al., 2010; Didžiulis and Žurek 2013; Failla–Siquier et al., 2017).

The characteristic drought-resistant forms of the species suggest that the natural aerial dispersal vector by migrating birds (see Reynolds et al., 2015; Green, 2016) may be an important factor in the introduction of alien species such as *C. sowerbii* into Bin El Ouidan, although in this reservoir, in order to limit the proliferation of algae as well as to enhance the biodiversity within the reservoir ecosystem, many exotic species including fish and aquatic plants (e.g. *Oncorhynchus mykiss* Walbaum, 1792; *Barbus barbatus* Linnaeus, 1758; *Micropterus dolomieu* Lacepède, 1802; *Sander lucioperca* Linnaeus, 1758; *Hypophthalmichthys molitrix* Valenciennes, 1844; *Ctenopharyngodon idella* Valenciennes, 1844 and *Cyprinus carpio* Linnaeus, 1758) started to be introduced in the reservoir one year after its construction (Rabii Souilem, pers. comm.).

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