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Research Article

Did the Indo-Pacific leptomedusa *Lovenella assimilis* (Browne, 1905) or *Eucheilota menoni* Kramp, 1959 invade northern European marine waters? Morphological and genetic approaches

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Abstract

Hydromedusae, morphologically resembling the Indo-Pacific leptomedusa *Lovenella assimilis* (Browne, 1905) (Cnidaria: Hydrozoa: Lovenellidae), are reported for the first time in both the eastern English Channel and the southern bight of the North Sea. Analyses of past zooplankton samples from a long-term monitoring program suggest that this non-indigenous species has been present in the eastern English Channel at least since 2007. Genetic analyses identified specimens as *Eucheilota menoni* based on nearly identical 18S ribosomal RNA gene, mitochondrial cytochrome oxydase subunit gene I (COI) sequences, and 16S Ribosomal RNA gene. Consequently, published morphological descriptions of *L. assimilis* and *E. menoni* were compared, and their species status is discussed with regard to morphological and genetic evidence. In conclusion we suggest synonymizing these 2 indistinguishable species.

Key words: non-indigenous species (NIS), jellyfish, Hydrozoa, *Lovenella assimilis, Eucheilota menoni*

Introduction

The Leptomedusa *Lovenella assimilis* (Browne, 1905) (Cnidaria: Hydrozoa: Lovenellidae) has been reported from tropical to temperate regions in Indo-Pacific waters (Kramp, 1968, cf. in Pires-Miranda et al. 2013; Figure 2) such as Sri-Lanka (Browne 1905; original description), the Bay of Bengal (Navas-Pereira and Vannucci 1991), China and Philippines (Chow and Huang 1958; Kramp 1961; Xu et al. 2008), Papua New Guinea (Bouillon 1984), New Zealand (Bouillon 1995), Japan (Hirano and Yamada 1985) and Red Sea (Schmidt 1973). *Lovenella assimilis* was never been reported outside the Indo-Pacific Oceans until a first citation in Europe, in the Bay of Biscay (Altuna 2010).

In this paper, we report for the first time the occurrence of medusae morphologically closely resembling *Lovenella assimilis* in the eastern English Channel and southern bight of the North Sea. Morphological and genetic analyses allowed to discuss: (i) the taxonomic, diagnostic characteristics; (ii) the validity of the taxonomic status from another lovenellid hydromedusa, *Eucheilota menoni* Kramp, 1959, which was already reported as an introduced species in European waters (Altuna 2009); and (iii) the correctness of available DNA sequences of both species in the online repositories.

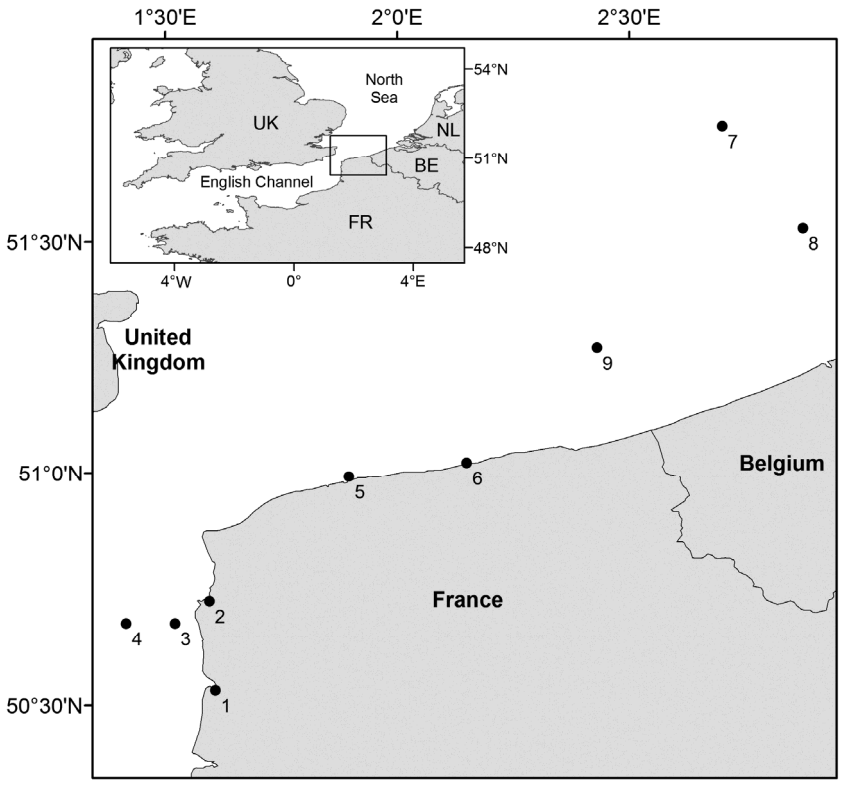

Figure 1. Sampling sites in the eastern English Channel and southern bight of the North Sea (See Table S1 for details).

Methods

Sampling and morphological identification

Sampling sites were located along the French coast of the Eastern English Channel and the Belgian coast (North Sea; Figure 1, supplementary Table S1). A WP2 net (aperture area: 0.25m² and 200-µm mesh size) and/or a WP3 net (aperture area 1m² and 1-mm mesh size) (Fraser 1968) were used depending on the location. Temperature (°C) and salinity were measured with CTD probes. Collected samples (Table S1) were preserved in buffered formalin (4% final concentration) for morphological analyses or in 90–100% ethanol for genetic analyses. In the laboratory, preserved hydromedusa specimens were morphologically identified according to Brown (1905), Chow and Huang (1958), Kramp (1959; 1961), Bouillon (1984), Hirano and Yamada (1985), and Bouillon et al. (1988). Samples collected since 2000 (stored formalin) from the French long-term monitoring Service d'Observation en Milieu LITtoral (SOMLIT, http://somlit.epoc.u-bordeaux1.fr/fr/) program were screened for the presence of *L. assimilis*.

Genetic analysis

The chromosomal 18S ribosomal RNA gene and the internal transcribed spacer 1–2 (ITS1-ITS2) sequence, the mitochondrial 16S ribosomal RNA gene and the mitochondrial cytochrome c oxidase subunit I (COI) gene sequences were used for DNA barcoding identification. Each specimen was briefly homogenized in 20 µL of nuclease-free water and the resulting suspension served as a template for the polymerase chain reactions (PCR). Each PCR reaction contained one template, KOD Xtreme hot-start DNA polymerase (Novagen-Merck, Darmstadt, Germany), and either set of universal primers for COI gene (Folmer et al. 1994), 16S rRNA gene (Cunningham et al. 1993), 18S rRNA gene (Leclère et al. 2009), or ITS sequence (Gardes and Bruns 1993). PCR products were purified with the Wizard SV gel and PCR Clean-Up system (Promega, Fitchburg, WI, USA), and subsequently cloned with the NEB PCR cloning kit (New England Biolabs, Ipswich, MA, USA). Insert-containing plasmids were extracted with illustra plasmidPrep mini spin kit (GE Life Sciences, Piscataway, NJ, USA) and analyzed by Sanger sequencing at Genoscreen (Lille, France).

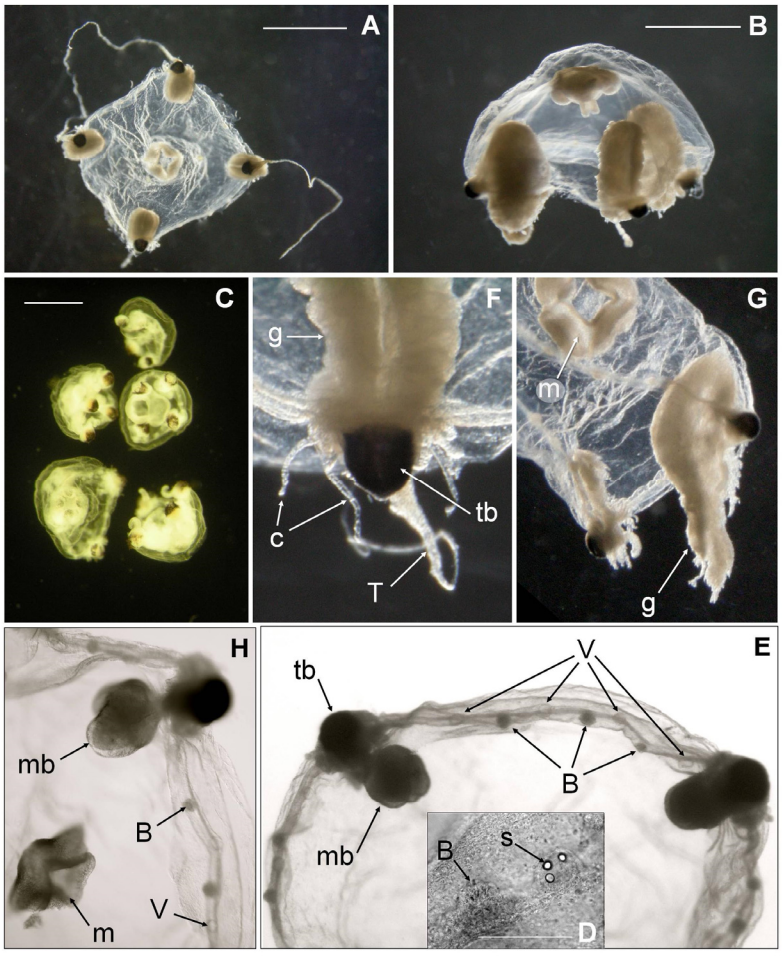

Figure 2. *Lovenella assimilis* (Browne, 1905), morphological specifications. Female, oral view (A); Female, lateral view, (B); Young specimens (C); Marginal bulb, and statocyst with 3 statoliths (D); Sequence of marginal bulbs and statocysts in an inter-radial quadrant (E); Longitudinally divided female gonad, tentacular bulb with black spot, and lateral clusters of tentacular cirri (F); Male gonad exceeding the edge of the umbrella (G); Medusal buds on the place of gonad (H).
g, gonad; m, manubrium; mb, medusal bud; B, marginal bulb; s, statolith; T, radial tentacle; tb, radial tentacular bulb; c, tentacular cirri; V, marginal vesicle.
Scale bar: A, B, C: 1 mm; H: 100 µm.
Photomicrographs by Jean-Michel Brylinski.

Obtained sequences were submitted to GenBank and accession numbers were indicated in Table S2.

The Basic Local Alignment Search Tool (BLAST) was applied for approximate taxonomic affiliation of 18S rRNA, 16S rRNA, ITS1-ITS2, and COI sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In particular, Megablast was used to search the non-redundant nucleotide collection database of GenBank, wherein all search parameters are set to default values. Multiple alignments were performed using the program Clustal Omega (Sievers et al. 2011). Prior to phylogenetic analysis, all sequences were trimmed to equal length using the Bioedit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). In order to construct phylogenetic trees and show taxonomic affiliations between our samples and closely related species, different analyses (neighbor joining, minimum evolution, and maximum likelihood) were performed using the MEGA 6 software (Tamura et al. 2004, 2013). All three methods provided very similar tree topologies (data not shown). The tree presented in this paper was based on neighbor-joining analysis using the p-distance model. Bootstrapping under parsimony criteria was performed with 1,000 replicates.

Results

Morphological description of examined material (Figure 2)

Based on pigment distribution, gonad shape, number of marginal bulbs, occurrence of cirri, number of statocysts and their concretions, and the cnidome, all studied specimens were identified as *Lovenella assimilis*.

Description: Umbrella fairly thick, 2.5 mm wide (1 to 3 mm for fixed specimens, Figure 2A to 2C), a little broader than high. Velum narrow. Stomach short, fleshy, with a quadrangular base.

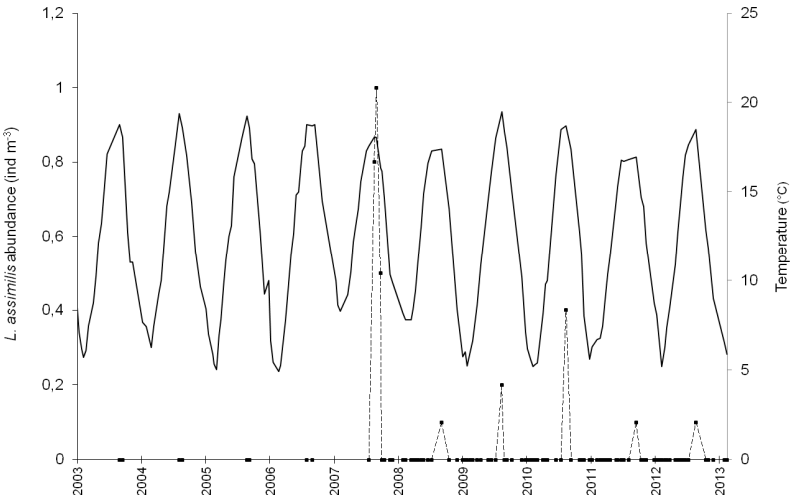

Figure 3. Temperature of seawater (Full line; °C; 5m depth) and abundance of *Lovenella assimilis* (Dotted line; ind.m-3) at the coastal SOMLIT station (Figure 1, nr 3).

Mouth generally wide open and quadrangular in outline, with four lips. Manubrium basis slightly pigmented with black granules at the inter-radial faces. 4 radial canals. 4 perradial tentacles with large basal and black pigmented bulbs. Upper part of these bulbs sometimes green-fluorescent in living specimens. Each bulb flanked by 2 clusters of lateral cirri. Each cluster with up to 6 cirri. 12 marginal bulbs (4 interradial and 8 adradial bulbs) without cirri. 16 marginal vesicles (statocysts), most of them with 3 concretions (1–3 statoliths, Figure 2D, Table S2). Hence, in each quadrant, between 2 tentacles (T), there are 3 marginal bulbs (B) alternating with 5 marginal vesicles (V) (Figure 2E). The formula TVBVBVBVT, with homogeneity between the four quadrants, can be considered as a standard formula. Irregularities in this formula with additional bulb and/or missing vesicle are also possible, resulting in differences between the quadrants (Table S2).

Gonads form large oval sacs along the outer half of the radial canals. Each gonad more or less divided into two by a median longitudinal line (Figure 2F). Gonads poorly developed in the small specimens (< 1 mm) but well developed in the large specimen (> 2.5 mm) and female gonads distinguished by spherical gametes (50 µm in diameter). Male gonads were slightly narrower than those of females and contained indistinguishable gametes. At maximum development, gonads protruded from the umbrella (e.g., Figure 2B and 2G). This phenomenon had never been reported in the literature and did not seem to only correspond to a preservation artifact because the external extension was as long as the fixed part of the gonad attached to the radial canal. Medusa buds located occasionally at the place of the gonads for asexual reproduction (Figure 2H) (Chow and Huang 1958; Bouillon 1984).

The cnidome was investigated based on the shape, size and location of the cnidocysts; It was very similar to the one described for *L. assimilis* by Hirano and Yamada (1985): Atrichous isorhizas (2 sizes) and merotrichous (2 sizes), and basitrichous (2 sizes).

DNA barcoding analysis

Six specimens were subjected to DNA barcoding analyses: specimen GENE-B was only analyzed for the mitochondrial 16S ribosomal RNA gene sequence; specimen GENE-0 was analyzed for the mitochondrial cytochrome c oxidase subunit I (COI) gene and the chromosomal 18S ribosomal RNA gene sequences; specimens GENE-1, GENE-2, GENE-4, and GENE-5 were analyzed for both mitochondrial 16S ribosomal RNA gene and COI gene sequences, and both chromosomal 18S ribosomal RNA gene and the internal transcribed spacers 1 & 2 (ITS1 & 2) sequences (Table S2). A total of 19 sequences were obtained and submitted to GenBank (sequence accessions were listed in Table S2). All sequences were BLAST searched for closely related species and phylogenetic analyzed for taxonomic affiliations as described in the Methods section. A brief summary of results can be found in Table S2. The analysis detail for each set of DNA barcoding is described below.

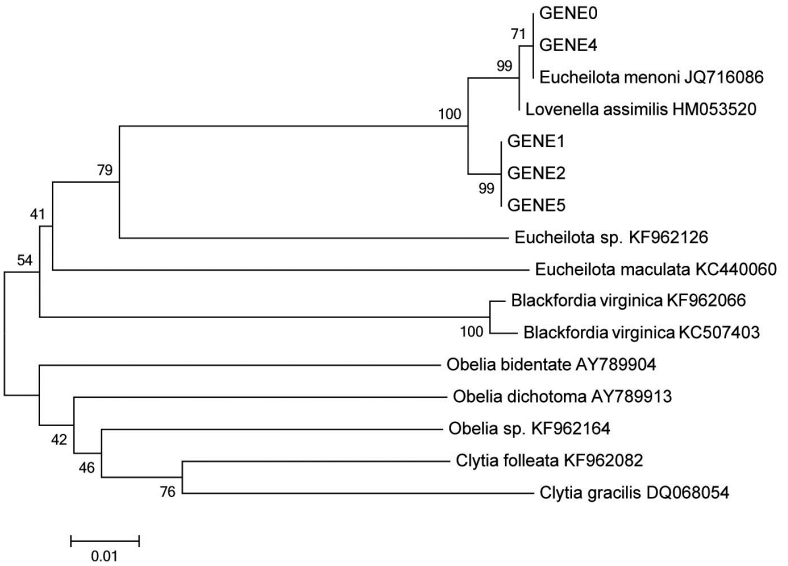

Figure 4. Phylogenetic tree based on COI sequences of our samples and closely related species. The phylogenetic relativeness between sequences was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.56313759 is shown. Bootstrap values were estimated from 1,000 replicates (Felsenstein 1985). The evolutionary distances were computed using the p-distance method (Nei and Kumar 2000). The phylogenetic tree was conducted in MEGA6 (Tamura et al. 2013).

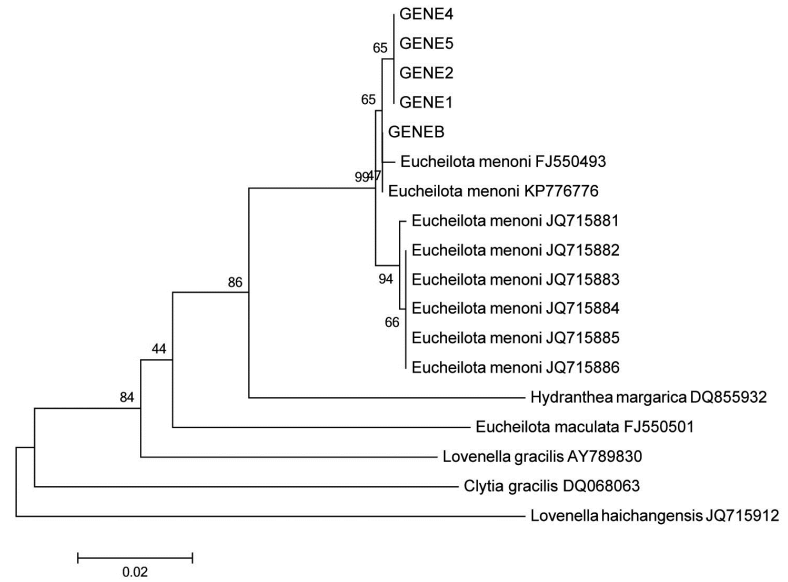

Figure 5. Phylogenetic tree based on 16S rRNA gene sequences of our samples and closely related species. The phylogenetic relativeness between sequences was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.38889628 is shown. Bootstrap values were estimated from 1,000 replicates. The evolutionary distances were computed using the p-distance method. The phylogenetic tree was conducted in MEGA6.

Mitochondrial cytochrome c oxidase subunit I (COI) gene. Specimens GENE-0, GENE-1, GENE-2, GENE-4, and GENE-5 could be separated into 2 groups according to their COI sequences: GENE-0 and GENE-4 are identical (haplotype 1), while GENE-1, GENE-2, and GENE-5 are also identical (haplotype 2). COI sequences of haplotype 1 specimens are 100% identical to the COI of another Indo-Pacific Hydrozoan, *Eucheilota menoni* (JQ716086), and only 1 bp different from the COI of *L. assimilis* (HM053520). COI sequences of haplotype 2 specimens have 7 bp differences from the COI of *E. menoni*, and have 6 bp differences from the COI of *L. assimilis*. However, the COI gene difference between *E. menoni* (JQ716086) and *L. assimilis* (HM053520) is just 1 bp. In other words, there are more differences in the COI gene between our haplotypes 1 and 2 than between *L. assimilis* and *E. menoni*. Based on the sequence alignment of our samples and closely related species, a neighbor joining tree was constructed (Figure 4). According to this tree,

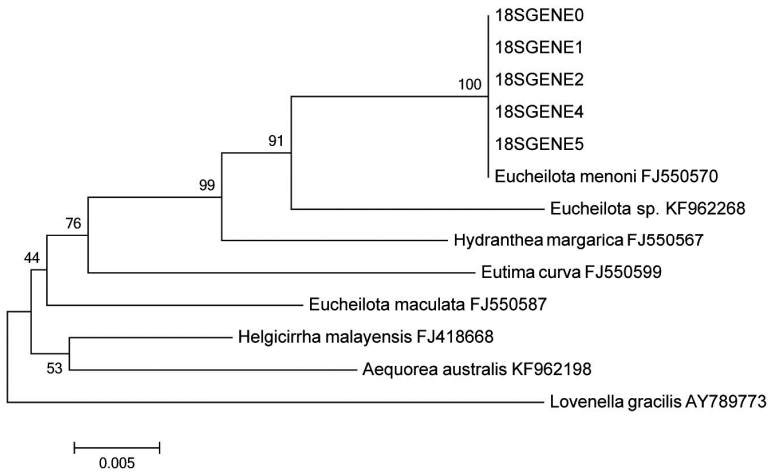

Figure 6. Phylogenetic tree based on 18S rRNA gene sequences of our samples and closely related species. The phylogenetic relativeness between sequences was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.15429562 is shown. Bootstrap values were estimated from 1,000 replicates. The evolutionary distances were computed using the p-distance method. The phylogenetic tree was conducted in MEGA6.

GENE-0 and GENE-4 (haplotype 1) are indeed more closely related to *E. menoni* and *L. assimilis* compared to haplotype 2 (GENE-1, GENE-2 and GENE5). Nevertheless, it is difficult to morphologically differentiate the two types (Table S2): GENE-1 and GENE-2 (haplotype 2) have 3 concretions in the vesicles, but GENE-5 (haplotype 2) and GENE-4 (haplotype 1) mostly have 2 concretions.

Mitochondrial 16S ribosomal RNA gene. 16S rRNA gene sequences of specimens GENE-1, GENE-2, GENE-4, and GENE-5 are identical, but differ in 2 bp from the sequence of specimen GENE-B. The result of BLAST search indicates that they are most closely related to *E. menoni* (FJ550493 and KP776776) with 2 bp differences. Based on sequence alignment of our samples and closely related species, a neighbor joining tree was constructed (Figure 5). Although recent studies recommended to use 16S ribosomal RNA gene as the preferential barcoding for Hydrozoa (Zheng et al. 2014; Lindsay et al. 2015), it is not as helpful in this study due to the lack of reference 16S ribosomal RNA sequence of *L. assimilis* in GenBank.

Chromosomal 18S ribosomal RNA gene. Chromosomal 18S rRNA sequences of specimens GENE-0, GENE-1, GENE-2, GENE-4, and GENE-5 are all identical to the sequence of *E. menoni* (FJ550570). Based on sequence alignment of our samples and closely related species, a neighbor joining tree was constructed (Figure 6). Similar to the 16S ribosomal RNA gene barcode, no 18S rRNA sequence of *L. assimilis* is available in GenBank.

Chromosomal internal transcribed spacer 1–2 (ITS1-ITS2) sequence. Beside the COI sequence of *L. assimilis* (HM053520), the only other *L. assimilis* sequence available in GenBank is HM053534 which contains information on the whole ribosomal region including the sequence for partial 18S rRNA gene, complete internal transcribed spacer (ITS1), 5.8S rRNA gene, internal transcribed spacer (ITS2), and partial 28S rRNA gene (usually shortened as ITS1-ITS2). Therefore, we included ITS1-ITS2 barcode in our analysis. The ITS1-ITS2 barcode of specimens GENE-1 and GENE-2 are identical while GENE-4 has 4 bp differences with GENE-5 and 4 bp differences with GENE-1 and 2. The ITS1-ITS2 barcode of GENE-5 also has 4 bp differences with GENE-1 and 2. According to the BLAST search result, sequences of closely related species are almost all indicated as "uncultured eukaryote", and originated from an unpublished study of Yu et al. (2014), which states that communities of planktonic eukaryotes can be influenced by the complicated condition in the north of South China Sea. The most closely related sequence with a species affiliation is "*Aequorea conica* Browne, 1905 (HM053544)", which only shared 92% identities with our sequence. A neighbor joining tree based on sequence alignment of our samples and closely related species is shown in Figure 7A. Unexpectedly, according to BLAST results, most closely related species affiliated with the *L. assimilis* ITS sequence (HM053534) were *Bolinopsis* sp. (94% identities) and *Mnemiopsis leidyi* A. Agassiz, 1865 (94% identities), both belonging to the Phylum of Ctenophora (Figure 7B). However, since no

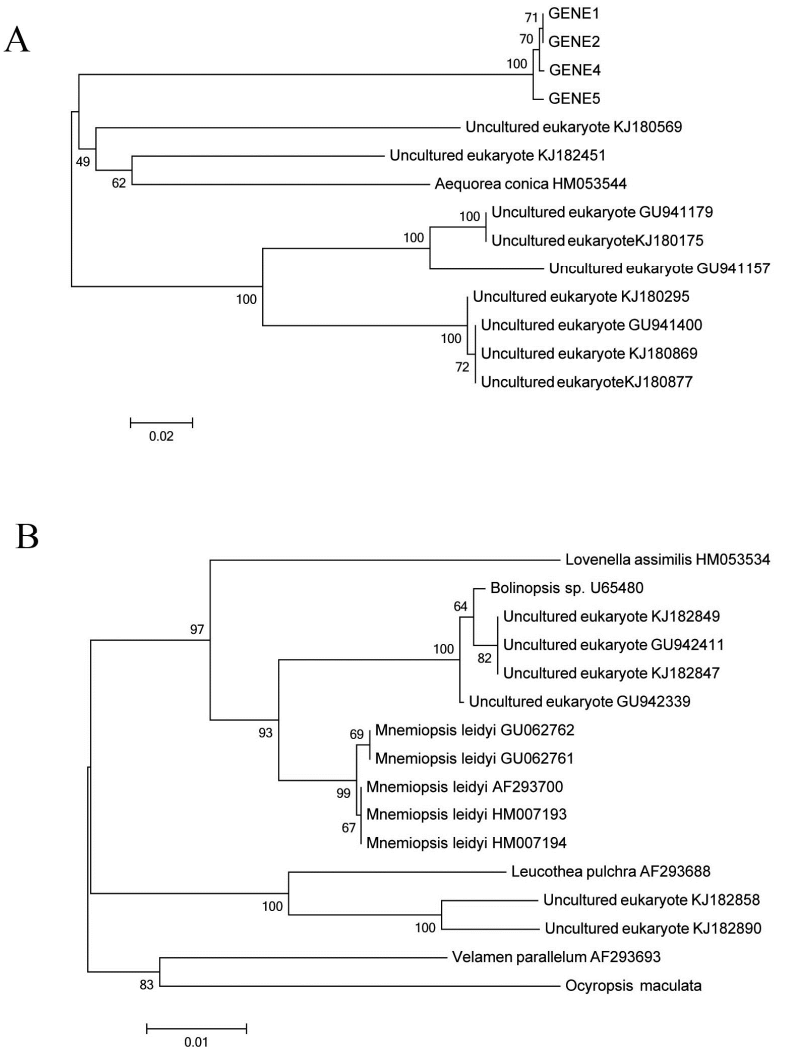

Figure 7. Phylogenetic tree based on ITS1-ITS2 sequences. (A) A neighbor joining tree of our samples and closely related species. (B) A neighbor joining tree of *Lovenella assimilis* (Accession HM053534) and closely related species. The phylogenetic relativeness between sequences was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.70846932 (for tree A) and 0.23975817 (for tree B) are shown. Bootstrap values were estimated from 1,000 replicates. The evolutionary distances were computed using the p-distance method. Both phylogenetic trees were conducted in MEGA6.

publication was associated with ITS sequence HM053534, it is unclear how this sequence was affiliated to *L. assimilis*.

Distribution in eastern English Channel and southern bight of the North Sea (Table SI and Figure 1)

The first record of this medusa type *L. assimilis* (based on morphological identification) was traced back to the end of summer 2007 in the eastern English Channel (coastal SOMLIT station; Figure 3). Since then, the species was repeatedly observed at this location around the end of summer (from August to the beginning of October) when seawater temperature reached its maximum annual value (Figure 3). At this location, the salinity was always higher than 34. The species appeared at the SOMLIT offshore station two years later (Table SI, nr 4), and since 2008 it was recorded at the long-term Gravelines station in August and September (Table SI, nr 6). The first observation in Belgian waters was in 2009 (Table SI, nr 9 and 7). However, it might have been present earlier, but the absence of regular zooplankton monitoring in Belgian waters hampers drawing this conclusion. The species occurred in low densities (< 1.5 ind.m^{-3}). In Gravelines (August 2008), as well as at the Belgian stations (October and November 2011), we observed some individuals with medusal buds instead of gonads (Figure 2H).

Discussion

Morphological descriptions of this species varied notably between authors (see for instance Browne 1905; Chow and Huang 1958; Kramp 1959; Hirano and Yamada 1985) (Table S3), nevertheless we identified our specimens as *Lovenella assimilis*. However, as the genetic identification did not fully confirm this, we refer to it as ?*Lovenella assimilis* (cf. in Kramp, 1959, p.246) and discuss this inconsistency.

The collected hydromedusae specimens represent the first record of the Indo-Pacific species ?*L. assimilis* along the Belgian and French coasts of the eastern English Channel and the southern bight of the North Sea. The hydromedusae of this region are perhaps the most intensely studied of the world, mainly due to the extensive studies of Russell (1953), who is unlikely to have overlooked this medusa. The specimens' morphology is consistent with the descriptions of Kramp (1961; 1968) and Hirano and Yamada (1985). The original description of Browne (1905) (he provisionally placed the specimen in the genus *Mitrocomium*) must cautiously be interpreted, as it was based on a single male specimen. Browne (1905), using a specimen from Type locality: Cheval Parr Bank, Sri Lanka, described about 5 marginal vesicles in each inter-radial quadrant and 5 to 7 marginal bulbs, "the central, interradial, bulb being much larger than others", as in Altuna (2009 - Figure 2). Kramp (1961; 1968) adjusted the description by limiting the number of bulbs to approximately 5, and noted "the median one the largest", using Browne's figure. In our specimens, the interradial bulb is barely larger than the others (adradial). Finally, Chow and Huang (1958) did not observe different sizes between the marginal bulbs. Hirano and Yamada (1985) observed 3 to 5 marginal bulbs and 2 to 6 vesicles in each inter-radial quadrant. Their drawing (Figure 1 p. 132) corresponds to our standard formula TVBVBVBVT. Chow and Huang (1958) observed 4–7 marginal bulbs in each quadrant and 4–6 marginal vesicles, with 2–3 statoliths. The differences between the descriptions of the cirri of the perradial bulbs might be primarily due to natural variability and/or age of the specimens. However, the difficulty in distinguishing the different elements of the complex may also explain these differences and result from the contraction of the cirri in fixed specimens. Additionally, the morphology of gonads, with longitudinal midline separation (Browne 1905), may potentially represent an important diagnostic characteristic of this species. Chow and Huang (1958) noted "four large gonads in a spherical shape" but did not cite nor illustrate this division. Browne (1905) reported male gonads to be "very large for the size of the medusa". In fact, our biggest specimen (2.5 mm, Figure 2B and 2G) showed a projection of the gonads beyond the umbrella margin.

Lovenella assimilis is morphologically similar to *Eucheilota menoni* (Lovenellidae), another Indo-Pacific medusa of the same size, and which was already reported in northern Spain (Altuna 2009). Nevertheless, *L. assimilis* differs from *E. menoni* by a higher number of statocysts (16 instead 8) each with more concretions (2-3 instead of 1) (Kramp 1968; Bouillon 1984). Moreover, *L. assimilis* possesses divided gonads (Browne 1905), which was not found or at least not recorded for *E. menoni*.

Schmidt (1973) found one *E. menoni* and two *L. assimilis* specimens at the same location in the Red Sea but the descriptions are indistinct. Their *E. menoni* specimen was only 1.5 mm wide and could rather be a young *L. assimilis*. In the same idea, our small specimen GENE-2 (Table S2) had mixed characteristics with only eight vesicles (characteristic of *E. menoni*) but each with three statoliths (characteristic of *L. assimilis*). Some Hydromedusae are known to increase the number of vesicles during growth and we have to consider our small individual (1.5 mm) as a young and incomplete specimen of ?*L. assimilis*.

Studies of the cnidome in a wide range of species of medusa has shown that cnidocysts may be used as supplementary characteristics of classification, but unfortunately in a less obvious way for Leptomedusae (Russell 1953). In Bouillon et al. (1988), the cnidomes of *E. menoni* and *L. assimilis* appeared very similar with 3 types of cnidocysts: atrichous isorhizas (2 sizes), merotrichous isorhizas (2 sizes), and microbasic mastigophores (1 size). Only the location (tentacular and rudimentary bulbs, tentacle, cirrus, oral lip) of the various types differed between species. The capsule size of cnidocysts varies widely, both in a given type within one individual and in different specimens of one species (Bouillon et al. 1988). So, the observed differences between the two species by these authors cannot be considered as significant. Hirano and Yamada (1985) determined another formula for *L. assimilis*: atrichous isorhizas (2 sizes), merotrichous isorhizas (2 sizes), and basitrichous isorhizas (2 sizes). Based on shapes, sizes and locations of cnidocysts, our specimens were similar to those of Hirano and Yamada (1985). The main difference is the presence of atrichous

isorhizas in the rudimentary bulbs, a feature which was not found (or not studied?) by these authors. Nevertheless, no data are available for *E. menoni* in this Japanese paper, and finally, the identification based on the cnidome description remains inconclusive for these two species. The criteria for the identification are the shape and the size of the capsule and the aspect of the coiled thread in the capsule or extruded after discharge. This last examination is difficult with optical microscopy and can be erroneous. For example, the merotrichous isorhizas of *L. assimilis* (Bouillon et al. 1988) were initially identified as a macrobasic mastigophore (Bouillon 1984). SEM (Scanning Electron Microscope) observations could bring more reliable indications in characterization of the species (Östman 2000).

Surprisingly, in spite of morphological traits referable to *L. assimilis*, the results of DNA barcoding analysis of specimens from the English Channel and the North Sea revealed high genetic similarities to available sequences in DNA repositories attributed to *E. menoni*. One possible explanation is that only two *L. assimilis* sequences (HM053520 and HM053534) are currently available in GenBank and, as mentioned above, only one sequence (HM053520, Wang et al. 2010, unpublished) seems to be reliable. In contrast, there are 10 sequences of *E. menoni* available in GenBank. The COI gene sequence of our haplotype 1 specimens was 100% identical to *E. menoni* (JQ716086) from offshore China (Zheng et al. 2014). Unfortunately, these authors did not describe the morphology of their specimen. Furthermore, the only reliable *L. assimilis* COI partial sequence is almost identical to the *E. menoni* COI sequence (only 1 bp difference). We also analyzed rDNA genes (16S and 18S rRNA gene sequence) from our specimens, but unfortunately, neither 16S nor 18S rRNA gene sequences of *L. assimilis* are available in the GenBank for comparison. However, there are 7 *E. menoni* 16S rRNA gene sequences in GenBank: FJ550493 (Leclère et al. 2009) and JQ715881-JQ715886 (Zheng et al. 2014). The 16S rRNA sequences of all our specimens differ by 2 bp from FJ550493 (Leclère et al. 2009). Within the sequence group JQ715881-JQ715886 (Zheng et al. 2014), there is at most only 1 bp different. Two or three bp were different compared to FJ550493, and 3 or 4 bp differed compared to our specimens. Considering the length of these 16S rRNA sequences (around 600 bp), these differences are very small, and all sequences share more than 99% sequence identities. As for the 18S rRNA gene sequence, all sequences of our specimens were identical to the sequence of *E. menoni* (FJ550570, Leclère et al. 2009). All these findings raise the question of whether *L. assimilis* and *E. menoni* are the same species.

The *E. menoni* records in the Bay of Biscay (Spain) were genetically identified as *E. menoni* (Altuna 2009), but morphological species description by Altuna (2009; his figure 2 and legend) was more in favor of *L. assimilis*, although gonads in his specimens were not divided longitudinally. In fact, Altuna (2009) obtained his *E. menoni* medusa from the polyp stage, but doubted his species identification (Altuna, personal communication) and later referred to his specimen as "*Lovenella cf. assimilis* (Browne, 1905)" in a more recent paper (Altuna 2010). However, Altuna did not cite his 2009 publication in this 2010 paper. Furthermore, the reliability of the *E. menoni* genetic reference (Voucher INVE33457 from New Zealand; Leclère et al. 2009) used by Altuna (2009) is thought to be questionable (Schuchert, personal communication). The COI gene sequence of our type 1 specimens was 100% identical to *E. menoni* (JQ716086.1) from offshore China (He et al. 2012, unpublished) but these authors unfortunately did not describe the morphology of their specimen. Hence, it cannot be disregarded that some sequences available in GenBank of *E. menoni* are based on misidentified specimens of *L. assimilis*.

Another possibility is that the two species are more closely related than previously thought or even conspecific, this despite them presently being classified as belonging to two different genera. The latter conclusion is only due to the lack of taxonomic revision, although both Bouillon (1984) and Kramp (1968) already pointed out the difficulty in distinguishing these species and suggested a revision of the *Eucheilota* species. Intraspecific variation due to varying environmental conditions may also have contributed to this confusion. The description of *L. assimilis* changed in literature, but the original description (Browne 1905) described the species as having 5 marginal bulbs (as *E. menoni*) and approximately 5 marginal vesicles, each with 2–3 otoliths (as *L. assimilis*), in each quadrant of the umbrella. This again supports the hypothesis that the two nominal species are in reality one (Schuchert, personal communication).

In conclusion, a thorough re-evaluation of their respective status, implying an exhaustive morphological description, including the cnidome, and DNA sequence examination of the Indian Ocean's native populations is needed. Our

observations confirm that today the use of COI/16S barcode sequences is an essential tool for such studies (Laakmann and Holst 2013; Zheng et al. 2014; Lindsay et al. 2015).

A non-indigenous species

Recently, Brylinski et al. (2012) discussed the possibilities of plankton being transported in ballast water from Asia to the southern bight of the North Sea. Indo-Pacific leptomedusae could be transported in the same way, and also fouling on the hull of ships during the polyp phase is possible. The introduction pathway of ?*Lovenella assimilis* by intercontinental ship transport is congruent with the importance of the eastern English Channel in the world ocean's traffic. The Channel area is recognized as the 2nd most invaded water body in Europe (Gollasch 2006). Moreover, the proximity of important European commercial harbors, such as Rotterdam, Antwerp, and Zeebrugge, and the relatively short transit duration (25 to 45 days) favors the introduction from Indo-Pacific waters to the French and Belgian coasts. Thirty Indo-pacific species have already been observed in this area (Dewarumez et al. 2011). Two hydromedusa species, *Nemopsis bachei* (L. Agassiz, 1849) and *Gonionemus vertens* (A. Agassiz, 1862), were imported from North-America, Japan, or China (Wolff 2005).

Lovenella assimilis and *Eucheilota menoni* were both reported in the Red Sea by Schmidt (1973) but there is no evidence of colonization in the Mediterranean Sea through Lessepsian migration (Gravili et al. 2013).

Altuna (2009; 2010) found polyps of "*Lovenella cf. assimilis"* in July 2007 in the Bay of Biscay (Spain) and assumed that the medusae "would be discovered sooner or later in plankton from other parts of Europe". Actually, our first observation of ?*L. assimilis* occurred in the eastern English Channel in August of the same year. The presence of two haplotypes suggests a multiple introduction of *?L. assimilis* in Europe in various areas before 2007 and its dispersal along coasts, favored by its high potential for asexual reproduction (Boero et al. 2002). In spite of its small size, this species is easy to find in a sample under a dissecting microscope because of the large black spot on each of the four tentacular bulbs. However, it is also often overlooked because of its low abundance. Indeed, we often found only one or two specimens by net-sample. Furthermore, in spite of the high potential effect of hydrozoan jellyfish as keystone planktonic predators (Piraino et al. 2002), the interest in small jellyfish is not always shared in the community of plankton researchers. As a result, we identified this medusa in 2011, while it was already present 5 years earlier.

Recurrent records of ?*L. assimilis* in summer and autumn since 2007, along with asexual reproduction features (well developed gonads and medusal buds) and young specimens, are consistent with a fully established population in the area. Therefore, although occurring in low abundance, ?*L. assimilis* may be considered as an established non-indigenous species (NIS) in the eastern English Channel and the southern bight of the North Sea.

This work confirms the importance of long-term planktonic surveys (i.e. the French SOMLIT network and IGA Gravelines survey) to infer and trace back the historical presence of new plankton species, which could be due to anthropogenic introduction or related to global change. This work also confirms the need for complementarity between genetic and morphological analyses in the study of hydromedusae (Leclère et al. 2009; Laakmann and Holst 2013) and adequate description of morphological characteristics in taxonomic studies (Boero and Bernardi 2014).

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The following supplementary material is available for this article:

Table S1. Records of *Lovenella assimilis* (Browne, 1905) in the eastern English Channel and the southern bight of the North Sea.

Table S2. Distribution (formula) of the tentacles (T), marginal bulbs (B), vesicles and number of statoliths (V3) in each quadrant of 6 specimens of *Lovenella assimilis* and genetic characteristics.

Table S3. Morphology of *Lovenella assimilis* following different authors. Comparison with *Eucheilota menoni*.

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