THE OSCARELLA SPONGE GENUS: A HIDDEN YET COLORFUL DIVERSITY

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ABSTRACT

The identification and ranking of new taxa are crucial for advancing our understanding of biodiversity. However, assigning samples to new taxa requires a cautious and rigorous approach. Historically, taxonomy has heavily relied on morphological traits, which can be subjective and may not always correspond to underlying genetic divergence. This challenge is especially pronounced in organisms with limited diagnostic morphological traits. A prime example is the poriferans of the genus *Oscarella* (Homoscleromorpha), where species delimitation often depends on insufficient morphological features and genetic data. In this study, we addressed these challenges by integrating a comprehensive genetic dataset, including both mitochondrial and nuclear markers, with systematic photographic documentation of 192 specimens collected in Western Mediterranean Sea (Bay of Marseille, France). This method allowed us to provide a more robust assessment of *Oscarella* diversity, leading to the identification of four species new to science. In addition, our phylogenetic multigene analyses enabled us to propose a meaningful evolutionary scenario for color polymorphism. Additionally, our data showed the critical limitation in current methodologies for *Oscarella* studies, such as the low resolution of the widely used *cytochrome c oxidase subunit I* (*cox1*/*COI*) gene, the lack of genetic data for many species, and the insufficient information regarding their geographical distribution. These findings underscore the need for more rigorous genetic frameworks and comprehensive datasets to enhance species delimitation and taxonomy.

Key words: Porifera, Genetic markers, Species delimitation, Taxonomy, Biodiversity

INTRODUCTION

In a context where the biodiversity remains largely unknow or under-estimated in many marine ecosystems despite their undeniable roles from an ecological, economic, and cultural point of view, the monitoring and appropriate local management of these later are challenging objectives[1]. Indeed, because they are more difficult to reach compared to most continental ecosystems (oceanographic vessels, diving gear) and because they are often under strict regulations, marine ecosystems suffer from a long-standing delay in their taxonomic description. Another factor contributing to the limbo of marine biodiversity is the recurrent clustering of non-vertebrate cryptic species under the names of presumably cosmopolitan species[2–5]. This has been triggered by the recurrent lack of clear morphological distinguishable traits in numerous invertebrate taxa, and the constant controversy of new formal species description only upon molecular characters. Among key marine invertebrate groups facing such challenges, sponges are a prime example[6,7].

Sponges (Porifera) are major components of many marine ecosystems. With other non-vertebrates such as corals and gorgons (Cnidaria), they build-up structural supports that offer habitats to numerous organisms. Accordingly, many sponge species appear as engineer whose life cycle and maintenance influence the entire ecosystem. In the Mediterranean Sea, approximately 1'000 sponge species are encountered with a high level of endemism[8]. Among them, *Oscarella* species (Homoscleromorpha) play important roles by covering up to 37% of the substrate covered by sponges in some coralligenous ecosystems[9]. The first *Oscarella* species that has been described is *O. lobularis* (Schmidt, 1862)[10]. It has been first identified in the Mediterranean Sea and then retrieved throughout the world[11–30]. Accordingly, *O. lobularis* was originally considered as a cosmopolitan species. To these days, 25 accepted *Oscarella* species have been described across the world[31]. Nevertheless, very little is still known about the real diversity of *Oscarella* species with no significant advancements in our understanding of their geographical distribution. In this context, the Bay of Marseille (Western Mediterranean Sea, France) is, in a way, the cradle of *Oscarella* research, as it is the location where *Oscarella* diversity is most thoroughly documented with seven species properly identified (**Table 1**)[32,7,33].

Identifying sponge species often relies on the description of their skeletons. Indeed, the composition, shape and size of small mineral structures, called spicules, and the density and reticulation of organic fibers vary greatly from one species to another[34]. Unfortunately, this approach is impossible in *Oscarella* since these sponges are devoid of skeleton[8]. Accordingly, species identification in this peculiar genus has been originally based on sponge morphology (*e.g.*, shape of the lobes, texture of the surface), cytological feature (*e.g.*, cell diversity and occurrence), tissue consistency (*i.e.*, fragile, soft or cartilaginous), and color[7]. However, the limited number of traits, their low variability, and their relative subjectivity are concerns that must be addressed to reliably describe *Oscarella* diversity. Therefore, from 2013 onwards, molecular analyses were systematically incorporated to the description of new species in this peculiar poriferan genus.

In the first genetic survey of *Oscarella*, two nuclear markers (*18S* and *28S ribosomal DNA*) and two mitochondrial markers (*ATP synthase membrane subunit 6*, *atp6* and *twin-arginine translocase component, tatC*) were used to study few samples collected around the world[35]. Although this approach was sufficient to discriminate most species and describe two new species, it failed to efficiently distinguish *O. lobularis* and *O. tuberculata*. In more recent studies, the *I3-M11* fragment of the widely used *cytochrome oxidase subunit 1* (*cox1*/*COI*) mitochondrial marker were used[36–40]. Unfortunately, it also failed to discriminate some closely related *Oscarella* species, such as *O. carollineae, O. filipoi* and *O. ruthae*[36]. All these results either reflect (i) that the described species actually represent single

species, (ii) that the markers used were not resolutive to discriminate closely related *Oscarella* species (e.g. mutation rate too low, incomplete lineage sorting)[41], or (iii) that *a priori* species identification of individuals upon morphology and tissue consistency is not that trivial.

In this study, we collected 192 specimens of *Oscarella spp.* from eight locations across the Bay of Marseille to conduct a comprehensive genetic survey of the local diversity. This barcoding approach included (i) the three previously used mitochondrial protein-coding markers (*atp6*, *cox1 and tatC*), (ii) a newly developed mitochondrial marker that includes the intergenic region between the *NADH dehydrogenase subunit 1* gene and the *ATP synthase membrane subunit 8* gene (*nad1-atp8*), and (iii) a newly developed nuclear marker which is an *Exon Primed Intron Crossing* (EPIC) locus (*epic12*). *EPIC* loci are introns retrieved in highly conserved genes that can be amplified to assess nuclear variation in animals for which no genetic information is available by the use of potentially universal or degenerated primers[42,43]. We compared these five markers and identified the most suitable ones for implementing a barcode approach aimed at efficiently identifying *Oscarella* species. In addition to species delimitation, partial mitochondrial genomes were obtained to further clarify some phylogenetic relationships and retrace the evolution of color polymorphisms. As a result, this study stands as the most comprehensive investigation of the *Oscarella* genus to date, both in terms of specimen sampling and the number of genetic markers.

MATERIALS AND METHODS

Specimen collection

Oscarella spp. specimens (n = 192) corresponding to different color morphs were collected at different locations across the Bay of Marseille (**Figure 1**). Each of these samples was associated with an identifier that includes the abbreviation of the sampling location and the sample number (*e.g.*, 3PP_01, **Supplementary Table 1**). Each animal was pictured (**Supplementary Figure 1**) and fixed overnight at 4°C in a solution containing 4% paraformaldhehyde (50% PBS-Tween 1‰, 25% sea water). The next day, samples were washed in PBS-Tween, transferred to increasing methanol concentrations and preserved in 100% methanol at -20°C. For many specimens, small pieces of fresh tissue were also flash frozen in liquid nitrogen and conserved at -80 °C.

Marker amplification, sequencing, and haplotype networks

Mitochondrial (*atp6*, *cox1*, *nad1-atp8*, *tatC, SNP*) and nuclear (*epic12*) markers were amplified using the GoTaq® G2 Flexi DNA polymerase (Promega). PCRs were done in 20µL (1X Buffer, 0.2mM dNTP, 1µM primers, 1.5mM MgCl2, 0.5u GoTaq, and 3µL of template). No DNA extraction was performed here. Instead, PCR template consisted in a small piece of fixed tissue (~3mm square) mashed in 1mL of water using a pestle (*i.e.*, crude extracts), as previously reported for *Hydra*[44]. Amplification was done by 30 PCR cycles. Primer sequences, amplicon sizes and PCR melting temperatures are provided in **Table 2**. PCR products were checked on 1% agarose gels and sent to Eurofins Genomics for Sanger sequencing. All sequences obtained in this study have been deposited on NCBI database (**Supplementary Table 2**). *Nad1-atp8* sequenced were aligned using MUSCLE[45] while other markers were manually aligned. Corresponding haplotype networks were obtained using the Minimum Spanning Network function of PopArt[46].

Mitochondrial genome amplification of new species, sequencing, and phylogenetic reconstruction

DNA extractions were done using the DNeasy® Blood & Tissue Kit (Qiagen) on 25mg of flash frozen tissue. The GoTaq® Long PCR Master Mix (Promega) was used (1X mix, 1µM primers, and 5µg DNA). Primer sequences, amplicon sizes and melting temperatures are provided in **Table 2**. PCR amplicons

were checked on 0.5% agarose gel, purified using the Qiagen DNA extraction kit and sequenced using the Oxford Nanopore Technologies services of Eurofins Genomics. Partial mitochondrial genomes have been deposited on NCBI database (**Supplementary Table 2**). The amplicon sequence encompassed eight of the mitochondrial protein-coding genes (*cox1*, *nad1*, *atp8*, *atp6*, *cox3*, *atp9*, *nad4*, and *nad6*). Protein coding genes were concatenated for each studied species. The resulting sequences were aligned with MUSCLE[45], and nonrelevant positions were removed by Gblocks with the settings set as follow, minimal conservation for a flank position at 85%, maximal contiguous non-conserved position at 8 bp, minimal block length at 10 bp and no gap allowed in final blocks[47]. This approach retained 98% of the original 6591 positions. The best nucleotide substitution model evolution was estimated using the jModelTest software[48]. The phylogenetic reconstruction was done in MEGA[49] using the Maximum Likelihood method, the General Time Reversible model, the Gamma Distributed substitution rate (4 categories), and a proportion of invariable sites (GTR + I + G). Branch robustness was tested by 1000 bootstrap replications (BS).

Semi-thin sections and light microscopy

Specimens from the collection were transferred to 100% ethanol. They were then embedded in Araldite resin. Semi-thin sections (300 nm in thickness) were cut on a Ultramicrotome RMC PowerTome XL equipped with a Diatome knife. Semi-thin sections were stained with a mixture of 1% toluidine blue and 0.2% methylene blue. Finally, they were observed under a LEICA ICC50 W light microscope and pictured using a ToupCam XCAM camera.

RESULTS

Comprehensive genetic survey of *Oscarella* species in the Bay of Marseille

PCR amplification of the *atp6* marker using crude macerates as templates was successful for the majority of specimens (95% amplification success). As a result, *atp6* was chosen as the primary marker for delimiting molecular operational taxonomic units (MOTUs). Analysis of *atp6* sequences from 183 newly collected specimens identified 11 distinct MOTUs, labeled MOTU_a to MOTU_k (**Figure 2A**). Comparisons with reference mitochondrial genomes identified MOTU_a and MOTU_d as *O. tuberculata*, MOTU_b as *O. lobularis*, MOTU_g as *O. viridis*, and MOTU_h as *O. microlobata*. In contrast, none of the *atp6* sequences obtained here matched *O. balibaloi* or *P. jarrei*. Accordingly, we added the sequences of available mitochondrial genomes for these two species into our *atp6* network (MOTU_l and MOTU_m, respectively). For the six remaining MOTUs, species identification could not be determined at this stage. However, both MOTU_e (10 samples) and MOTU_j (one sample) branched directly on the main *O. tuberculata* haplotype (MOTU_a) without any additional connection (**Figure 2A**).

To (i) confirm the *atp6* data, (ii) assess whether the remaining six MOTUs represent new taxa, and (iii) obtain genetic information for the nine specimens where *atp6* amplification failed, we targeted three additional mitochondrial markers: *nad1-atp8* (66% of specimens sequenced), *tatC* (40% of specimens sequenced), and *cox1* (14% of specimens sequenced) (**Supplementary Figure 2**). Overall, partitions obtained from these markers, except *cox1*, were either fully consistent (*e.g.*, MOTU_b was recovered with *tatC*) or compatible (*e.g.*, MOTU_b split into three closely related haplotypes using *nad1-atp8*) with the *atp6* data (**Figure 2B**, **Supplementary Table 1**, **Supplementary Figure 2**). Additionally, all specimens for which *atp6* amplification failed were identified as *O. balibaloi* thanks to *nad1-atp8* and *tatC*, while MOTUs a, d, e, and j grouped together as *O. tuberculata*. These specimen clustering patterns were further corroborated by results from the newly developed *epic12* nuclear marker since all *epic12* data are also consistent or compatible with *atp6* MOTUs (**Figure 2B**, **Supplementary Table 2**, **Supplementary Figure 2**). Unfortunately, *epic12* amplification was unsuccessful for *O. microlobata*.

The combined analyses of partitions from all five markers strongly suggests that many of the identified MOTUs represent distinct taxa, likely distinct species (*i.e.,* similar partitions). Indeed, no alleles were shared between MOTUs (except between MOTU_b and MOTU_c for *cox1*) despite their sympatric distribution within the Bay of Marseille (**Supplementary Table 1**). Therefore, at least ten *Oscarella* species inhabit the bay, of which four are new to science. We propose the following names for these new species: *O. veronicae* sp. nov. (MOTU_c), *O. sabinae* sp. nov. (MOTU_k), *O. fragilis* sp. nov. (MOTU_i), and *O. lunettae* sp. nov. (MOTU_f). A formal description of these species is provided in the *Systematics* section.

Phylogenetic relationships reveal a complex of four polychromatic species

Analysis of the haplotype networks suggested that four species within the *Oscarella* genus are closely related. Specifically, *O. tuberculata* and *O. lobularis* consistently clustered with *O. sabinae* sp. nov. and *O. veronicae* sp. nov (**Figure 2A, Supplementary Figure 2**). Whatever the marker considered (*atp6*, *nad1-atp8*, *tatC*, *cox1* or *epic12*), the genetic distances between haplotypes within this complex were extremely short, compared to the genetic distances between this complex and the closest neighboring species (*e.g.*, 0,4% vs 5,2% with *atp6*). Similarly, *O. viridis* and *O. lunettae* sp. nov. tended to be close to each other across all markers (*e.g.*, 1,6% with *atp6*).

To further confirm the relationships among *Oscarella* species, we sequenced 9874 bp of the mitochondrial genome for one specimen of each new species. The resulting phylogenetic tree strongly supported (BS value = 100%) the close relationship between *O. tuberculata*, *O. lobularis*, *O. sabinae* sp. nov., and *O. veronicae* sp. nov. (**Figure 2C**). The phylogenetic analysis also confirmed that *O. lunettae* sp. nov. is the sister species to *O. viridis* (BS value = 100%). Finally, *O. fragilis* sp. nov. formed a distinct lineage, diverging before the emergence of the *O. carmela* / *O. malakovi* group (BS value = 100%), which are two North Pacific species.

Because color played a crucial role in species identification in the *Oscarella* genus, we had a look to color polymorphism in each species (**Figure 2B, Supplementary Figure 1**). *O. tuberculata*, *O. lobularis* but also *O. veronicae* sp. nov. displayed a broader range of colors, including pink and blue specimens. Interestingly, the only *O. sabinae* sp. nov. specimen was also a very bright pink while other *Oscarella* species exhibited a range of colors from white to bright or rusty orange, including various shades of light brown, beige and yellow. Accordingly, polychromatic species with pink, violet/purple and blue morphotypes form a monophyletic group. As a result, color can be a reliable criterion for distinguishing species within versus outside the complex.

DISCUSSION

Towards a standardized approach for species identification in the *Oscarella* genus

PCR and amplicon sequencing were integrated into research laboratories as a reliable method for species identification, ranking, and the discovery of new species across diverse taxa by the late 1990s and early 2000s. Although this molecular approach offered a more standardized and precise alternative to traditional morphological methods, this barcoding approach was first employed in the identification of new *Oscarella* species only in 2013[35]. Nevertheless, *Oscarella* diversity is sometimes assessed using *atp6* and *tatC* markers[35] and sometimes using the very broadly used *cox1* (*I3-M11*) marker[36–38,50]. This absence of consistent markers within the small community studying *Oscarella* thus prevents comparison between studies and the reconstruction of phylogenetic relationships within this genus clearly (**Table 1**). As shown here, *atp6* appears to be a more suitable marker than *cox1* to discriminate closely related *Oscarella* species. Indeed, *cox1* failed to discriminate *O. lobularis* from *O. veronicae* sp. nov. in the present study but also *O. carollineae*, *O. filipoi*, and *O. ruthae* in a previous study[36]. This is particularly concerning especially since *O. ruthae* exhibits significant morphological variability, with six

distinct morphotypes. This underscores the broader pitfall in using *cox1*/*COI* marker for discriminating species in various taxa[51,52]. Although *atp6* or *nad1-atp8* mitochondrial markers appeared as good alternatives to *cox1*, we recommend that future species descriptions in *Oscarella* include, at a minimum, partial mitochondrial genomes and the nuclear marker *epic12* in addition to photographic evidence. To achieve a uniform dataset, the resampling of species for which no reliable genetic data currently exists is required (at least 13 accepted species out of 24). Unfortunately, this task will be particularly challenging for species lacking both genetic data and photographic documentation (**Table 1**). For instance, the *O. imperialis* species exclusively relies on the cytological description of one specimen and previous studies, such as our, were unable to find it[35,7]. Even though a very small fragment of the holotype was provided to the National Museum of Natural History in Paris (MNHN, France), it was not possible to amplify any DNA sequences using various protocols and primers (data not shown) or to identify without doubt similar specimens in the field. Consequently, in the absence of both photographic documentation and genetic data for this species, it would be prudent to remove it from the list of accepted species due to the lack of feasible comparison. This case study thus highlights the critical need for re-evaluating certain species and the necessary of genetic data from neotypes for accurate and enduring species identification not only in the *Oscarella* genus due to the lack of skeleton but probably also in many other taxa[53,54].

Beyond type localities: expanding the geographical knowledge of *Oscarella* species

Besides the lack of robust reference genetic framework for each species, another limbo of *Oscarella* diversity lies in their poorly defined geographical distributions. For most species, the only available data are those provided in their original descriptions, which limit our understanding of their distribution to the type locality. However, *O. lobularis* and *O. tuberculata* stand as exceptions. *O. lobularis* was first described in the Adriatic Sea (Croatia)[10], and has since been reported throughout the Mediterranean Sea, including the Aegean Sea (Greece)[14,15]; the Levantine Sea (Israel)[11]; the coast of Tunisia[13,16]; the Strait of Gibraltar (Spain)[20]; the Gulf of Lion (France)[24–26]; and the Tyrrhenian Sea (Italy)[12,19,23,29]. Additional reports also suggest its presence in the North Atlantic Ocean[17,18,21,22,27,28,30]. Similarly, *O. tuberculata* has also been tentatively recorded across the Mediterranean Sea, following its initial identification in the Adriatic Sea (Croatia)[55] with subsequent reports from the Ionian Sea (Malta)[56]; the Aegean Sea (Greece)[57]; the Levantine Sea (Israel)[58]; and the Gulf of Lion (France)[59,60]. However, most of these studies primarily consist of species occurrence lists at specific locations, often lacking supporting genetic data or even photographic documentation. Therefore, these reports should not be considered as definitive evidence of the species presence but rather as potential sightings of an *Oscarella* species. In some instances, basic photographs are provided, though the reliability of specimen assignment to a specific species remains questionable. For example, the image provided by Idan et al. for *O. tuberculata* specimens observed at a depth of 97 meters likely represents a new species, as the specimen morphology and the depth of the Herzliya site do not align with the known characteristics of *O. tuberculata*[11,7]. Thus, there is very few solid evidence supporting the presence of *O. lobularis* or *O. tuberculata* beyond their original sampling sites. Acquiring genetic data from *Oscarella* samples across the Mediterranean Sea and beyond would greatly enhance our understanding of the geographical distribution of each species which is a prerequisite for efficient conservation management (endemic vs cosmopolitan species). Furthermore, this will undoubtedly lead to the discovery of numerous new *Oscarella* species for a better overview of *Oscarella* diversity. This is particularly demonstrated by our study, which identified four new species despite extensive prior research in the Bay of Marseille (*Oscarella* species richness increased by 57%). This approach is essential given that, out of the four poriferan lineages, the Homoscleromorpha represents the smallest one, with only 136 accepted species, constituting less than 1.4% of the world's sponge diversity[11].

Color polymorphism in *Oscarella*: Evolutionary insights and biochemical mysteries

In this study, we provide compelling evidence that both *Oscarella tuberculata* and *Oscarella lobularis* are polychromatic species, exhibiting bright pink, blue, and purple/violet morphotypes. These findings corroborate previous assumptions of their color diversity[7]. In contrast, other species within the *Oscarella* genus tend to exhibit more subdued coloration, such as cream, yellow, orange, or brown[36–38,61,35,32,62–64,7,33]. This suggests that the striking color polymorphism seen in *O. tuberculata* and *O. lobularis* likely resulted from a singular evolutionary event within the genus. The discovery of new species in Bay of Marseille supports this hypothesis. Notably, the blue and pink morphs of *O. veronicae* sp. nov. and the pink specimen of *O. sabinae* sp. nov. form a monophyletic group with *O. lobularis* and *O. tuberculata*, while the orange-colored *O. fragilis* sp. nov. and the yellow *O. lunettae* sp. nov. branch outside this cluster. This suggests that the evolution of color polymorphism may have been confined to a specific lineage. Further exploration of the Brazilian species *O. ruthae*, which is presented as the sister group to *O. tuberculata* and *O. lobularis* upon cob phylogeny, could provide additional insight into this evolutionary process as it shows color polymorphism, though no blue morphs were observed[36].

Despite the vibrant colors seen in many sponges, the biochemical nature of these pigments remains largely unexplored. So far, the pigments identified in sponges, including those responsible for yellow, red, or black coloration, are often specialized metabolites such as quinones, alkaloids, terpenes, or carotenoids[65]. Interestingly, yellow pigments appear disproportionately represented in the literature. In contrast, blue pigments remain poorly documented, not only in sponges but across the animal and plant kingdoms. In many animals, such as chameleons and butterflies, blue coloration typically arises from structural mechanisms (e.g., nanocrystals or microscopic scales) that manipulate light also called structural coloration, rather than from pigments themselves[66]. Only a few natural organic blue pigments have been therefore identified, such as anthocyanins in plants[67], GFP-like chromoproteins in cnidarians[68,69], and tunichrome peptides in tunicates[70]. Given the distinct nature of these pigments, further investigation into the molecular mechanisms behind coloration in sponges, especially species like *Oscarella*, could unlock broader applications in cell biology and biotechnology. For example, sponge pigments may have potential uses as industrial pigments or as reporters for gene expression studies.

SYSTEMATICS

Phylum: Porifera Grant, 1836.
Class: Homoscleromorpha Bergquist, 1978.
Order: Homosclerophorida Dendy, 1905.
Family: Oscarellidae Lendenfeld, 1887.
Genus: *Oscarella* Vosmaer, 1884.

***Oscarella fragilis* sp. nov.**

Holotype: *Muséum National d'Histoire Naturelle de Paris*, MNHN – XXX XXX (in progress): Collected from France, Mediterranean Sea, Marseille, Jarre (43.19°N, 5.36°W), at a depth of 14-17 m by Dorian Guillemain on 2023-04-26 (JAR_01, **Figure 3A**). Specimen fixed in 4% paraformaldehyde and preserved in absolute methanol.

Paratype: Muséum National d'Histoire Naturelle de Paris, MNHN – XXX XXX (in progress): Collected from France, Mediterranean Sea, Marseille, Tiboulen du Frioul (43.2799°N, 5.2860°W), at a depth of 30-35 m by Dorian Guillemain on 2023-06-28 (TIB_11, **Supplementary Figure 1**). Specimen fixed in 4% paraformaldehyde and preserved in absolute methanol.

Morphological diagnosis: *O. fragilis* sp. nov. is an encrusting sponge characterized by its irregular growth forms, covering areas of 15–20 cm² with a thickness of 15–25 mm. The specimens exhibit distinct coloration: one is pale orange, while the other is bright orange. Although the entire surface of this species is lobed, the lobes form transparent, very membranous lamellae, which is a feature that starkly contrasts with those of other species in the genus (**Figure 3A** and **3A'**).

Molecular diagnosis: Molecular analysis provides strong evidence for recognizing *O. fragilis* sp. nov. as a distinct species, with its genetic sequences showing high divergence from all other known *Oscarella* species (**Figure 2**).

Ecology and distribution: One specimen was found encrusting red algae, while the other was encrusted on another organism, possibly the bryozoan *Smittina cervicornis* (**Supplementary Figure 1**). The first specimen was collected at a depth of 14–17 m, while the second was found at 30–35 m. Given the limited data, it is currently premature to draw definitive conclusions about the ecology and distribution of this species.

***Oscarella lunettae* sp. nov.**

Holotype: *Muséum National d'Histoire Naturelle de Paris*, MNHN – XXX XXX (in progress): Collected from France, Mediterranean Sea, Marseille, Levant (43.09288°N, 5.37365°W), at a depth of 40 m by Dorian Guillemain on 2024-03-07 (LEV_04, **Figure 3B**). Specimen fixed in 4% paraformaldehyde and preserved in absolute methanol.

Paratype: *Muséum National d'Histoire Naturelle de Paris*, MNHN – XXX XXX (in progress): Collected from France, Mediterranean Sea, Marseille, Levant (43.09288°N, 5.37365°W), at a depth of 40 m Dorian Guillemain on 2024-03-07 (LEV_05, **Supplementary Figure 1**). Specimen fixed in 4% paraformaldehyde and preserved in absolute methanol.

Other material examined: Station Marine d'Endoume, IMBE: three additional specimens collected from Levant (43.09288°N, 5.37365°W) and Tiboulen du Frioul (43.2799°N, 5.2860°W) (**Supplementary Table 1** and **Supplementary Figure 1**). Specimen fixed in 4% paraformaldehyde and preserved in absolute methanol.

Morphological diagnosis: *O. lunettae* sp. nov. is an encrusting sponge characterized by irregular growth forms, covering areas up to 80 cm² with a thickness ranging from 10 to 20 mm. All specimens exhibit a yellowish or cream-colored base with distinct orange coloration at the apex of their lobes (**Figure 3B**). Numerous ostia, which are easily visible under low magnification, perforate the sponge's surface (**Figure 3B'**). The consistency of *O. lunettae* is relatively soft yet not fragile.

Molecular diagnosis: Molecular data strongly support the recognition of *O. lunettae* sp. nov. as a distinct species, as its sequences show significant divergence from all currently available *Oscarella* data (**Figure 2**). Phylogenetic analyses suggest that it is closely related to *O. viridis*, positioning it as its sister species.

Ecology and distribution: Like other *Oscarella* species found in the Bay of Marseille, *O. lunettae* sp. nov. is a sciaphilous species, typically found in coralligenous environments. To date, it has been observed at only two locations: Levant and Tiboulen du Frioul. While *O. lobularis* and *O. tuberculata* are also present

in these locations, *O. lunettae* has not been detected elsewhere, suggesting that it may occupy a slightly different ecological niche. All specimens were collected at depths between 30 and 40 m, suggesting that *O. lunettae* might thrive below the thermocline, where temperatures are more stable.

***Oscarella sabinae* sp. nov.**

Holotype: *Muséum National d'Histoire Naturelle de Paris*, MNHN – XXX XXX (in progress): Collected from France, Mediterranean Sea, Marseille, Maire Island (43.2096°N, 5.3353°W), at a depth of 8-18 m by Dorian Guillemain on 2024-06-19 (MAI_95, **Figure 3C**). Specimen fixed in 4% paraformaldehyde and preserved in absolute methanol. No other material has been examined.

Morphological Diagnosis: The unique specimen of *O. sabinae* sp. nov. observed so far measured between 10-15 cm². The surface is lobate, often exhibiting irregularities providing it a cauliflower-like appearance (**Figure 3C'**). The color of the specimen was a deep pink, more akin to *O. lobularis* than to *O. tuberculata* or *O. veronicae* sp. nov. While the specimen was extremely soft, it was not fragile.

Molecular Diagnosis: Molecular analysis of *O. sabinae* sp. nov. revealed distinct haplotypes for all mitochondrial markers, differentiating it from other species within the complex (**Supplementary Figure 2**). Comparative analysis of partial mitochondrial genomes identified a total of 19 diagnostic positions unique to this species, two of them are found in *nad1-atp8*. Additionally, the *epic12* nuclear data confirmed the distinctiveness of *O. sabinae* sp. nov.

Ecology and Distribution: Out of 192 samples collected, only one specimen was identified as *O. sabinae* sp. nov., indicating that this species may be rare or that the sampling area may not represent its primary habitat. Consequently, it is premature to detail the ecology and distribution of this species based on the available data.

***Oscarella veronicae* sp. nov.**

Holotype: *Muséum National d'Histoire Naturelle de Paris*, MNHN – XXX XXX (in progress): Collected from France, Mediterranean Sea, Marseille, Maire Island (43.2096°N, 5.3353°W), at a depth of 6-10 m by Dorian Guillemain on 2023-04-12 (MAI_35, **Figure 3D**). Specimen fixed in 4% paraformaldehyde and preserved in absolute methanol.

Paratype: *Muséum National d'Histoire Naturelle de Paris*, MNHN – XXX XXX (in progress): Collected from France, Mediterranean Sea, Marseille, Maire Island (43.2096°N, 5.3353°W), at a depth of 17-20 m by Dorian Guillemain on 2023-05-04 (MAI_56, **Supplementary Figure 1**). Specimen fixed in 4% paraformaldehyde and preserved in absolute methanol.

Other material examined: Station Marine d'Endoume, IMBE: 23 additional specimens collected from various locations across Marseille Bay, including 3PP (43.09°N, 05.36°W), Maïre Island (43.2096°N, 5.3353°W), Mugel (43.1001°N, 5.3618°W), Tiboulen du Frioul (43.2799°N, 5.2860°W), and Vesse (43.2026°N, 5.1539°W) (**Supplementary Table 1** and **Supplementary Figure 1**). Specimen fixed in 4% paraformaldehyde and preserved in absolute methanol.

Morphological diagnosis: *O. veronicae* sp. nov. is an encrusting sponge with irregular growth forms covering areas up to 20-30 cm² and with a thickness of 10-15 mm. Most specimens display a bright or

light blue coloration (**Figure 3D**), although exceptions include yellow (2 out of 25 specimens) or dark pink similar to *O. tuberculata* (one specimen). Many specimens exhibited a cauliflower-like morphology closely resembling that of *O. sabinae* sp. nov (**Figure 3C'** and **3D'**). The sponge is relatively soft but not fragile in consistency. Note that in contrast to *O. fragilis* sp. nov., *O. lunettae* sp. nov. and *O. sabinae* sp. nov., vacuolar cells are numerous in *O. veronicae* sp. nov. (**Figure 3A''**, **3B''**, **3C''**, and **3D''**).

Molecular diagnosis: Mitochondrial markers were found to differentiate *Oscarella veronicae* sp. nov. from related species, though they did not exhibit mutations exclusive to this species. However, comparison of partial mitochondrial genomes between *O. lobularis*, *O. tuberculata*, *O. veronicae* sp. nov., and *O. sabinae* sp. nov. identified a unique mutation specific to *O. veronicae* sp. nov. (position 7'562, A -> G). This mutation was validated through PCR amplification across various specimens from each species (**Supplementary Figure 3**). In contrast, nuclear data provided a clearer distinction, with the *epic12* marker revealing four *O. veronicae* sp. nov. specific mutations despite its short length (51 bp in these species) (**Supplementary Figure 2**).

Ecology and distribution: *O. veronicae* sp. nov. is a sciaphilous species consistently found in sympatry with *O. tuberculata* and *O. lobularis*. It inhabits the cavities of coralligenous walls or the entrances of submarine caves at depths ranging from 5 to 35 m. To date, no data are available to confirm the presence or absence of this species elsewhere in the Mediterranean Sea or along the French Atlantic coasts.

CONCLUSION

In conclusion, this study highlights the importance of integrating genetic data and standardized methodologies in the identification and taxonomy of *Oscarella* species. By employing a robust dataset that includes mitochondrial and nuclear markers, alongside comprehensive photographic documentation, we were able to enhance the resolution of species delimitation within this genus devoid of skeleton. Our findings revealed the existence of four new species in a very limited area (Bay of Marseille), thus shedding light on the underexplored diversity of *Oscarella* and the evolution of color morphs. Moreover, we exposed critical limitations in current taxonomic practices, such as the low resolution of the cytochrome c oxidase subunit I (cox1/COI) gene and the scarcity of genetic data for many species. These challenges underscore the need for more rigorous and consistent genetic frameworks, not only for accurate species identification but also to better understand the geographical distribution of *Oscarella*. This study paves the way for future research aimed at expanding the genetic datasets for poorly documented species, ultimately contributing to the broader goal of biodiversity conservation.

FIGURE AND TABLE LEGENDS

Table 1: Chronological list of *Oscarella* accepted species according to Word Porifera Database. Species names underlined in blue indicate those found in the Bay of Marseille, Gulf of Lion, Western Mediterranean Sea. The table includes references for the original descriptions, along with the corresponding sampling locations for each species. Additionally, it provides information on whether informative images were included in the original descriptions and whether genetic data are available. The data presented in this study are highlighted in red. Note that, according to all phylogenetic studies, including this one, *Pseudocorticium jarrei* should be renamed *Oscarella jarrei*.

Table 2: Information regarding PCR primers, Tm and amplicon size.

Figure 1: Sampling locations across the Marseille Bay and number of samples per location.

Figure 2: Species Delimitation Analysis of Oscarella spp. mapped onto a Maximum Likelihood (ML) Phylogenetic Tree. (A) Haplotype network based on the *atp6* marker. Each haplotype (a, b, c, etc.) is depicted as a circle, with the size of the circle proportional to the number of specimens represented. Numbers between haplotypes indicate the number of mutations separating them. (B) Schematic representation of species partitions derived from single-locus sequences, either mitochondrial (*atp6*, *nad1-atp8*, etc.) or nuclear (*epic12*), along with the proportion of color morphs within each partition (color). Detailed specimen information is provided in **Supplementary Table 1** and other haplotype networks are shown in **Supplementary Figure 2**. (C) Maximum likelihood (ML) phylogenetic tree of *Oscarella* species based on eight mitochondrial protein-coding genes (*cox1*, *nad1*, *atp8*, *atp6*, *cox3*, *atp9*, *nad4*, and *nad6*). Only nodes with bootstrap values greater than 95% (1000 BS replicates) are shown. Dots on the tree indicate the inferred ancestral color state of the common ancestor of *Oscarella* and the last common ancestor of *O. lobularis*, *O. sabinae* sp. nov., *O. tuberculata*, and *O. veronicae* sp. nov. Note that *Pseudocorticium jarrei* is proposed to be renamed *Oscarella jarrei*.

Figure 3: External and internal morphology of *Oscarella* species new to science. (A to A'') *Oscarella fragilis* sp. nov. (B to B'') *Oscarella lunettae* sp. nov. (C to C'') *Oscarella sabinae* sp. nov (D to D'') *Oscarella veronicae* sp. nov. (A'', B'', C'' and D'') Histological semi-thin sections. Abbreviations: c.c., choanocyte chamber; ex, exopinacocyte, i.c., inhalant canal, and v.c. vacuolar cells.

Supplementary Table 1: List of *Oscarella spp*. specimens. It contains information regarding the sampling, the PCR results (haplotypes), and its assignment to a given species.

Supplementary Table 2: Accession numbers of mitochondrial genomes and haplotypes. Species names with an asterisk correspond to partial mitochondrial genomes. Species names underlined in blue indicate those obtained in the present study.

Supplementary Figure 1: Picture of each specimen. Abbreviatons: 3PP, 3PP; JAR, Jarre; LEV, Levant; MAI, Maïre; MEJ, Mejean; MUG, Mugel; TIB, Tiboulen du Frioul; and VES, Vesse.

Supplementary Figure 2: Haplotype networks supporting the delimitation of four new *Oscarella* species. Haplotype networks based on the *atp6*, *nad1-atp8*, *tatC*, *cox1* mitochondrial markers and *epic12* nuclear marker. Each haplotype (a, b, c, etc.) is depicted as a circle, with the size of the circle proportional to the number of specimens represented. Numbers between haplotypes indicate the number of mutations separating them. Colors correspond to species delimitation.

Supplementary Figure 3: Sequence alignment highlighting the unique diagnostic mutation found in the partial mitochondrial genome of *O. veronicae* sp. nov. This sequence, we called *SNP*, was amplified and sequenced for several specimens belonging to the species complex. The mutation specific to *O. veronicae* sp. nov. is marked by a black arrow.

AUTHOR CONTRIBUTIONS

DG conducted the majority of the sampling. QS captured the images. EG and QS prepared the samples and performed all gene amplifications. QS carried out the phylogenetic analysis and constructed the haplotype networks. QS, ER, CB, and EG contributed to writing the paper. QS supervised the entire project.

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