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Article

Isolation and Identification of Luminescent Bacteria in Deep Sea Marine Organisms from Sicilian Waters (Mediterranean Sea)

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Abstract: Luminescent bacteria are a fascinating component of marine microbial communities, often related to the light emissions in deep sea marine organisms. They are mainly affiliated with specific phylogenetic groups, such as *Photobacterium*, *Vibrio*, and *Photorhabdus*, and are sometimes involved in symbiotic relationships. However, the luminescence of some marine organisms remains a poorly understood process, and it is not always certain whether their luminescence is attributable to associated luminescent bacteria. In this study, for the first time, luminescent bacteria were isolated from two deep sea organisms, namely, the cephalopod *Neorossia caroli* and the teleost *Chlorophthalmus agassizi*. The isolation was carried out on glycerol-supplemented medium, and the search for the *lux*AB gene was performed on all isolates as a complementary tool to the culture-dependent techniques to detect bioluminescence by molecular approach. The optimum of salinity, temperature, and pH was evaluated by physiological tests for all isolates. The production of extracellular polymeric substances was also preliminarily screened. A total of 24 luminescent isolates were obtained, with an abundance from *C. agassizi* specimens. All the isolates were taxonomically characterized and were related to different species of *Photobacterium*, with the exception of *Vibrio* sp. CLD11 that was from *C. agassizi*. The *lux*AB gene was detected in about the 90% of the analysed strains.

Keywords: luminescent bacteria; deep sea; *lux*AB; bioluminescence; *Chlorophthalmus agassizi*; *Neorossia caroli*

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1. Introduction

Luminescent bacteria are ubiquitous in marine environments, brackish waters, and soils [1,2], and some species can establish symbiotic relationships with other marine organisms [3,4]. This topic has attracted increasing interest during the last century [5–8]. Bioluminescence is the peculiar process of light emission from living organisms, involved in many ecological dynamics, and is so widespread in nature that 76% of marine pelagic meso- and macro-organisms are bioluminescent [9].

In a marine environment, the sunlight filters down through the water layers, gradually diminishing about 10-fold for every 75 m of descent, until all visible light disappears below 1000 m [10]. Several deep sea organisms adopted bioluminescence as an adaptive strategy for living in the lower level (in the twilight zone, i.e., at a depth of 200–1000 m) due to the absence of light in this environment. This phenomenon could be used as a sexual selection tool to attract the counterpart during the reproduction period as well as being used as a support for predation and camouflage strategies, as a defense mechanism, and for communication [10–16].

Light-emitting marine organisms include the members of several taxonomical groups, from bacteria to more evolved animals such as fish and cephalopods. In Eukaryotes, bioluminescence could be intrinsic (autogenic bioluminescence) if derived from chemical compounds produced in special tissues of animals, or extrinsic (bacteriogenic bioluminescence) if bacterially mediated, originating from a symbiotic relationship with luminous bacteria hosted in specialized organs [10,12,17,18]. In most cases, bioluminescent species developed organized luminescent organs and photophores that can host photocytes or luminous symbiotic bacteria. Regarding the symbiotic bioluminescence of fish, bacteria are often hosted in specific areas of the fish, such as ventral luminous organs (e.g., Macrouridae, Moridae, and Chlorophthalmidae), luminous lures (e.g., Ceratiidae and Melanocetidae), subocular organs (e.g., Anomalopidae), and esophageal pouches (e.g., Leiognathidae) [7,17,19,20].

Several studies indicated that symbiotic bacteria are also hosted in cephalopod light organs or structures [21–26]. Specific tissues are usually formed during embryonic development and are poised to receive luminous bacteria from the environment [21,27,28]. The light organ is then colonized by highly specific symbiotic bacteria, becoming fully functional [25]. The bacteria in the photophores produce wavelengths of light similar to those in downwelling moonlight. This light appears to counterilluminate the squid's ink sac, making it more difficult for visual predators to see [29–31].

Different aspects of the symbiotic bioluminescence need deeper investigation and an ecological interpretation. Luminous bacteria forming specific symbioses with fishes and cephalopods provide the host with a light that could be used for several functions, and at the same time, the host provides the bacteria with an ideal growth environment [10]. The presence of symbiotic bacteria also seems to be necessary for the correct morphogenesis and development of luminous organs, and in many cases, the relationship is obligate [32–34]. More interestingly, the relationship was proven to have a certain grade of specificity since animals are able to select the specific symbiont bacteria [35,36] and actively eliminate non-luminous bacteria. In the association between *Aliivibrio fischeri* and the bobtail squid *Euprymna scolopes*, the host was demonstrated to be able to detect at a molecular and physiological level if its symbionts are bioluminescent or not and to reject non-luminescent strains of *A. fischeri* [37,38]. It is also well-known that even if luminous organs are mostly colonized by a unique bacterial species, co-existence with a more taxonomic group could occur. Indeed, the light organs of a *Sepiola* spp. host mixed the bacterial population of *A. fischeri* and *A. logei* [24]. The co-occurrence of different luminous bacteria in the same light organ was also observed both in some species of Perciform fish, i.e., the co-symbionts *Photobacterium mandapamensis* and *Photobacterium leiognathi* [39], and in loliginid squids [40]. Evidence for parallel speciation patterns among sepiolid squids and their luminous symbiotic bacteria were also provided by experimental studies, indicating a hierarchy of symbiont competency that reflects the phylogenetic relationships of the partners [41]. Changes in the taxonomic composition of symbiotic luminescent bacteria could also occur in response to seasonal variation, which was observed in the in-fish intestinal luminescent microflora that mainly contain *P. phosphoreum* members in the summer while containing more *A. logei* members in the winter [42].

Although several studies examined the symbiotic relationship between luminous bacteria and fish or cephalopods, they often focused on the same host species. For this reason, it is important to improve our current knowledge on other poorly investigated taxa equipped with luminescent organs. In this study, we examined two deep sea demersal Mediterranean species, the Carol bobtail squid (*Neorossia caroli*, Sepiolidae, Cephalopoda) and the shortnose greeneye (*Chlorophthalmus agassizi*, Chlorophthalmidae, Osteichthyes). The Carol bobtail squid is the most bathyal species among sepiolids, which can live at depths greater than 1700 m (but more commonly found between 200 and 700 m) and shows a preference for deep muddy bottoms colonized by *Isidella elongata* [43]. *C. agassizi* is a demersal teleost that lives on muddy and clay bottoms (up to 1000 m depth) [44]. Our study evaluated the presence of luminous bacteria in association with *N. caroli* and *C. agassizi*, examining organisms collected in the Strait of Messina and in the southern Tyrrhenian

Sea. These analyses were carried out through an integrated approach, coupling molecular techniques and culture-dependent methodologies. We also screened the physiological capabilities of luminous strains and searched for the *lux*AB gene, which is responsible for bioluminescence production. Finally, we preliminarily investigated the biotechnological ability of isolates as potential exopolysaccharides producers.

2. Materials and Methods

2.1. Sample Collection

Overall, fourteen *N. caroli* (Figure 1a) and thirteen *C. agassizi* (Figure 1b) specimens were collected by bottom trawling between 550 and 650 m deep in the Gulf of Patti (southern Tyrrhenian Sea) and were used for the isolation of luminescent bacteria. Additional individuals (n = 13) of *C. agassizi* were also collected from the nearby area of the Strait of Messina, where fresh deep sea fauna is usually found on the shore after stranding phenomena due to upwelling currents [45]. Each individual was collected and kept in a sterile tube at 4 °C until its arrival in the laboratory. We used disposable gloves while measuring each to the nearest 0.1 mm (mantle length ML for *N. caroli*; total length TL for *C. agassizi*) and weighing it (body mass W to the nearest 0.1 g). All manipulation procedures for further analysis were carried out through tweezers that were previously cleaned with absolute alcohol.

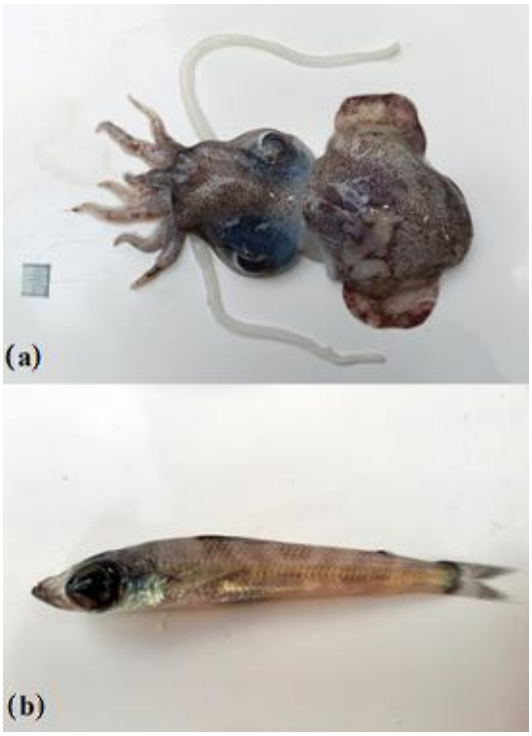

Figure 1. The deep sea demersal species considered in this study. (**a**) *Neorossia caroli* (Sepiolidae, Cephalopoda) and (**b**) *Chlorophthalmus agassizi* (Chlorophthalmidae, Osteichthyes).

2.2. Preliminary Treatment of Samples

In laboratory, each specimen was firstly washed slightly with sterile seawater to remove transient and loosely attached bacteria and impurities, and it was then kept cold to prevent decomposition and avoid the growth of foreign bacteria.

In aseptic conditions, the following different tissues/portions were collected from the specimens using sterile scalpels and forceps:

- *N. caroli*: small portions of the siphon, ventral mantle, eye, and the mantle invagination
- *C. agassizi:* the ventral perirectal light organ located in the abdomen just before the anal opening

2.3. Isolation of Luminous Bacteria

All tissue portions were cut and washed with sterile seawater and then homogenized using Ultraturrax (T10 basic ULTRA-TURRAX®, IKA, Staufen, Germany). This operation was carried out in ice to avoid tissue damages due to overheating. The luminescent bacteria were isolated from the pooled homogenates of each portion of interest from all specimens. Aliquots (100 µL) of serially diluted pooled homogenates were spread plated in two replicates on Sea Water Complete (SWC) plates (composition per liter: 5 g bacteriological peptone, 3 g yeast extract, 3 mL glycerol, and 15 g agar, in a mixture of 250 mL distilled water and 750 mL seawater) [46]. Plates were incubated at RT for 24 h and observed for the appearance of luminescent colonies in a dark room. Luminescent colony-forming units (CFU/g) were counted, randomly picked up from each pool, and further re-streaked onto SWC plates three times under the same conditions.

2.4. Physiological Tests

The growth of isolates was tested on a luminescence agar medium (LA, composition per liter: 30 g NaCl, 5 g yeast extract, 10 g peptone (Bacto Peptone, Life Technologies, Carlsbad, CA, USA), and 15 g agar in 1000 mL distilled water), LSW70 medium (composition per liter: 10 g tryptone, 5 g yeast extract, and 15 g agar, in a mixture of 350 mL seawater and 650 mL distilled water), and Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS, Condalab, Madrid, Spain) medium.

The growths on the luminescence agar medium [47] and LSW70 medium [48] were tested to assess which medium was more optimal for growth and the presence/absence of bioluminescence in comparison to the SWC medium. TCBS is a selective medium for the isolation and detection of *Vibrio cholerae* and other *Vibrio* species, and it was used here to test the growth of all isolated luminous bacteria. All isolates were streaked onto TCBS medium plates and incubated for 24 h at 35 °C. The appearance of yellow-colored colonies was recorded as an indication of the presence of *Vibrio* sp. members. All isolates were grown at different temperatures, salinities, and pH values to establish the growth range and the optimum. The growth at different temperatures was first assayed on SWC broth by incubation at 4,10, 15, 20, and 30 °C for 24 h. Once the optimum temperature was identified, the bacterial growth was tested by adjusting the pH values in the range of 5 to 10 in SWC broth. Finally, once the optimum pH was also identified, different concentrations of NaCl were tested on a SWC agar plate (50–100–150–180 g/L; *w*/*v*). All tests were performed in triplicate.

As an additional preliminary screening, the potential production of extracellular polymeric substances (EPSs) was evaluated by observing the occurrence of mucous phenotypes during growth in solidified SWC that was supplemented with glucose (1%, *w*/*v*) and sucrose (1%, *w*/*v*) [49].

2.5. PCR Amplification of 16S rDNA for Bacterial Isolates

DNA was extracted from single colonies of each strain through lysis by heating at 95 °C for 10 min. Amplification of 16S rRNA gene was performed with a thermocycler (BioRad C100 touch thermocycler) using bacterial specific universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The reaction mixtures contained for each sample: 1 µL of DNA, 1 µL of each forward and reverse primer (10 µM), 1 µL of dNTP mix (10 mM), 5 µL of reaction buffer 10×, 0.4 µL of polymerase (Hot Start DNA Polymerase), and 2 µL of $MgCl_2$ in a final volume of 50 µL with MilliQ water. Negative controls for DNA extraction and PCR setup (reaction mixture without a DNA template) were also used in every PCR run.

The PCR program was as follows: an initial polymerase activation at 95 °C for 15 min, 35 cycles of 30 s at 95 °C for denaturation, 30 s at 51 °C for annealing phase, 30 s at 72 °C for elongation, and a final elongation for 5 min at 72 °C. The PCR products were visualized on agarose gel electrophoresis (1%, *w*/*v*) in TAE buffer (0.04 M Tris-acetate, 0.02 M acetic acid, and 0.001 M EDTA).

2.6. Sequencing and Analysis of 16S rRNA Gene

Sequencing was carried out at the Eurofins Genomics Laboratory (Milan, Italy). The sequences of isolates were compared with 16S rRNA gene sequences in the NCBI GenBank and the EMBL databases using BLAST to detect the next relatives of isolates through the "Seqmatch" and "Classifier" programs of the Ribosomal Database Project II (http://rdp.cme.msu.edu/ accessed on 25 June 2022) [50].

The program "Clustal W" was used to align sequences [51] to the most similar orthologous sequences retrieved from the database. Each alignment was checked manually, corrected, and then analyzed using the maximum likelihood method according to the model of Jukes–Cantor distances [52]. A phylogenetic tree was constructed using the MEGA 5 (Molecular Evolutionary Genetics Analysis) software [53]. The robustness of the inferred trees was evaluated by 400 bootstrap re-samplings.

2.7. LuxAB Gene Amplification

The *Lux*AB gene was searched for all bacterial isolates. The gene was amplified by using the specific Primers Lux AB-66F (3′-CAAATGTGRAAAGGTCGTTTTAATTTTGG-5′) and 611R (3′-AACRAAATCWYKCCATTGRCCTTTAT-5′) [54]. The reaction mixtures were carried out as described above, and the PCR program was as follows: an initial polymerase activation at 95 °C for 1 min, 30 cycles of 15 s at 95 °C for denaturation, 15 s at 37 °C for an annealing phase, 10 s at 72 °C for elongation, and a final elongation for 5 min at 72 °C. The PCR products were confirmed by visualization on agarose gel electrophoresis (1.5%, *w*/*v*) in TAE buffer (0.04 M Tris-acetate, 0.02 M acetic acid, and 0.001 M EDTA).

3. Results

The *N. caroli* specimens ranged between 32.1 and 68.0 mm in size (ML) and between 22.7 and 90.5 g in weight (mean ML ± s.d. = 47.9 ± 10.9 mm; mean W ± s.d. = 42.9 ± 20.7 g). The *C. agassizi* specimens measured between 41.7 and 184.7 mm in size (TL) and between 0.44 to 48.6 g in weight (mean ML ± s.d. = 91.9 ± 52.0 mm; mean W ± s.d. = 11.7 ± 15.5 g).

3.1. Isolation of Luminous Bacteria

A total of 24 strains were isolated from the two species. Overall, eight luminous bacterial isolates were from *N. caroli* specimens: five were isolated from the siphon, two from the ventral part of mantle, and one from mantle invagination. A total of 17 isolates were from *C. agassizi*. All strains showed growth on the LA medium, and 22 out of the 24 strains were capable of growth on LSW-40. Only one strain was able to grow on the TCBS medium. Complete data are shown in Table 1.

Table 1. Number of isolates obtained from SWC plates. Growth of isolates on the LSW40 Agar, luminescent agar, and TCBS Agar plates.

Sample	Isolates	Growth Test		
	SWC	TCBS	LSW	LA
Neorossia caroli				
Siphon	5	0	4	5
Ventral mantle	2	0	2	2
Mantle invagination	1	0	1	1
Chlorophtalmus agassizii				
Perianal gland	16	1	16	17
Total isolates	24	0	22	24

3.2. Physiological Tests

The results of the physiological tests are shown in Figure 2. The optimum growth temperature was identified to be 20 °C. More than half of the tested strains showed growth at 4 °C, and as many as 72% managed to grow up to 40 °C, even though at higher temperatures the bioluminescence was inhibited. The isolates CLD1, CLD5, CLD7, CLD9,

CLD10, CLD12, and NE1 were not able to tolerate temperatures higher than 30 °C. The results of the different pH values tests showed that, in general, isolates tolerate acidity conditions poorly. The optimum condition was detected to be 7 pH value, and 3% of the tested bacterial strains were able to grow in a range between 6 and 10 pH values, in some cases without bioluminescence. Only two strains (CLD1 and CLD5) showed growth at pH 5 and maintained their bioluminescence. Salinity tests showed that all bacterial strains grew up to a final concentration of 50 g/L NaCl in the isolation media. However, in some cases, the bioluminescence was inhibited by the salt addition at the final NaCl concentration of 50 g/L, namely, in the case of isolates CLD6, NE6, and NE7. No strain was able to tolerate salinity concentration higher than 50 g/L.

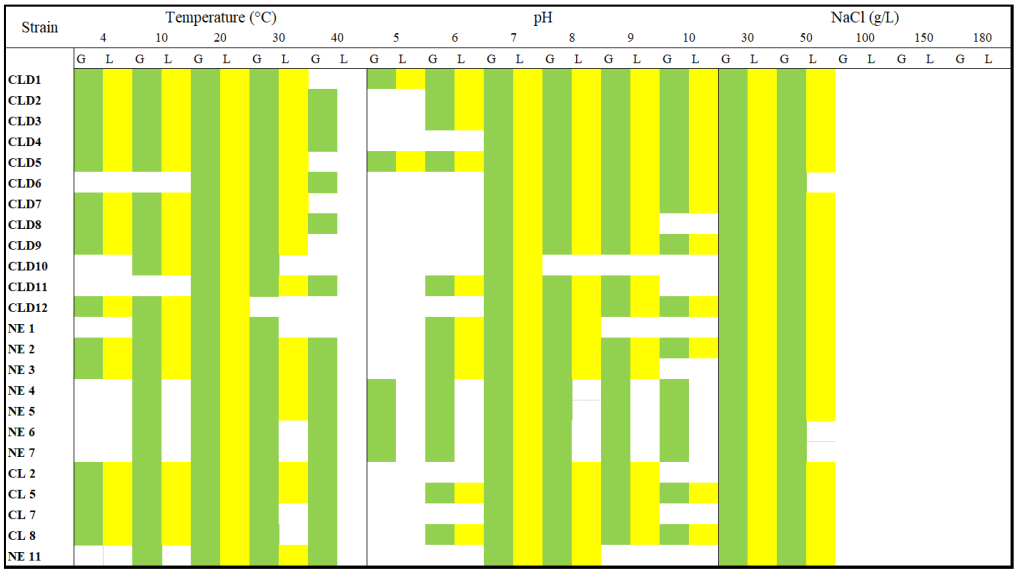

Figure 2. Growth (green cells) and presence/absence of luminescence (yellow cells) of isolates at different temperatures, pH values, and salinities.

The results from the preliminary screening for EPS production showed that 12% of the bacterial strains presented a mucous phenotype. In detail, the isolates CL2, CLD10, and CLD12 showed a more mucoid aspect in the culture medium plates supplemented separately with both glucose and sucrose (final concentration 1%, *w*/*v*). In the cultures in liquid broth with the same conditions, a very dense and milky mucus was observed.

3.3. Phylogenetic Identification

The comparative sequence analysis indicated that the luminous bacteria were closely related to known bacteria with 16S rRNA gene similarity ≥98%. All isolates were affiliated to Gammaproteobacteria and were specifically identified as *Photobacterium* genus members, with the exception of one *Vibrio* member (Table 2). The phylogenetic analysis showed that a high species diversity was found on the 25 bacterial strains identified. Overall, three species of *Photobacterium* (*P. kishitanii*, *P. leiognathi*, and *P. phosphoreum)* and one species of *Vibrio* (*V. campbelli*) were identified. The genomic DNA were successfully extracted, and the *lux*AB gene was amplified in 22 strains of all isolates (Table 2; Figure 3).

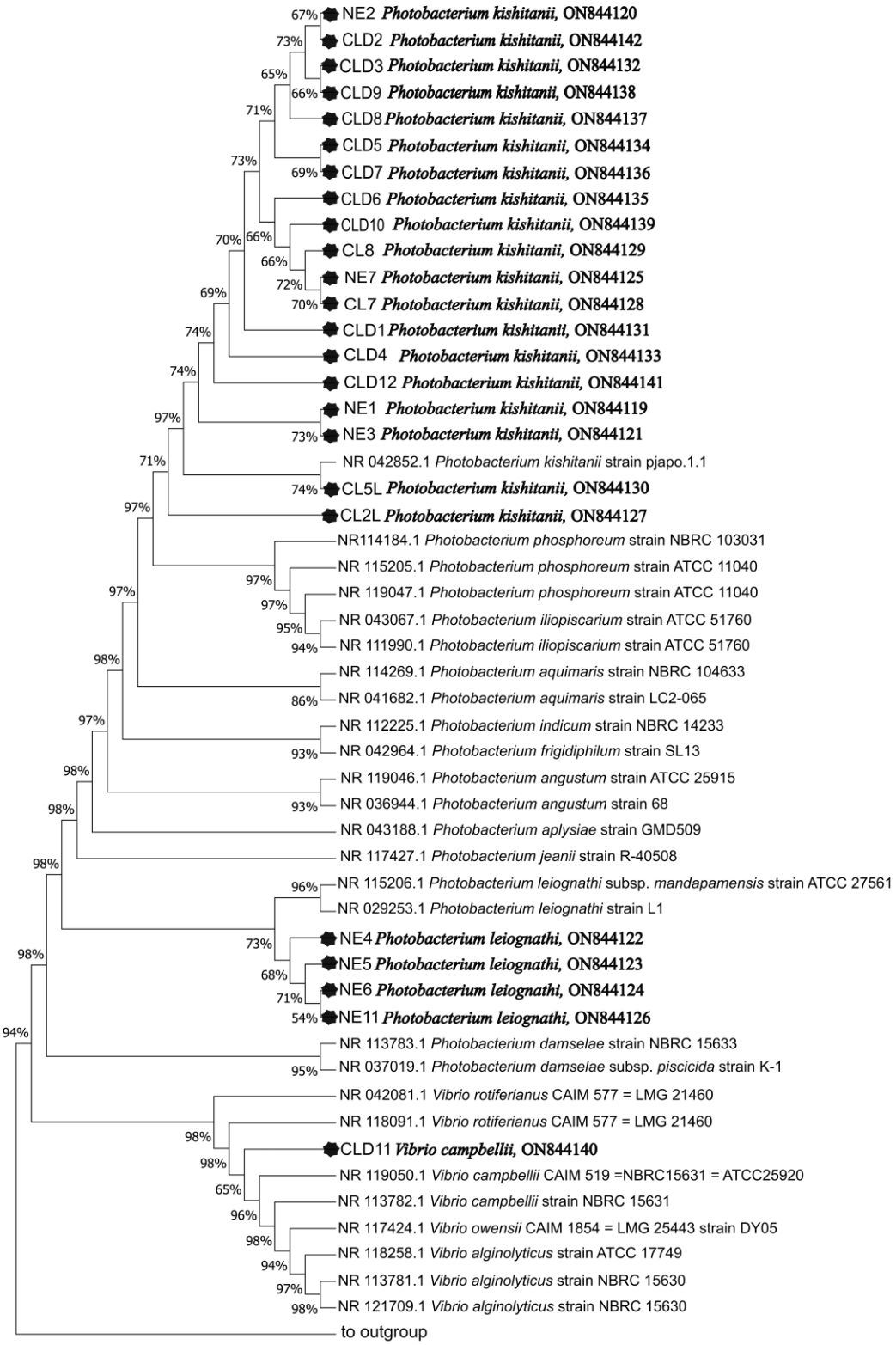

Figure 3. Rooted phylogenetic tree calculated by a Jukes–Cantor distance estimation algorithm showing the affiliation of the most-representative bacterial isolates to the most closely related sequences from either cultivated or cloned bacteria. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree was outgrouped with the 16S rRNA gene sequence of *Methanocaldococcus jannaschii* DSM2661. The representative isolates of this study are indicated by black polygons.

Table 2. Phylogenetic affiliation of luminescent bacteria from the deep sea organisms of the Strait of Messina and the occurrence of the *lux*AB gene.

Strain	AN	Next Relative by GenBank Alignment (AN **, Organism)	Isolation Matrix	Hom § (%)	*lux*AB Gene
		Neorossia caroli			
NE1	ON844119	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	siphon	99	+
NE2	ON844120	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	ventral	98	+
NE3	ON844121	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	ventral	99	+
NE4	ON844122	NR_115206.1, *Photobacterium leiognathi subsp. mandapamensis*	siphon	99	-
NE5	ON844123	NR_029253.1, *Photobacterium leiognathi* strain L1	siphon	99	+
NE6	ON844124	NR_029253.1, *Photobacterium leiognathi* strain L1	siphon	99	+
NE7	ON844125	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	siphon	98	+
NE11	ON844126	NR_029253.1, *Photobacterium leiognathi* strain L1	invagination	99	+
		Chlorophtalmus agassizi			
CL2L	ON844127	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	99	+
CL7	ON844128	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	98	+
CL8	ON844129	NR_115205.1, *Photobacterium phosphoreum* strain ATCC 11040	perianal gland	98	+
CL5L	ON844130	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	99	+
CLD1	ON844131	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	99	+
CLD2	ON844142	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	98	-
CLD3	ON844132	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	98	+
CLD4	ON844133	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	98	+
CLD5	ON844134	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	98	+
CLD6	ON844135	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	98	+
CLD7	ON844136	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	99	+
CLD8	ON844137	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	99	+
CLD9	ON844138	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	99	+
CLD10	ON844139	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	99	+
CLD11	ON844140	NR_113782.1, *Vibrio campbellii* strain NBRC 15631	perianal gland	99	+
CLD12	ON844141	NR_042852.1, *Photobacterium kishitanii* strain pjapo.1.1	perianal gland	99	+

** Accession number; § Homology.

The nucleotide sequences were deposited into the GenBank database under the accession nos. ON844119–ON844142.

4. Discussion

This study is the first to provide evidence of the presence of associated luminous bacteria in the cephalopod *N. caroli* and the fish *C. agassizi*. In our samples, on a total of 17 isolates, we found different associated bacteria belonging to three *Photobacterium* genera, namely, *P. kishitanii, P. leiognathi,* and *P. phosphoreum,* and one belonging to the genus *Vibrio*, namely, *V. campbellii*. In particular, *P. kishitanii* and *P. leiognathi* were associated with *N. caroli*, whereas *P. kishitanii, P. phosphoreum*, and *V. campbellii* were associated with *C. agassizi*.

To date, all known luminescent bacteria involved in symbiotic luminescence with fish and cephalopods are Gammaproteobacteria affiliated with the *Vibrio*, *Photobacterium*, and *Shewanella* genera.

The presence of symbiont luminous bacteria belonging to *Photobacterium* and *Vibrio* has been observed in sepiolids equipped with extrinsic bioluminescence [26,40,55–63]. The sepiolid *Euprymna scolopes* is the most-investigated species for the study of bioluminescence in cephalopods [64]; its luminous organ is a morphologically complex structure, containing crypts that house bioluminescent strains of *Vibrio* as well as reflective tissue, a lens, and a shutter mechanism [65,66]. According to [59], the *E. scolopes–V. fischeri* partnership is ecologically obligate, and juvenile squids develop a light organ with a superficial ciliated epithelium containing "appendages" that help recruit environmental bacteria. The colonization of epithelium crypts by free-living *V. fischeri* leads to light organ morphogenesis.

While studies on *E. scolopes* focused on several aspects of the association with luminous bacteria by describing the morphology of their luminous organs, the characterization of

symbiont bacteria, and their selection mechanism from the environment [59], the other sepiolids are poorly investigated. The symbiont bacteria *P. leiognathi* was found in the light organ of *Rondeletiola minor* in the Mediterranean Sea [63] as well as in *Sepiolina nipponensis* and *Uroteuthis noctiluca* [67]. A recent study [26] on the phylogenomics of Sepiolida reported that *N. caroli* lacks luminescence. In our study, we found *P. kishitanii* and *P. leiognathi* associated with *N. caroli*, which was not previously observed. This finding suggests a possible association between the sepiolid and luminous bacteria, but it is not easy to establish whether these luminous bacteria are real symbionts or not. While all procedures in our study were carried out scrupulously, taking care to treat different tissues separately to avoid cross-contamination, it is not possible to exclude the seawater origin of transient luminous bacteria. A similar scenario was described by [68] for the *P. leiognathi* symbionts that can follow one of two paths upon encountering a host: simply transit for short periods along the specimen, or become symbiont inhabitants. In line with this, the presence of *Vibrio* sp. members was reported in the mantle cavity of *E. scolopes* from seawater [69] but then became representative of the local bacterial population and was able to colonize the host [58]. However, while the mantle cavity and/or the siphon are used for swimming propulsion by pumping water, the finding in our study of luminous bacteria in mantle invaginations is quite interesting because these anatomical parts were more preserved and thus potentially ideal to host symbionts that find these parts a good growth habitat. Given that *N. caroli* lacks light organs, the association between *Photobacterium* and this sepiolid may be a non-obligate relationship and instead facultative, as suggested by a recent study [70], demonstrating the non-species-specific association of five luminous bacteria with the non-luminous squid *Sepioteuthis lessoniana*. Future studies will be devoted to ascertain the occurrence of a symbiotic relationship between these bacteria and *N. caroli* by examining a higher number of specimens and by coupling a metagenomics approach to the traditional culture-dependent strategy.

With regard to *C. agassizii*, our study is the first to characterize the presence of luminous bacteria in the perianal gland of this deep sea fish. To the best of our knowledge, few studies investigated the presence of luminous symbionts in congeneric species [71] or observed the presence of non-identified luminous bacteria in the perianal gland of *Chlorophthalmus albatrossis* from Japanese waters through histological and morphological analyses of the light organ and simple luminescence tests. Our findings are in line with more specific studies that characterized the symbiont bacteria of Chlorophthalmidae, finding that they belonged to the genus *Photobacterium*. Indeed, Dunlap and Ast [7] observed the presence of *Photobacterium phosphoreum* and *Photobacterium iliopiscarium* through a phylogenetic identification of a total of 47 bioluminescent isolates obtained from the perianal gland of *C. albatrossis*. Later, other authors [72,73] reported *P. kishitanii* as the symbiotic luminous strain in four species of Chlorophthalmidae (*C. albatrossis, C. acutifrons*, *C. borealis,* and *C. nigromarginatus*). The finding of *P. kishitanii* in *C. agassizii* from Mediterranean waters (our study) supports the hypothesis formulated by Saito et al. [73], that all members of the genus *Chlorophthalmus* can also harbor the same bacterial species regardless of the distribution of the host. The presence of the *V. campbelli* strain is likely attributable to a temporary transit or to a relationship other than a symbiotic one. Luminous Vibrios are considered etiologic agents of luminous vibriosis in aquaculture industries worldwide [71]. *V. campbelli* is a Gram-negative bacterium widely distributed in marine environments [74], initially misidentified as *Vibrio harveyi* [75,76]. More recently, different studies reported its role as the pathogen responsible for the luminescent disease in farmed shrimp and the mortality in fish and shellfish [74–78]. However, to the best of our knowledge, this is the first observation of *V. campbelli* in *C. agassizii* organisms.

One important issue in the study of luminescent bacteria is their difficult maintenance in laboratory conditions since it requires many precautions and care to keep these bacteria alive and luminescent. In addition to the importance of investigation on their ecological role, different biotechnological and industrial applications of luminous bacteria have recently been highlighted. For instance, bioluminescent bacteria were proposed as indicators or

biosensors for measuring the pollution level and toxicity of chemicals in environmental samples or for detecting possible DNA-tropic and oxidative stress-causing agents [79–81].

It is important to broaden our knowledge about the optimal growth conditions for and maintenance of these bacteria. All isolates tested in our study showed a wide growth-temperature range while better tolerating basic conditions of pH rather than acidic conditions. Salinity was proven to be the greatest factor to affect bioluminescence, with several isolates able to grow at increasing NaCl concentrations but unable to produce luminescence. Moreover, based on our results, the bacterial strains affiliated with *P. kishitanii* and *leiognathi* were strongly inhibited by pH values greater than 9. Recently, some luminous bacteria were reported to be producer of EPSs [80]. In this study, the *P. kishitanii* members provided promising results for the production of EPS with the addition of both glucose and sucrose to the culture medium.

The search for the *lux*AB gene was performed to assess if it could be used as tool for the monitoring and detection of luminous bacteria. The luciferase enzyme catalyzes the light emission reaction in luminescent bacteria, and the *lux*AB gene encodes the *alpha* subunit of luciferase [82–84]. Approximately 90% of luminous isolates showed the presence of the *lux*AB gene, confirming the *lux*AB gene search as suitable screening approach.

Our proposed investigative approach was proven suitable to carry out the study and could also be used in future studies aiming to improve the biotechnological potential of luminous bacteria (i.e., extracellular polymeric molecules production, pathogenicity tests, and adaptative strategies).

5. Conclusions

For many deep sea marine organisms able to produce luminescence, it is hard for scientists to identify whether this function is related to the presence of symbiotic luminescent bacteria. For the first time, we succeeded in isolating luminescent bacteria in two underexplored species, namely, the cephalopod *Neorossia caroli* and the teleost *Chlorophthalmus agassizii*. We obtained strains affiliated with the species *P. kishitanii*, *P. phosphoreum*, and *P. leiognathi* and only one microorganism of the genus *Vibrio*. Moreover, we elucidated some physiological and biotechnological aspects of luminous isolates that were previously poorly investigated as a topical biotechnological issue.

In the future, studies should continue to search for these luminescent symbionts in invertebrate and vertebrate organisms living in deep sea environments, identify their ecological role to assess if they occur in symbiotic relationships as consortia of different taxonomic group, detect the stimulation mechanisms at the base of the luminescent process and the possible quorum-sensing processes, and understand the symbiont bacteria selection by the host and its evolutive scenarios. Further studies are also necessary for improving our knowledge about the biotechnological potential of luminescent bacteria by investigating the production and the optimization of EPSs.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The nucleotide sequences were deposited into the NCBI GenBank database under the accession numbers ON844119–ON844142.

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