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## Dinoflagellate diversity among nudibranchs and sponges from French Polynesia: Insights into associations and transfer



*La diversité des dinoflagellés chez les nudibranches et les éponges de Polynésie française : associations et transfert*

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## ABSTRACT

Symbioses with the dinoflagellate *Symbiodinium* are widespread among marine invertebrates and protists, especially in nutritionally demanding habitats, such as tropical coral reefs, where they play a major role in ecosystem survival. Moreover, apart from corals and sea anemones, many of the *Symbiodinium* species and clades involved in these partnerships remain to be characterized. This study provides new insights into nudibranch and sponge associations with *Symbiodinium* by sequencing regions of the *Symbiodinium* 28S rDNA and the host mitochondrial *COI* oxidase. Specimens were sampled between 2011 and 2013 from locations around the islands of Moorea and Tahiti, French Polynesia. Our results revealed that some of the sponges and nudibranchs harbored typical *Symbiodinium* from clade B or C while others harbored new, undescribed *Symbiodinium*-like dinoflagellates. A detailed analysis of the different life stages of the nudibranch *Phestilla lugubris* and of its specific coral prey, *Porites rus*, suggests a prey-predator horizontal transfer of the symbiont and its vertical inheritance from the parent to the eggs.

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## R É S U M É

Les symbioses à zooxanthelles, dinoflagellés du genre *Symbiodinium*, sont largement répandues chez les invertébrés marins et les protozoaires, notamment dans les eaux oligotrophes, où ils jouent un rôle important dans la survie des récifs coralliens. De plus, excepté chez les coraux et les anémones, les différentes espèces et clades de *Symbiodinium* impliqués dans ces associations restent à être caractérisés. Notre étude, grâce à l'utilisation des marqueurs génétiques 28S ADNr des *Symbiodinium* et la sous-unité 1 de la cytochrome oxydase mitochondriale (*COI*) de l'hôte, procure de nouvelles données quant aux associations des éponges et des nudibranches avec les *Symbiodinium*. L'échantillonnage a

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eu lieu autour de Moorea et de Tahiti (Polynésie française), entre 2011 et 2013. Nos résultats révèlent que certaines espèces d'éponges et de nudibranches possèdent les clades B et C communément retrouvés, chez les coraux notamment, alors que d'autres présentent de nouveaux dinoflagellés « *Symbiodinium*-like ». Une analyses détaillée des *Symbiodinium* à différents stades du cycle de vie du nudibranche corallivore *Phestilla lugubris* et de son corail hôte *Porites rus* suggèrent l'existence d'un transfert horizontal de *Symbiodinium* proie-prédateur et un transfert vertical du parent *P. lugubris* à ses œufs.

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## 1. Introduction

The high number of marine invertebrate hosts that have evolved towards establishing symbiotic relationships with photosynthetic dinoflagellates of the genus *Symbiodinium* [1] suggests the highly valuable competitive metabolic advantage provided by such associations [2]. This is especially true in the shallow and nutrient-poor tropical waters. Indeed, the symbiotic dinoflagellates can provide up to 90% of the host's energetic requirements, in the form of photosynthetic products [3]. *Symbiodinium* were initially considered as belonging to one single species, *Symbiodinium microadriaticum* [4]. To date up to nine different clades (from A to I) of *Symbiodinium* have been identified, with each clade being itself composed of numerous sub-clades [5,6]. Clades A, C and D have been reported as present in hard corals from Moorea [7]. Despite our developed knowledge on coral-*Symbiodinium* associations, very little is known about associations involving other invertebrate groups, such as sponges and nudibranchs.

Marine sponges (Porifera) represent the second largest biomass on tropical reefs after corals [8]. They play an important role in removing detritus and organic nutrients from water [9]. Although marine sponges are known to contain a range of microbial symbionts, e.g., ammonium-oxidizing archaea, sulfate-reducing bacteria, and cyanobacteria with a photosynthetic function [10,11 and references within], only a few of them have been reported to harbor *Symbiodinium*. The sponge *Haliclona* sp. is characterized by the presence of dinoflagellates [9], but whether these are from the genus *Symbiodinium* or not still needs to be addressed from a molecular point of view. To our knowledge, *Symbiodinium* clades have only been characterized in excavating sponges from the genus *Cliona* [12]. Those sponges acquire their symbionts when digesting the coral substratum they live in. Other than these two types of sponges, barely anything is known about sponge-*Symbiodinium* associations.

Nudibranchs (Mollusca) are among the most ecologically and morphologically diverse of all gastropod taxa [13]. Some of them have been identified as having *Symbiodinium* in their tissues. These are mostly carnivorous nudibranchs feeding on sponges, anemones [14], hydroids, or corals [15], which themselves contain *Symbiodinium*. These nudibranchs are referred to as “solar powered” and are assumed to obtain their *Symbiodinium* directly from their prey [16]. The dinoflagellates they obtain are stored alive within the epithelial cells of the

finely branched digestive system, which leads into the skin layers of their intermittently flattened cerata. This system enables the host to adjust the symbionts' exposure to sunlight [15]. This mutualistic relationship with symbiotic algae, and its subsequent host adaptation, have been studied only on the genera *Phylloidesmium* [17] and *Pteraeolidia* [18].

The focus of this study was to identify sponge- and nudibranch-*Symbiodinium* associations, to assess their specificity, and to find out more about their acquisition by transfer as nudibranchs and sponges are crucial in the sense that they could represent important *Symbiodinium* reservoirs and vectors.

## 2. Materials and methods

### 2.1. Specimen collection and storage

Nudibranch and sponge samples were collected using a combination of SCUBA from vessels, shore-based SCUBA, and snorkel from locations around the islands of Moorea (17.5333°S, 149.8333°W) and Tahiti (17.6667°S, 149.4167°W), French Polynesia, between 2011 and 2013 (Table 1). In addition, a fragment of a *Porites rus* coral colony – harboring eggs and adults of the nudibranch *Phestilla lugubris* – was collected from the field and kept for one month in an aquarium (open water flow). Eggs, juveniles and adult nudibranchs were separated from their coral substratum. All specimens were preserved in 85% ETOH at 4 °C.

### 2.2. DNA extraction, Amplification and sequencing

Whole genomic DNA was extracted from the ethanol-preserved tissue. A CTAB protocol, modified after Mieog et al. [19], was used. Specific genomic regions typically used as a reference for barcoding were amplified by PCR.

To confirm the taxonomic identification of the collected species, “universal” primers [20] were used to amplify by PCR a 658 bp DNA region of the cytochrome *c* oxidase subunit 1 gene (*COI*) from either the nudibranch or sponge mitochondrial genome. To detect symbionts, a close to 580 bp region of the *Symbiodinium* large ribosomal subunit RNA (28S rDNA) was amplified with Richter's universal primers [21]. Amplifications by PCR were performed following the manufacturer's protocol (Promega Corporation, Madison, WI). Amplicons were purified and sent for sequencing of both strands to Macrogen

**Table 1**  
Characteristics of the specimens presented in this study.

	Location, depth	Replicates	Dinoflagellates	Accession No.
<i>Lamellodysidea herbacea</i> OTU QM2538	Tahiti, 35m	3	<i>Symbiodinium</i> Clade C	KJ500000
<i>Aeolidiella alba</i>	Moorea, fringing reef, 8 m	6	Clade B	KJ500001
<i>Porites rus</i>	Moorea, fringing reef, 1.5 m	1	Clade C	KJ499999
<i>Phestilla lugubris</i> Adults	Moorea, fringing reef, 1.5 m	6	Clade C	KJ499999
Juvenils		6		
<i>Haliclona</i> sp. OTU QM4715	Moorea, 17 m	7	<i>Dinophysis</i>	KJ499997
<i>Phycopsis</i> sp. OTU QM1640	Tahiti, 20 m	6	<i>Dinophyceae</i> sp.	KJ499996
<i>Flabellina</i> sp.	Moorea, barrier reef, 5 m	4	<i>Gymnodinium</i>	KJ50002

([www.macrogen.com](http://www.macrogen.com)). Complementary sequences were edited and assembled into contigs using Genious pro 5.5.6 software (Biomatters Ltd, New Zealand). Multiple sequence alignment was performed using MEGA5 software [22]. Gene sequences were deposited in GenBank.

### 2.3. Species determination and phylogenetic reconstruction

The amplified sequences of the partial *COI* and 28S rDNA regions were compared with the sequences available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI) by using its World Wide Web site (<http://www.ncbi.nlm.nih.gov>), and the BLAST-algorithm [23]. Publicly available sequences exhibiting the highest similarities with our data have been retained to construct the dinoflagellate tree. The phylogenetic reconstruction was performed by the maximum-likelihood method with the nucleotide substitution model “General Time Reversible” using a discrete Gamma distribution (best model fitted) embedded in the MEGA5 software for dinoflagellate 28S data set. 28S rDNA sequences of species of the phylum Apicomplexa were chosen as outgroup for the dinoflagellate tree (Fig. 1).

## 3. Results and discussion

The aim of the present study was to uncover new *Symbiodinium* associations in two marine invertebrate hosts: nudibranchs and sponges in order to examine their specificity, as well as their mode of transfer as stated above. Firstly, we confirmed the taxonomic identification of the collected species by sequencing the hosts’ *COI* region. Novel sequences of nudibranch *COI* genes from Tahiti and Moorea were submitted to GenBank (KJ522456–KJ522466). Secondly, 28S rRNA amplicons were sequenced to detect the potential presence of the symbionts in the host tissue. We chose the 28S rDNA for the simple discrimination taxonomy because of its robustness for molecular taxonomy of the currently recognized *Symbiodinium* clades [24].

### 3.1. *Symbiodinium*

Hill et al. [12] showed by cloning that excavating cionaid sponges from the Indo-Pacific region harbor

*Symbiodinium* from clades A, C, and G, with the majority of sponge hosts harboring only one clade. This report presents the first evidence that a filtering sponge (OTU QM2538) harbors clade C *Symbiodinium* (KJ500000) (Fig. 1). To date it has only been frequently observed that *L. herbacea*, and other related sponges, host filamentous cyanobacterium *Oscillatoria spongellae* [25]. The fragment (KJ500000) showed similarity to *Symbiodinium* C90 (JN558047) hosted by foraminifera *Sorites* sp. [26] Except for the fact that type C90 has previously been found in corals [7], relatively little is known about this association.

Concerning nudibranchs, this study is also the first to date to examine the presence of *Symbiodinium* in numerous species. The 28S rDNA dinoflagellate primers successfully amplified a 565 bp fragments in different species of nudibranch tested as well as in the coral *Porites rus*. Our results show for the first time that the nudibranch *Aeolidiella alba* harbors a clade B representative (KJ500001) with a high similarity to the subclade B1 found in the soft coral *Plexaura kuna* (JN558057). Clade B had never been found before in French Polynesia, but previous studies had only looked at hard corals [7, unpublished work of the CRIOBE]. It is noteworthy that Venn et al. revealed the abundance of *Symbiodinium* clades A and B in a tropical sea anemone [27]. Moreover, members of the genus *Aeolidiella* feed on anemones, which means that *A. alba* could have taken up clade B from their prey. It would therefore be interesting to find out more about the *Symbiodinium* associated with anemones of French Polynesia.

Finally, this study shows the first direct sequence comparison of the *Symbiodinium* found in a nudibranch and its coral substratum, except Ziegler et al. [28]. All life stages of the nudibranch *Phestilla lugubris* (KJ522463), from eggs, to juveniles and to adults (Fig. 1) were collected, and sequence analysis revealed a new 28S rRNA gene of a so far unknown subclade C (KJ499999). Not only was the same clade detected in eggs, juveniles and adults, but the sequence alignment also revealed a 100% identity along 522 bp. This significant result supports that *P. lugubris* is very likely to be a case of direct inheritance of the symbionts (vertical transfer). In addition, the associated invertebrate host species – the coral *Porites rus* – on which the predator *P. lugubris* feeds, was analyzed. Sequencing data revealed that both the nudibranch and the coral carry

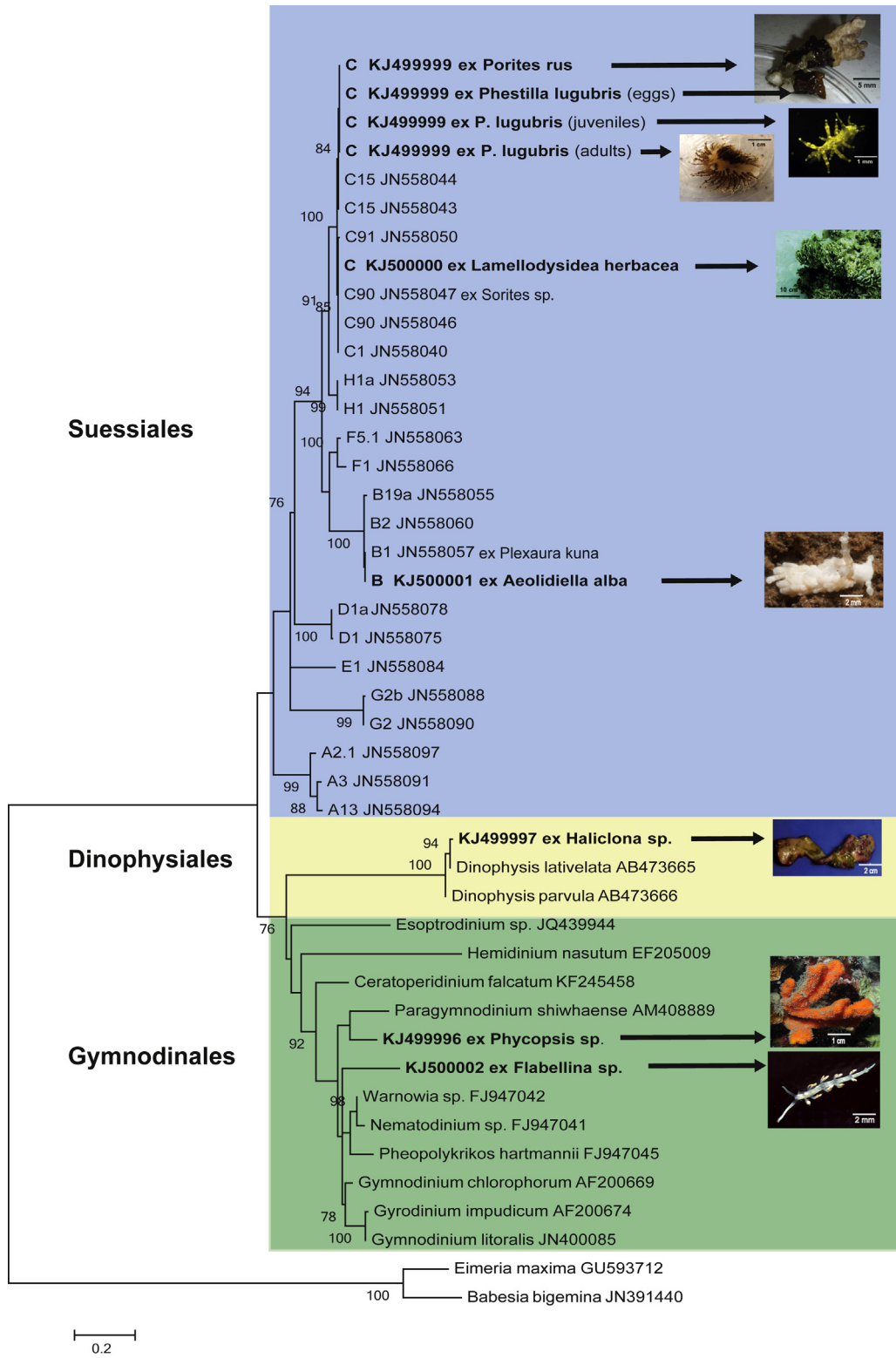


Fig. 1. (Color online.) Maximum-likelihood reconstruction of the Dinoflagellate 28S nuclear ribosomal DNA genotypes of nudibranch and sponge specimens from the islands Moorea and Tahiti in French Polynesia. Controls of the order Suessiales, Gymnodinales, and Dinophysiales were included in the analysis, as well as outgroup organisms of the phylum Apicomplexa. Node numbers indicate percentage bootstrap support from 500 replicates. Nodes without bootstrap values were supported by less than 75% of the replicates.

exactly the same clade of *Symbiodinium* (KJ499999) (Fig. 1). The novelty of this work comes from the fact that those two species had never been described as interacting together, and that all life stages of *P. lugubris* occur on *P. rus*. This argues in favor of the existence of a horizontal transfer of symbionts, from coral to adult nudibranchs, which in turn will transfer its symbionts to their eggs.

### 3.2. Dinophysis/Gymnodinium

The size of the obtained PCR fragments did not always correspond to the expected 565 bp 28S rDNA size: in addition, another 700 bp amplicon was sometimes detected. The amplified 700 bp fragment (KJ499997) of the sponge *Haliclona* sp. (OTU QM4715) had a similar sequence (94%) to the free-living dinoflagellate *Dinophysis laticellata* (AB473665) [29] (Fig. 1). The sponge *Phycopsis* sp. (OTU QM1640), which was expected to be *Symbiodinium*-free, was found to host species (KJ499996) with a 28S gene sequence similar to a genus of a new planktonic mixotrophic dinoflagellate *Paragymnodinium shiwhaense* n. gen., n. sp. isolated from coastal waters off Western Korea (AM408889) (Fig. 1). The latter has recently been described by Kang et al. [30].

The partial 28S DNA sequence (KJ500002) amplified from the nudibranch *Flabellina* sp. suggests that it could either be a new genus or a new species within the order *Gymnodiniales* or genus *Gymnodinium* (Fig. 1).

Two possible hypotheses could explain these new observations: (i) *Dinophysis* and *Gymnodinium* are free-living and use host tissues as habitats to aggregate and reproduce or (ii), host tissues contain symbiotic lineages of *Dinophysis* and *Gymnodinium*. Histological analyses should shed more light on this. To date, only planktonic *Dinophysis* and *Paragymnodinium* are referenced. It is known that dinoflagellates *Gymnodinium* spp. and *Pfisteria piscicida* kleptoplastids (notably chloroplasts) are photosynthetically active for only a few days, while kleptoplastids in *Dinophysis* spp. can continue to function for two months [31] and therefore could be an alternative pathway to provide energy to the host.

### 3.3. Associations

Correlation between the clades present in various hosts from a single location reveals that clades A, C and D were found in corals from Moorea's lagoon [7]; clade B and C were found in the studied nudibranch and sponge specimens from Moorea's and/or Tahiti's lagoon (Table 1). Since nudibranchs can move from one location to another and release healthy symbionts to the environment [15], this study reveals their ability to act as potential reservoirs and vectors of viable *Symbiodinium* for the whole reef ecosystem. They may be able to provide the corals or anemones they feed on with *Symbiodinium*, by discharging them in their vicinity, allowing, for example, symbiont switching after a bleaching event or in case of changing environmental conditions. A more comprehensive set of data concerning the clades present, their uptake, turnover and seasonal flexibility in nudibranchs and sponges, together with their coral substrates or preys, will enable

the assessment of their role in buffering the coral reefs' response to climate change and ocean acidification.

To conclude, this work provides numerous new elements, which will serve to better characterize the nature of the relationships involving *Symbiodinium* and invertebrate host species. Other areas of interest should now be considered, such as the study of metabolic exchanges between host and symbionts: the rate and nature of nutrient exchanges has not yet been studied in sponges and nudibranchs. The extent to which they rely on the dinoflagellates for survival is still under investigation. Also, the question of the stability and specificity of the relationship needs to be studied more extensively. Indeed, it has been recently demonstrated in corals [7] that flexible hosts exhibit a higher sensitivity to environmental changes; they have a higher propensity to bleach and higher mortality rates. Hosts, which have a high degree of fidelity towards their symbionts, may be more environmentally resilient. Compared to corals, sponges and nudibranchs have alternate and quite efficient feeding mechanisms, which make the role of *Symbiodinium* clades less important in their acclimatization to changing environments. Further investigations on the sponges and nudibranchs' ability to switch from one *Symbiodinium* clade to another linked to their fitness in a changing environment will determine if specific associations are required for survival.

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