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Base composition of DNA from symbiotic dinoflagellates: a tool for phylogenetic classification

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Abstract. DNA of eight endosymbiotic dinoflagellates (zooxanthellae) from seven different host species has been analyzed as to its thermal characteristics and base composition by means of spectrophotometry and high performance liquid chromatography. All algae under investigation contain both methylcytosine and hydroxymethyluracil in addition to the bases typical of nuclear DNA. As a result, melting temperatures are decreased, suggesting lower contents of guanine plus cytosine than actually present. True percentages of guanine plus cytosine plus methylcytosine range from about 43 to 54 mol%. They are unique for the symbionts from different hosts, indicating phylogenetic separation of the taxa comparised within the genus *Symbiodinium*.

Key words: Dinoflagellates – DNA composition – Hydroxymethyluracil – Methylcytosine – Speciation – *Symbiodinium* – Symbiosis – Zooxanthellae

Two morphologically distinct forms of dinoflagellates are currently known among eukaryotic endosymbionts in the marine environment. The group of amphidinioid zooxanthellae is composed of several species of the genus *Amphidinium* (Blank and Trench 1986; Trench and Blank 1987), while the gymnodinioid type was for a long time regarded as representing a single pandemic species of symbiotic dinoflagellate (cf. Freudenthal 1962; Taylor 1974), including varieties (cf. Taylor 1984) or nomina nuda (Hollande and Carré 1974; Duclaux 1977). It has now been established that gymnodinioid symbionts are also heterogeneous, and that their species belong to the genus *Gymnodinium* (Spero 1987) as well as to the taxon *Symbiodinium* (Trench and Blank 1987).

Symbiodinium has been recorded free-living outside its hosts only twice (Loeblich and Sherley 1979; Taylor 1983). Studies were performed in situ, or on cultured symbionts in vitro. However, isolated zooxanthellae grow very slowly. It is difficult to bring them into culture, and even harder to bring them into mass culture. Therefore, it is not surprising that little emphasis has been placed on studying their DNA composition. To our knowledge, work including symbiotic dinoflagellates for DNA analyses is very rare (Franker 1970; Rae 1976). Data obtained from these studies have never served as arguments for phylogenetic discussions. Thus, even now the bulk of descriptions of symbiotic dinoflagellates is based on their morphology, with some more sophisticated techniques like behavioral, biochemical and physiological characterizations and karyotyping in tandem with three-dimensional reconstructions employed for the recognition of different species within the genus *Symbiodinium* (for review, see Blank and Trench 1985a, b; Trench and Blank 1987).

Besides the need for identifying zooxanthellae from different hosts as to their G+C contents, DNA of dinoflagellates in general bears interesting evolutionary aspects due to the occurrence of partially high amounts of methylated bases like methyladenine, methylcytosine and especially hydroxymethyluracyl, substituting the bases customarily present in eukaryotes (cf. Rae and Steele 1978). We have therefore analyzed the DNA base composition of gymnodinioid zooxanthellae from seven different host species of five different orders of marine invertebrates. In order to meet representative selection of symbionts, we studied *Symbiodinium*-like dinoflagellates from a jellyfish, sea anemones, a zoanthid, and a stony coral, where the algae occur as intracellular cytosymbionts each enclosed within a single host vacuole in nature, as well as giant clams harboring intercellular endosymbionts in their haemal sinuses. Also, care was taken for choosing worldwide distribution of the hosts from which the symbionts had been originally isolated, using algae of invertebrates collected from Caribbean waters as well as from the Indopacific, with one identical host species originating from Palau and the Eniwetok atoll.

Our aim was to resolve three questions: (1) would different G+C compositions be uncovered in the DNA of zooxanthellae from different hosts; (2) are modified bases substituting for the canonical bases and to what extent; and

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Abbreviations: dA, deoxyadenosine; dC, deoxycytidine; dG, deoxyguanosine; dT, deoxythymidine; m5dC, 5-methyldeoxycytidine; hmdU, 5-hydroxymethyldeoxyuridine; rC, ribocytidine; Br8G, bromine-8-guanosine; A, adenine; C, cytosine; G, guanine; T, thymine; m5C, 5-methylcytosine; hmU, 5-hydroxymethyluracil; G + C, guanine plus cytosine plus 5-methylcytosine; HPLC, high performance liquid chromatography; T_m, temperature at the midpoint of hyperchromic shift; CTAB, N-cetyl-N,N,N-trimethylammonium bromide; EDTA, ethylenediamine-tetraacetic acid, disodium salt; TRIS, tris-(hydroxymethyl)-aminomethane; 1 × SSC, standard saline citrate (0.15 M NaCl + 0.015 M trisodium citrate, pH 7.0)

Table 1. Cultures of zooxanthellae employed

Symbiont taxon	Host taxon	Collection site	Original isolation
Division Pyrrophyta	Phylum Cnidaria		
Class Dinophyceae	Class Scyphozoa		
Order Gymnodiniales	Order Rhizostomeae		
Family Gymnodiniaceae	Family Cassiopeidae		
Symbiodinium microadriaticum[a-d]	*Cassiopea xamachana*	Florida	S. S. Chang
	Class Anthozoa		
	Order Actiniaria		
	Family Actiniidae		
Symbiodinium sp.[b]	*Condylactis gigantea*	Jamaica	S. S. Chang
	Family Aiptasiidae		
Symbiodinium sp.[b]	*Aiptasia pulchella*	Hawaii	N. J. Colley
Symbiodinium sp.[b]	*Aiptasia tagetes*	Puerto Rico	D. A. Schoenberg
	Order Zoanthinaria		
	Family Zoanthidae		
Symbiodinium pilosum[c]	*Zoanthus sociatus*	Jamaica	D. A. Schoenberg
	Order Sceleractinia		
	Family Acroporidae		
Symbiodinium kawagutii[c]	*Montipora verrucosa*	Hawaii	R. York
	Phylum Mollusca		
	Class Bivalvia		
	Order Veneroida		
	Family Tridacnidae		
Symbiodinium sp.[b]	*Tridacna maxima*	Palau	R. K. Trench
Symbiodinium sp.[b]	*Tridacna maxima*	Eniwetok	S. S. Chang

[a] Cf. Freudenthal (1962)
[b] Cf. Blank and Trench (1986)
[c] Cf. Trench and Blank (1987)
[d] Type species

(3) could one or both of these characters serve as a tool for phylogenetic classification of zooxanthellae.

Materials and methods

Origin and maintenance of cultures

Unialgal stock cultures of *Symbiodinium microadriaticum* Freudenthal emend. Trench et Blank, *S. kawagutii* Trench et Blank, *S. pilosum* Trench et Blank, and five dinoflagellates regarded as *Symbiodinium* sp. were provided to us by R. K. Trench, University of California at Santa Barbara (Table 1). All cultures were maintained in modified ASP-8 A medium as described by Blank (1987a).

Mass cultures were started with a primary inoculum of 5 ml, that was inoculated into 100 ml and transferred further to 750 ml and finally 4 l medium. Six parallel cultures of each isolate were grown simultaneously at 27° C on a 14 h light: 10 h dark regime at an illumination of about 2 klx and a relative humidity of 80 to 90%, except for the last stage where they were kept at 25° C under continuous light of 3 klx and were bubbled with compressed air supplemented with 3% CO_2. Preceding each transfer, cultures were checked for purity by phase contrast microscopy. Each stage was then maintained for two weeks to allow sufficient proliferation time up to the late log-phase of growth.

Preparation of DNAs

Algae were harvested from 24 l of culture in a WKF continuous-flow centrifuge during 2 h at 11,000 rpm and 4° C, and were freed from any bacteria by repeated washings in TRIS-EDTA buffer (0.05 M TRIS, 0.03 M EDTA, pH 7.0). Cells were then mechanically disrupted with glass beads as described by Kerfin and Kessler (1978); time necessary for complete disruption varied between 40 and 90 s. The cell extract was separated from the glass beads by several buffer washes through a sinter-filter. Isolation and purification of DNA was after Marmur (1961), using CTAB, RNase and pronase for removal of polysaccharides, RNA and proteins, as described by Huss et al. (1986).

Total DNA yield from 24 l algal culture ranged between 0.1 and 1.5 mg in most cases. DNA from *S. microadriaticum* was about 5 mg and could be separated further in nuclear and chloroplast fractions by means of CsCl density gradient centrifugation for 40 h at 40,000 rpm and 20° C in a Beckman L-M 8 ultracentrifuge. The clear separation yielded about 1.5% chloroplast DNA. We have to assume contamination with about 1 to 2% plastidial DNA in those preparations, which were not subject to density gradient separation.

Analysis of base compositions

Melting profiles of DNA were determined in 1 × SSC, using a Gilford 2600 spectrophotometer equipped with a thermoprogrammer. G + C percentages were then calculated from the resulting T_m-values by the equation of De Ley (1970). DNA from *Escherichia coli* was employed as reference. For HPLC measurements (cf. Kuo et al. 1980; Ebert 1985), DNA was digested further by nuclease and alkaline phosphatase, and analyzed at 30° C in TRIS/HCl buffer, using a reversed-phase Supelcosil LC-18-S 250 × 4.6 mm column with the appropriate nucleosides as a standard

double-checked against *Escherichia coli* and calf thymus DNA.

Results

Nuclear DNA

All symbiotic dinoflagellates under investigation contain DNA composed of six major components. Figure 1 shows an HPLC chromatogram which demonstrates typical base separation. Smaller quantities can be identified as clearly as larger ones (Table 2). We have not adjusted any ratios when the measured percentages of pyrimidine bases were not balanced with equal amounts of purine bases. These unexpected cases are explained by minor contamination with chloroplast DNA and partial deamination.

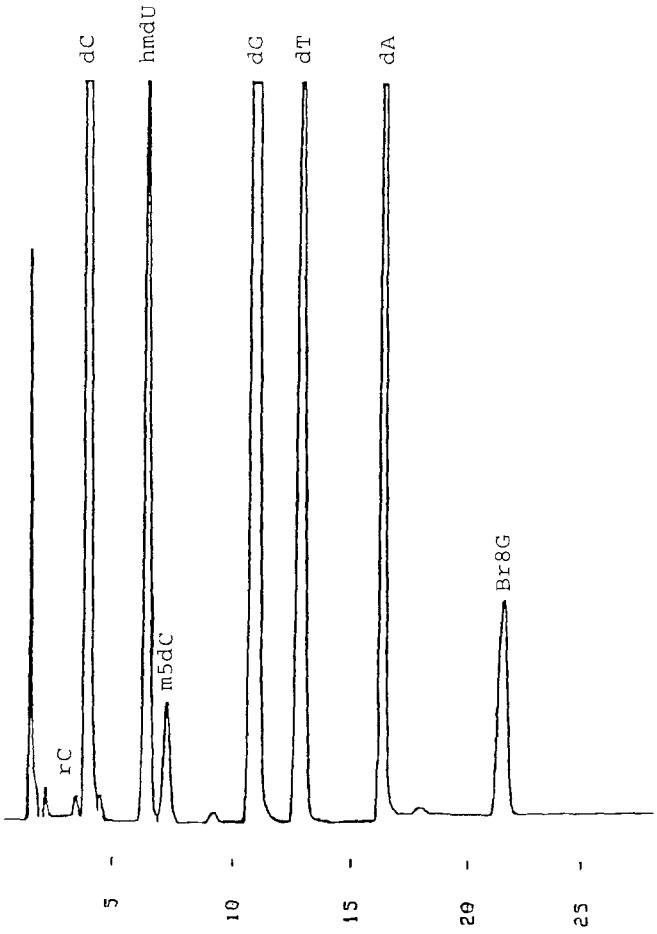

Fig. 1. HPLC chromatogram showing distribution of nucleosides in nuclear DNA of *Symbiodinium* sp. from *Tridacna maxima* collected off Palau. Due to clear separation of contents, even low quantities can be discerned. Minor amounts of ribonucleosides are present in this preparation as indicated by ribocytidine. Bromine-8-guanosine is included as internal standard

Methylcytosine and hydroxymethyluracil replace parts of cytosine and thymine, respectively. The degree of substitution differs among the algae isolated from different hosts and depends on the substituting base. Methylcytosine is present in relatively small amounts, ranging from about 1 to 3 mol%. Hydroxymethyluracil occurs in respectable quantities. It may replace up to half of actual thymine, covering a percentage of about 5 to 12 mol% of DNA. Large ratios of hydroxymethyluracil are combined with large or small amounts of methylcytosine, and vice versa.

Methylated bases have an influence on both buoyant density and melting point of DNA (Rae 1976). Our results from HPLC analyses confirm that G+C figures as calculated from the thermal behavior are not representative if the investigated DNA contains modified bases (Table 3). Thermostability and, therefore, calculated G+C percentage decreases, as replacement with substituted bases increases. Actual G+C contents are 3 to 18 mol% above the photometrically determined values.

True G+C percentages are heterogeneous among the algae harbored by different hosts as well as between the two isolates from an identical host species collected at different localities. The smallest level occurs at about 45 mol% and has been found in *Symbiodinium* sp. from *Aiptasia tagetes* and in *S. kawagutii*, respectively; the largest amount of about 54 mol% is present in the isolate from *A. pulchella*. G+C contents of the other five zooxanthellae range in between. With one exception, G+C ratios are correlated with hydroxymethyluracil percentages, while apparently no correlation exists for methylcytosine.

When the different amounts of G+C and methylated bases are taken into joint consideration, none of the symbionts has an identical DNA composition. There is even a slight difference between the two isolates from *Tridacna maxima* that originated from different locations in the Indopacific. Best fit in all characteristics is obtained between the type species *S. microadriaticum* and *Symbiodinium* sp. inhabiting *Condylactis gigantea*, which were both derived from the Caribbean.

Different DNA compositions were also reported for algal isolates from the California sea anemone *Anthopleura*

Table 2. HPLC analyses of nuclear DNA from zooxanthellae

Symbiont species	Host species	Origin	dA (mol%)	dT (mol%)	hmdU (mol%)	dG (mol%)	dC (mol%)	m5dC (mol%)
S. microadriaticum	*C. xamachana*	Florida	25.8	12.3	11.4	26.0	23.5	1.0
Symbiodinium sp.	*C. gigantea*	Jamaica	25.6	13.0	11.4	25.5	23.4	1.1
Symbiodinium sp.	*A. pulchella*	Hawaii	ND	ND	6.9[a]	ND	ND	3.3[a]
Symbiodinium sp.	*A. tagetes*	Puerto Rico	29.2	22.7	4.9	22.1	19.2	1.9
S. pilosum	*Z. sociatus*	Jamaica	26.4	13.1	11.5	25.2	22.1	1.7
S. kawagutii	*M. verrucosa*	Hawai	27.5	21.2	4.9	23.8	20.6	2.0
Symbiodinium sp.	*T. maxima*	Palau	26.5	12.9	11.0	25.4	23.1	1.1
Symbiodinium sp.	*T. maxima*	Eniwetok	25.5	12.2	11.3	23.4	25.8	1.8

[a] Approximation due to small DNA yield
ND, Not determined

Table 3. Thermal properties and base ratios of nuclear DNA from zooxanthellae

Symbiont species	Host species	Origin	T_m[a] (°C)	Calculated G+C[b] (mol%)	Actual G+C[c] (mol%)	Replacement of C+m5C by m5C (%)	Replacement of T+hmU by hmU (%)
S. microadriaticum	*C. xamachana*	Florida	86.9	42.9	50.5	4.1	48.1
Symbiodinium sp.	*C. gigantea*	Jamaica	86.3	41.2	50.0	4.5	46.7
Symbiodinium sp.	*A. pulchella*	Hawaii	84.4	36.6	54.2[d]	11.4[d]	24.6[d]
Symbiodinium sp.	*A. tagetes*	Puerto Rico	86.1	40.8	43.2	9.0	17.8
S. pilosum	*Z. sociatus*	Jamaica	84.9	37.8	49.0	7.1	46.7
S. kawagutii	*M. verrucosa*	Hawaii	87.3	43.8	46.4	8.8	18.8
Symbiodinium sp.	*T. maxima*	Palau	86.6	41.9	49.6	4.5	46.0
Symbiodinium sp.	*T. maxima*	Eniwetok	ND	ND	51.0	6.5	48.1

[a] Analyzed in 1 × SSC
[b] Derived from T_m by the equation of De Ley (1970)
[c] Total of G+C+m5C derived from HPLC analyses
[d] Approximation due to small DNA yield
ND, Not determined

elegantissima (Franker 1970) and the giant clam *Hippopus hippopus* collected at the Great Barrier Reef (Rae 1976). By means of paper and thin-layer chromatography, G+C ratios of these symbiotic dinoflagellates were assessed at 35.4 mol% for the previous and 46.2 mol% for the latter. It is clear that different DNA organization of the symbionts is reflected in different karyotypes. Chromosome numbers and corresponding G_2 volumes of 97 (1.6 μm^3), 78 (4.5 μm^3), 50 (3.7 μm^3), and 26 (3.2 μm^3) have been ascertained for the zooxanthellae of *Cassiopea xamachana, Zoanthus sociatus, Anthopleura elegantissima*, and *Montipora verrucosa*, respectively (cf. Blank and Trench 1985a, b; Trench and Blank 1987).

Chloroplast DNA

We also analyzed the G+C percentage of chloroplast DNA from *S. microadriaticum* and determined it as 33.8 mol% from calculations based on thermal behavior. Plastidial DNA does not contain modified bases (Franker 1970). The G+C content of chloroplast DNA from *S. microadriaticum* can therefore be compared directly with the paper chromatographic value found for chloroplast DNA of *Symbiodinium* sp. from *A. elegantissima*, which contains 29.2 mol% G+C (Franker 1970).

The plastids of these two algae are quite distinct as to their ultrastructure (cf. Blank and Trench 1985b; Blank 1986). The chloroplast of *S. microadriaticum* is characterized by parallel thylakoid arrangement, double-stalked pyrenoid and clustered carboxysomes. The chloroplast of *A. elegantissima* algae has parallel and peripheral thylakoid arrangement (girdle lamellae), but no carboxysomes; its pyrenoid is connected to it by four to six stalks. Hence, phenotypical differences among these organisms are corroborated by different compositions of nuclear as well as chloroplast DNA.

Discussion

Recognition of species among gymnodinioid zooxanthellae has long been hampered for lack of genetic evidence. There are some notes on sexual stages (cf. Freudenthal 1962; Taylor 1973). Based on our studies, however, no generative production is involved in the life history of *Symbiodinium* (Blank 1987b), as has been indicated by Fitt and Trench (1983). Thus, crossing experiments can not be performed for the assessment of sibling species, as is the case in *Chlorella*. Investigations of nuclear DNA as employed for characterizing this green alga (cf. Douglas and Huss 1986; Huss et al. 1986) seemed therefore quite appropriate for zooxanthellae. In addition, we analyzed chloroplast DNA from *S. microadriaticum*, and tested if the amounts of substituting bases present in nuclear DNA of dinoflagellates would be of taxonomic value.

This study shows that DNA composition is different among the gymnodinioid symbionts isolated from different hosts, opposing the idea of one pandemic species inhabiting tridacnids (Taylor 1969) or marine invertebrates in general (cf. Taylor 1974, 1984). In fact, our results explain why zooxanthellae harbored by different hosts have so many distinct characters: (1) morphology of gymnodinioid and coccoid cells; (2) karyotypes; (3) plastidial and mitochondrial structures; (4) isoenzyme patterns; (5) sterol compositions; (6) isoelectric forms of peridinin-chlorophyll$_a$-proteins; (7) CO_2 metabolism; (8) photoadaptation; (9) life cycle physiology; and (10), one of the most important criteria in symbiotic systems, infectivity (for review, see Blank and Trench 1985a, b; Trench and Blank 1987).

Our data indicate that geographical separation is of similar importance to evolutionary differentiation of zooxanthellae as is their host specificity. The observed diversification among the different isolates parallels the situation reported for the taxa comprised within the free-living dinoflagellate *Crypthecodinium cohnii*. Genetic analysis could resolve sexually incompatible species of *C. cohnii*-like algae collected at distant localities (cf. Beam and Himes 1977, 1982), which are also distinct in biochemical markers (Beam and Himes 1987). These results have been corroborated by the patterns of restriction endonuclease cleavage of ribosomal RNA genes. The G+C percentages of two different species from the *C. cohnii*-complex were determined as 41.3 mol% and 56.9 mol%, respectively (Steele and Rae 1980). We have pointed out that a similar difference of G+C ratios is present in our organisms, accounting for speciation within the genus *Symbiodinium*.

Significance of modified bases in dinoflagellate DNA is poorly understood (cf. Loeblich 1984). Rae and Steele (1978)

gave four explanations for large amounts of hydroxymethyluracil being present in dinoflagellate DNA: (1) involvement in chromosome structure; (2) role in a modification-restriction system; (3) requirement for nuclear metabolism of dinoflagellates; or (4) vestige of an earlier important function. If only one of these explanations is of any importance, then its different amounts recorded for the different symbionts can be regarded as another tool in the assessment of speciation, in addition to G+C percentages of nuclear and chloroplast DNA.

With buoyant density and thermokinetics being unfit for analyses of dinoflagellate DNA, paper or thin-layer chromatography was the only way of choice some years ago. However, base detection can be crucial and remains critical if small amounts shall be uncovered by this technique. Franker (1970) reported on methylcytosine as the only modified base in an algal isolate derived from *Anthopleura elegantissima*. Rae (1976) has observed only hydroxymethyluracil in the symbionts of *Hippopus hippopus* and in the free-living dinoflagellate *Amphidinium carterae*; yet both methylcytosine and hydroxymethyluracil were found in DNA from *C. cohnii* and *Prorocentrum cassubicum*. Methyladenine replacing adenine at a level of about 10% was detected in *Peridinium triquetrum* in addition to hydroxymethyluracil, but no methylcytosine was observed in the DNA from this dinoflagellate (Rae 1976).

We have not detected methyladenine in our organisms but did find methylcytosine and hydroxymethyluracil in all zooxanthellae under investigation. In a companion study, we have uncovered both bases also in nuclear DNA from an unidentified cryptomonad at levels as low as 0.9 mol% for methylcytosine and 0.3 mol% for hydroxymethyluracil. The analyzed G+C content of 57.3 mol% falls well within the range of cryptomonad DNA and is slightly smaller than the ratios described for *Chroomonas* sp. and *Rhodomonas lens*, for which no modified bases were reported (Rae 1976). To our knowledge, these bases are not regarded as typical of cryptomonads. The presence of hydroxymethyluracil is especially surprising as its only natural occurrence other than in dinoflagellates has become known from certain bacteriophages infecting *Bacillus subtilis* (cf. Kallen et al. 1962). It might be speculated if even more eukaryotes contain hydroxymethyluracil in tiny amounts, but were not yet investigated by means of HPLC analyses.

In conclusion, we have uncovered different G+C compositions as well as different quantities of substituted bases in zooxanthellae harbored by different hosts. We have then established that both G+C ratios and the different amounts of modified bases may be regarded as tools for phylogenetic discussions. These characteristics should therefore be included in the criteria employed for taxonomic descriptions of *Symbiodinium* species, for the advancement of recognizing symbiotic taxa of dinoflagellates.

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