



GENETIC DIVERSITY OF PYRAMIMONAS FROM RYUKYU ARCHIPELAGO, JAPAN (CHLOROPHYCEAE, PYRAMIMONADALES)

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GENETIC DIVERSITY OF *Pyramimonas* FROM RYUKYU ARCHIPELAGO, JAPAN (CHLOROPHYCEAE, PYRAMIMONADALES)

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and Daphne Georgina Faria¹

Key words: *Pyramimonas*, nuclear SSU rDNA, genetic diversity, Ryukyu Archipelago.

ABSTRACT

The genus *Pyramimonas* Schmarada was traditionally described on observations of its periplast and internal structure of different flagellar apparatus or eyespot orientations and currently comprises of ca. 60 species that are divided into six subgenera: *Pyramimonas*, *Vestigifera*, *Trichocystis*, *Punctatae*, *Hexactis*, and *Macrura*. In order to understand the genetic diversity and phylogenetic relationships of genus *Pyramimonas* members, we analyzed nuclear SSU rDNA molecular data from 41 strains isolated from different locations of the Ryukyu Archipelago. Phylogenetic analyses revealed that strains could be segregated into six clades, four of which represented existing subgenera: *Pyramimonas*, *Vestigifera* McFadden, *Trichocystis* McFadden, and *Punctatae* McFadden, and two undescribed subgenera. Most recovered subgenera were represented by established and confirmed species of *Pyramimonas* along with several potentially undescribed species with the exception of one undescribed subgenus. Many of the newly isolated strains were closely related with *Pyramimonas aurea* and *P. olivacea*, and were considered to belong to subgenus *Punctatae*. However, nuclear SSU rDNA phylogenetic trees clearly showed that they are genetically different and can be divided into two subgenera. Subgenus *Punctatae* comprised of *Pyramimonas olivacea* and related strains whilst, *P. aurea* and related strains belonged to an undescribed subgenus which is tentatively referred to as undescribed subgenus 1 which shared a sister-group relationship with subgenus *Punctatae*. The genetic similarity based on

nuclear SSU rDNA ranged from 98.2% to 99.9% and 97.3% to 98.3% within and between subgenera, respectively. The present work shows high level of genetic diversity for the genus *Pyramimonas* from the Ryukyu Archipelago, Japan.

I. INTRODUCTION

The genus *Pyramimonas* Schmarada was first described in 1850 based on *P. tetrahyinchus* Schmarada a freshwater species isolated from a ditch on Coldham's Common, Cambridge, United Kingdom. Subsequently, species isolated were assigned to several generic names, including *Pyramidomonas* Stein, *Chloraster* Ehrenberg, *Asteromonas* Artari, and *Polyblepharides* Dangeard. The genus had shown to be artificial as certain freshwater species were moved to the genus *Hafniomonas* Ettl et Moestrup. Genus *Hafniomonas* resembles *Pyramimonas* in cell shape but differs with respect to lack of flagellar and body scales, different cell symmetry, flagellar root, and details of cytokinesis [18].

To date ca. sixty species have been recorded worldwide with species mostly from the marine plankton, but few benthic [9, 16, 19, 34]. The class Prasinophyceae was first formally described by Moestrup and Throndsen in 1988 [20] and comprised of unicellular green algae possessing scales on their flagella and body surface. Prasinophytes (previously class Prasinophyceae) has placed great emphasis on the architecture and structure of the various scale types that are distributed on the flagella and cell surface [25, 27]. Hence resulting in the detailed study of the type species with respect to the ultrastructure, scale morphology and scale morphogenesis [1, 12, 21, 31].

Early work on the genus began with light microscopy (LM) however, LM observations alone failed to assess the existing variability within species mainly because of the large degree of overlap that existed with respect to cell shape, internal organization and dimensions [32]. Subsequently, the genus was emended and four subgenera were categorized based on electron microscopic observations [16, 17]. However, a modified classification of the genus at the subgeneric level was

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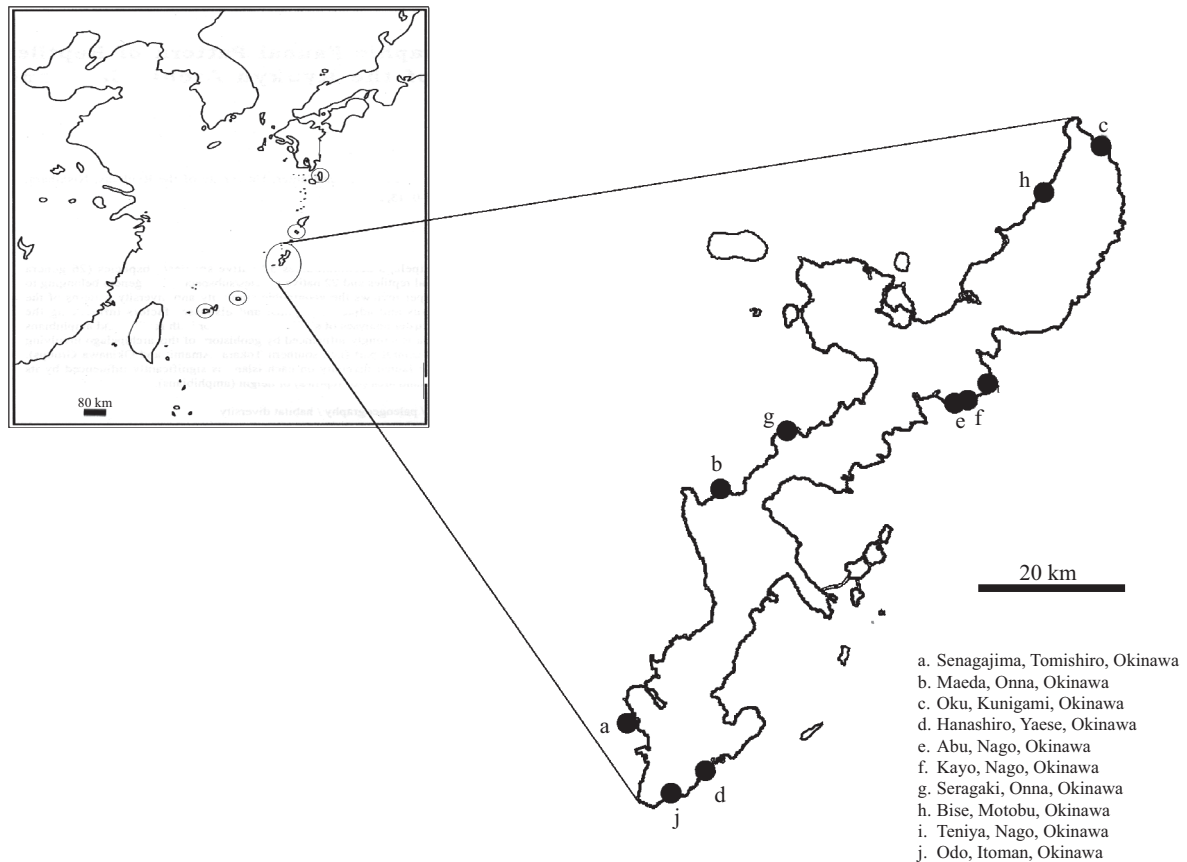


Fig. 1. Map of Ryukyu Archipelago, Japan (inset). Detailed sampling on Okinawa-jima Island.

put forth [9] which was based on four different flagellar apparatus or eyespot orientations and two new subgenera were proposed. Currently the genus is classified into the following six subgenera:

1. *Pyramimonas*
2. *Vestigifera* McFadden
3. *Trichocystis* McFadden
4. *Punctatae* McFadden
5. *Hexactis* Hori, Moestrup & Hoffmann
6. *Macrura* Hori, Moestrup & Hoffmann

Molecular phylogenetic analyses of the genus *Pyramimonas* and prasinophytes was carried out using nuclear encoded rDNA gene [18S rDNA; 6, 22, 24, 30, 35, 36], plastid encoded rDNA gene [16S; 15], and ribulose-1, 5-biphosphate carboxylase oxygenase gene [*rbcL*; 3, 4, 22, 30, 35]. However, relations between the subgenera have remained unresolved [30].

The Ryukyu Archipelago located in southwestern Japan of the western Pacific Ocean consists of over 900 islands and islets between mainland Japan and Taiwan (Fig. 1; inset) and this region have not been investigated for the genus *Pyramimonas*. These islands are known to sustain rich and diverse flora and fauna that is mainly attributed to the Kuroshio Current and hence considered a hotspot of endemism.

The objective of the present study was to investigate genetic diversity and phylogenetic relationships among *Pyramimonas* species using larger taxon sampling by isolating strains from different locations around Ryukyu Archipelago, Japan using nuclear SSU rDNA and discuss the congruence between phylogeny and morphology based delineation.

II. MATERIALS AND METHODS

1. Sampling

Bottom sand and water samples were collected from 15 locations throughout the Ryukyu Archipelago: Iriomote-jima Island (1), Miyako-jima Island (1), Okinawa-jima Island (10; Fig. 1 for details on locations), Okinoerabu-jima Island (2), and Tanegashima (1). Samples were enriched with IMK (Nippon Pharmaceutical, Co. Tokyo, Japan) + GeO₂ medium and cultured at 24 ± 2°C under 14:10 light:dark cycle and 40 μmol photon m⁻² s⁻¹ from cool white fluorescent tubes.

2. Strain Identification and Culture Establishment

Forty-one unialgal culture strains of typical inversely pyramidal shaped flagellates were isolated by micropipetting and cultures were established. Table 1 lists strain names and collection information of isolated strains from Ryukyu Archipelago.

Table 1. List of *Pyramimonas* strains isolated in this study, with their strain name, geographic origin.

Strain	Geographic Origin	Collection yy/mm/dd	Longitude:Latitude
7-Py	Senagajima, Itoman, Okinawa	2003/04/01	26.177N: 127.641E
Te2Py	Teniya, Nago, Okinawa	2003/05/01	26.565N: 128.140E
Py4	Maeda, Onna, Okinawa	2004/01/22	26.444N: 127.767E
Py5	Maeda, Onna, Okinawa	2004/01/22	26.444N: 127.767E
Py8	Maeda, Onna, Okinawa	2004/01/22	26.444N: 127.767E
Hae5Py5	Haemida, Iriomote, Taketomi, Okinawa	2004/03/07	24.271N: 123.829E
Ok2P3	Oku, Kunigami, Okinawa	2004/03/23	26.849N: 128.284E
M1Py1	Maeda, Onna, Okinawa	2004/04/06	26.443N: 127.768E
Ab4-11	Abu, Nago, Okinawa	2004/07/02	26.538N: 128.095E
Ab4-13	Abu, Nago, Okinawa	2004/07/02	26.538N: 128.095E
Sa5P5	Hanashiro, Yaese, Okinawa	2004/08/30	26.115N: 127.745E
Sera3-4-4	Seragaki, Onna, Okinawa	2004/09/15	26.509N: 127.864E
Mad5-2	Maeda, Onna, Okinawa	2005/02/02	26.444N: 127.767E
Mad 6-5	Maeda, Onna, Okinawa	2005/02/02	26.444N: 127.767E
Ka5	Kayo, Nago, Okinawa	2005/03/09	26.547N: 128.108E
Ka10	Kayo, Nago, Okinawa	2005/03/09	26.547N: 128.108E
Ka1	Kayo, Nago, Okinawa	2005/03/09	26.547N: 128.108E
Mae3P1-2	Maeda, Onna, Okinawa	2005/03/14	26.444N: 127.767E
MD6-19	Maeda, Onna, Okinawa	2005/06/13	26.444N: 127.767E
BiP5	Bise, Motobu, Okinawa	2005/07/22	26.710N: 127.880E
Tni4Py1	Teniya, Nago, Okinawa	2005/08/07	26.565N: 128.140E
Tni4Py2	Teniya, Nago, Okinawa	2005/08/07	26.565N: 128.140E
Tni4Py3	Teniya, Nago, Okinawa	2005/08/07	26.565N: 128.140E
Tni4Py4	Teniya, Nago, Okinawa	2005/08/07	26.565N: 128.140E
MK7P4	Maeda, Onna, Okinawa	2006/03/17	26.444N: 127.767E
Mk7P27	Maeda, Onna, Okinawa	2006/03/17	26.444N: 127.767E
Mk7N1	Maeda, Onna, Okinawa	2006/03/17	26.444N: 127.767E
MIYI-8	Aragusuku, Miyako, Okinawa	2006/06/17	24.762N: 125.426E
SergOP7-2	Seragaki, Onna, Okinawa	2006/06/26	26.509N: 127.864E
SergOP6-1	Seragaki, Onna, Okinawa	2006/06/26	26.509N: 127.864E
SergOP9	Seragaki, Onna, Okinawa	2006/06/26	26.509N: 127.864E
OEF06-4	Furusato, Wadamari, Kagoshima	2006/07/28	27.362N: 128.626N
OEF013	Furusato, Wadamari, Kagoshima	2006/07/28	27.362N: 128.626N
TUP12	Urata, Nishinoomote, Kagoshima	2006/08/11	30.823N: 131.038E
TU8	Urata, Nishinoomote, Kagoshima	2006/08/11	30.823N: 131.038E
TU6	Urata, Nishinoomote, Kagoshima	2006/08/11	30.823N: 131.038E
TU2	Urata, Nishinoomote, Kagoshima	2006/08/11	30.823N: 131.038E
OD6P2	Odo, Itoman, Okinawa	2006/09/07	26.088N: 127.708E
OD6P1	Odo, Itoman, Okinawa	2006/09/07	26.088N: 127.708E
OEO3-3	Okidomari, Shinjyo, China, Kagoshima	2007/07/29	27.400N: 128.560N
M4-1	Maeda, Onna, Okinawa	2008/05/05	26.444N: 127.767E

Strain names in bold are lost.

3. DNA Extraction, Amplification, Sequencing, and Alignments

Forty-one strains were cultured, harvested, and DNA extracted following the protocol by Suda [30]. The amplification and sequencing primers used for the nuclear SSU rDNA are those reported by Nakayama *et al.* [24]. Strains were sequenced for their partial nuclear SSU rDNA and compared with previous available sequence data. The SSU rDNA se-

quence data was aligned in BioEdit 7.0.5.3 [7].

4. Phylogenetic Analyses

A data set of 1,617 base pairs was utilized for phylogenetic analyses. The sequences of 23 strains used as outgroup taxa to root the phylogenetic tree because these taxa are considered close relatives of the genus *Pyramimonas* [4, 15]. Table 2 lists all taxa/strains used for the phylogenetic analyses.

Table 2. List of *Pyramimonas* strains used in this study, with their taxon/strain name, geographic origin and GenBank accession numbers for SSU rDNA sequences.

Subgenus	Taxon/Strain	Geographic Origin	GenBank accession number
<i>Punctatae</i>			
<i>Pyramimonas olivacea</i>	M 1668		FN562442
<i>Pyramimonas olivacea</i>	Shizugawa	Shizugawa, Miyagi, Japan	AB017122
	7-Py	Senagajima, Itoman, Okinawa	AB853999
	Ka1	Kayo, Nago, Okinawa	AB854004
	Mk7P27	Maeda, Onna, Okinawa	AB854016
	OD6P1	Odo, Itoman, Okinawa	AB854017
	SergOP6-1	Seragaki, Onna, Okinawa	AB854028
	OEO3-3	Okidomari, Shinjyo, China, Kagoshima	AB854019
	Mk7N1	Maeda, Onna, Okinawa	AB854014
	Py8	Maeda, Onna, Okinawa	AB854025
	Sa5P5	Hanashiro, Yaese, Okinawa	AB854026
	Sera3-4-4	Seragaki, Onna, Okinawa	AB854027
	Py5	Maeda, Onna, Okinawa	AB854024
	Tni4Py1	Teniya, Nago, Okinawa	AB854032
<i>Pyramimonas</i>			
<i>Pyramimonas propulsa</i>	NIES 251		AB017123
<i>Pyramimonas tetrarhynchus</i>	SCCAP K 0002		FN562441
	M4-1	Maeda, Onna, Okinawa	AB854008
	MD6-19	Maeda, Onna, Okinawa	AB854012
	Te2Py	Teniya, Nago, Okinawa	AB854026
	MK7P4	Maeda, Onna, Okinawa	AB854015
<i>Trichocystis</i>			
<i>Pyramimonas parkeae</i>	Hachijo	Hachijo Is., Tokyo Japan	AB017124
<i>Pyramimonas parkeae</i>	CCMP 726		FN562443
<i>Pyramimonas australis</i>		Antarctica	AJ404886
<i>Pyramimonas</i> sp.	RCC2500		JN934689
<i>Pyramimonas</i> sp.	RCC2009		JF794047
Uncultured prasinophyte	BL010625.18		AY425322
	TU6	Urata, Nishinoomote, Kagoshima	AB854037
Undescribed subgenus 1			
<i>Pyramimonas aurea</i>	MBIC10862		AB052289
<i>Pyramimonas mucifera</i>	WitsPyrami		EU330223
	Hae5Py5	Haemida, Iriomote, Taketomi, Okinawa	AB854003
	M1Py1	Maeda, Onna, Okinawa	AB854007
	OEF013	Furusato, Wadomari, Kagoshima	AB854021
	TU8	Urata, Nishinoomote, Kagoshima	AB854038
	Tni4Py2	Teniya, Nago, Okinawa	AB854033
	Mad 6-5	Maeda, Onna, Okinawa	AB854010
	Ka5	Kayo, Nago, Okinawa	AB854005
	Ab4-11	Abu, Nago, Okinawa	AB854000
	BiP5	Bise, Motobu, Okinawa	AB854002
	Mae3P1-2	Maeda, Onna, Okinawa	AB854011
	SergOP9	Seragaki, Onna, Okinawa	AB854030
	Ka10	Kayo, Nago, Okinawa	AB854006
	Ab4-13	Abu, Nago, Okinawa	AB854001
	SergOP7-2	Seragaki, Onna, Okinawa	AB854029
	TU2	Urata, Nishinoomote, Kagoshima	AB854036
	TUP12	Urata, Nishinoomote, Kagoshima	AB854039

Table 2. (Continued)

Subgenus	Taxon/ Strain	Geographic Origin	GenBank accession number
Undescribed subgenus 2			
	MIYI-8	Aragusuku, Miyako, Okinawa	AB854013
	Ok2P3	Oku, Kunigami, Okinawa	AB854022
	Tni4Py4	Teniya, Nago, Okinawa	AB854035
	Mad5-2	Maeda, Onna, Okinawa	AB854009
	OD6P2	Odo, Itoman, Okinawa	AB854018
	Py4	Maeda, Onna, Okinawa	AB854023
<i>Vestigifera</i>			
<i>Pyramimonas disomata</i>	M1802		FN562440
<i>Pyramimonas disomata</i>	Singapore	Changi, Singapore	AB017121
<i>Pyramimonas</i> sp.	RCC1987		JN934670
<i>Pyramimonas gelidicola</i>	O. Gorman Rocks	Antarctica	HQ111510
<i>Pyramimonas</i> sp.	RCC2015	Beaufort Sea	JF794048
<i>Pyramimonas gelidicola</i>	Highway Lake	Antarctica	HQ111509
<i>Pyramimonas gelidicola</i>	Ace Lake	Antarctica	HQ111511
	OEFO6-4	Furusato, Wadamari, Kagoshima	AB854020
	Tni4Py3	Teniya, Nago, Okinawa	AB854034
Outgroup			
<i>Halosphaera</i> sp.	Shizugawa	Shizugawa, Miyagi, Japan	AB017125
<i>Halosphaera</i> sp.	M1670		FN562439
<i>Cymbomonas tetramitiformis</i>	Shizugawa	Shizugawa, Miyagi, Japan	AB017126
<i>C. tetramitiformis</i>	M1669		FN562438
<i>Pterosperma cristatum</i>	Yokohama	Yokohama, Kanagawa, Japan	AB017127
<i>P. cristatum</i>	NIES 221		AJ010407
<i>Prasinopapilla vacuolata</i>	MBIC10879		AB183649
<i>Mantoniella antarctica</i>		Lützow Holmbukta, Antarctica	AB017128
<i>Micromonas pusilla</i>	M1681		FN562452
<i>Mantoniella squamata</i>	CCAP 1965/1		X73999
<i>Mamiella</i> sp.	Shizugawa	Shizugawa, Miyagi, Japan	AB017129
<i>Mamiella gilva</i>	PLY 197		FN562450
<i>Bathycoccus prasinus</i>	SCCAP K-0417		FN562453
<i>Osterococcus tauri</i>			Y15814
<i>Monomastix</i> sp.	M0722		FN562447
<i>M. opisthostigma</i>	M2844		FN562445
<i>M. minuta</i>	NIES-255		FN562446
<i>Dolichomastix tenuilepis</i>	M1680		FN562449
<i>Crustomastix stigmatica</i>	pras1		AJ629844
<i>C. didyma</i>	MBIC10709		AB183628
<i>Prasinococcus capusulatus</i>	MBIC11011		AB058384
<i>P. capsulatus</i>	CCMP1202		AF203401
<i>Prasinoderma coloniale</i>	M1681		FN562437

1) Bayesian Analysis

Bayesian phylogenetic analysis for nuclear SSU rDNA dataset was implemented in MrBayes 3.1.2 [10]. MrModeltest2 [26] was used to find the optimal model of DNA substitutions for the construction of Bayesian tree. The general time reversible plus invariant sites plus the gamma-distributed model (GTR+I+G) was selected as the best-fit model for

the nuclear SSU rDNA. The model parameters are summarized (Table 3). The Markov chain Monte Carlo (MCMC) process was set to two chains, for one million generations. Sampling frequency was assigned at every 100 generations. After analysis, the first 2,000 trees were deleted as burn-in and the consensus tree was constructed. Bayesian trees were visualized with FigTree v1.4.0. Bayesian posterior

Table 3. Evolutionary models, log likelihood values (-lnL), and model parameters selected by hLRTs results of MrModeltest2 [26] and MEGA 5 [33] for nuclear SSU rDNA.

Phylogeny	Large	Small
Number of taxa	81	62
Nuclear SSU rDNA		
Model parameter/Data type	Nuclear SSU rDNA	
Model	GTR+I+G	K2+G+I
-lnL	9137.496	2681.572
I	0.5738	0.7023
G	0.5175	0.5540
Base frequencies		
A	0.2531	0.2500
C	0.2097	0.2500
G	0.2710	0.2500
T	0.2662	0.2500
Rate Matrix (G/T=1.0)		
A/C	0.7582	0.0500
A/G	1.7667	0.1400
A/T	0.9723	0.0500
C/G	0.9042	0.0500
C/T	3.8298	0.1400
Characteristics		
Nt	1,617	788
Nc	1,175	660
Nv	434	127
PI	318	81

Abbreviations: -lnL, negative log likelihood; A, C, G and T, frequency of nucleotides; I, fraction of invariant nucleotides; G, Shape parameter of gamma (G) distribution rates; A-C, A-G, A-T, C-G, C-T and G-T, rates of reversible nucleotide substitutions; hLRTs, hierarchical Likelihood Ratio Tests; GTR, General Time Reversible; K2, Kimura 2-parameter; Nt, total number of sites compared; Nc, total number of conserved sites; Nv, total number of variable sites; PI, number of parsimony informative sites.

probabilities (PP) more than 0.50 were indicated at branch nodes.

2) Maximum Likelihood (ML)

A maximum likelihood (ML) tree was constructed using MEGA 5 with K2+G+I nucleotide substitution model. The tree was rooted to two strains of *Cymbomonas tetramitiformis*. Bootstrap proportions (BP) of more than 50 were indicated at branch nodes.

5. Molecular Data Analyses

The corrected pairwise (p -) genetic distances were calculated with Kimura two-parameter model in MEGA 5 [33].

Sequence characteristics, including total aligned positions (N_t), conserved positions (N_c) and variable (N_v), parsimony informative site (PI) were analyzed in MEGA 5. Based on these sequence similarity was calculated as pair wise genetic distance $\{(1-\text{each pair-wise distance value}) \times 100\}$ within and between subgenera (Tables 4 and 5).

III. RESULTS

1. Sampling Effort and Morphological Characterization of *Pyramimonas* Strains

This study is the first to carry out large-scale sampling of the Ryukyu Archipelago; Japan initiated from 2003 to 2008. As a result, forty-one strains for the genus *Pyramimonas* were isolated from 15 locations throughout the Ryukyu Archipelago with strains isolated in the following order: Okinawa-jima Island (32 from 10 locations), Tanegashima (4 from 1 location), Okinoerabu-jima Island (3 from 2 locations), Iriomote-jima Island and Miyako-jima Island (1 each from a single location) (Table 1).

2. Taxon Sampling and Choice of Molecular Marker

Nuclear SSU rDNA was used to assess the phylogenetic relationships among *Pyramimonas* subgenera. Taxon sampling of 81 and 62 species for large and small phylogeny respectively did yield congruent phylogenetic relationships regardless of the different evolutionary rates among lineages (Table 3).

3. Characteristics of the Nuclear rDNA of Genus *Pyramimonas*

Newly isolated 41 strains from diverse habitats of the Ryukyu Archipelago contained 33 new sequences of nuclear SSU rDNA. Genetic variations within subgenera were investigated using DNA similarity scores (Table 4). Within subgenera, similarity scores were highest and lowest for undescribed subgenus 2 (99.9%) and *Trichocystis* (98.2%), respectively. The pair wise differences were lowest for undescribed subgenus 2 (0-3) and highest for *Trichocystis* (0-47). Several of the sequences generated were identical (with zero bp difference). For instance, within the subgenus *Punctatae* strains Sera3-4-4, Sa5P5 and SergOP6, and Tni4Py1 and Py5 were identical. This was also true for members of the subgenus *Pyramimonas* (MD6-19 and M4-1), undescribed subgenus 1 (Ab4-13 and SergOP7, Tu2 and TUP12, and *P. aurea* AB052289 and MIPYI), and undescribed subgenus 2 (Py4 and OD6P2). Between subgen-era, similarity scores were highest between undescribed subgenus 1 and *Vestigifera* (98.3%), and lowest between *Punctatae* and *Trichocystis*, and *Punctatae* and undescribed subgenus 2 (97.3%) (Table 5).

4. Distance Relationships of *Pyramimonas* Subgenera

Within subgenera, nucleotide divergences were measured for nuclear SSU rDNA (Fig. 2). The percentage p -distances or genetic divergence for the genus *Pyramimonas* ranged from

Table 4. Comparisons of within subgenera for the genus *Pyramimonas* based on nuclear SSU rDNA gene sequences.

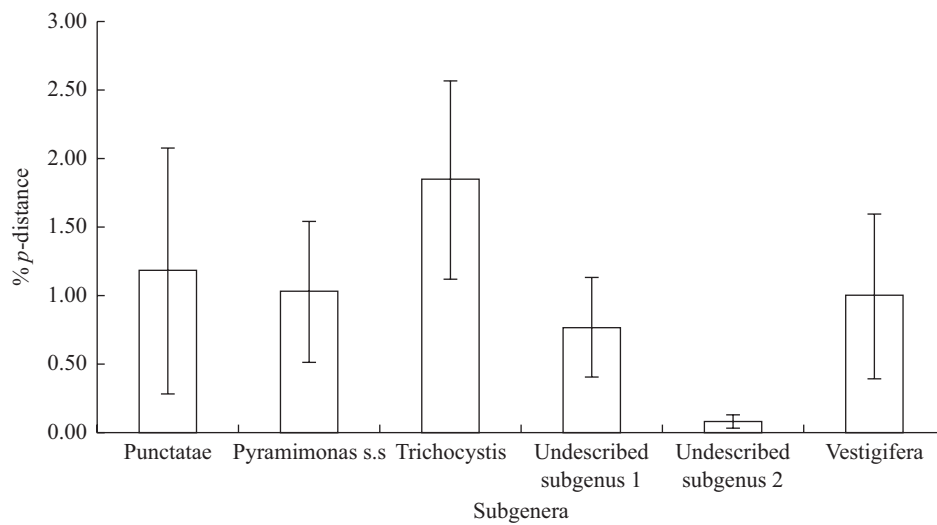
Subgenera	Nuclear SSU rDNA					
	N	Length (bp)	Similarity (%)	Similarity*	<i>p</i> -distance	Pair wise differences
<i>Punctatae</i>	14	1,617	97.4-100.0	98.8	0.00-0.03	0-40
<i>Pyramimonas</i>	6	1,617	98.4-100.0	99.0	0.00-0.02	0-28
<i>Trichocystis</i>	6	1,617	97.0-99.7	98.2	0.00-0.03	4-47
Undescribed subgenus 1	17	1,617	98.4-100.0	99.2	0.00-0.02	0-26
Undescribed subgenus 2	6	1,617	99.8-100.0	99.9	0.00-0.00	0-3
<i>Vestigifera</i>	9	1,617	98.0-99.9	99.0	0.00-0.02	1-32

Pair wise (*p*) distances were calculated based on the Kimura two-parameter model in MEGA 5; *N*, number of isolates; * represents average scores.

Table 5. Table of distances (below diagonal) and percentage similarity (above diagonal) between *Pyramimonas* subgenera based on nuclear SSU rDNA.

Subgenus	[Percentage Similarity]					
	[1]	[2]	[3]	[4]	[5]	[6]
[1] <i>Punctatae</i>		97.9	97.4	98.7	97.4	98.2
[2] <i>Pyramimonas</i>	0.02		97.3	98.2	97.3	97.8
[3] <i>Trichocystis</i>	0.03	0.03		97.6	97.4	97.4
[4] Undescribed subgenus 1	0.01	0.02	0.02		97.7	98.3
[5] Undescribed subgenus 2	0.03	0.03	0.03	0.02		97.4
[6] <i>Vestigifera</i>	0.02	0.02	0.03	0.02	0.03	

[Distances]

**Fig. 2. Genetic divergence of genus *Pyramimonas* nuclear SSU rDNA sequences based on corrected *p*-distances. Genetic distance between each sequence pair was calculated using Kimura two-parameter model, where representative members of the *Pyramimonas* subgenera were compared.****Table 6. Percentage difference within *Pyramimonas* subgenera based on nuclear SSU rDNA.**

Subgenus	Within subgenera differences (%)
<i>Punctatae</i>	0-2.6
<i>Pyramimonas</i>	0-1.8
<i>Trichocystis</i>	0.3-3.0
Undescribed subgenus 1	0-1.7
Undescribed subgenus 2	0-0.2
<i>Vestigifera</i>	0.1-2.0

0%-2.6%. Table 5 lists percentage difference within *Pyramimonas* subgenera in nuclear SSU rDNA. For instance, nucleotide divergence was highest for subgenus *Trichocystis* (1.85) and lowest for undescribed subgenus 2 (0.09).

5. Molecular Phylogenetic Analyses of Genus *Pyramimonas*

In order to assess the molecular phylogeny for the genus *Pyramimonas* of all isolated strains were sequenced for their nuclear SSU rDNA that generated forty-one nuclear SSU

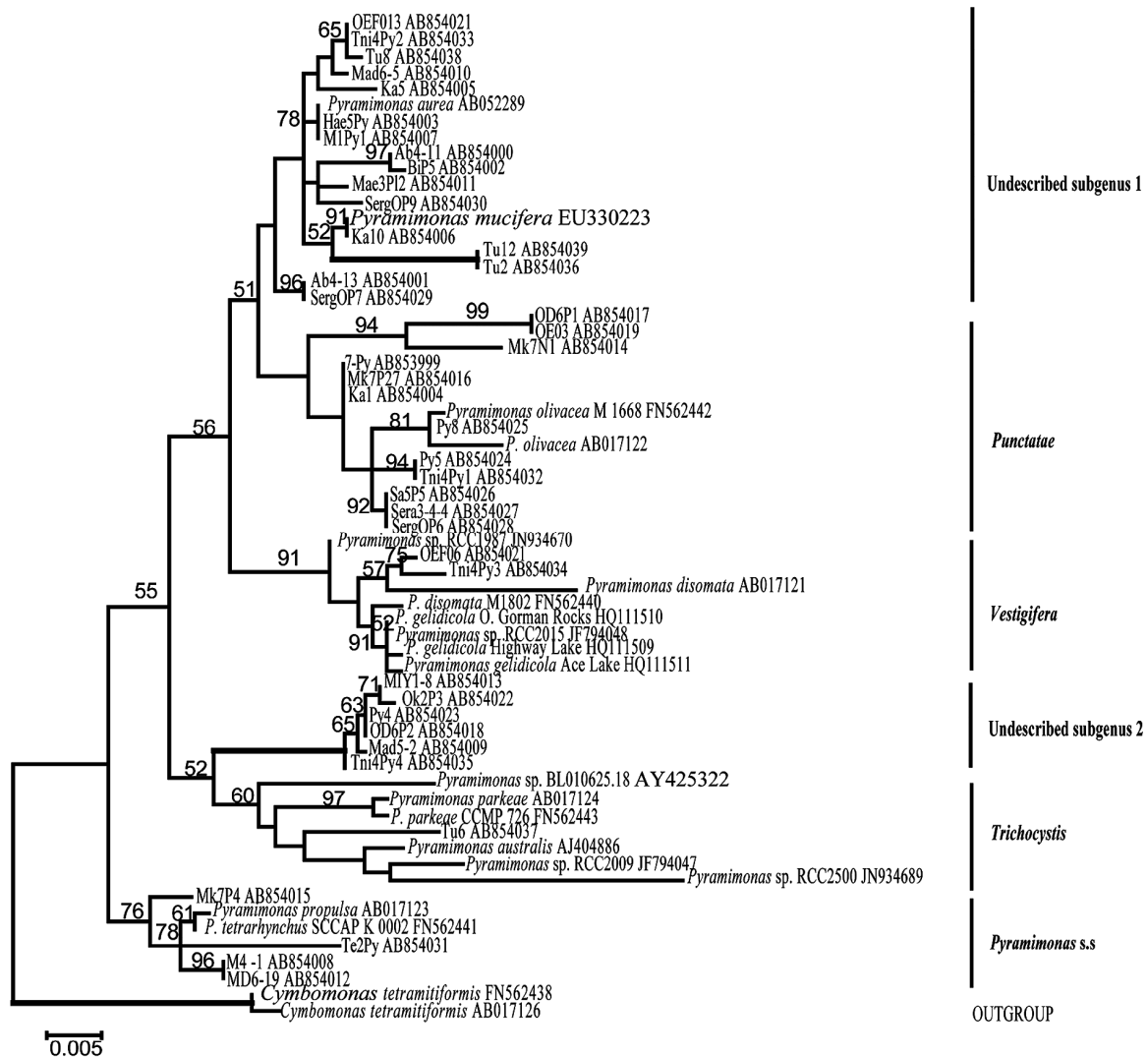


Fig. 4. Maximum likelihood tree for the genus *Pyramimonas* inferred from nuclear SSU rDNA sequences, including sixty-two strains. K2+G+I nucleotide substitution model was used for the analysis. Maximum likelihood scores for the tree were calculated at $-\ln L = 2681.6$ and 2 strains of *Cymbomonas tetramitiformis* were used as outgroup. The numbers at the nodes display BP (> 50) in likelihood analysis. Monophyly with bootstrap proportions=100 are shown as thick branches.

(*Pyramimonas aurea* and related strains), Undescribed subgenus 2, and *Vestigifera* (*Pyramimonas disomata*, *Pyramimonas gelidicola*, and potentially undescribed species). The topology of these subgenera was as follows: Subgenus *Pyramimonas*, undescribed subgenus 2, and *Trichocystis* shared a common ancestor separate from species of the subgenera *Punctatae*, undescribed subgenus 1, and *Vestigifera*. Subgenus *Trichocystis* displayed sister-group relationship with undescribed subgenus 2, and these were sister groups to subgenus *Pyramimonas*. Subgenus *Punctatae* was the sister group of undescribed subgenus 1, and these were sister groups to *Vestigifera*. Sequences from new isolates from the Ryukyu Archipelago were represented as follows among the six subgenera: Undescribed subgenus 1 (16 from 17 sequences) $>$ *Punctatae* (12 from 14 sequences) $>$ *Vestigifera* (2 from 9 sequences) *Pyramimonas* (4 from 6 sequences) $>$ *Trichocystis*

(1 from 6 sequences) $>$ undescribed subgenus 2 (5 new sequences). Subgenus *Pyramimonas* clade with six sequences (four from the present study) formed a robust monophyletic clade (PP=1.00, represented by bold line; Fig. 3). Undescribed subgenus 2 and *Vestigifera* also formed robust monophyletic clades (PP=1.00). *Punctatae*, undescribed subgenus 1, and *Trichocystis* with very strong support values (PP=0.99) also formed monophyletic clades.

The second dataset comprised of 62 sequences with 660 constant positions (83.6%), 127 variable positions (16.1%), and 81 positions that were parsimoniously informative (10.3%). The second dataset comprised of members only from the genus *Pyramimonas* and a maximum likelihood tree was generated that was rooted to two strains of *Cymbomonas tetramitiformis* (Fig. 4). Unlike the larger dataset, the smaller phylogeny dataset has two additional sequences, *Pyrami-*

monas mucifera strain WitsPyrami and the environmental sequence BL010625.18. In the phylogenetic tree, the genus *Pyramimonas* was paraphyletic and comprised of six subgenera. The composition of all genera were identical to the larger phylogeny dataset except undescribed subgenus 1 (*Pyramimonas aurea*, *Pyramimonas mucifera* WitsPyrami EU330223, and related strains), and *Trichocystis* (*Pyramimonas parkeae*, *Pyramimonas australis*, *Pyramimonas* sp. BL010625.18 AY425322, and potentially undescribed species). The tree topology was as follows: Subgenus *Pyramimonas* displayed sister-group relationship to undescribed subgenus 2 and *Trichocystis*, and *Punctatae*, Undescribed subgenus 1, and *Vestigifera*. The only subgenus that was recovered monophyletic was undescribed subgenus 2 (BP=100). Subgenus *Vestigifera* displayed strong support (BP=91) and was monophyletic. Subgenera *Punctatae* and undescribed subgenus 1 were not supported whilst, subgenera *Trichocystis* and *Pyramimonas* with BP=60 and 76 respectively, were moderately supported. Regardless of the support, the topology of six subgenera in both the large and the small phylogeny were congruent.

IV. DISCUSSION

1. Sampling Effort and Distribution Implications of *Pyramimonas* Species

The genus *Pyramimonas* is known cosmopolitan genus with species isolated from diverse parts of the world [22] including freshwater, brackish, and marine habitats [2] however, most species are from the marine plankton, with few reports from the benthic [9, 16, 19, 34]. The members of this genus are also well represented in the picoplankton [36] where significant genetic diversity at the subgeneric level was recently reported for the Mediterranean Sea.

The present study is the first to carry out large-scale sampling of the Ryukyu Archipelago; Japan initiated from 2003 to 2008. As a result, forty-one strains for the genus *Pyramimonas* were isolated from 15 locations throughout the Ryukyu Archipelago with strains isolated in the following order: Okinawa-jima Island (32 from 10 locations), Tanegashima (4 from 2 location), Okinoerabu-jima Island (3 from 2 locations), Iriomote-jima Island and Miyako-jima Island (1 each from a single location) (Table 1). Most isolated strains were from bottom sand and water samples contrary to earlier studies that have reported few benthic *Pyramimonas* species. The wide diversity observed with regard to isolated species around the Ryukyu Archipelago can be mainly attributed to the Kuroshio Current. A similar wide genetic diversity was also observed for another prasinophyte *Nephroselmis* from the Ryukyu Archipelago (Faria *et al.* unpublished) and hence the present study location can be considered a hotspot of endemism.

2. Taxon Sampling and Choice of Molecular Marker

To date ca. 60 species of *Pyramimonas* have been described with approximately 34 species studied both by light and elec-

tron microscopy [9]. Generally, the use of nuclear SSU rDNA has proven to be advantageous because significant sequence data is available in public databases. However, only nine species have been analyzed for nuclear SSU rDNA in comparison of the *rbcL* gene (18 species). This trend can be attributed to the use of *rbcL* gene for subgeneric classification that has shown to be well correlated to pyrenoid structure types [9] and monophyletic clades recovered from *rbcL* sequences [22, 30]. Furthermore, Suda [30] opines that this association stems from RuBisCo being concentrated in the pyrenoid. However, nuclear SSU rDNA was used in the present study to assess the genetic diversity and phylogenetic relationships among *Pyramimonas* subgenera. The use of nuclear SSU rDNA for phylogenetic studies has often suffered from limited taxon sampling [5] and it is well established that sampling taxa more densely results in homoplasy, and therefore improves accuracy of phylogenetic inferences [8, 28]. Hence emphasis was laid on larger taxon sampling which in part was achieved by isolating strains from different locations around the Ryukyu Archipelago, Japan (41 sequences) and available nuclear SSU rDNA sequence data (19 sequences) was retrieved from public database (e.g., GenBank) and utilized to discuss the congruence between phylogenetic relationships and morphology based delineation.

3. Characteristics of the Nuclear rDNA and Distance Relationships of *Pyramimonas*

In order to assess genetic divergence, *p*-distances of nuclear SSU rDNA gene sequences were determined from taxa/strains from all six subgenera and it was observed that subgenus *Trichocystis* and undescribed subgenus 2 was most and least divergent, respectively. This could be explained as undescribed subgenus 2 had the highest similarity scores, whilst *Trichocystis* recorded the lowest. Furthermore, pair wise differences for the undescribed subgenus 2 were lowest (0-3) and highest for *Trichocystis* (0-47). Additionally, pair wise differences aided in segregating identical strains within several subgenera. The percentage *p*-distances or genetic divergence for the genus *Pyramimonas* ranged from 0%-2.6%, which is much lower than those, reported for other genera viz., genus *Cryptoglana* 1.1%-4.7% wherein gene divergence was used to reclassify *Cryptoglana* species [11].

Individual subgenera percentage *p*-distances (Table 6) was lowest for undescribed subgenus 2 (0%-0.2%) and highest for *Trichocystis* (0.3%-3.0%). Murray *et al.* [23] has reported that in some *Amphidinium* species, different strains of the same species were 0.2%-1.0% different. In the present study, undescribed subgenus 2 with 0-3bp differences is potentially a monospecific subgenus. The base pair differences between Tu6 and *Pyramimonas parkeae* CCMP 726 FN562443 was 47bp and hence a potentially new species. Setting boundaries for the subgenera of the genus, *Pyramimonas* is highly unlikely now, as few species in particular only 9 confirmed species have been sequenced for their nuclear SSU rDNA. Furthermore, Murray *et al.* [23] states that estimating bounda-

ries, using sequence divergence is problematic and the authors consider this, as it is unlikely that all existing genotypes could have been sampled.

4. Molecular Phylogenetic Analyses of Genus

Pyramimonas

The class Prasinophyceae comprises of unicellular green algae covered with flagella and body scales [14]. The validity of this class was questioned and extensive molecular phylogenetic studies revealed that the class 'Prasinophyceae' was non-monophyletic composed of several independent lineages [6, 15, 24, 29] and consequently, the 'class Prasinophyceae' is no longer accepted (informally referred to as 'prasinophytes') [13]. The independent lineages currently recognized are Pyramimonadales (clade I), Mamiellophyceae (clade II) [15], Nephroselmidophyceae (clade III), Chlorodendrales (clade IV), Pycnococaceae (clade V), Prasinococcales (clade VI), *Picocystis* (clade VII), clade VIII, and clade IX.

Our molecular phylogenetic analysis based on large taxon sampling comprised of 81 sequences with members from Clade I (Pyramimonadales), Clade II (Mamiellophyceae), and Clade VI (Prasinococcales) belonging to the prasinophytes [6, 15, 24, 36] (Fig. 3). The phylogenetic tree was rooted to 23 sequences of Clade II and Clade VI. Sister-group relationship was observed between clade II and clade I that was also observed in previous studies [13, 35]. Clade I with 100% support was divided into two lineages. The first lineage comprised of *Halosphaera*, *Cymbomonas*, *Pterosperma*, and *Prasinopapilla*. The monophyly of the former three genera was well supported and they shared a sister-group relationship with the second lineage, genus *Pyramimonas*. Suda [30] reported that *rbcL* gene based studies failed to infer this relationship. Genus *Pyramimonas* was monophyletic and comprised of six subgenera: *Punctatae* (*Pyramimonas olivaceae* and related strains; Table 2), *Pyramimonas* (*Pyramimonas propulsa*, *Pyramimonas tetrarhynchus* and potentially undescribed species), *Trichocystis* (*Pyramimonas parkeae*, *Pyramimonas australis*, *Pyramimonas* sp. BL010625.18 AY425322, and potentially undescribed species), undescribed subgenus 1 (*Pyramimonas aurea*, *Pyramimonas mucifera* WitsPyrami EU330223, and related strains), undescribed subgenus 2, and *Vestigifera* (*Pyramimonas disomata*, *Pyramimonas gelidicola* and potentially undescribed species). Most of the species within subgenera mentioned above are in close agreement with the classification put forth by Hori *et al.* [9] and the subsumption of subgenus *Punctatae* into subgenus *Pyramimonas* is invalid. The topology of the subgenera recovered in the larger taxon sampled tree were as follows: Subgenus *Pyramimonas*, undescribed subgenus 2, and *Trichocystis* shared a common ancestor separate from species of the subgenera *Punctatae*, undescribed subgenus 1, and *Vestigifera*. *Trichocystis* displayed sister-group relationship with undescribed subgenus 2, and these were sister groups to subgenus *Pyramimonas*. *Punctatae* was the sister group of undescribed subgenus 1,

and these were sister groups to *Vestigifera*. Most subgenera have very strong support at the nodes with the exception of undescribed subgenus 1 hence; the phylogenetic relationships among different subgenera recovered in the present study are sound however, far from complete as many of the confirmed species are yet to be sequenced and analyzed.

Several studies [4, 22, 30, 36] have reported on the monophyly of the genus *Pyramimonas*. Daugbjerg *et al.* [4] reported five subgenera four of which were established while the fifth subgenus was proposed for *Pyramimonas* sp. L34834. This species was later described as *P. formosa*, and placed under subgenus *Punctatae*. Suda [30] also proposed a new subgenus for this species. Suda [30] described *Pyramimonas aurea* in 2004 and the author placed the species under the subgenus *Punctatae* however, the author discussed that *Punctatae* includes *P. olivaceae*, whilst *Pyramimonas aurea* along with *Pyramimonas mucifera* (belonging to *Punctatae*) would be moved to a new subgenus. In the present study (Fig. 3) shows *Pyramimonas mucifera* WitsPyrami EU330223 in a clade with *P. aurea* and related species whilst, *Pyramimonas olivaceae* belongs to *Punctatae*, regardless of the fact that these strains have consistently displayed double eyespots and numerous puncta with a variable nature of their single basal pyrenoid i.e., either surrounded by a double or numerous starch sheath. Guillou *et al.* [6], reported that environmental sequence BL010625.18 from Blanes library from June 2001 was closely related to *Pyramimonas australis* and *P. propulsa* which was also reported by Viprey *et al.* [36] along with two new environmental sequences from the Mediterranean coast. In our present study, we included the environmental sequence BL010625.18 that displayed a close relationship with species *Pyramimonas australis* and *P. propulsa*, and strain Tu6.

V. SUMMARY

This study accounts for a wide genetic diversity for the genus *Pyramimonas* from the Ryukyu Archipelago, Japan represented by four of the existing subgenera and two undescribed subgenera. However, the lack of nuclear SSU rDNA sequences in public databases like GenBank is a major hindrance for the phylogenetic comparative studies. It would be interesting to sequence existing confirmed species for their nuclear SSU rDNA in order to understand the phylogenetic relationships and further resolve the phylogeny of the genus *Pyramimonas*. Additionally, *rbcL* gene sequences should also be sequenced and the two genes should be analyzed individually and in unison for a better phylogenetic resolution, and the potential use of new genes should also be considered. Furthermore, a more congruent sampling approach should be considered in order to alleviate unresolved phylogeny not only for the genus *Pyramimonas* but genera at large. Essentially, morphology and phylogeny studied together is the way of the future rather than studies carried out in isolation.

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