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Title	Diversity of Vibrios in the Coral Reef Ecosystem of Ishigaki Island, Japan
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Degree Grantor	北海道大学
Degree Name	博士(水産科学)
Dissertation Number	甲第12660号
Issue Date	2017-03-23
DOI	https://doi.org/10.14943/doctoral.k12660
Doc URL	https://hdl.handle.net/2115/67148
Type	doctoral thesis
File Information	AKM_Amin.pdf

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Diversity of Vibrios in the Coral Reef Ecosystem of Ishigaki Island, Japan

(石垣島周辺海域のサンゴ生態系における ビブリオの多様性)

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2017 (平成 29 年)

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Dedicated to my beloved parents and wife

List of Abbreviations

AAI	Average amino acid identity
ANI	Average nucleotide identity
CAIM	Collection of Aquacultural Important Microorganisms
CECT	Colección Española de Cultivos Tipo
DDBJ	The DNA Data Bank of Japan
DDH	DNA-DNA hybridization
DOC	Dissolved organic carbon
DNA	Deoxyribonucleic acid
DSM	DSMZ-Deutsche Sammlung von Mikroorganismen
EMBL	The European Molecular Biology Laboratory
GC	Guanine-cytosine
GGDC	Genome to genome distance calculator
JCM	Japan Collection of Microorganisms
LMG	Laboratorium voor Microbiologie
MEGA	Molecular evolutionary genetics analysis
ML	Maximum-parsimony
MLSA	Multilocus sequence analysis
MP	Maximum-parsimony
NJ	Neighbor-joining
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
QIIME	Quantitative insights into microbial ecology
RAST	Rapid annotation using subsystem technology
SCUBA	Self-contained underwater breathing apparatus
SDA	Split decomposition analysis
TCBS	Thiosulfate-citrate-bile salt-sucrose
VBNC	Viable but non-culturable
WGS	Whole genome sequence

Chapter 1. General Introduction

Currently, reefs are being observed as endangered ecosystems both on a global and local level by the synergic interactions of several stressors. At the global level, coral reefs are considered as one of the most sensitive ecosystems to climate change and other natural and artificial stressors. The recognized natural detrimental factors associated with global corals are temperature fluctuations, nutritional imbalance, toxic chemicals, storms and outbreak of diseases whereas, detected artificial stressors are overfishing, pollution and so on (Vega-Thurber et al., 2009; Tout et al., 2015). Increasing temperatures due to climate change may enhance the emergence and prevalence of both opportunistic and notorious coral pathogenic microbes, such as vibrios, by enhancing proliferation as well as triggering the activity of virulence factors and pathogenicity. It is important to know local potential factors to affect coral health in the global warming era.

1.1. *Vibrio* and climate change

Vibrionaceae family belong to Gammaproteobacteria class that includes six genera (Gomez-Gil et al., 2014) of Gram-negative, facultative anaerobic, straight or curved rods, mostly oxidase and catalase positive, halophilic and motile in features. They are usually free-living, commensals to marine organisms and play a significant role in nutrient cycling of natural aquatic environments. The great flexibility in *Vibrio* lifestyles allows many lines to adapt to marine substrates and animals with versatile ecological implications for vibrios' dynamics in the ocean. The metabolic diversity and forward adaptability of vibrios to changing environments may cause seasonal and geographical variations in *Vibrionaceae* populations, in response to climate change and marine circulations (Mansergh and Zehr, 2014). Vibrios are also important marine bacteria which have substantial environmental and human health significance. The bacteria are also reported to be highly influenced by the consequences of environmental alteration and climate change (Colwell, 1996; Froelich et al., 2013; Jacobs et

al., 2014; Takemura et al., 2014; Vezzulli et al., 2015; Vezzulli et al., 2016). These bacteria are recognized as pathogens not only in humans (Baker-Austin et al., 2012; Geng et al., 2014; Orata et al., 2014; Kubota, 2015) but also in other marine vertebrates and invertebrates including corals (Ben-Haim and Rosenberg, 2002; Becker et al., 2004; Frans et al., 2011; Geng et al., 2014; Munn, 2015). In marine and estuarine environments, elevated temperatures increase the abundance of vibrios and pathogenic *Vibrio* outbreaks are correlated to thermal anomalies (Table 1.1). A lot of reports are currently available regarding increased *Vibrio* infection due to climate change driven raised sea surface temperature throughout the world (Paz et al., 2007; Martinez-Urtaza et al., 2008; Baker-Austin et al., 2010; Baker-Austin et al., 2012; Tout et al., 2015). It has been determined that an increase in sea temperature of 3.7°C enhances*Vibrio* relevant illness risk by 2-3 times (Sterk et al., 2015).

Specifically in the reef system, variation of physico-chemical conditions has distinct impacts on the coral *Vibrio* community. Environmental stresses disrupt the coordinated activities of the host corals and ultimately pose a great risk to coral metabolism by altering bacterial community structure and facilitating the abundance of opportunistic pathogens including vibrios. Earlier reports have demonstrated that coral relevant vibrios are sensitive to environmental changes (Roder et al., 2014; Munn, 2015), and dynamics in coral associated microbial populations is animportant factor allowing the reefs to rapidly acclimate to changes in the environment (Reshef et al., 2006). Therefore, there is special interest in determining factors correlating to dominant abundance of vibrios in different aquatic ecosystems in relation to public health, economic and ecological aspects. Among all the environmental factors, temperature is a critical one that greatly influences the abundance of vibrios (Table 1.1) and also affects the other elements of the ecosystem (algal photosynthesis, DOC availability etc.), as a consequence of environmental perturbations and climate change (Froelich et al., 2013; Jacobs et al., 2014; Takemura et al., 2014). For instance, in south-

eastern Australia, seawater temperatures are increasing at a more rapid rate compared to other parts of the world which has resulted in a high abundance of pathogenic vibrios (Siboni et al., 2016). Beyond temperature, dissolved organic carbon (DOC) has also been found to be connected to bacterial community alterations in reef systems, where it may specifically stimulate the activity and growth of heterotrophic bacteria (Nelson et al., 2013) including reduction of dissolved oxygen thus favouring coral pathogens (Rosenberg and Ben-Haim, 2002; Kline et al. 2006; Vega Thurber et al., 2014; Meyer et al., 2016)

Eutrophication (high loads of nutrients) also creates suitable environments for harmful bacteria in reef systems and may hasten disease progression. Similarly, anthropogenic stressors can influence coral bacterial communities by deteriorating the health status of reef systems (Zieger et al., 2016). Looking at the history of Japanese coral degradation, climate mediated thermal bleaching is a vital coral declining factor. The consequences of world climate change on reefs, the rapid and destructive phenomenon of causing coral damage is defined as 'coral bleaching'. Over the past eight decades, coral bleaching has become an important issue and the first relates report 'Temperature Induced Coral Bleaching' was published in 1931(Yonge and Nichols, 1931). During coral bleaching, the loss of endosymbiotic algae and/or its photosynthetic pigments responsible for coral color disappear and whitening follows. Coral bleaching is sometimes referred as a disease although it is an interacting process between the coral pathogens and triggering ecological factors which is also considered to be an indicator of global processes. On the other hand, the early warning indicators of coral bleaching can be certain vibrios (Table 1.2) or environmental drivers that are highly susceptible to environmental changes.

Table 1.1. *Vibrio* outbreaks (infectious diseases) related to increasing temperature

Country/region	Year	Host	Type of infection	*Vibrio* species	Ref.
Peru	1991	human	cholera	*V. cholarae, V. parahaemolyticus*	Martinez-Urtaza et al., 2008
Bangladesh	1992	human	cholera	*V. cholerae*	Huq et al., 2005
Israel	1996	human	cholera	*V. vulnificus*	Paz et al., 2007
Chile	1998	human	cholera	*V. parahaemolyticus*	Gonzalez-Escalona et al., 2005
Finland	2000	human	wound infection	*V. cholarae, V. parahaemolyticus*	Baker-Austin et al., 2012
Sweden, Finland	2001	human	wound infection	*V. cholarae, V. parahaemolyticus, V. parahaemolyticus*	Baker-Austin et al., 2012
Germany, Finland	2002	human	wound infection	*V. cholarae, V. vulnificus*	Baker-Austin et al., 2012
Finland, Germany, Sweden	2003	human	wound infection	*V. cholarae, V. vulnificus*	Baker-Austin et al., 2012
Sweden, Finland	2005	human	wound infection	*V. cholarae, V. parahaemolyticus*	Baker-Austin et al., 2012
Germany	2006	human	wound infection	*V. vulnificus*	Frank et al., 2006
Sweden	2006	human	wound infection	*V. cholerae*	Andersson and Ekdahl, 2006
Netherlands	2006	human	wound infection	*V. alginolyticus*	Schets et al, 2006
Denmark	2006	human	wound infection	*V. alginolyticus, V. parahaemolyticus*	Andersen, 2006
Finland, Sweden, Germany	2009	human	wound infection	*V. cholarae, V. parahaemolyticus, V. alginolyticus*	Baker-Austin et al., 2012
Netherlands	2009	human	wound infection	*V. cholarae, V. parahaemolyticus, V. alginolyticus*	Schets et al., 2011
Netherlands	2010	human	wound infection	*V. cholarae, V. parahaemolyticus, V. alginolyticus*	Baker-Austin et al., 2012
United kingdom	2011	human	wound infection	*V. alginolyticus*	Reilly et al., 2011
Kuwait	1989	grouper fish	gastro-enteritis	*V. harveyi*	Austin and Zhang, 2006
Venezuela	1993	shrimp	hepatopencrease lesions	*V. harveyi*	Alvarez et al., 1998
Spain	1999-2000	seabass	body and fin infection	*V. harveyi*	Puzalte et al., 2003
Japan	2003-2007	abalone	foot-gill infection	*V. harveyi*	Fukui et al., 2009
Caribbean sea	1997	coral	white-band disease	*V . harveyi*	Ritchie and Smith, 1998
Carrebean sea	1999	coral	yellow-blotch disease	*V. harveyi, V. alginolyticus, V. proteolyticus*	Cervino et al., 2004
Mediterranean sea	1999, 2003	soft coral	not mentioned	*Vibrio* spp.	Vezzulli et al., 2010
Mediterranean sea	2003	benthic communities	tissue lesions	*V. coralliilyticus*	Bally and Garrabou, 2007; Garrabou et al., 2009
Caribbean sea, Indo-pacific	2005	coral	rapid tissue necrosis	*V. alginolyticus, V. harveyi*	Luna et al., 2007
Easterm Mediterranean sea	2006, 2008	coral	coral lesions	*V. mediteraney, V. coralliilyticus*	Rubio-Portillo et al., 2014
Caribbean sea	2007	coral	black-band disease	*Vibrio* spp.	Arotsker et al., 2009

Table 1.2. Coral bleaching events and related vibrios

Disease	Causative agent	Coral host	Location	Ref.
Bleaching	*V. shiloi*	*Oculina patagonica*	Eastern Mediterranean	Kushmaro et al., 1997
Bleaching and lysis	*V. coralliilyticus*	*Pocillopora damicornis*	Indian Ocean, Red Sea	Ben-Haim et al., 2003
Bleaching like disease	*V. harveyi*	*Acropora hyacinthus*	Vatia Bay, American samoa	Wilson et al., 2012
Bleaching like disease	*V. owensii*	*Montipora capitata*	Kaneohe Bay, Hawaii	Ushijima et al., 2012
Bleaching	*Vibro* spp.	*Montastrea annularis*	Caribbean/Great Barrier reef	Ritchie et al., 1994

It is now evident that temperature-induced coral bleaching has caused mass coral decline throughout the world including the Indian ocean, the Pacific Ocean and the south Atlantic Ocean, the Caribbean and Japan reef areas (Goreau et al., 2000; Loya et al., 2001; Berkelmans et al., 2004; Guest et al., 2012; Chimetto Tonon et al., 2015). The putative causes of coral bleaching include several factors such as photoinhibition, oxidative stress, thermal shock, abundance of harmful microbiota etc. (Rosenberg et al., 2009), where thermal stress is critically significant. Other recognized coral-bleaching influencing abiotic and biotic factors are ocean acidification (Anthony et al., 2008), salinity variation (Kerswell and Jones, 2003), nutrient enrichment (Wiedenmann et al., 2013), and sedimentation (Glynn, 1996). Moreover, some local stressors (pollutants, nutrient loads, sedimentation etc.) are also considered as variables influencing localized bleaching events. The important considerable output of bleaching events is the shifting of coral-relevant bacterial communities and reduction of antimicrobial activity in the holobiont (Bourne et al., 2008; Ritchie, 2006), especially the alarming dominance of coral pathogens specifically vibrios (Muller et al., 2008; MaClanahan et al., 2009; Littman et al., 2011; Munn, 2015) and thereby a clear link is established among coral bleaching, elevated seawater temperature and other coral disease outbreaks (i.e, white band, yellow blotch). In the worst case scenario, the aforesaid multiple factors may interact before, during and after bleaching events by antagonistically and/or synergistically and finally leading to the proliferation of coral pathogens and simultaneously enhancing coral degradation.

1.2. *Vibrio* as a coral holobiont

'Holobiont' is a complex multi-partner symbiotic entity where the inter-organisomal associations appear to have co-evolved and are quite dynamic. Reef-building corals are the basis of reef ecosystems and act as a holobiont that possesses diversified visible and invisible organisms including the representatives from all three domains of life (Rohwer et al., 2002;

Rosenberg et al., 2007). The familiar coral-associated eukaryotes are symbiotic dinoflagellates (*Symbiodinium*), endolithic algae, fungi, sponges dominant in coral tissue and skeleton, whereas a high abundance and diversity of bacteria and archaea are present in the tissue, mucus and skeleton of corals. Importantly, reef system provides suitable niches for bacteria, as different coral layers invite numerous bacterial communities. Interactions among host-associated bacterial communities are critical for the overall health of the coral holobiont. The bacteria and archaea play important roles in reef systems especially in nutrient cycling and coral immunity although some bacteria are detrimental to coral.

In detail, corals and its associated 'coral holobionts' contain diversified extracellular and intracellular microbes, distinct from those available in water columns and may be niche specific, frequently showing spatial variability at geographic scales (Rohwer et al., 2002; Kvennefors et al., 2012). Almost all the surfaces of reef systems possess microbes, dominantly bacteria, adapting to the specific environmental conditions of each niche by directly competing or co-habiting with other bacteria and host coral. Usually, corals grow in oligotrophic environments where nutrient concentrations are low and therefore, bacteria may assimilate required limiting nutrients to create a suitable environment for coral growth and in turn, corals facilitate bacterial growth by releasing fixed carbon, used as sole energy source by reef associated bacteria and thus the mutualistic relation is established between coral and related bacteria (Shashar et al., 1994; Wild et al., 2004; Rix et al., 2016). More specifically, in open coral systems, both the obligate endosymbionts and other microbes may closely interact with the other holobiont members in a temporally dynamic fashion (Rohwer et al., 2002; Ainsworth et al., 2008). Although the coral host metabolism is the vital contributor in regulating coral associated bacterial communities, ecological factors also play important roles (Kelly et al., 2014; Pantos et al., 2015) as coral-homeostasis is greatly affected by the altered environment. In this era of global warming, prokaryotes have to survive through establishing

different types of relationships with their surroundings in novel or changed environment following different ecological strategies such as phenotypic plasticity, behavioral modifications or migration to more favorable niches. In coral reef ecosystems, vibrios appear to be a considerable proportion of the microbiota in both healthy (Koren and Rosenberg, 2006) and infected reefs (Weil et al., 2006; Bourne et al., 2008; Kooperman et al., 2007; Munn, 2015). Indeed, vibrios represent a vital portion of coral microbiomes, representing more than 68% in some corals (Koren and Rosenberg, 2006). Changed ecological parameters may also be linked to the shift of bacterial communities and may lead to the dominance of opportunistic *Vibrio* pathogens by shutting down the emergent functions and diminishing the homeostasis of the coral holobiont with ecosystem-scale cosequences. Therefore, it is interesting as well as important to improve the understanding of *Vibrionaceae* bacteria and their behavior as coral-partners via the study of their diversity, dynamics and ecological interactions within the holobiont that will promote comprehensive knowledge of mechanisms, shaping the coral holobiont on ecological and evolutionary time scales.

The contributions of vibrios in coral-holobionts may be positive or negative and are probably influenced by environmental variables. Despite several vibrios establishing mutualistic relationships with coral host through nitrogen fixation (Lesser et al., 2004; Chimetto et al., 2008; Olson et al., 2009), chitin decomposition (Ducklow and Mitchell, 1979), antimicrobials provision (Ritchie, 2006), swarming inhibition (Krediet et al., 2013), and quorum sensing inhibition (Teplitski and Ritchie, 2009); some are recognized as opportunistic coral pathogens (Luna et al., 2010; Ushijima et al., 2012; Zhenyu et al., 2013; Munn, 2015; Vezulli et al., 2015). A few vibrios are dominant as opportunistic as well as notorious pathogenic coral holobionts (Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003; Garren et al., 2014; Tout et al., 2015; Munn, 2015) whereas a wide range of *Vibrionaceae* members (*Vibrio*, *Photobacterium*, *Enterovibrio*) are isolated from the tissue and mucus of healthy-corals

(Thompson et al., 2005; Dinsdale et al., 2008; Chimetto et al., 2009; Alves et al., 2010; Haldar et al., 2011). Some vibrios also seem to be examples of part of the normal bacteria community in reef systems rather than being linked to disease (Thompson et al., 2005), and have even been found to play defensive roles against coral pathogens (Ritchie, 2006).

1.3. Recent progress on the assessment of *Vibrio* diversity

Microbiota represent the vast majority of the planet's biodiversity and we now know that most phyla in the tree of life are comprised of microbial taxa due to recent advances in sequence based phylogeny (Langille et al., 2013; Martiny et al., 2013; Yarza et al., 2014; Garrity, 2016; Hug et al., 2016; Sangal et al., 2016). Species is the basic unit of taxonomy. Accurate identification of bacterial species with appropiate nomenclature is obviously important in all branches of microbiology.

Studies on diversity, population biology and genetics of vibrios is an emerging to know their cosmopolitan spread in the nature. Until now, multiple studies (Rehnstam et al., 1993; Eilers et al., 2000; Heidelberg et al., 2002; Chimetto Tonon et al., 2015; Vezzulli et al., 2015) on '*Vibrio* ecology' have focused on the specific taxon, leading to an extensive body of literature on their genetics and ecology, although the diversity and dynamics of co-occurring *Vibrio* populations have rarely been addressed. The increasing progress in the study of *Vibrio* diversity, genomics, and evolution has forced bacteriologists to update the *Vibrio*-phylogeny with their ecological and evolutionary status. However, taxonomic information still seems to be insufficient to push forward the clear elucidation of *Vibrio*-diversity, dynamics and evolution. For *Vibrio* species identification, *pyrH* gene (uridylate kinase) is recognized as a good discriminate single molecular marker at species level (Chimetto et al., 2009; Alves et al., 2010; Gregoracci et al., 2012; Tall et al., 2013; Chimetto Tonon et al., 2015; Amin et al.,

2016) as this gene fulfilled the criteria necessary to be a good predictor of the whole *Vibrio* genome.

Description as well as characterization of *Vibrionaceae* diversity in the environment including their ecological features, distribution and abundances with novel species addition is currently creating growing interest. Recent advancements in microbial phylogeny have led to a frontier understanding of the role of microorganisms in the links of host-microbes interactions focusing on the diversity and functional capacity of microbiota in nature including reef systems (Bayer et al., 2013; McFall-Ngai et al., 2013; Chimetto Tonon et al., 2015; Amin et al., 2016). The phylogeny based mathematical modeling can also play a crucial role in this regard in connecting disciplines toward a better understanding of complex biological, physical, evolutionary and environmental interactions of bacterial ecological systems. In particular, the quantitative mathematical model, AdaptML is capable of combining genetic and ecological information of the analyzed samples by identifying the genetically and ecologically distinct bacteria at population level (Chimetto Tonon et al., 2015; Amin et al., 2016). The evolutionary hidden Markov model of AdaptML predicts bacterial populations as 'group of related strains' that share a common projected habitat which reflects their relative abundances in the studied environmental habitat categories. So, AdaptML establishes the evolutionary history of habitat differentiation and thus incorporates the phylogeny, sampling sites and sampling time of studied bacteria.

1.4. General aims of the study

Ishigaki Island, located in the southern part of Okinawa prefecture in Japan, is considered to be a sub-tropical climate zone for terrestrial environments although its' marine system is considered as tropical due to the strong influence of Kuroshio-warm ocean current (Shimomura and Naruse, 2015). According to the previous history of Ishigaki reef (Harri et

al., 2014), total coral area was decreased by severe bleaching events in 1978. After rapid recovery in 2003, the coral area further declined from 2004 to 2008 due to repeated bleaching events and typhoons (Kayanne et al., 2002; Dadhich et al., 2012; Harii et al., 2014). The synergistic impacts of thermal anomalies, sedimentation and typhoon-mediated physical damage selectively threatened the Ishigaki reefs. Beyond what little information of reef declining stressors in Ishigaki coral Island is available, the microbial structure as well as the impact of environmental stressors to bacterial populations and their dynamics are quite unknown today. A comprehensive elucidation of *Vibrionaceae* diversity, their dynamics and environmental drivers of their spatial and temporal evolution are essential pre-requisites for addressing human and coral health issues. The present study has been conducted with the following aims; 1) to elucidate the spatiotemporal diversity dynamics of vibrios in the Ishgaki coral reef system, and 2) to understand environmental drives affecting the *Vibrio* diversity. I also described new *Vibrio* taxa found through the research.

Chapter 2. The First Temporal and Spatial Assessment of *Vibrio* Diversity of the Surrounding Seawater of Coral Reefs in Isihigaki, Japan

2.1. Abstract

Coral reefs perform a major role in regulating marine biodiversity and serve as hotspot for highly dynamic and diverse microbiomes as holobionts. Corals around Ishigaki, however, are at risk due to tremendous stressors including elevation of seawater temperature, eutrophication and so on. However, no information is currently available on *Vibrionaceae* populations in Ishigaki coral reef ecosystems including the message how *Vibrio* diversity fluctuates spatially and temporally. The aims of this chapter are to characterize *Vibrionaceae* diversity and to elucidate spatiotemporal *Vibrio* diversity dynamic at both community and population levels and to assess the environmental drivers correlated to *Vibrio* abundance and diversity. The *Vibrio* community identified based on *pyrH* gene phylogeny of 685 isolates from seawater directly connecting to Ishigaki coral holobionts consisted of 22 known and 12 potential novel *Vibrionaceae* species. The most prominent species were *V. hyugaensis*, *V. owensii*, and *V. harveyi* followed by *V. maritimus/V. variabillis*, *V. campbellii*, *V. coralliilyticus* and *P. rosenbergii*. The *Vibrio* community fluctuations, assessed by PCoA with UniFrac distance and clustering with Euclidiean distance were varied less not only by year but also by site. Interstingly, significant positive correlation was observed between rising seawater temperature and the abundance of *V. campbellii* (r=0.62; P<0.05) whereas the opposite was observed for *V. owensii* (r=-0.58; P<0.05) and the C6 group of *V. hyugaensis* (r=-0.62; P<0.05). AdaptML-based microhabitat differentiation revealed that *V. harveyi*, *V. campbellii*, *P. rosenbergii*, and *V. coralliilyticus* populations were less-ecologically distinctive whereas *V. astriarenae* and *V. ishigakensis* were ecologically diverse. This knowledge could be important clue for the future actions of coral conservation.

2.2. Introduction

Coral reef ecosystem consists of a flexible consortium of eukaryotic and prokaryotic organisms as a holobiont and are rich in compounds for interacting each other as cross talks, protection of their territory and as triggers for these symbiotic association dynamics (Shnit-Orland et al., 2009; Kvennefors et al., 2012; Kooperman et al., 2007). Coral hosts large, diverse and specific microbial populations, which have both important beneficial and harmful roles for the host. (Ritchie and Smith, 2004; Rosenberg et al., 2007). More specifically, corals provide three habitats for bacteria (the surface mucus layer, coral tissue and calcium carbonate skeleton) each of which harbors a distinct bacterial population (Koren & Rosenberg, 2006). Among the bacterial populations, vibrios have been recognized as important members of coral holobionts (Rohwer and Kelly, 2004; Vezzulli et al., 2010; Vezzulli et al., 2012; Baker-Austin et al., 2012; Moreira et al., 2014; Rubio-Portillo et al., 2014; Munn, 2015). Some vibrios may establish mutualistic partnership with corals by releasing nutrients and secondary metabolites (Ritchi, 2006; Chimetto et al., 2008; Chimetto et al., 2009), while others play a major role in the disruption of coral health (Munn, 2015).

Coral and coral reefs are in steep decline worldwide due to the combined effects of various stressors such as global warming, pollution, overfishing and infectious diseases (Hughes et al., 2003; Weil et al., 2006; Dinsdale et al., 2008; Vezzulli et al., 2012; Baker-Austin et al., 2012). Rising seawater temperature related to global climate change is a big threat to coral health and is ultimately linked to increasing coral disease such as bleaching events (Tout et al., 2015). Impressive progress has been reported regarding characterization of the coral microbiota over the last decade with its altered composition often correlated to the appearance of signs and diseases and/or bleaching that ultimately suggest a link between microbes, global coral health and stability of reef ecosystems (Krediet et al., 2013). Among them, coral vibriosis is a well known disease, of which occurrences are greatly influenced by

increasing seawater temperature (Vezzulli et al., 2012; Baker-Austin et al., 2012; Munn, 2015). In particular, at warm temperatures, *V. shiloi* expresses a cell-surface adhesion protein that is required for bacterial attachment to the coral surface and simultaneously expresses Toxin-P that ultimately inhibits photosynthesis of the coral-endosymbiotic algae and super-oxide dismutase required for survival of this pathogen inside the coral (Rosenberg et al., 2007; Munn, 2015).

The coral reef ecosystem in Ishigaki Island in southwestern Japan is surrounded by well developed fringing corals with a variety of mangroves and sandy or rocky shores (Sano, 2000; Shimomura and Naruse, 2015). Recently, these reefs have been severely stressed due to typhoons, coral bleaching and soil pollution. Red soil derived turbid seawater in Okinawa, Ishigaki and Iriomote coral reef seawater adversely affects the photosynthesis of coral symbiont algae (Zooxanthelae) and also interfered with the settlement of coral larvae on the ocean bed which ultimately influenced coral damage (Kakuma and Kamimura, 2011). Due to this, eutrophication including inflow of red soil is an important threat to Ishigaki corals. More specifically, accumulation of red soil and high nutrient inputs due to land development since 1972 have severely affected the Ishigaki coral reef (Hasegawa, 2011). Rivers from the northern part of Ishigaki coral reef originating from agricultural watershed may have detrimental effects on corals because exposure to silt and nutrient rich sediments may badly stress corals (Weber et al., 2006). In addition, run-off from soils after typhoons can easily damage coral as well (Harii et al., 2014). Presently, several reports are available regarding coral-microbiota from different parts of the world (Rohwer et al., 2002; Rosenberg et al., 2007; Chimetto et al., 2009; Alves Jr et al., 2010; Krediet et al., 2013; Wild et al., 2004; Chimetto Tonon et al., 2015) but little is known about the Ishigaki coral reef microbial communities. The aims of this chapter are to characterize the *Vibrio* diversity spatially and

temporally, and to elucidate which environmental determinants (temperature or DOC) correlate the *Vibrio* diversity dynamics.

2.3. Materials and Methods

2.3.1. Water sampling and isolation of vibrios

Seawater samples were collected in 2012, 2013 and 2014 (June-July) from five sites (Figure 2.1) off the Ishigaki coral reef, Okinawa, Japan. The sampling sites were Miyara (24°20.5489' N, 124°13.0408' E), Osaki (24°25.4171' N 124°04.4956' E), Taketomi (24°20.5260' N, 124°05.6443' E), Sekisei outer (24°21.7557' N, 124°02.7190' E) and Iriomote (24°23.208' N 123°55.681' E) at depths of 3-4 meters. More specifically, these seawater samples were within 2 m of coral holobionts and were collected by SCUBA diving and stored in clean containers and/or sterilized tubes. No specific permissions were required for the water sampling activities in these locations. The seawater samples were then brought back to the laboratory for bacterial isolation. A total of 0.2 mL of the seawater sample was directly spread on to TCBS agar plate (Nissui Pharmacy, Tokyo, Japan) and incubated at 25°C for 48 hours. After incubation, numbers of individual colonies were counted manually to estimate 'viable *Vibrio* counts'. All colonies except those showing lack of growth were purified twice on the TCBS agar plates. The purity was checked using the ZoBell 2216E agar plate at 25°C. These strains were preserved in cryo-vials using the ZoBell broth supplemented with 20% (v/v) glycerol at -80°C.

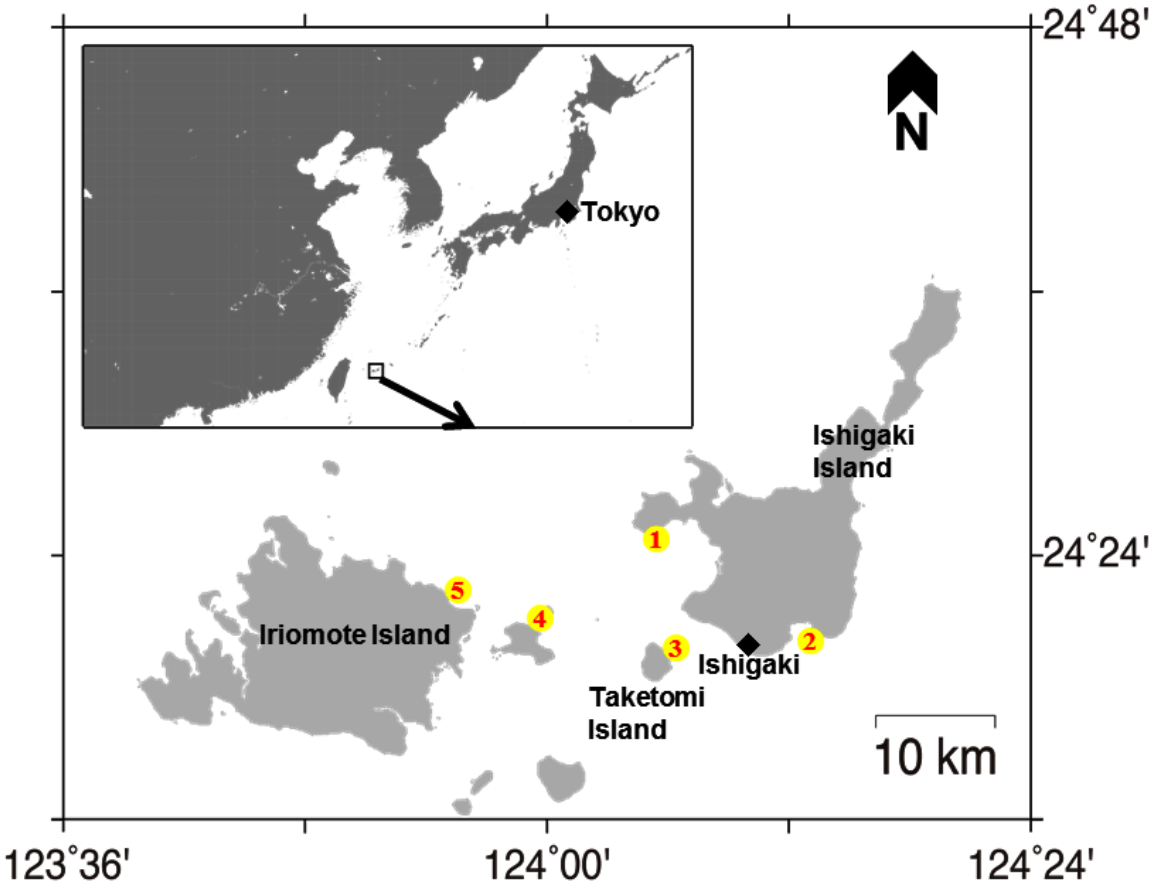

Figure 2.1. Map showing the sampling locations. Sites are marked with a yellow circle. 1. Osaki; 2. Miyara; 3. Taketomi; 4. Sekisei outer; 5. Iriomote in Ishigaki coral reef ecosystem.

2.3.2. Molecular phylogenetic affiliation on the basis of *pyrH* gene sequences to *Vibrionaceae* species groups

Molecular phylogenetic affiliation of all *Vibrio* isolates (*n*=685) was performed on the basis of *pyrH* (uridylate kinase) gene sequences. A 550 bp of *pyrH* gene was amplified using pyrH80F and pyrH530R primers (Sawabe et al., 2007) as a colony suspension or a purified DNA as a PCR template. Genomic DNA was purified using a Promega Wizard Genomic DNA extraction system according to the protocol provided by the manufacturer. The PCR cycle consisted of an initial denaturation step at 95°C for 180 s and 30 cycles of an denaturation (95°C for 60 s), an annealing step (50°C for 60 s) and an extension step (72°C for 60 s). The PCR products were analyzed on 1.5% agarose gel and the PCR products producing a single band on agarose gels were purified using Promega Gel and PCR purification system (Promega, Madison, WI). Approximately 50 ng of template was directly sequenced using the pyrH80F or pyrH530R primer and a BigDye terminator sequencing kit version 3.1 (Life Technologies, Carlsbad, CA) according to the protocol recommended by the manufacturer. DNA sequencing was performed with an Applied Biosystems model 373A automated sequencer. The same primers (pyrH80F or pyrH530R) were used for the sequencing.

Using the well curated *pyrH* gene sequences, which could cover more than 145 known described species of *Vibrionaceae*, molecular phylogenetic affiliations of Ishigaki *Vibrio* isolates were performed. To retain the *pyrH* tree topology within *Vibrionaceae*, *pyrH* gene sequence of *E. coli* W3110 (NC_010473) was included in the dataset as an out group. The sequences were aligned using Clustal X version 2.1 (Larkin et al., 2007) and the alignment was checked by eye and corrected manually. The position used for the affiliation corresponded to positions 171 to 543 in the *V. cholera* O1 Eltor N16961 (AE003852). *pyrH* gene phylogeny was reconstructed by neighbor-joining method (Saitou and Nei, 1987) with

bootstrap values of 500 replicates implemented to MEGA programs version 6.06 (Tamura et al., 2013). Evolutionary distances were corrected using the Jukes-Cantor method. Clustering to a described species with more than 98.4% *pyrH* gene similarity was used for the final affiliation for species identification of Ishigaki coral reef isolates. For the affiliation of closed species in clusters harboring *V. harveyi*, *V. owensii*, *V. hyugaensis*, *V. communis*, *V. campbellii* and *P. damselae* subsp. *piscicida*, a >99.7% cutoff was adopted to affiliate species. All *pyrH* and 16S rRNA genes sequences used in this study have been deposited in BaMBa under data package identification number pmeirelles.20.1. To further affiliate unassigned *pyrH* clusters to new species candidates, 16S rRNA gene sequences of 38 representative strains from *pyrH* unassigned clusters were obtained according to Al-saari et al. (2015). In brief, the amplification primers (24F and 1509R) used for PCR amplification gave a 1.5 kb long PCR product and corresponded to positions 25 to 1521 in the *E. coli* sequence. A 99.7% cut-off was used for the final affiliation of Ishigaki coral reef isolates to the new *Vibrionaceae* species candidate.

2.3.3. Inferred habitat associations of *Vibrio* community

To infer habitat associations of the *Vibrio* community in Ishigaki coral reef during a three-year (2012-2014) assessment, PCoA based on unweighted UniFrac distance, hierarchical cluster analysis of *Vibrio* community composition and AdaptML was performed.

UniFrac distance was calculated using 658 *pyrH* gene sequences of *Vibrio* isolates and Quantitative Insights Into Microbial Ecology (QIIME) 1.9 software package (Caporaso et al., 2010) according to Yamazaki et al. (2016) except the use of *pyrH* gene phylogenetic tree. Relationships among *Vibrio* communities in accordance with sampling sites and years were visualized using PCoA. Hierarchical clustering analyses were performed with R (R Development Core Team 2011), except where indicated. Abundance and multivariate figures

were plotted with packages ggplot2 and reshape (Wickham 2007, 2009). The hierarchical cluster was constructed using Ward's minimum variance method (performed using "hclust" R function), which aims at finding compact, spherical clusters, on a Euclidian dissimilarity matrix (calculated using "dist" R function). *Vibrio* diversity indices (Shannon entropy and Shannon evenness [i.e., Hill's Ratio]) and richness were calculated with the vegan R package (Oksanen et al., 2005).

The mathematical model (AdaptML), which uses a Hidden Markov Model, was used to predict the phylogenetic bounds of ecologically distinct *Vibrio* populations and their habitat composition (distribution among environmental categories). 'Clusters of vibrios' sequences were obtained using the software AdaptML as described previously (Hunt et al., 2008). In brief, the software combines genetic information embedded in sequence-based phylogenies and information about the ecology, herein the source and place of isolation, in order to identify genetically-and ecologically-distinct bacterial populations. AdaptML algorithms can account for environmental parameter discretization schemes and are based on the model concept of habitat (a place and related features that determines microbial distribution). Habitats are characterized by discrete probability distributions describing the likelihood that a strain adapted to a habitat will be sampled from a given ecological state, i.e. at a particular location in the water column. A maximum likelihood model is used for the evolution of habitat association on the tree (Hunt et al., 2008). The habitat-learning and clustering steps of AdaptML were performed using the default settings. Confident assignments are shown to fit ecological populations predicted by the model. The model threshold value was set at 0.05 and *E. coli* W3110 was used as an out-group. The bootstrap percentages analysis was rerun 100 times with the same phylogenetic tree to verify the stability of the predictions. The circular tree figure was drawn using online iTOL software (Letunic and Bork, 2007). To prevent numerical instabilities in AdaptML's maximum likelihood computations, branches with zero

length were assigned to the minimal observed non-zero branch length: 0.001. Clades supported in 80% of bootstraps are shown.

2.3.4. Correlation between *Vibrionaceae* species groups and environmewntal determinants

Seawater environmental determinants including temperature and DOC were assessed. Water temperature was measured directly using a thermometer. DOC was determined using the method described by Grasshoff et al. (2009). Pearson correlation between each *Vibrio* abundance and environmental determinant was calculated using Microsoft Excel software.

2.4. Results

2.4.1. Diversity of vibrios in Ishigaki coral reef seawater

Investigation of *Vibrio* diversity in Ishigaki coral reef seawater using *pyrH* gene phylogeny discovered a total of 44 clusters (Table 2.1, Figure 2. 2); 32 clusters were affiliated to 22 known *Vibrionaceae* species including 10 *V. hyugaensis* and 2 *P. damselae* subsp. *piscicida* subclusters, and the other 12 were affiliated to novel species candidates in *Vibrionaceae*. A total of 658 strains were identified as known *Vibrionaceae* species to *V. alfacsensis, V. alginolyticus, V. astriarenae, V. azureus, V. campbellii, V. communis, V. coralliilyticus*-*V. neptunis, V. harveyi, V. hyugaensis, V. "ishigakensis", V. mediterranei, V. nigripulchritudo, V. orientalis, V. owensii, V. ponticus, V. pelagius, V. rotiferianus, V. tubiashii, V. variabillis/ V. maritimus, Photobacterium aphoticum, P. damselae* subsp. *piscicida* and *P. rosenbergii*.

However, 26 strains belonged to C121 (*n*=5), CV39 (*n*=2), CV50 (*n*=4), CV58 (*n*=1), CV96 (*n*=1), CV97 (*n*=1), CV172 (*n*=1), C4I174 (*n*=4), C4II189 (*n*=1), C4II259 (*n*=1), C4III282 (*n*=3) and C4V358 (*n*=1) clusters which were not affiliated to any known vibrios species (Figure 2.2, Table 2.1). *V. hyugaensis* was the largest group of the *Vibrio* community in

Ishigaki coral reef seawater (*n* = 200, 29.2%) and was mostly diversified into 10 different *pyrH* clusters (Table 2.1).

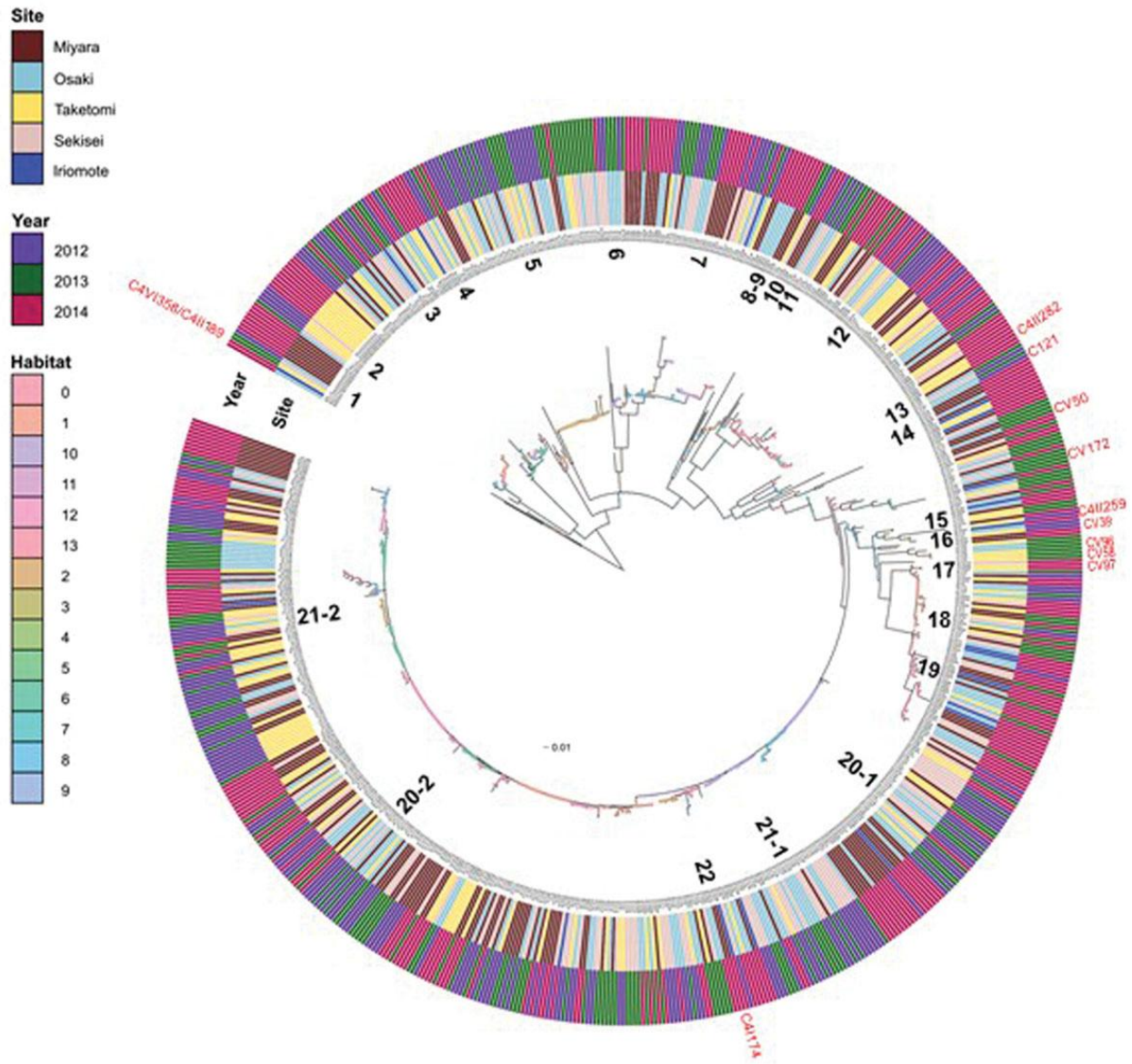

Figure 2.2. **Inferred habitats predicted by AdaptML model using *pyrH* gene sequences of *Vibrio* isolates from Ishigaki coral reef seawater.** Inner and outer ring indicate sampling sites and sampling years respectively. Predicted habitats are shown as nodes on the tree (habitat legend matches the colored circles). The closest named *Vibrio* species to numbered *Vibrio* populations' are as follows- 1, *P. aphoticum*; 2, *V. astriarenae*; 3, *V. ishigakensis*; 4, *V. pelagius*; 5, *P. rosenbergii*; 6, *V. mediterranei*; 7, *V. variabillis/maritimus*; 8, *V. nigripulchritudo*; 9, *V. orientalis*; 10, *V. tubiashii*; 11, *V. alginolyticus*; 12, *V. coralliilyticus/neptunis*; 13. *V. ponticus*; 14. *V. azureus*, 15, *V. alfacsensis*; 16, *V. orientalis*; 17, *P. damsella* subsp. *piscicida*; 18, *V. harveyi*; 19, *V. campbellii*; 20-1, *V. owensii*-1; 20-2, *V. owensii*-2; 21-1, *V. hyugaensis*; 21-2, *V. hyugaensis*; 22, *V. communis*. Red marked strains, outside of the outer ring indicate the positions of putative new species candidates.

Table 2.1. List of strains isolated from coral reef seawater at different sampling locations of Ishigaki Island

Species name	Total No. of isolates	**Sampling locations**				
	No. (%)	Miyara	Osaki	Taketomi	Sekisei	Iriomote
V. owensii	148 (21.6)	55	28	35	29	1
V. variabillis/maritimus	60 (8.8)	22	20	4	13	1
V. hyugaensis (C6)	52 (7.6)	17	15	8	12	0
V. coralliilyticus/neptunis	49 (7.2)	15	9	15	10	0
V. hyugaensis (C46)	41 (6.0)	4	17	3	15	2
P. rosenbergii	41 (6.0)	5	15	8	13	0
V. campbellii	36 (5.3)	9	12	1	8	6
V. harveyi	31(4.5)	9	5	11	5	1
V. hyugaensis (C68)	31 (4.5)	14	8	2	6	1
V. ishigakensis (C1)	29 (4.2)	6	10	6	6	1
V. hyugaensis (C58)	25 (3.7)	21	0	3	1	0
V. astriarenae (C7)	23 (3.4)	8	1	12	2	0
V. hyugaensis (C71)	14 (2.0)	3	1	7	2	1
V. hyugaensis (C16)	13 (1.9)	3	4	2	4	0
V. hyugaensis (C49)	13 (1.9)	3	4	5	1	0
V. pelagius	9 (1.3)	2	2	1	3	1
*V. hyugaensis (*C164)	9 (1.3)	1	5	0	3	0
V. communis	5 (0.7)	3	0	0	2	0
V. tubiashii	5 (0.7)	3	1	0	1	0
V. alginolyticus	5 (0.7)	0	2	1	2	0
V. ponticus	3 (0.4)	1	1	0	1	0
P. aphoticum	3 (0.4)	0	1	1	1	0
V. rotiferianus	3 (0.4)	0	1	1	0	1
P. damsellae (CV38)	3 (0.4)	0	1	2	0	0
V. nigripulchritudo	1 (0.1)	1	0	0	0	0
V. alfacsensis	1 (0.1)	0	0	1	0	0
V. orientalis	1 (0.1)	0	0	0	1	0
V. azureus	1 (0.1)	0	1	0	0	0
V. mediterranei	1 (0.1)	0	1	0	0	0
V. hyugaensis (C156)	1 (0.1)	0	0	0	1	0
V. hyugaensis (C4I100)	1 (0.1)	1	0	0	0	0
P. damsellae (CV125)	1 (0.1)	0	0	0	1	0
C121*	5 (0.6)	3	2	0	0	0
C4I174*	4 (0.6)	3	0	0	0	1
CV50*	4 (0.6)	2	2	0	0	0
C4III282*	3 (0.4)	0	0	3	0	0
CV39*	2 (0.3)	0	0	2	0	0
C4II259*	2 (0.3)	0	1	0	0	1
CV172*	1 (0.1)	0	1	0	0	0
CV58*	1 (0.2)	0	1	0	0	0
CV96*	1 (0.1)	0	0	1	0	0
CV97*	1 (0.1)	0	0	1	0	0
C4II189*	1 (0.1)	0	1	0	0	0
C4V358*	1 (0.1)	0	0	0	0	1

*new species candidates

The occupation rates of each *V. hyugaensis* cluster were C71 (2.0%), C68 (4.5%), C6 (7.6%), C16 (1.9%), C49 (1.9%), C58 (3.7%), C46 (6.0%), C156 (0.1%), C164 (1.3%) and C4I100 (0.1%). *V. owensii*, a potent coral pathogen, was present in 21.6% of the total isolated vibrios. The third most abundant *Vibrio* group was *V. variabilis*/*maritimus* which was recorded in 8.8% of the isolated vibrios. Other prominent vibrios were *Photobacterium rosenbergii* (6.0%), *V. campbellii* (5.3%) and *V. harveyi* (4.5%). Some other recognized potential coral pathogens, *V. coralliilyticus* (*n*=49, 7.2%) and *V. alginolyticus* (*n*=5) were also found. Less significant amounts of identified vibrios (Table 1.1) were *V. ponticus* (*n*=3, 0.4%), *V. pelagius* (*n*=9, 1.3%), *V. tubiashi* (*n* = 5, 0.7%), *V. rotiferianus* (*n* = 3, 0.4%), *P. aphoticum* (*n* = 3, 0.4%) and two recent newly described species, *V. astriarenae* (3.4%) and *V. ishigakensis* (4.2%). *V. nigripulchritudo* (*n*=1), *V. alfacsensis* (*n*=1), *V. orientalis* (*n*=1), *V. azureus* (*n*=1) and *V. mediterranei* (*n*=1) were less highlighted vibrios.

2.4.2. *Vibrio* community dynamics

Unweighted FastUniFrac analysis revealed no-apparent grouping of *Vibrio* communities not only site by site but also year by year (Figure 2.3A). However, a simple cladogram constructed on the basis of relative abundances in the *Vibrio* community illustrated two apparent clusters in the *Vibrio* communities of Iriomote 2014, Osaki 2014, Sekisei 2014, Taketomi 2014 and Miyara 2013 and those of Taketomi 2012, Miyara 2014, Sekisei 2012 and Osaki 2013 (Figure 2.3B). Signatures of the former and the latter communities showed significant abundances ($P<0.05$) of *V. campbellii*, *V. hyugaensis* (C6 group), and *V. owensii* respectively. Vibrios from Sekisei 2013 and Osaka 2012 were sub-clustered using a relative higher abundance of *P. rosenbergii*.

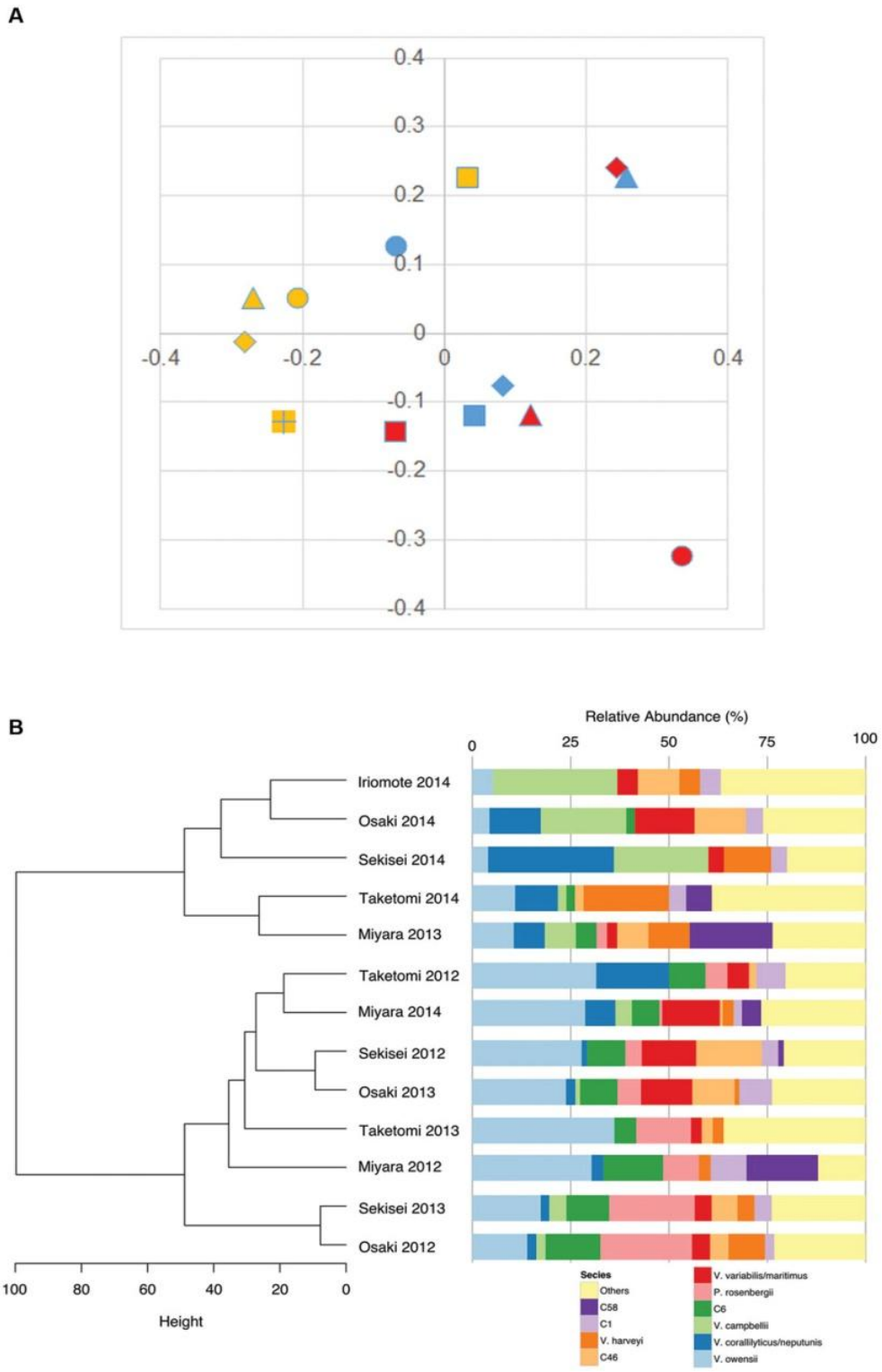

Figure 2.3. *Vibrio* community diversity dynamics in Ishigaki coral reef seawater. (A) multidimensional scaling plot (FastUnifrac:PCoA), isolates indicating colors and shapes: blue, 2012; red, 2013; yellow, 2014; dot, Taketomi; square, Miyara; diamond, Osaki; triangle, Sekisei; square (+), Iriomote; (B) dendrogram clustering of vibrios according to their relative abundances.

2.4.3. Ecologically distinct populations among *Vibrio* isolates

The AdaptML analysis showed that the *Vibrio* isolates are distributed in 8 habitat spectra (H-0, H-1, H-2, H-6, H-8, H-10, H-12 and H-13) (Figures 2.2 and 2.4). Habitats are the part of an ecosystem (a spectrum of environment types) from where microbial populations are isolated. *V. owensii*, *V. harveyi*, *V. hyugaensis* (C71, C46, C6, C16, C49, C58 and C68 groups), *V. campbellii*, *V. ishigakensis*, *V. variabillis/V. maritimus*, *V. coralliilyticus/V. neptunis*, *P. rosenbergii* and *V. pelagius* were observed from all the sites although *V. alfacsensis*, and *V. nigripulchritudo* were found only from Taketomi and Miyara respectively (Figure 1.4). Another two vibrios, *V. azureus* and *V. mediterranei* were specific for Osaki and one more single *Vibrio*, *V. orientalis*, was recorded solely from Sekisei reef site. The spatiotemporal distribution of *V. campbelli* was likely to support the temperature dependent abundance from 2014 in these study areas (Figure 2.4). From all the predicted habitat spectra, H-10 and H-12 showed the strains which were isolated from all the sampling sites and sampling years with major isolates from Sekisei-2012 (41.7%) and Miyara-2014 (41.2%), respectively. Other habitats (H-0, H-1, H-2, H-6, H-8, H-13) showed the isolates (Figure 2.5) from several sampling sites and years but not from all. The major isolates of H-0, H-1, H-2, H-6, H-8, and H-13 were obtained from Osaki-2014 (34.9%), Taketomi-2014 (34.9%), Osaki-2012 (20.2%), Osaki-2013 (33.7%), Miyara-2014 (78.6%) and Taketomi-2012 (55%), respectively.

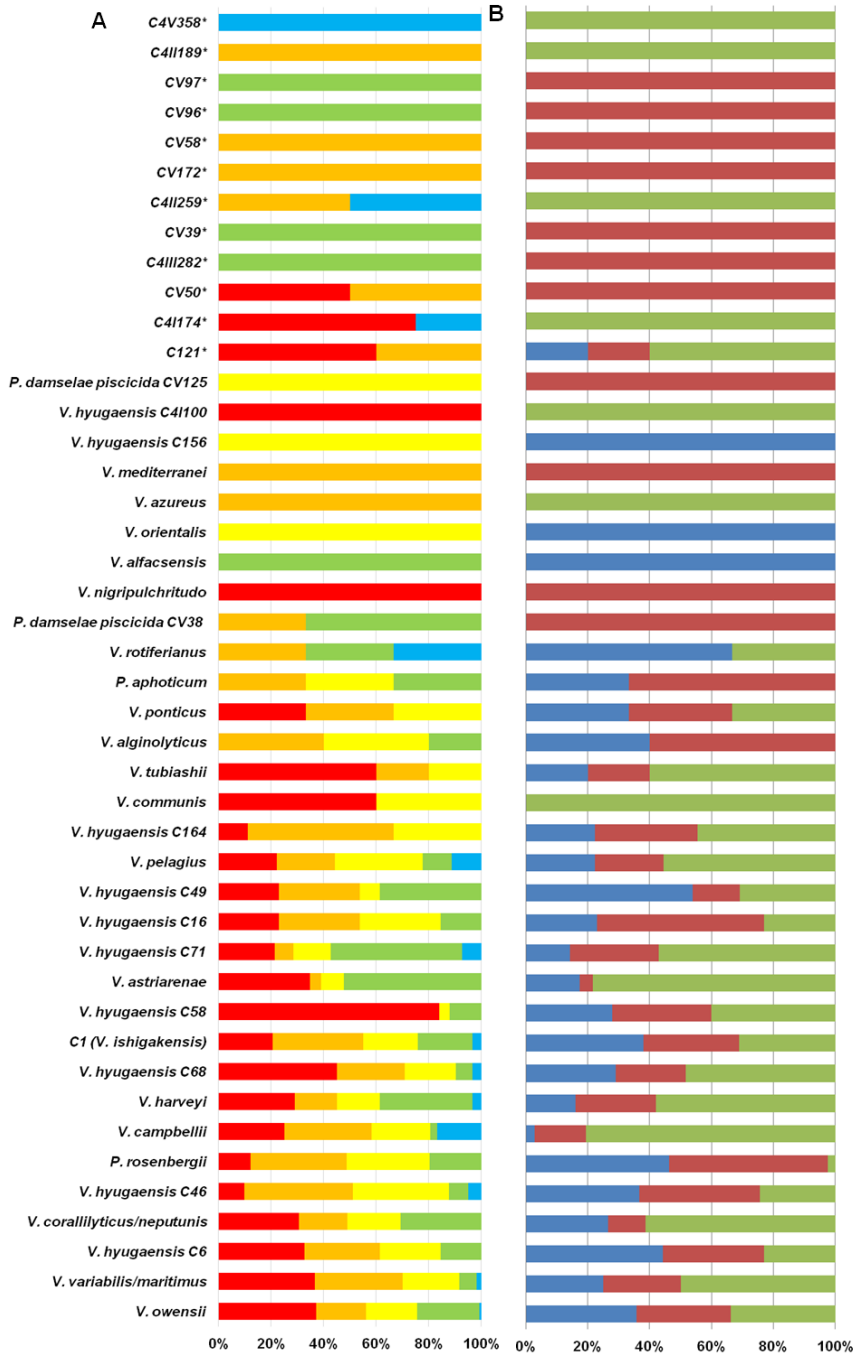

Figure 2.4. Spatiotemporal *Vibrio* population dynamics in Ishigaki coral reef seawater. (A) spatial *Vibrio* population dynamics (red, brown, yellow, green, sky blue colors indicate sampling sites of Miyara, Osaki, Sekisei Outer, Taketomi and Iriomote respectively); (B) Temporal *Vibrio* population dynamics (blue, violate and green colors indicate sampling year of 2012, 2013 and 2014 accordingly).

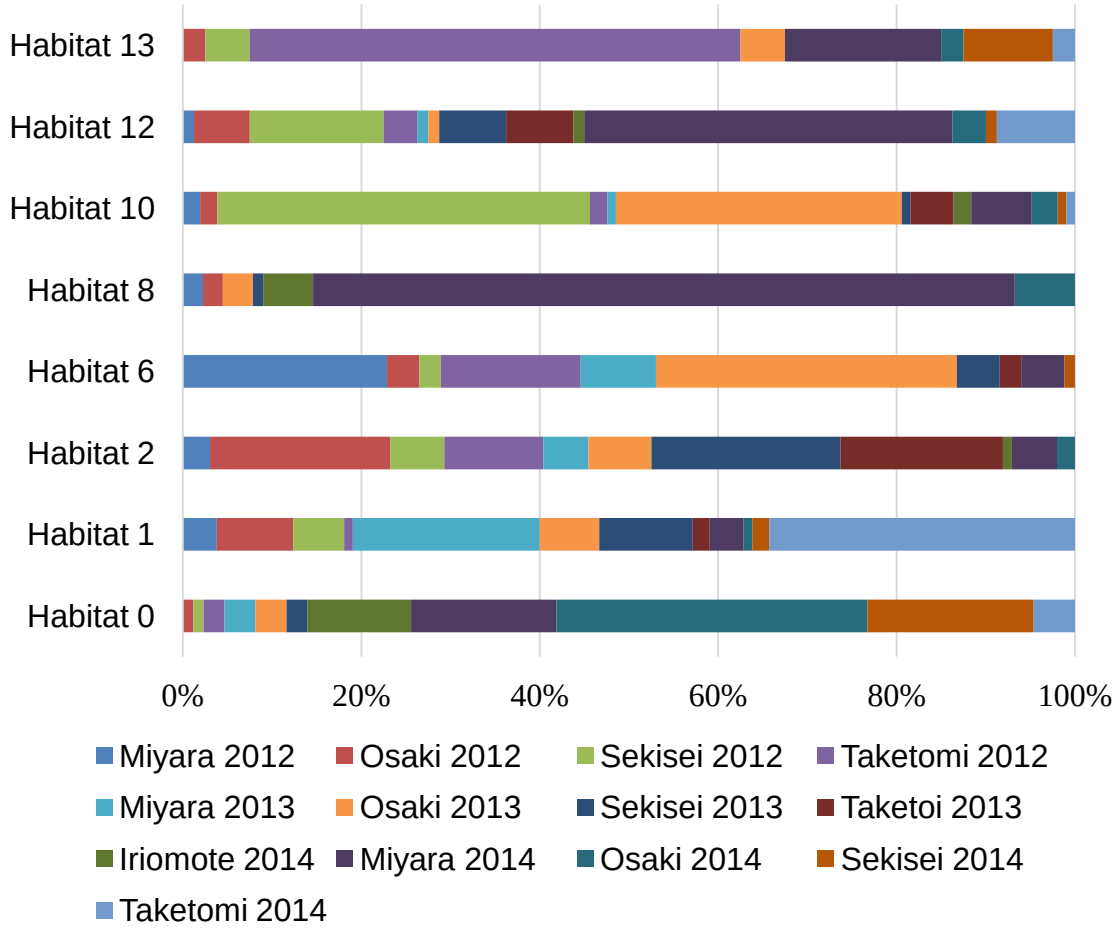

Figure 2.5. Distribution of the environmental categories that compose AdaptML predicted each of the 8 habitats. Color legends indicate the *Vibrio* isolating different sampling sites and years.

Table 2.2. Distribution (number) of vibrios that compose each of the eight habitats inferred by AdaptML

Vibrios	H-0	H-1	H-2	H-6	H-8	H-10	H-12	H-13
V. owensii	1	0	13	36	24	26	43	5
V. variabillis/maritimus	7	1	6	0	17	25	0	4
V. hyugaensis (C6)	3	22	0	4	2	3	16	2
V. coralliilyticus/neptunis	16	5	0	4	4	0	0	20
V. hyugaensis (C46)	2	11	0	0	1	23	3	1
P. rosenbergii	0	0	40	1	0	0	0	0
V. campbellii	34	1	0	0	0	0	1	0
V. harveyi	3	25	0	0	3	0	0	0
V. hyugaensis (C68)	2	5	0	5	9	10	0	0
V. ishigakensis (C1)	2	2	3	12	0	10	0	0
V. hyugaensis (C58)	0	2	12	1	7	1	0	2
V. astriarenae (C7)	0	9	0	0	7	0	0	7
V. hyugaensis (C71)	2	4	0	3	1	0	4	0
V. hyugaensis (C16)	0	2	10	0	1	0	0	0
V. hyugaensis (C49)	3	0	0	4	0	0	5	1
V. pelagius	4	0	4	0	1	0	0	0
V. hyugaensis (C164)	0	1	0	1	2	2	3	0
V. communis	1	0	0	0	3	1	0	0
V. tubiashii	0	0	2	0	3	0	0	0
V. alginolyticus	0	0	5	0	0	0	0	0
V. ponticus	0	0	0	0	0	3	0	0
P. aphoticum	0	0	3	0	0	0	0	0
V. rotiferianus	0	0	3	0	0	0	0	0
P. damsellae (CV38)	0	0	3	0	0	0	0	0
V. nigripulchritudo	0	1	0	0	0	0	0	0
V. alfacsensis	0	0	0	0	0	0	0	1
V. orientalis	0	0	0	0	0	1	0	0
V. azureus	1	0	0	0	0	0	0	0
V. mediterranei	0	0	0	1	0	0	0	0
V. hyugaensis (C156)	0	0	0	0	0	0	1	0
V. hyugaensis (C4I100)	0	0	0	0	0	1	0	0
P. damselae (CV125)	0	0	0	1	0	0	0	0
C121*	0	0	0	0	5	0	0	0
C4I174*	0	0	0	0	4	0	0	0
CV50*	0	0	0	4	0	0	0	0
C4III282*	0	3	0	0	0	0	0	0
CV39*	0	0	2	0	0	0	0	0
C4II259*	1	0	0	0	1	0	0	0
CV172*	0	0	0	1	0	0	0	0
CV58*	0	0	1	0	0	0	0	0
CV96*	0	0	1	0	0	0	0	0
CV97*	0	0	1	0	0	0	0	0
C4II189*	1	0	0	0	0	0	0	0
C4V358*	1	0	0	0	0	0	0	0

* Novel species candidates.

The adapted predominant *Vibrio* species (Table 2.2) in H-0 were *V. harveyi* (9.7%), *V. campbellii* (94.4%), *V. ishigakensis* (6.9%), *V. variabillis/V. maritimus* (11.7%), *V. coralliilyticus/V. neptunis* (32.7%) and *V. pelagius* (44.4%). The highlighted vibrios in H-1 were *V. harveyi* (80.6%) and *V. hyugaensis* C6 group (42.3%), *V. hyugaensis* C58 group (48.0%), *V. hyugaensis* C46 group (26.8%) and *V. astriarenae* (43.5%). H-2 comprised mostly of *V. owensii* (8.8%), *V. hyugaensis* C16 group (76.9%) and *P. rosenbergii* (97.6%). Two prevalent vibrios in H-6 were *V. owensii* (24.3%) and *V. ishigakensis* (44.8%). The most abundant vibrios in H-8 were *V. owensii* (16.2%) and *V. variabillis/V. maritimus* (28.3%). The dominant vibrios in H-10 were *V. owensii* (17.6%), *V. hyugaensis* C68 group (32.3%), *V. hyugaensis* C46 group (56.1%), *V. ishigakensis* (31.0%) and *V. variabillis*-*V. maritimus* (41.7%). In H-12 the prominent vibrios were *V. owensii* (29.1%) and *V. hyugaensis*-C6 (30.8%). H-13 was found to contain *V. coralliilyticus/V. neptunis* (40.8%) and *V. astriarenae* (30.4%). Moreover, several potential novel vibrios (CV39, CV58, CV96, CV97) and one opportunistic coral pathogen *V. alginolyicus* (100%) were retrieved specifically from H-2.

AdaptML could also estimate the evolutionary history of ecological differentiation (Hunt et al., 2008). Interestingly, the recent ongoing adaptive radiations (with a shallow branch: meaning frequently changing adaptations) were observed as more likely to be in *V. astriarenae*, *V. ishigakensis*, *V. mediterranei*, *V. variabilis/V. maritimus*, *V. coralliilyticus/V. neputunis* and *V. communis*/*V. owensii*, *V. hyugaensis*, however no such radiations were observed in *V. campbellii*, *V. harveyi* and *P. rosenbergii* (Figure 2.2).

2.4.4. Correlation between environmental variables and *Vibrio* abundances

Vibrio campbellii abundance significantly increased (r=0.62; P<0.05) along with rising seawater temperature whereas *V. owensii* (r=-0.58; P<0.05) and the C6 group of *V. hyugaensis* (r=-0.62; P<0.05) abundances significantly decreased (Table 2.3) with an increase in seawater temperature. Moreover, the correlation assessment between vibrio abundances and dissolved organic carbon (DOC) availability illustrated (Table 2.3) that *P. rosenbergii* and the *V. hyugaensis* (C6 group) increased along with increased DOC whereas *V. owensii*, *V. campbellii* and *V. coralliilyticus* showed the opposite results.

Table 2.3. Correlation coefficient (*r*) and significant levels (*P*-values) obtained from the correlation analysis between major vibrio abundances and environmental variables

Vibrios	**Environmental parameter**	**Correlation coefficient (*r*-value)**	**Significance (*P*-value)**
V. owensii	Temperature	-0.58	0.038
V. campbellii	Temperature	0.62	0.023
P. rosenbergii	Temperature	-0.39	0.18
V. coralliilyticus	Temperature	0.16	0.60
V. hyugaensis (C6)	Temperature	-0.62	0.0013
V. owensii	DOC	-0.58	0.64
V. campbellii	DOC	-0.55	0.13
P. rosenbergii	DOC	0.60	0.086
V. coralliilyticus	DOC	0.25	0.52
V. hyugaensis (C6)	DOC	0.54	0.13

2.5. Discussion

Ishigaki coral reef ecosystems are known to be the largest and the most diverse reef ecosystems in Japan, but are in now extremely vulnerable. In this era of global warming, the surrounding seawater of coral reefs are one of the most important ecological niches as well as heat carriers. It is worth investigating the evolution of spatiotemporal *Vibrio* dynamics in oceanic fields not only for the conservation of fragile coral reefs but also for public health and aquaculture issues (Rubio-Portillo et al., 2014; Tout et al., 2015). A three year survey was performed to elucidate *Vibrio* diversity using well curated *pyrH* gene sequence set to achieve fine scale discrimination (Thompson et al., 2005; Thompson et al., 2007b; Tall et al., 2013; Chimetto Tonon et al., 2015; Amin et al., 2016). The *pyrH* gene set allows not only to affiliate *Vibrio* isolates to known described *Vibrionaceae* species or currently unknown new species candidates but also to elucidate the structure of regional populations of specific species and how they have diverged. The findings of this study demonstrated that the *Vibrio* community of the coral surrounding seawater consisted of at least 22 known described species and 12 undescribed species candidates (26 isolates) which is likely to be a more diversified *Vibrio* community compared to those reported from tropical and temperate coral reef environments (Chimetto et al., 2008; Alves Jr et al., 2010; Raina et al., 2009; Chimetto Tonon et al., 2015). Unfortunately, assessment of the Ishigaki coral reef microbiota was not included due to lack of coral samples, the seawater around Ishigaki coral reefs is likely to share major *Vibrionaceae* communities of reported microbiomes *V. communis, V. owensii, V. mediterranei, V. shiloi, V. harveyi, V. alginolyticus, V. campbellii, V. maritimus, V. variabilis, V. tubiashii, V. coralliilyticus, V. pelagius* consisting of coral holobionts (Alves Jr et al., 2010; Chimetto Tonon et al., 2015). High abundance of *Harveyi* clade (*V. hyugaensis, V. communis, V. owensii, V. harveyi, V. campbellii*) and lower abundance of *Splendidus* clade species (*V. pelagius*) are the specific clues to help define subtropical Ishigaki coral reef

microbiota (Hunt et al., 2008; Tall et al., 2013). *Coralliilyticus* clade species (*V. coralliilyticus, V. neptunis*), *Mediterranei* clade species (*V. mediterranei*, *V. maritimus, V. variabillis*) and *P. rosenbergii* are secondary significant members (>5% constitution) of the Ishigaki coral reef seawaters.

The availability of at least 12 novel species candidates (CV172, CV50, C4I174, CV96, CV58, CV39, CV97, C4II282, C121, C4II259, C4II189 and C4V358) in the Ishigaki coral reef ecosystem suggests important evidence of *Vibrionaceae* evolution. Relevant subsequent studies (Thompson et al., 2005; Rosenberg et al., 2007; Reis et al., 2009; Moreira et al., 2014; Chimetto Tonon et al., 2015) reported that coral and surrounding seawater are prime sources in isolating novel vibrios and is an important habitat for studying the microevolution of vibrios. Comparing previously recorded vibrios from coral and reef ecosystems (Chimetto et al., 2009; Alves Jr. et al., 2010; Chimetto Tonon et al., 2015), this study investigated mostly diversified populations including numerous vibrios unique to our studied reef locations. The retrieved unique *Vibrio* populations were *V. hyugaensis, V. ishigakensis, V. astriarenae, P. damsella* subsp. *piscicida, P. aphoticum, V. nigripulchritudo, V. alfacsensis,* and *V. azureus* suggesting that Ishigaki coral reef locations are hotspots for versatile and dynamic vibrio populations and may serve as diversified ecological niches.

This spatiotemporal survey also revealed that the community and population level dynamics in the surrounding seawater of coral holobionts in Ishigaki reef ecosystems can determine generalists and specialists (Figures 2.2 and 2.4). *V. owensii* and *V. hyugaensis* populations were considered to be the dominant generalist populations in the coral seawater ecosystems. The other prevalent generalists (Table 2.1, Figure 2.2) in Ishigaki reef seawater were *V. variabillis, V. harveyi, V. campbellii, P. rosenbergii, V. coralliilyticus, V. astriarenae,* and *V. ishigakensis*.

Numerous previous reports suggested that vibrios from *Harveyi* clade appear to have ecologically diversified passively by invading new niches (Chimetto et al., 2008; Preheim et al., 2011; Chimetto Tonon et al., 2015). The observed diversities of *V. owensii* and *V. hyugaensis* populations were likely to be higher than those of *V. harveyi* and *V. campbellii* (Figure 2.2). *V. owensii* was isolated from cultured crustaceans in Australia as a member of *Harveyi* clade (Cano-Gomez et al., 2010). This species was also isolated from *Acropora* white syndrome lesions in American Samoa and is considered to be a putative coral pathogen (Wilson et al., 2012; Ushijima et al., 2012) and was also isolated from '*Montipora capitata*' diseased-coral (Tissue loss disease: *Montipora* White Syndrome) in Hawaiian coral reefs. *V. hyugaensis* was isolated from a seawater sample collected in Miyazaki prefecture in Japan (Urbanczyk et al., 2015). It is a luminous *Vibrio* and several light-producing bacteria were reported from seawater samples taken from different sites in Miyazaki prefecture between 2010 and 2012 (Urbanczyk et al., 2014).

Specialist *Vibrio* populations in Ishigaki reef seawater showed site-specific distributions (Table 2.1). *V. azureus* and *V. mediterranei* were isolated specifically from Osaki, whereas, *V. nigripulchritudo*, *V. alfacsensis*, and *V. orientalis* were noted as region-specific in Miyara, Taketomi, and Sekisei, respectively. Bacteria as coral holobionts may be host-specific or may display spatial variability which might show protective activities against pathogens or other detrimental agents by occupying entry niches and space (as competitors) or by production of antibiotic compounds (Mullen et al., 2004; Rohwer et al., 2002, Kelman et al., 2006; Ritchie, 2006). Moreover, the mathematical model AdaptML generated the environmental grouping (Table 2.2) of the Ishigaki reef seawater *Vibrio* populations and detected both ecologically restricted and ecologically distinctive populations (Hunt et. al., 2008). Two proposed novel vibrios isolated from this location, *V. astriarenae*, *Agarivorans* clade (Al-saari et al., 2015), and *V. ishigakensis*, *Halioticoli* clade (Gao et al., 2016), were found in more ecologically

diverse populations (Figure 2.2). The former is agarolytic whereas the latter is non-motile and alginolytic. Benthic flora might determine the specific niches for these species.

One of the more intriguing findings disclosed by this study was the availability of versatile and dominant putative coral pathogenic vibrios, *V. owensii* and *V. coralliilyticus* occupied 21.6% and 7.2% respectively in our total analyzed vibrios (Table 2.1, Figure 2.2). This may be due to higher global oceanic temperatures. Temperature influenced pathogenic potential of coral pathogenic vibrios may lead to an increase in incidences of diseases in marine environments. Swimming motility, swarming motility and protease activity have been shown to be related to the virulence of *V. owensii* (Ushijima et al., 2012). According to Kimes et al. (2012) for *V. coralliilyticus*, an increase in seawater temperature to more than 27ºC plays a direct role in triggering virulence genes and other factors related to host degradation, secretion, antimicrobial resistance and motility. The mechanism of virulence includes motility and chemotaxis that involve post colonization with subsequent production of coral tissue damaging 'zink-metalloproteases' (Ben-Haim et al., 2003). Recently, Tout et al. (2015) demonstrated that raised-seawater temperature increase natural *Vibrio* abundances, particularly the notorious coral pathogen *V. coralliilyticus*, in coral ecosystems. However, this study findings on *V. coralliilyticus* abundance were not significantly correlated to seawater temperature rises. Beyond the previously mentioned potential coral pathogens, I also identified *V. harveyi* (4.5%) and *V. alginolyticus* (0.7%) from the seawater. *V. alginolyticus* was found only in the single habitat H-2 suggesting that this bacterium may have a new ecological niche-partitioning in Ishigaki reef seawater. Other results associated with coral pathogenic vibrios (Luna et al., 2010; Zhenyu et al., 2013; Munn, 2015) show that most of them are opportunistic in nature and show their virulence under certain environmental conditions by overwhelming the host-defense, overgrowth and tissue destruction.

Environmental variables, particularly temperature is considered to be the prominent triggering factor in *Vibrio* ecology, population dynamics, physiological stress response and evolution (Sato et al., 2009). Elevated temperatures and high loads of nutrients particularly DOC have direct impacts on coral holobiont microbial communities and ultimately make them diverse, abundant and even niche or host specific (Kline et al., 2006; Paz et al., 2007). Other findings similarly demonstrated that elevated nutrients (i.e, phosphate, nitrate, ammonia) and DOC in coastal waters have been a cause of reef decline (Bruno et al., 2003; Kline et al., 2006). In this assessment, only specific *Vibrionaceae* populations correlated with environmental parameters (e.g. temperature, DOC). *V. campbellii* abundance was significantly correlated to rising seawater temperatures, where *V. owensii* and C6 group of *V. hyugaensis* showed significant negative correlations with increasing seawater temperature. The optimum growth temperature of *V. hyugaensis* was reported to be 26^{o}C and it could not grow at 37^{o}C, which might reflect its low tolerance to high temperature. These findings collaborated previous reports that thermal anomalies and nutrient-rich environment including DOC are well-known ecological triggers, involved in proliferation and increased abundance of holobiont microbial communities in coastal environments globally (Ducklow et al., 1986; Wild et al., 2004; Barott and Rohwer, 2012) along with reef ecosystems in southern Japan (Goto et al., 2010). Roder et al. (2014) also noted that coral associated bacterial communities including potential pathogens emerge and dominate during environmental stress and the shift of bacterial community may be a direct effect of temperature on growth of specific members of the microbial community (Garren et al., 2014).

Chapter 3. *Thaumasiovibrio occultus* gen. nov., sp. nov. and *Thaumasiovibrio subtropicus* sp. nov. within the family *Vibrionaceae*, isolated from coral reef seawater of Ishigaki Island, Japan

3.1. Abstract

Two phylogenetically distinct *Vibrionaceae* strains C4II189^T and C4V358^T were isolated from the reef seawater of Ishigaki Island, Japan, in 2014. Molecular phylogenetic analyses on the basis of 16S rRNA gene and multilocus sequence analysis (MLSA) using eight protein coding genes did not show any clustering of these strains with any other known *Vibrionaceae* genera. Similarities obtained with the known *Vibrionaceae* members were below 94.7% for the 16S rRNA gene and below 83% for the eight-gene MLSA. Both strains showed low enough similarities to consider them as two distinct species. Genome based taxonomy with average nucleotide identity (ANI) and *in silico* DNA-DNA Hybridization (DDH) calculation, in addition to experimental DDH, also showed the genetic dissimilarities of two species from any other known genera of *Vibrionaceae* with less than 27.2% experimental DDH, 71.7% genome based ANI, and 23% *in silico* DDH similarities. The whole genome average amino acid identity (AAI) and number of core genes (>90% amino acid identity and alignment) of the two new species was higher than those of representative strains of each genus. All aspects of phylogenetic, phenotypic and genetic cohesions between the two *Thaumasiovibrio* spp. (Thau.ma.si.o.vi'bri.o. Gr. adj. thaumasios, unusual, unique; N.L. masc. n. vibrio, a motile bacterium; N.L. masc. n. *Thaumasiovibrio*, an unusual bacterium) were high enough to propose them as the new genus in the *Vibrionaceae*. Here, we proposed a seventh genus *Thaumasiovibrio* gen. nov. with two new species of *T. occultus* sp. nov. strain C4II189^T (=DSM 101554^T=JCM 31629^T) and *T. subtropicus* sp. nov. strain C4V358^T (=DSM 101555^T=JCM 31630^T), of the family *Vibrionaceae*.

3.2. Introduction

In this century, bacterial systematics has become a dynamic scientific area integrating classical experimental observations of morphological, biochemical, physiological and genetic features, and state-of-art technologies involving molecular phylogeny and genomics (Wayne et al., 1987; Stackebrandt et al., 2002; Thompson et al., 2014; Konstantinidis and Tiedje, 2005; Sawabe et al., 2007, 2013; Amaral et al., 2014; Kim et al., 2014; Rosselló-Móra and Amann, 2015; Garrity, 2016). Two recent landmarks which could strongly affect bacterial systematics are 1) a proposal of rational taxonomic boundaries for high taxa of bacteria and archaea on the basis of the huge number of 16S rRNA gene entries in the public database (Yarza et al., 2014), and 2) maturation of species threshold boundary on the basis of genome sequence comparison (Thompson et al., 2014; Chun and Rainey, 2014; Oren and Garrity, 2014; Rosselló-Móra and Amann, 2015; Varghese et al., 2015; Whitman et al., 2015; Garrity, 2016). Due to these efforts, we have met not only well-accepted criteria combinations for species delineation with 95-96% average nucleotide identity of shared genes (ANI) and 98.7% of 16S rRNA gene sequence identity but also 94.5%, 86.5%, 82.0%, 78.5%, and 75.0% threshold for high taxa of genus, family, order, class, and phylum on the basis of nearly complete 16S rRNA gene sequences (Yarza et al., 2014, Rosselló-Móra and Amann, 2015). These thresholds on the basis of 16S rRNA gene sequences are universal which means they are applicable to uncultured lineages (Yarza et al., 2014). Several efforts to better assess definite standards of genome cohesions in higher taxonomic ranks have also been made instead of ANI and the 16S rRNA gene chronometer. Genome wide average amino acid identity (AAI) (Konstantinos and Tiedje, 2005; Sangal et al., 2016) could be a better candidate, e.g. 66-86% of genome wide AAI value observed in the transient zone for genus-family boundaries (Konstantinos and Tiedje, 2005), and 87.8% AAI value using 255 core genes separating potential genera in the complex *Rhodococcus* taxa (Sangal et al., 2016).

The family '*Vibrionaceae*' is one of the first bacterial families where advanced microbial molecular taxonomy has been applied (Kita-Tsukamoto et al., 1993; Thompson et al., 2005; 2009; Sawabe et al., 2007; 2013; Al-saari et al., 2015; Gao et al., 2016). *Vibrionaceae* species were also defined as a group of strains that share >95% MLSA identity and super-tree analysis, >96% AAI, ≤ 10 genome signature dissimilarity (Thompson et al., 2009; Sawabe et al., 2013; Gomez- Gil et al., 2014). Currently, the family *Vibrionaceae* contains the following six genera: *Vibrio*, *Photobacterium*, *Salinivibrio*, *Enterovibrio*, *Grimontia*, and *Aliivibrio* proposed in 1854, 1889, 1996, 2002, 2003, and 2007 respectively, consisting of over 160 species (Gomez-Gil et al., 2014). The 8-genes MLSA succeeded in delineating 23 clades, a *Salinivibrio-Grimontia-Enterovibrio* super-clade and four orphan clade (Sawabe et al., 2013; Al-saari et al., 2015), some of which correspond to "genus" such as the *Fischeri* clade to the genus *Aliivibrio* (Urbanczyk et al., 2007). After the current descriptions of three *Grimontia* species, *Echinimonas* is the only genus consisting of a single species (Gomez-Gil et al., 2014). Today the family *Vibrionaceae* has become effective taxa for testing thresholds of higher taxa such as the genus boundary testing with multiples species.

In the extensive assessments of *Vibrio* diversity correlated with the coral health in Ishigaki, Japan, two *Vibrionaceae* strains of a quite unique lineage on the basis of *pyrH* gene sequences were isolated (Amin et al., 2016). As these strains could not normally be affiliated to any known genera in *Vibrionaceae*, we tried to adapt the reported genus criteria on the basis of 16S rRNA gene threshold, 8-gene MLSA network analysis, and AAI comparison. Finally, we attempted to fulfill the monophyly, genetic and phenotypic coherences of these two phylogenetically distinct new *Vibrionaceae* strains. Here, we propose *Thaumasiovibrio* gen. nov. for these strains. Phylogenetic, genetic, and phenotypic characterization of two new species, *Thaumasiovibrio occultus* sp. nov. (oc.cul'tus. L. adj. occultus, hidden; referring to resistant to the discovery of the species in the diverse active coral reef ecosystem) and *T.*

subtropicus sp. nov. (sub.tro'pi.cus. N.L. adj. subtropicus, subtropical, referring to the isolation site of the Ishigaki coral reef) are provided. The type species of the *Thaumasiovibrio* gen. nov. is *T. occultus* strain C4II189^T (=DSM 101554^T=JCM 31629^T).

3.3. Materials and methods

3.3.1. Bacterial strains

Thaumasiovibrio strains C4II189^T and C4V358^T were isolated from coral reef seawater of Ishigaki Island, Okinawa, Japan (Amin et al., 2016). The seawater samples were taken from Osaki (24°25.4178' N 124°04.4892' E, 3 m depth) and Iriomote (24°23.208' N 123°55.681' E, 6 m depth) reef points by SCUBA diving using an underwater pump. Specific permissions were not necessary for the seawater sampling activities in these locations. Bacterial isolation was done using TCBS medium (Nissui Pharmacy, Tokyo, Japan). After twice isolation on TCBS at 25°C for 24 h, the isolates were further purified on ZoBell 2216 E agar plate using the same temperature. These strains were stored at -80°C with 20% glycerol-supplemented broth.

3.3.2. Molecular phylogenetic analysis on the basis of 16S rRNA gene

16S rRNA gene amplification and sequencing were performed according to a standard protocol described in Al-saari et al. (2015) and Gao et al. (2016). In brief, the amplification primers (24F and 1509R) were used for PCR amplification that gave a 1.5 kb long PCR product and corresponded to positions 25 to 1521 in the *E. coli* sequence. The 16S rRNA genes of C4II189^T and C4V358^T were sequenced using an Applied Biosystems model 373A automated sequencer. Six DNA primers (24F, 800F, 1100F, 520R, 920R, 1509R) were used in the sequencing reactions (Sawabe et al., 1998; Al-saari et al., 2015; Gao et al., 2016).

For phylogenetic analysis, sequences were aligned and studied using Clustal X version 2.1 (Larkin et al., 2007) and MEGA programs version 6.06 (Tamura et al., 2013). The alignment was checked by eye and corrected manually. In all phylogenetic analyses, we used the sequences determined in this study and 16S rRNA gene sequences obtained from the public database. The full dataset included 16S rRNA gene sequences of 157 known *Vibrionaceae* species and *Escherichia coli* K-12. Molecular phylogenetic analyses based on 16S rRNA gene sequences were conducted using three different algorism of NJ, MP, and ML methods (Saitou and Nei, 1987). For figure 3.1, the tree was reconstructed using NJ with bootstrap calculation of 1000 replicates. Evolutionary distances were corrected using the Jukes-Cantor method. Trees were drawn using the MEGA6. Only nodes supported by three different algorithms were shown with the bootstrap value.

3.3.3. Genome sequence

Genomic DNAs of C4II189^T and C4V358^T were purified using NucleoSpin Tissue (MACHEREY-NAGEL GmbH & Co., Düren, Germany) with the standard protocol. Draft genome sequence of the strains C4II189^T and C4V358^T were obtained using the MiSeq platform. A paired-end library was constructed using the Nextera XT DNA Library Preparation Kit. The genome sequences were assembled using Platanus ver. 1.2.1. (Kajitani et al., 2014) into 230 and 271 scaffolds, respectively.

3.3.4. MLSA

MLSA was conducted in the same manner as in Sawabe et al. (2007, 2013). The eight MLSA genes were retrieved from the available *Vibrionaceae* genomes. As the 8- housekeeping gene phylogeny (*gapA, gyrB, ftsZ, pyrH, recA, rpoA, mreB* and *topA*) is the recognized most powerful method for delineating *Vibrio* clade and species that reduces the misidentication of *Vibrio* clade ancestry, so finally, eight protein-coding genes of the candidate strains were

analyzed. MLSA used 8-housekeeping gene coding proteins are: *gapA* (glyceraldehyde-3-phosphate dehydrogenase A), *gyrB* (DNA gyrase subunit B), *ftsZ* (filamenting temperature-sensitive mutant Z), *pyrH* (urydilate kinase), *recA* (rec A protein), *rpoA* (RNA polymerase alpha subunit A), *mreB* (rod shaping protein), and *topA* (DNA-topoisomerase subunit A).

The sequences were aligned in the ClustalX program ver. 2.1 (Larkin et al., 2007) using a data set of eight protein-coding genes for *Vibrionaceae* (Baumann and Schubert, 1984), and then corrected manually. The domains used to reconstruct the phylogenetic tree were regions of the *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, and *topA* genes of *Vibrionaceae*: positions 196-631, 226-861, 442-1026, 390-898, 175-543, 430-915, 385-762, and 571-990 (*V. cholerae* O1 Eltor N16961 (AE003852) numbering), respectively. The MEGA6 (Tamura et al., 2013) was used to calculate the sequence similarity (Sawabe et al., 2007, 2013). SDA was performed using SplitsTree version 4.14.3, with a neighbor net drawing and Jukes-Cantor correction (Bandelt and Dress, 1992; Huson and Bryant, 2005). Each aligned set was used to reconstruct the network using the tool "concatenate sequences from files" implemented in the program.

3.3.5. Genome comparisons

General genome features including G+C content were obtained though RAST server (version 4.0) (Aziz et al., 2008). ANI values (%) were calculated using C4II189^T and C4V358^T genomes against genomes of type strains; *E. calviensis* DSM 14347^T, *E. coralii* CAIM 912^T, *G. hollisae* ATCC 33564^T, *G. celer* CECT 9029^T, *G. indica* AK16^T, *G. marina* CECT 8713^T, *Salinivibrio costicola* subsp. *costicola* LMG 11651^T, *S. "socompensis"* S10B^T, *P. angustum* ATCC 25915^T, *P. aphoticum* DSM 25995^T, *P. aquae* CGMCC 1.12159^T, *P. aquimaris* NCCB 100386^T, *P. damselae* subsp. *damselae* CIP 10276^T, *P. gaetbulicola* KCTC 22804^T, *P. galatheae* S2753^T, *P. ganghwense* DSM 22954^T, *P. halotolerans* DSM 18316^T, *P. iliopiscarium* ATCC 51760^T, *P. jeanii* R-40508^T, *P. kishitanii* ATCC BAA-1194^T, *P.*

leiognathi ATCC 2552^{T}, *P. phosphoreum* ATCC 11040^{T}, *P. sanctipauli* A-394^{T}, and *P. swingsii* CAIM 1393^{T} using the orthANI program (Lee et al., 2015). The *in silico* DDH values were calculated using the same data set for ANI% calculation with the use of the GGDC 2.1 (Meier-Kolthoff et al., 2013). AAI value was calculated according to Konstantinos and Tiedje (2005). Circos (Krzywinski et al., 2009) was used for drawing the linkage plot of the core genes showing >90% amino acid identity and alignment amongst representative strains of genera *Enterovibrio, Grimontia, Photobacterium*, and *Salinivibrio*.

3.3.6. DNA-DNA hybridization

DNAs of all the selected strains were prepared using the procedures by Marmur (Marmur, 1961), with minor modification. DNA-DNA hybridization experiments were performed by the same way as described in (Sawabe et al., 1998) using a fluorometric direct binding method in microdilution wells (Ezaki et al., 1988). DNAs of the strain C4V358^{T} was labeled with photobiotin (Vector Laboratories, Inc., Burlingame, CA). Total strain used in DNA-DNA hybridization experiment were C4V358^{T}, C4II189^{T}, *G. hollisae* (LMG 17718^{T}), *E. novergicus* (LMG 18839^{T}), *P. sanctipauli* (CAIM 1892^{T}), *P. ganghwense* (CAIM 512^{T}), *P. rosenbergii* (CAIM 911^{T}), *P. lutimaris* (CAIM 1851^{T}), *P. swingsii* (CAIM 1393^{T}), *S. sharmiensis* (CAIM 1888^{T}), *S. costicola vallismaris* (CAIM 735^{T}), *S. proteolyticus* (CAIM 1855^{T}). Unlabeled single-stranded DNA from target strains were immobilized in microdilution wells (Immuron 200, FIA/LIA plate, black type, Greiner labotechnik, Germany), then 20 ng of labeled DNA was added to each microdilution well and the hybridization was performed under optimal condition following pre-hybridization (Meinkoth and Wahl, 1984). The hybridization of the biotinylated DNA to immobilized DNAs was performed under optimal condition following pre-hybridization and detected by fluorometry after binding streptavidin-β-galactosidase to labeled DNA. 4-Methylumbelliferyl-β-D-galactopyranoside (6 x 10^{-4} M; Wako, Osaka, Japan) was added to each well as fluorogenic

substrate for β-galactosidase and incubated at 30°C. Then, fluorescence intensity of each well was measured using microplate reader (Infinite 200, Tecan, Switzerland) at wave length of 360 nm for excitation and 465 nm for emission. DNA-DNA homology was calculated according to Ezaki et al. (1989).

3.3.7. Phenotypic characterization

The phenotypic features of *Thaumasiovibrio* strains were determined as described previously comparing to those of representative type strains of *Enterovibrio*, *Grimontia*, *Salinivibrio*, and the *Rosenbergii* clade species of *Photobacterium* (Baumann and Schubert, 1984; Hidaka and Sakai, 1968; Ostle and Holt, 1982; West et al., 1977; Holt et al., 1994; Al-saari et al., 2015; Gao et al., 2016). In total, 88 features including morphological and biochemical tests of carbon source assimilation (51 carbons sources) were conducted.

3.3.8. Nucleotide sequence accession numbers

The genome sequences are available at DDBJ/EMBL/GenBank under the accession numbers of BDGP01000001-BDGP01000230, and BDGQ01000001-BDGQ01000271 for *Thaumasiovibrio occultus* DSM 101554^T (C4II189) and *T. subtropicus* DSM 101555^T (C4V358).

The GenBank-deposited accession numbers of Sanger sequenced 16S rRNA genes for *Thaumasiovibrio occultus* DSM 101554^T (C4II189) and *T. subtropicus* DSM 101555^T (C4V358) were KX 713152 and KX 713153, respectively.

3.4. Results

3.4.1. The molecular phylogeny

The 16S rRNA gene sequence analysis of two novel *Thaumasiovibrio* species candidates (C4II189^T and C4V358^T) using the latest data set including 156 species of *Aliivibrio, Echinimonas, Enterovibrio, Grimontia, Photobacterium, Salinivibrio,* and *Vibrio* in the family *Vibrionaceae* revealed a reliable clustering of the two *Thaumasiovibrio* species apart from known species in any *Vibrionaceae* genera supported by three different phylogenetic algorithms (Figure 3.1). The 16S rRNA gene similarity between C4II189^T and C4V358^T was 95.7%, of which value was sufficiently lowered the threshold (98.7%) for species delineation (Kita-Tsukamoto et al., 1993; Tindall et al., 2010; Yarza et al., 2014; Rosselló-Móra and Amann, 2015). *Vibrionaceae* species which gave the highest similarity were *P. panuliri* (KC990827) (96.9%) followed to *P. aquimaris* (NR_114269) (96.4%), and *P.jeanii* (GU065210) (96.2%) for C4II189^T, and that for C4V358^T was C4II189^T (95.6%) followed to *P. jeanii* (95.4%), and *P. damselae* subsp. *damselae* (AB032015) (95.2%). The phylogenetic position of the *Thaumasiovibrio* species was seemed to be branching at the deeper position of the sister clade consisted of three genera of *Enterovibrio, Grimontia* and *Salinivibrio* (Figure 3.1). The *Thaumasiovibrio* was seemed to be rather apart from genera *Aliivibrio, Echinimonas* and *Vibrio.*

16S rRNA gene phylogeny was unlikely to be sufficient to demonstrate monophyly in the *Vibrionaceae* family. MLSA is one of the most powerful tool for delineating novel species and monophyletic group (clade) description in the family of *Vibrionaceae* (Thompson et al., 2005; Thompson and Swings, 2006; Sawabe et al., 2007; Thompson et al., 2007a; Sawabe et al., 2013). Therefore, we further performed the 8-genes MLSA to clarify whether *Thaumasiovibrio* species C4II189^T and C4V358^T form a new clade. The MLSA of the two

Thaumasiovibrio species C4II189^{T} and C4V358^{T} strains showed these two formed a distinct clade separated from not only all the known clades but also any genera of the *Vibrionaceae* family (Figure 3.2). A significant sub-branching of the *Thaumasiovibiro* clade from the *Enterovibrio-Grimontia-Salinivibrio* super-clade (Sawabe et al., 2013) was also observed, which also strengthens the monophyly of the *Thaumasiovibiro* on the basis of 16S rRNA gene phylogeny (Figure 3.1).

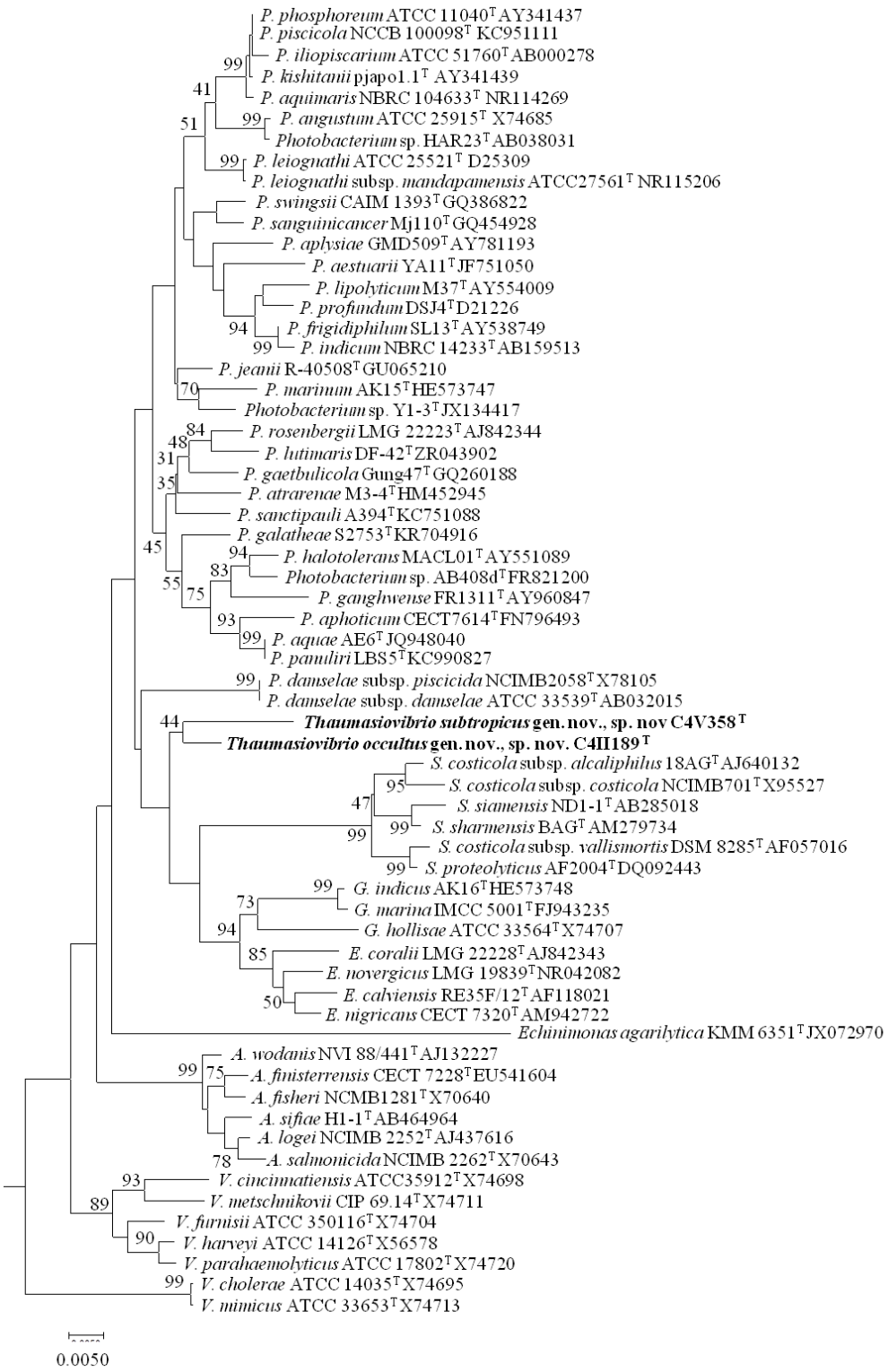

Figure 3.1. Rooted phylogenetic tree on the basis of 16S rRNA gene sequences. This figure combines the results of three analyses, neighbor-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML). The topology shown was obtained by NJ, and the percentage values are the results of a bootstrap analysis using 1000 replications. Branches also obtained in the ML and MP analyses are only indicated with bootstrap value.

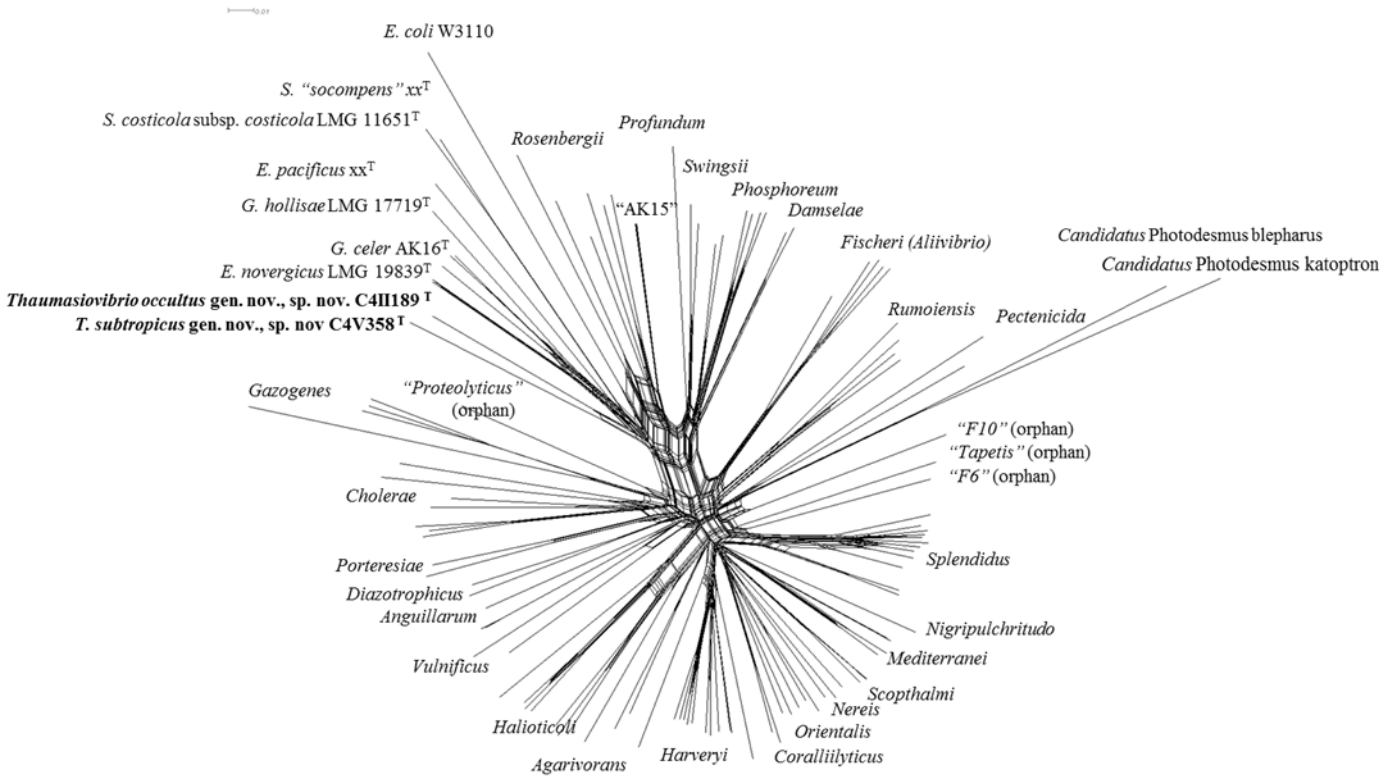

Figure 3.2. Concatenated split network tree based on eight protein coding gene loci. The *gapA*, *gyrB*, *ftsZ*, *mreB*, *pyrH*, *recA*, *rpoA*, and *topA* gene sequences of 120 taxa were concatenated including the representative of novel genus *Thaumasiovibrio* species (C4II189^T and C4V358^T). Phylogenetic tree was generated using the SplitsTree4program. Two novel species of this study formed a cluster not associated any known clade from the family *Vibrionaceae* proposed previously.

3.4.2. Genome-based taxonomy

Estimation of genome based ANI was done between the novel species candidates and towards the representative species (25 species) from all the related genera of *Vibrionaceae* family. The ANI between C4II189 and C4V358 was 71.9% (Table 3.1). The ANI betwenn these novel species candidates and other representative species from *Enterovibrio*/*Gimontia*/*Salinvibrio* genera were >71%. On the other hand, calculated ANI range of the novel bacteria with different photobacteria was 69.5%-71.7%. The *in silico* DDH values between the proposed two novel vibrios (Table 3.1) and towards the related species of *Vibrionaceae* family-belonged genera were 20.8% and >22%, respectively.

Table 3.1. Average nucleotide identity (ANI), average amino acid (AAI) and *in silico* DDH values between the proposed two novel vibrios and the related species

Strains	Accession numbers	ANI (%)		*in silico* DDH (%)		AAI (%)	
		C4II189	C4V358	C4II189	C4V358	C4II189	C4V358
Thaumasiovibrio occultus DSM 101554^T (C4II189)	BDGP01000001-BDGP01000230	-	71.9	-	20.8	-	71.0
T. subtropicus DSM 101555^T (C4V358)	BDGQ01000001-BDGQ01000271	71.9	-	20.8	-	71.5	-
Enterovibrio calviensis DSM 14347^T	NZ_JHZA00000000.1	69.9	69.4	21.6	21.0	61.8	61.0
E. coralii CAIM 912^T	NZ_LNTY00000000.1	70.5	69.5	20.9	21.0	66.5	65.6
E. pacificus CAIM 1920^T	NZ_LYBM00000000.1	69.2	68.8	21.2	22.1	65.6	65.0
Grimontia hollisae ATCC 33564^T	NZ_CP014056.1 and NZ_CP014055.1	70.3	69.9	21.9	23.0	66.8	66.4
G. celer CECT 9029^T	NZ_FIZX00000000.1	70.4	69.5	22.2	21.3	66.2	65.6
G. indica AK16^T	NZ_ANFM00000000.2	70.5	69.6	22.0	21.3	66.3	65.6
G. marina CECT 8713^T	NZ_FIZY00000000.1	70.4	69.7	21.1	21.0	66.1	65.7
Photobacterium angustum ATCC 25915^T	NZ_JZSO00000000.1	70.3	70.0	21.6	20.9	67.0	67.0
P. aphoticum DSM 25995^T	NZ_LDOV00000000.1	71.7	70.3	22.1	21.3	67.7	67.3
P. aquae CGMCC 1.12159^T	NZ_LDOT00000000.1	70.7	70.0	21.9	21.2	67.3	67.0
P. aquimaris NCCB 100386^T	NZ_LNQZ00000000.1	70.0	70.0	22.6	21.7	66.8	66.6
P. damselae subsp. *damselae* CIP 102761^T	NZ_ADBS00000000.1	70.4	70.4	23.7	22.9	67.4	67.3
P. gaetbulicola KCTC 22804^T	NZ_CP005973.1 and NZ_CP005974.1	70.8	70.2	23.3	23.2	67.5	68.1
P. galatheae S2753^T	NZ_JMIB00000000.1	70.5	69.8	21.7	22.0	66.8	66.6
P. ganghwense DSM 22954^T	NZ_LDOU00000000.1	71.1	70.0	22.8	20.9	67.5	67.5
P. halotolerans DSM 18316^T	NZ_AULG00000000.1	70.5	69.8	20.2	20.2	66.9	66.8
P. iliopiscarium ATCC 51760^T	NZ_JZSQ00000000.1	69.9	69.9	21.3	20.4	66.7	66.7
P. jeanii R-40508^T	NZ_LVHF00000000.1	71.0	70.6	22.2	21.1	67.3	67.5
P. kishitanii ATCC BAA-1194^T	NZ_JZSP00000000.1	69.7	69.8	21.7	20.6	66.7	66.4
P. leiognathi ATCC 25521^T	NZ_JZSK00000000.1	70.5	70.2	21.1	20.8	67.2	67.1
P. phosphoreum ATCC 11040^T	NZ_JZSJ00000000.1	69.9	69.9	21.6	21.2	66.7	66.4
P. sanctipauli A-394^T	NZ_JGVO00000000.1	71.0	70.4	22.1	21.7	67.4	67.7
P. swingsii CAIM 1393^T	NZ_LELC00000000.1	70.5	70.2	21.2	20.3	67.1	67.2
Salinivibrio costicola subsp. *costicola* LMG 11651^T	NZ_AQOF00000000.1	69.2	68.9	23.0	20.8	65.2	65.3
S. socompensis S10B^T	NZ_AQOE00000000.1	69.1	68.8	21.4	21.3	65.1	65.1

3.4.3. Experimental DNA-DNA hybridization

Experimental DDH using C4II189^T as probe showed 45.9% DNA-DNA relatedness against strain C4V358^T and lower values against the other species (3.4%-27.2%) (Table 3.2). The DDH experiments using the set of type strains, of which genomes are not available in public databases, could cover the weak points of above mentioned *in silico* genome comparisons. These reciprocal DDH using C4V358^T as a probe also showed 3.7% to 24.1% values, which indicates that C4II189^T and C4V358^T were separate species (Wayne et al., 1987), and the *Thaumasiovibrio* species were less genomic coherent to representative species in genera *Enterovibrio*, *Grimontia*, *Photobacterium*, and *Salinivibrio*.

Table 3.2. DND-DNA relatedness of the proposed two novel vibrios against the related *Vibrionaceae* species

Strains	**DNA-DNA relatedness (%)**	
	C4II189	C4V358
Thaumasiovibrio occultus DSM 101554^{T} (C4II189)	100.0	24.1
Thaumasiovibrio subtropicus DSM 101555^{T} (C4V358)	45.9	100.0
G. hollisae LMG 17718^{T}	5.7	3.7
E. novergicus LMG 18839^{T}	7.8	21.2
P. sanctipauli CAIM 1892^{T}	27.2	10.9
P. ganghwense CAIM 512^{T}	15.2	16.1
P. rosenbergii CAIM 911^{T}	14.4	8.4
P. lutimaris CAIM 1851^{T}	26.5	21.2
P. swingsii CAIM 1393^{T}	20.2	19.5
S. sharmensis CAIM 1888^{T}	13.0	15.9
S. costicola subsp. *vallismaris* CAIM 735^{T}	20.2	6.1
S. proteolyticus CAIM 1855^{T}	3.4	8.2

3.4.4. Phenotypic coherence

The differentiating characteristics of these novel bacteria between them, in particular to the *Rosenbergii* clade species, which is closely related taxon to the new species studied, were observed in pigmentation, growth temperature range, oxidase, catalase, acetoin production, indole production, amylase, gelatinase, nitrate reduction, utilization of 39 carbon sources (Table 3.3). Among 88 phenotypic traits tested, 85% was similar between two *Thaumasiovibrio* species. The similarity was likely to be high enough to propose a phenotypic coherence of two *Thaumasiovibrio* species comparing those similarities against *Photobacterium*, *Enterovibrio*, *Grimontia*, and *Salinivibrio* (below 72%). Increasing number of *Vibrionaceae* species rapidly reduces effective diagnostic features of the genus definition. Only negative catalase activity seemed to be the common feature of the two *Taumasiovibrio* species differentiating the other genera (Table 3.4). The inter-genus phenotypic differences (Table 3.4) observed by this study included several biochemical features which were susceptibility to O/129 (150 μg), catalase reaction, indole production, arginine dehydolase along assimilation of D-mannitol.

Table 3.3. Phenotypic characteristics for distinguishing *Thaumasiovibrio occulutus* gen. nov., sp. nov., *Thaumasiovibrio subtropicus* sp. nov., and their related species. Taxa are indicated as: (1) *T. occultus* C4II189^T, (2) *T. subtropicus* C4V358^T, (3) *P. sanctipaulii* CAIM 1892^T, (4) *P. rosenbergii* CAIM 911^T, (5) *P. lutimaris* CAIM 1851^T, (6) *P. marinum* CAIM 1887^T, (7) *P. ganghwense* CAIM 512^T, (8) *G. hollisae* LMG 17718T, (9) *E. norvegicus* LMG 18839T, (10) S. *costicola* subsp. *costicola* LMG 11651^T

Characteristics	1	2	3	4	5	6	7	8	9	10
Pigmentation	–	+ (brown/ soluble)	+ (yellow /soluble)	–	+ (yellow /soluble)	–	–	–	+ (yellow /soluble)	–
Growth on TCBS	+G	+G	+	+Y	+Y	+G	–	–	+G	–
Growth at										
4°C	–	–	–	–	+	–	–	–	+	–
37°C	–	+w	+	+	–	+	+	+	–	+
40°C	–	–	–	+	–	+	+	+	–	–
Growth in NaCl										
1%	+w	+w	+	+	+w	+	+	+	+	–
3%	+	+	+	+	+	+	+	+	+	+w
6%	–	–	+	+	+w	+	+	+	+	+
8%	–	–	–	–	–	–	+	+	–	+
10%	–	–	–	–	–	–	+	–	–	+
Oxidase	+	–	+	+	+	+	+	+	+	+
Catalase	–	–	+	+	+	+	+	+	+	+
Acetoin production	–	–	–	–	–	–	+	–	–	+
Indole production	–	–	+	+	+	–	–	+	–	–
Production of										
Amylase	–	–	–	–	–	+	+	–	–	–
Gelatinase	+	+	–	–	–	+	+	–	+	+
Nitrate reduction	–	+	+	+	+	+	+	+	–	+
Arginine dihydrase	–	–	+	+	+	+	+	–	–	–
Gas from D-glucose	–	–	–	–	–	+	–	–	–	–
Utilization of										
D-Mannose	+	+	+	+	+	+	–	+	–	–
D-Galactose	–	–	+	+	+	–	–	+	+	–
D-Fructose	+	+	+	+	+	+	+w	+w	–	–
Sucrose	–	–	–	+	–	–	–	–	–	–
Maltose	–	–	+	–	–	+	–	–	–	–
Melibiose	–	–	+	–	+	–	–	–	–	–
Lactose	–	–	+	+	+	–	–	–	+	–
D-Gluconate	–	–	–	+	+	–	+	+	–	+
N -Acetylglucosamine	+	+	–	–	–	–	–	–	–	–
Citrate	+	+	–	–	–	–	–	–	–	–
D-Mannitol	–	+	+	–	+	–	–	–	+	–
Glycerol	+	–	+	+	+	+	+	+	+	–
γ-Aminobutyrate	–	–	–	–	–	–	–	+	+	–
D-Sorbitol	–	+	–	–	–	–	–	–	+	–
DL-Malate	+	+	–	–	–	–	–	–	–	–
Xylose	–	–	–	+	+	–	–	–	–	–
D-Glucose	+	+	+	–	+	+	–	+	–	–
Trehalose	–	–	+	+	+	+	–	–	–	–
Glucronate	–	+	+	+	+	–	–	–	–	–
Acetate	+	+	–	–	+	+	+	+	–	–
D-Glucosamine	+	+	+	+	–	+	–	–	+	–
Pyruvate	+	+	+	+	+	–	+	+	+	–
Cellobiose	–	+	+	–	–	–	–	–	+w	–
L-Proline	–	+	+	+	+	+	+	+	+	–
L-Glutamate	+	+	–	–	–	–	–	–	–	–
Propinonate	–	+	–	–	–	–	+	+	–	–
Arabinose	–	–	–	+	–	–	–	+	–	–
Glycerate	+	+	+	+	+	–	–	+	+	–
D-Raffinose	–	–	–	+	–	–	–	–	–	–
D-Ribose	–	–	+	+	–	+	+	+w	–	–
Salicine	–	–	+	–	+	+	–	–	–	–
DL-Lactate	+	+	–	–	–	–	–	–	–	–
L-Alanine	+	+	+	+	+	+	+	+	+	–
L-Arginine	–	–	–	+	–	+	+	+	+	–
L-Citrulline	–	–	–	–	–	–	+	–	+	–
Glycine	–	–	+	+	–	+	+	+	+	–
Histidine	–	+	+	+	+	+	+	+	–	+
L-Ornithine	–	–	–	+	+	+	+	+	+	–
L-Serine	–	+	+	+	+	+	+	+	+	–

All species were gram nagative, motile with a polar flagellum, fermentative, and require salt for growth. All species were positive for growth on 15, 20, 25, 30°C, growth in 1, 3% NaCl broth, DNase, lipase (Tween 80), and utilization of fumarate, L-asparagine. All species were negative for swarming, alginase, agarase, κ-carrageenase, lysine and ornithine decarboxylase, bioluminescence, and utilization of succinate, aconitate, L-tyrosine, α-ketoglutarate, δ-aminovarate, putrescine, amygdalin, D-galacturonate, rhamnose.

Table 3.4. Useful features for differentiating genera in the family *Vibrionaceae.* +, positive; -, negative; w, weak; d, different

Characteristic	***Thaumasiovibrio***	***Aliivibrio***	***Enterovibrio***	***Grimontia***	***Photobacterium***	***Salinvivrio***	***Vibrio***
D-mannitol fermentation	v	+	−	v	−	v	v
Gelatinase activity	+	v	−	v	v	+	v
Voges-Proskauer	−	v	−	−	v	+	v
Indole production	−	v	v	+	v	−	v
Arginine hydolase	−	v	+	−	v	+	v
Ornithine decarboxilase	−	−	−	−	−	−	v
Susceptibility to O/129 (150 μg)	+	+	v	+	v	+	v
Growth at							
10% NaCl	−	−	−	v	−	+	−
40°C	−	−	−	−	−	+	−
Catalase	−	+	+	+	v	+	v
DNA G + C content (mol%)	47.2-49.4	38-42	47.1-49.5	48.4-51.0	39-53.6	49-51	38.7-51.3

3.4.5. Assessment of the genus threshold of the *Thaumasiovibrio* against the other genera in *Vibrionaceae*

The phylogenetic and phenotypic cohesions of two *Thaumasiovibrio* species could support to propose the seventh genus in the family *Vibrionaceae*. In fact, the inter-genus similarities on the basis of 16S rRNA gene sequences between *Thaumasiovibrio* and the other genera were below 94.7%, which fulfilled a recently reported taxonomic thresholds (minimum sequence identity 94.8%, threshold sequence identify 94.5%) for "Genus" delineation of *Bacteria* and *Archaea* (Tindall et al., 2010; Yarza et al., 2014; Rosselló-Móra and Amann, 2015) (Table 3.5). In addition of this, genome wide AAI between two *Thaumasiovibrio* spp. 71.0-71.5%, of which value was >3.8% higher than the other type strains of the representative genera (Table 3.1). As the AAI amongst representative strains including type species of each genus were 65.0-69.6, the genus threshold on the basis of AAI for *Vibrionaceae* could be found between 69.6 to 71.5% (Figure 3.3). More interestingly, the AAIs between *E. pacificus* and *G. hollisae* type strain were 71.8 to 72.2, higher than the value obtained by the comparison of two *Thaumasiovibrio* species. Considering to the inter-genus 16S rRNA (95%) (Table 3.5), 8-genes MLSA network (Figure 3.2), and the AAI (>70%), *Enterovibrio* and *Grimontia* should be classified to the same genus. The number of core genes defined as >90% amino acid identity and alignment between two *Thaumasiovibrio* spp. was higher than those of the representative strains of the related genera of *Enterovibrio*, *Grimontia*, *Photobacterium*, and *Salinivibrio* (Figure 3.4). Lowest conserved genes were visualized among the compared genomes of *T. occultus* (C4II189) and *S. costicola* subsp. *costicola* where as comperative more conserved genes were investigated between *T. occultus* (C4II189) and *P. phosphoreum*.

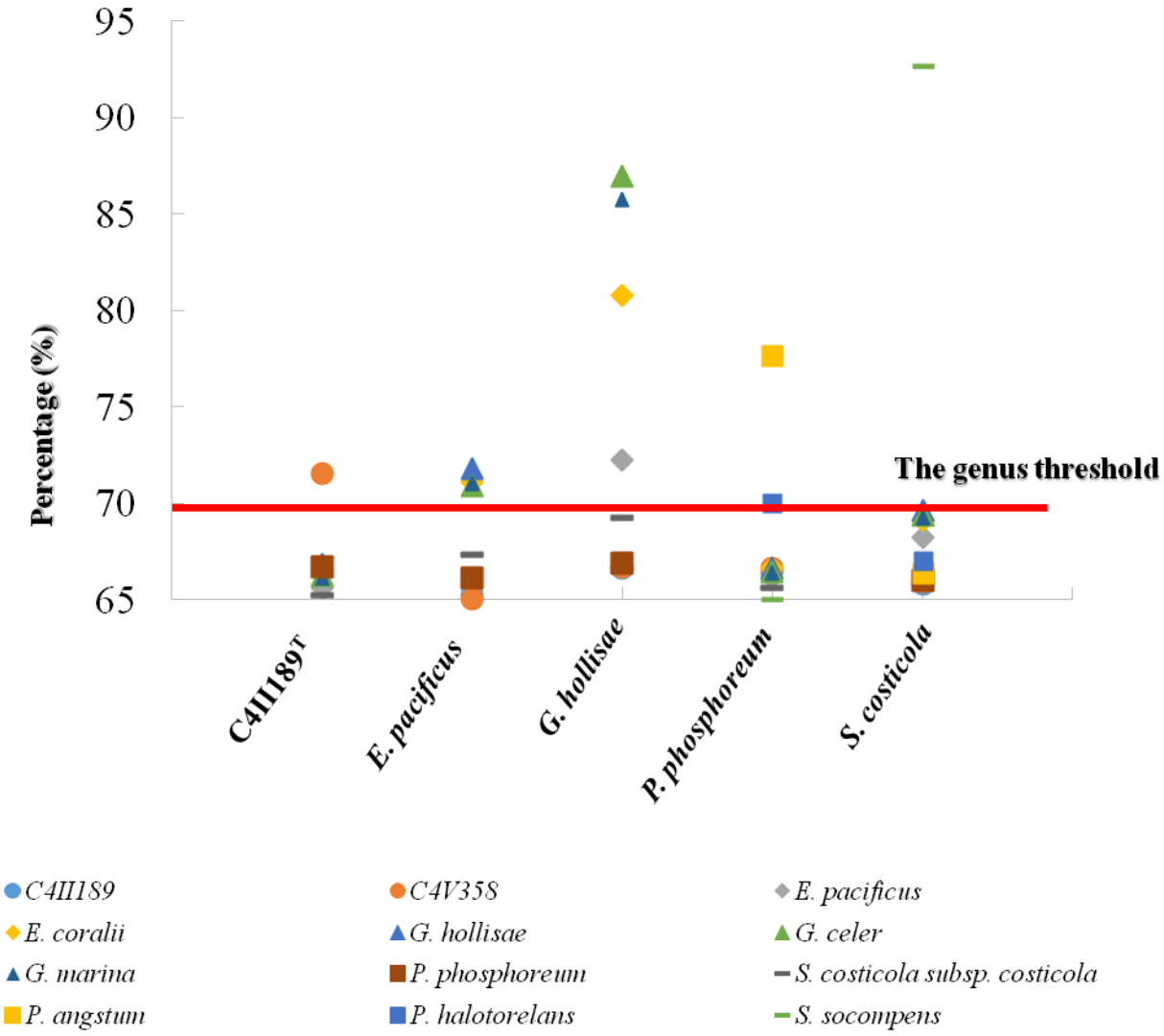

Figure 3.3. Inter-genus average amino-acid identity (AAI%).

Table 3.5. Inter-genus similarity on the basis of 16S rRNA gene sequence between *Thaumasiovibrio* gen. nov. and the other genera of the family *Vibrionaceae*

Genera	*Vibrio*	*Entero-vibrio*	*Grimontia*	*Photo-bacterium*	*Aliivibrio*	*Salinivibrio*	*Thaumasio-vibrio*
Enterovibrio	92.5						
Grimontia	92.1	95.0					
Photobacterium	93.5	93.6	93.4				
Aliivibrio	94.4	91.8	91.0	93.7			
Salinivibrio	90.8	92.6	92.3	91.7	90.2		
Thaumasiovibrio	93.5	94.2	94.2	94.7	93.0	92.9	
Echinimonas	89.7	89.1	88.3	90.3	90.3	88.9	90.1

Figure 3.4. Relationship of core genes amongs representative strains of five genera; *Thaumasiovibrio, Enterovibrio, Grimontia, Photobacterium*, and *Salinivibrio*. The number of core genes defined as >90% amino acid identity, green bars indicate genomes/ contigs/scaffolds.

3.4. Discussion

16S rRNA gene sequence analysis revealed that candidate two isolates (C4II189 and C4V358) formed two separate lines (Figure 3.1) with the species of genera *Photobacterium*, *Enterovibrio*, *Grimontia* and *Salinvibrio* of *Vibrionaceae*-family. The 16S rRNA gene sequences of C4II189 and C4V358 indicated their taxonomic positions (Figure 3.1, Table 3.5) as a new genus due to their lower sequence similarities (94.2%) than the established most recent boundary (>95%) of new genus circumscription (Tindall et al., 2010; Yarza et al., 2014; Rosselló-Móra and Amann, 2015) analyzed with the species of different genera belonging to *Vibrionaceae* family.

The MLSA showed the novel strains formed a distinct clade separated from all known clades formed by the members *Vibrionaceae* family (Figure 3.2). The updated MLSA network of this study (Figure 3.2) might also suggest 1) a clear separation of *Salinivibrio* clade but blend of the *Enterovibrio* and *Grimontia*, 2) the later synonym of "*Grimontia marinum"* AK15 to *Photobacterium marimum*, and 3) forming the candidate clade "Photodesmus" consisting *Candidatus* "Photodesmus katoptron" and *Candidatus* "Photodesmus blepharus". We still need more efforts to add all member of these genera, particularly of type strains of *Enterovibrio*, *Photobacterium*, *Grimontia*, and *Salinivibrio*, to the MLSA to perform an entire clade definition of the family *Vibrionaceae*, but even in the data set including all representative of the five *Photobacterium* clades, the two *Thaumasiovibrio* species form a monophyletic clade separated from major described clades of *Vibrionaceae*. The 8-gene AAI between the two species were 86.1%, which was higher than not only similarities against representative species of each genus but also previously observed values within each clade (>85%) (Sawabe et al., 2007, 2013).

As genomic average ANI of 95% corresponds to DNA-DNA hybridization values of 70% (Goris et al., 2007), two genome based tools, ANI and *in silico* DDH, are currently used common indexes in bacterial taxonomy (Stackebrandt et al., 2002; Richter and Rosselló-Móra, 2009; Meier-Kolthoff et al., 2013; Kim et al., 2014; Varghese et al., 2015). Genome based ANI estimations of the two *Thaumasiovibrio* spp. against the representative species of different genera of *Vibrionaceae* family (Table 3.1) showed less enough cut-off level (>95%) for species description (Goris et al., 2007; Richter and Rosselló-Móra, 2009). I used all available genome sequences in public databases of *Grimontia*, *Salinivibrio*, and *Photobacterium* species (totally 25 species). Similarly, GGDC revealed *in silico* DDH values of the *Thaumasiovibrio* species against above mentioned 25 species were sufficiently lower than the delineation threshold (>70%) to propose new bacterial species (Table 3.1) (Stackebrandt et al., 2002; Richter and Rosselló-Móra, 2009). This finding strongly supports that C4II189^T and C4V358^T are new species in *Vibrionaceae*. The determined DNA G+C content based on draft genomes (*in silico*) of these *Thaumasiovibrio* strains were 49.4% and 47.2% for C4II189^T and C4V358^T, respectively. *Thaumasiovibrio* spp. could be defined as a high G+C clade in *Vibrionaceae* accompanied by *Enterovibrio-Grimontia* and *Salinivibrio* clades (Table 3.4).

Phenotypic coherence was found in the studied two novel species candidates with their genetic (16S rRNA gene, MLSA, genomic) features. The comparative analysis of physiological, morphological, and biochemical characteristics of both novel species against the closely related species (Table 3.3) as well as genera (Table 3.4) showed that they were well separated from each other and towards related known described genera and species of *Vibrionaceae* family. Both of the novel bacteria were catalase negative. The low resistance to the active oxygen species of the species may cause the delay of discovery of the *Thaumasiovibrio* species until now.

Unfortunately, we could isolate only two strains of *Thaumasiovibrio* from the coral reef waters only in 2014 amongst totally 658 strains during the efforts of three years survey (Amin et al., 2016). Some vibrios have strong association to host animals but are less abundant in environments (Gomez-Gil et al., 2014), the ecology of the *Thaumasiovibiro* species, e.g association of corals or the other marine organisms, is worth studying in the local subtropical marine environments. Finally, I propose *Thaumasiovibrio* gen. nov. with *Thaumasiovibrio occultus* sp. nov. and *T. subtropicus* sp. nov. for the unusual rare vibrios in the marine tropical environments in Ishigaki, Japan. Unique phylogenetic features of these proposed novel bacteria, disclosed by this study would provide new insights not only for '*Vibrionaceae*' taxonomy and ecophysiology but also for reef system-*Vibrionaceae* interactions.

3.4.1. Description of (Thau.ma.si.o.vi'bri.o. Gr. adj. thaumasios, unusual, unique; N.L. masc. n. vibrio, a motile bacterium; N.L. masc. n. *Thaumasiovibrio*, an unusual bacterium)

Thaumasiovibrio strains are Gram-negative, facultatively anaerobic, motile rod. Sodium ion is essential for growth. No endospores are formed. They are mesophilic and neutrophilic chemo-organotroph. Flagellation is observed when the bacterium is cultivated on solidified and/or in liquid media. No swarming is observed. Green colonies on TCBS. No growth occurs at 4°C, and 40°C. The bacterium is grown in 1% to 3% NaCl broth. Does not produce gas from glucose. Negative for lysine decarboxylase, arginine dihydrolase and ornithine decarboxylase. Catalase, acetoin and indole productions are negative. Susceptible to the vibriostatic agent O/129 (150 µg/disc). No bioluminescence is observed, and does not required organic growth factors. Isolated from the seawater of Ishigaki coral reef. The range of DNA G+C content is 47.2-49.4%. Type species is *Thaumasiovibrio occultus* DSM 101554^T= JCM 31629^T=$C4II189^T$.

3.4.2. Descriptions of *Thaumasiovibrio occultus* sp. nov. (oc.cul'tus. L. adj. occultus, hidden; referring to resistant to the discovery of the species in the diverse active coral reef ecosystem)

Cells are 1.0-1.2 μm long and 0.7-0.8 μm width when the bacterium is grown on ZoBell 2216E agar. Colonies on ZoBell 2216E agar are beige, circular, smooth and convex grown in 2 mm diameter after 3 days' culture. Growth at 15-30°C. No pigmentation is observed. Major phenotypic features are, oxidase positive, assimilation of glycerol. The bacterium does not reduce nitrate, and assimilate D-mannitol, glucronate, cellobiose, L-proline, L-glutamate, propionate, histidine, and L-serine. The DNA G+C content is 49.4%. Type strain is *Thaumasiovibrio occultus* DSM 101554^{T}= JCM 31629^{T}=C4II189^{T}.

3.4.3. *Thaumasiovibrio subtropicus* sp. nov. (sub.tro'pi.cus. N.L. adj. subtropicus, subtropical, referring to the isolation site of the Ishigaki coral reef)

Cells are 1.9-2.0 μm long and 0.9-1.0 μm width when the bacterium is grown on ZoBell 2216E agar. Colonies on ZoBell 2216E agar are beige, circular, smooth and convex grown in 3 mm diameter after 3 days culture. Growth at 15-37°C. Major phenotypic features are oxidase negative, production of soluble brown pigments, nitrate reduction, assimilation of D-mannitol, D-sorbitol, glucronate, cellobiose, histidine, and L-serine. The DNA G+C content is 47.2%. Type strain is *Thaumasiovibrio subtropicus* DSM 101555^{T}= JCM 31630^{T}=C4V358^{T}.

Chapter 4. General Discussion

Ishigaki coral reefs are currently under threat from several stressors mainly due to global warming and anthropogenic eutrophication (Stone et al., 1999; Kayanne et al., 2002; Kawahata et al., 2004; Dadhich et al., 2012; Hasegawa, 2011; Harii et al., 2014; Shimomura and Naruse, 2015). As the corals provide a variety of micro-habitats for diversified microbes including vibrios (Rosenberg et al., 2007), microbes cause both beneficial and harmful interaction to the host coral (Rubio-Portillo et al., 2014). Some coral-associated bacteria play an active role in maintaining coral nutrition and health, e.g. nitrogen fixation, swarming and quarum sensing inhibition, antibiotics provision and so on (Ritchie, 2006; Chimetto et al., 2008; Teplitski and Ritchie, 2009; Krediet et al., 2013; Bourne and Webster, 2013), whereas several vibrios are pathogenic to corals, due to their relationships being affected by thermal anomalies (Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003; Garren et al., 2014; Munn, 2015; Vezzulli et al., 2012; Tout et al., 2015). Rising seawater temperatures associated with climate change are a significant threat to coral health, and also cause an increase in coral diseases by opportunistic *Vibrio* pathogens living in coral ecosystems (Munn, 2015; Tout et al., 2015). It is important to understand the abundance, diversity and dynamics of vibrios in coral reef ecosystems, as they are considered to be an early indicator of environmental stress and deteriorated coral health (Dinsdale et al., 2008). In the case of the Ishigaki coral reef environment, there are few reports on *Vibrio* diversity, their dynamics and relevant environmental determinants. This study aims to elucidate spatiotemporal *Vibrio* diversity and dynamics both at community and population levels along with the assessment of environmental drivers correlated to *Vibrio* diversity. To achieve the aim, I created curated *pyrH* gene sequences data sets covering over 90% (146 out of 157) of known *Vibrionaceae* species, and knowledge of the *Vibrio* diversity and dynamics of the Ishigaki coral reef system

compared to the other reports has been successfully accumulated, in particular, using PCoA and AdaptML for community and population analyses, respectively.

Firstly, I assessed the *Vibrio* community composition of the Ishigaki coral reef sites based on *pyrH* gene sequences of 685 strains isolated from seawater collected at five different reef sites over three years (2012 to 2014) off Ishigaki Island, Japan. The *Vibrionaceae* community in the reef environment of Ishigaki Island consists of 22 known and 12 potential novel *Vibrionaceae* species including several unique vibrios. *V. alginolyticus, V. campbellii, V. communis, V. coralliilyticus/V. neptunis, V. harveyi, V. mediterranei, V. owensii, V. ponticus, V. pelagius, V. rotiferianus, V. tubiashii, V. variabillis/V. maritimus*, and *Photobacterium rosenbergii* are commonly observed vibrios in coral ecosystems including coral holobionts. *V. astriarenae, V. azureus, V. hyugaensis, V. ishigakensis, V. nigripulchritudo, V. alfacsensis, P. damsella* subsp. *piscicida*, and *P. aphoticum* might have been observed for the first time in this study of coral ecosystems. The findings suggest that Ishigaki coral reef ecosystems are reservoirs of more diversified vibrios comparing to tropical (Chimetto Tonon et al., 2015) and temperate (Bourne and Muun, 2005; Raina et al., 2009; Siboni et al., 2016) reef systems (Figure 4.1). In other words, the Ishigaki coral reef areas harbor versatile and dynamic *Vibrio* populations consisting of both potential pathogenic and symbionts providing a variety of ecological niches.

The *Vibrio* community fluctuations assessed by PCoA with UniFrac distance and clustering with Euclidiean distance were unlikely to be related neither by year nor by site. Two clusters following a sub-cluster were seperated by the relative higher abundances of *V. campbellii* and *V. hyugaensis*-C6 (cluster-1), *V. owensii* (cluster-2) and *Photobacterium rosenbergii* (sub-cluster) from the analyzed Ishigaki reef seawater isolates. In detail, two apparent clusters in the *Vibrio* communities were observed; one consisted of those of Iriomote 2014, Osaki 2014, Sekisei 2014,

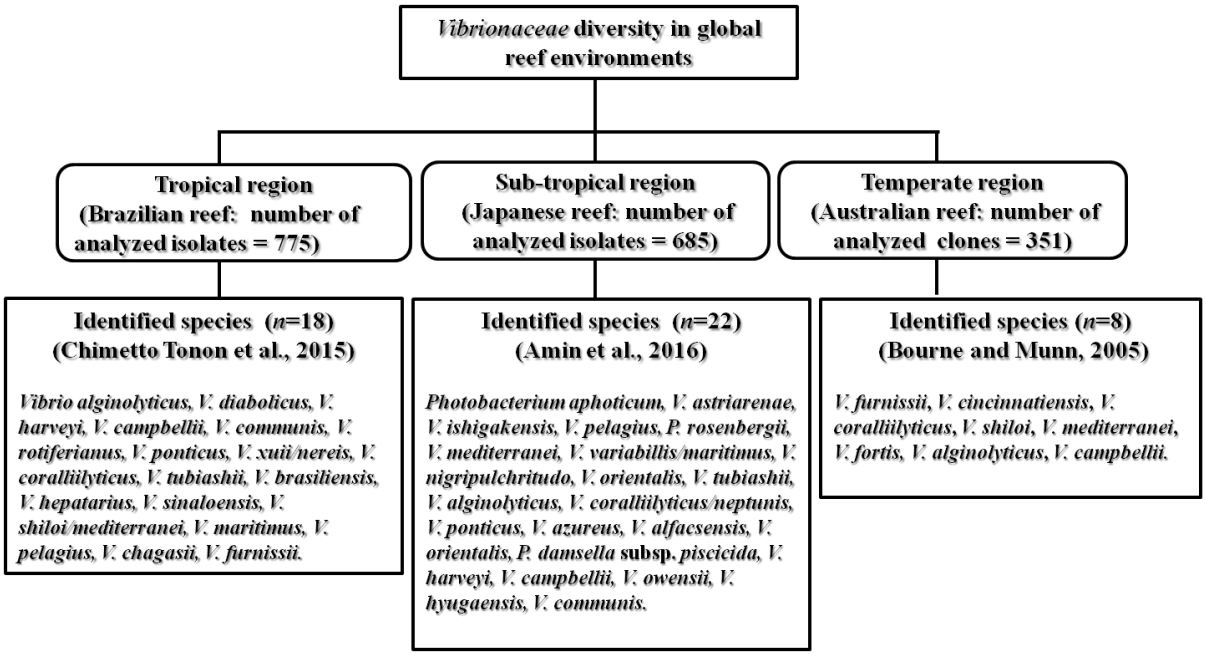

Figure 4.1. Differences in *Vibrionaceae* community in different coral reef environments.

Taketomi 2014 and Miyara 2013, and the other is from those of Taketomi 2012, Miyara 2014, Sekisei 2012, and Osaki 2013. Vibrios from Sekisei 2013 and Osaka 2012 were sub-clustered using a relative higher abundance of *P. rosenbergii*. Based on previous knowledge (Hunt et al., 2008; Tall et al., 2013), high abundances of *Harveyi* clade (*V. hyugaensis*, *V. communis, V. owensii, V.harveyi, V.campbellii*) and lower abundances of *Splendidus* clade species (*V. pelagius*) are the specific clues to help define subtropical Ishigaki coral reef microbiota. A couple of earlier reports (Chimetto et al., 2008; Preheim et al., 2011; Chimetto Tonon et al., 2015) also suggested that the vibrios from the *Harveyi* clade appear to have ecologically diversified passively invading new niches.

However, a recent report showed different results to my findings regarding the microbial community dynamic observations in estuarine water (Siboni et al., 2016), reef seawater and coral host (Ziegler et al., 2016). Significantly different *Vibrio* community dynamics were observed not only in the anthropogenic affected central Red Sea reef system (Ziegler et al., 2016) but also in seawater from different parts of the world (Baker-Austin et al., 2012; Vezzulli et. al., 2012; Takemora et al., 2014; Matteucci et al., 2015; Vezzulli et al., 2016; Siboni et al., 2016). These different results (significant different *Vibrio* community dynamics) from my study findings (only 2 main clusters, not so different *Vibrio* community dynamics) might be due to the impacts of geographical and environmental variations. Less spatiotemporal *Vibrio* community dynamics in the Ishigaki coral reef system suggest the presence of mostly identical environmental conditions even in different sampling points.

In the case of population levels, *V. hyugaensis* forms the largest *Vibrio* population (10 possible polulations consisting of 29.2% *Vibrio* communities), defined by the *pyrH* gene cluster, in Ishigaki coral reef environment. *V. owensii* (21.6%) forms the second largest population. In this study, *V. owensii* and *V. hyugaensis* showed generalist behavior (commonly isolated from all the sampling sites) which was consistent with other studies

conducted in the tropical reef systems (Chimetto Tonon et al., 2015). *V. owensii* is reported to be a potential coral pathogen (Ushijima et al., 2012). This coral pathogen was isolated as the causative agent of 'Montipora White Syndrome (MWS)' disease from the Hawaiian reef *Montipora capitata*. MWS is a tissue loss diseases observed in corals throughout the Hawaiian archiopelago that caused vast degradation of the Hawaiian reefs. Note that, *V. owensii* was also isolated from '*Acropora* White Syndrome Lesions (AWSL)' of American Samoa (Wilson et al., 2012). In addition to corals, this bacterium was noted as a pathogen for crustaceans in Australia (Cano-Gomez et al., 2010). Corals may be more at risk from the generalist *V. owensii*. On the other hand, the specialist *Vibrio* populations (isolated only from specific sampling site) of Ishigaki reef seawater (*V. alfacsensis, V. nigripulchritudo, V. azureus, V. mediterranei, V. orientalis*) were found to be completely different in my study area than those from other coral reef ecosystems. Although *V. shiloi* was reported as a specialist *Vibrio* (Chimetto Tonon et al., 2015) from tropical Brazilian reef systems (St. Sebastian region), this bacterium was not identified from the sub-tropical Ishigaki coral reef environment indicated that some vibrios may have evolved in closely associated habitats as coral holobionts. *V. splendidus* (Vezzulli et al., 2015) persists in marine environment through adopting multiple survival strategies including biofilm formation and attachment to the marine chitin substrates.

AdaptML based *Vibrio* population diversity dynamic studies have also detected some vibrios as being ecologically diverse. From all the identified known vibrios, *V. astriarenae* and *V. ishigakensis* were found as ecologically more diverse. Ishigaki reefs might determine the specific niches for these divergent *Vibrio* populations. The characterized phenotypic features of *V. astriarenae* and *V. ishigakensis* (Al-saari et al., 2015; Gao et al., 2016) showed multiple enzymatic activities such as amylase, lipase, DNase, gelatinase, alginate lyase and so on. The most ecologically divergent *V. hyugaensis* also possessed some extra ordinary phenotypic

features (Urbanczyk et al., 2015) including bright luminous and decarboxilase activities for ornithine and lysine. These important features may positively enhance the divergent distributions of these bacteria. For example, bacterial luminescence has both physiological and ecological benefits. Bioluminescence-symbiosis provides nutrients and respiratory facilities for rapid microbial growth and multiplication that ultimately create opportunities for co-evolutionary events that may also be influenced by ecological states (Dunlap and Kita-Tsukamoto, 2006; Dunlap et al., 2008). Moreover, the mathematical model AdaptML is capable to connect bacterial populations based on their phylogenetic and ecological information (Hunt et al., 2008). Although my study did not find the significant connections (genetic relation) among the analyzed *Vibrio* populations on a spatiotemporal basis, another relevant study (Chimetto Tonon et al., 2015) observed significant genetic congruence among the multi-sourced vibrios (i.e. seawater, coral host etc.) generated by AdaptML. The lack of well separated genetic connectivity among the vibrios from different reef ecosystems of Ishigaki Island may be caused by the geographic feature of the isolated island having nutrient rich coral ecosystems; the one world to vibrios.

The results of this study also reveal that Ishigaki coral reef is home to both coral associated beneficial (*V. harveyi*, *V. campbellii*, *V. alginolyticus*) and harmful vibrios (*V. coralliilyticus*, *V. owensii*). There is a lot of evidence relating to beneficial roles of *V. harveyi*, *V. campbelli*, and *V. alginolyticus* to coral hosts due to the fixation of nitrogen (Lesser et al., 2004; Chimetto et al., 2008). Coral holobionts including some nitrogen fixers and these bacteria provide considerable amount of nitrogen for metabolic activities of the coral host that ultimately facilitate the growth of corals mostly in oligotrophic environments (Capone et al., 2001). According to Ritchie (2006), some reef associated vibrios may remain as mutualistic partners in the coral holobiont family through releasing nutrients and secondary metabolites to coral. It is important to note that the above mentioned vibrios (except *V. campbellii*) have

also been recognized as coral pathogens in different tropical and temperate reef systems (Cervino et al., 2004; Ushijima et al., 2012; Sweet et al., 2014). These vibrios might act as opportunistic pathogens for corals and associated holobionts along with other marine organisms (Gomez-Gil et al., 2004; Fukui et al., 2009; Munn, 2015).

Climate change-derived thermal stress-influenced bleaching events were previously recorded from the Southern Japanese coral reefs in 1997-98 and 2001 (Stone et al., 1999; Kayanne et al., 2002) and specifically from Ishigaki Island in 2007 (Dadhich et al., 2012). Coral bleaching is a disease (Rosenberg et al., 2009) that is affected by biotic and environmental factors. Several vibrios (*V. shiloi*, *V. coralliilyticus*, *V. owensii*, *V. harveyi*) are thought to be important causative agents in coral bleaching (Kushmaro et al., 1996; Ben-Haim et al., 2003; Wilson et al., 2012; Ushijima et al., 2012). This study also investigated bleaching related vibrios including known coral pathogen, *V. coralliilyticus* along with other recognized potential coral pathogens (*V. owensii*, *V. harveyi*) as dominant vibrios from Ishigaki coral reef Island. Among these, *V. coralliilyticus* is a widespread dominant coral pathogen globally. The modes of pathogenicity to the host include chemotaxis (Winn et al., 2013), swarming and motile behavior (Stocker, 2011), extracellular-protease toxicity (Ben-Haim et al., 2003) and so on. Currently this pathogen is being monitored as a potentially devastating coral pathogen in different parts of the world including Indo-Pacific reefs (Sussman et al., 2008), Mediterranean corals (Vezzulli et al., 2010), Caribbean corals (Vizcaino et al., 2010), and Brazilian corals (Alves Jr et al., 2010). A lot of previous reports (Ben-Haim et al., 2003; Vezzulli et al., 2010; Tout et al, 2015; Munn, 2015) showed that the pathogenicity of *V. coralliilyticus* in corals is linked with rising seawater temperature, suggesting that warm seawater might influence outbreaks of *V. coralliilyticus* associated vibriosis.

Secondly, I tried to elucidate correlation between environmental variables and *Vibrio* abundances in the Ishigaki reef areas. Several vibrios showed significant correlation with

rising seawater temperature. Although significant positive correlation between the abundances of coral pathogenic vibrios and rising seawater temperature has been observed as a normal phenomenon (Rosenberg and Falkovitz, 2004; Takemura et al., 2014; Garren et al., 2015), *V. campbellii*, a non-coral pathogen, correlates with increased seawater temperature in the Ishigaki reef Island. Considering the coral and vibrios interactions in the reef system, *V. campbellii* is recognized as a beneficial *Vibrio* for corals that can fix nitrogen (Chimetto et al., 2008). However, there is also data about the pathogenicity of this *Vibrio* for marine organisms (Gomez-Gil et al., 2004) other than corals. A recently published draft genome of an environmental *V. campbellii* strain (Amaral et al., 2012) uncovered a complete proteorodopsin gene cluster including another complete type II, III secretion system genes along the genes related to type I, IV secretion systems indicating the advantage of the fitness in the mesotrophic and heterotrophic environments and also conferred a special adaptive advantage to planktonic cells in marine water column and to the cells of marine organisms. Another two vibrios, *V. owensii* and *V. hyugaensis* established significant negative correlations with rising seawater temperature in the Ishigaki reef environment. Between these, *V. owensii* is already known as an opportunistic pathogen for Hawaiian corals in the United States of America (Ushijima et al., 2012), whereas, *V. hyugaensis* is a very recently described novel *Vibrio* from the coastal seawater off Japan (Urbanczyk et al., 2015) and has not been reported as coral pathogen yet. These results indicate that the abundance and dynamics of these vibrios is a temperature dependent phenomenon.

Finally, in Chapter 3, this study also placed multiple novel species candidates (12 new species candidates with 26 isolates) from which a new genus (*Thaumasiovibrio*) of *Vibrionaceae* family is proposed, revealing that the reef system could be a hotspot of *Vibrionaceae* evolution. Similar observations (Reis et al., 2009; Moreira et al., 2014; Chimetto Tonon, 2015) were also reported from other reef systems. The proposed new genus,

Thaumasiovibrio belonged two phylogenetically unique two strains which never been affiliated to any known taxa of *Vibrioneaceae* are isolated. Applying the most recent advanced microbial taxanomic approaches fulfilling monophyly, genetic and phenotypic cohesion to them, I concluded to propose a new genus in *Vibrionaceae* with two new species *Thaumasiovibiro occultus* gen. nov., sp. nov, and *Thaumasiovibrio subtropicus* sp. nov. These species are catalase negative and showed green colonies in TCBS which are their important distinguishing criteria from the existing genera of *Vibrionaceae* family. Note that, two new *Vibrio* species have been described previously from the isolates used in this study. One is *V. astriarenae* which forms a newly proposed clade, *Agarivorans* clade, with *V. agarivorans* in the *Vibrionaceae* family (Al-saari et al., 2015). Agarolytic traits are a unique feature of the species. Another is *V. ishigakensis*, which is the 10th species belonging to the *Halioticoli* clade (Gao et al., 2016). The species shows not only alginolytic traits but also a variety of sugars and high temperature adaptation. So this species could provide important clues to elucidate the evolutionary history of *Halioticoli* clade strains. In this chapter, a new *Vibrionaceae* genus is proposed with two species showing TCBS grown (green) catalase negative strains applying almost all the latest advanced techniques including genomics (Richter and Rossello´-Mo´ra, 2009; Qin et al., 2014; Thompson et al., 2014; Chun and Rainey, 2014; Oren and Garrity, 2014; Rosselló-Móra and Amann, 2015; Varghese et al., 2015; Whitman et al., 2015; Garrity, 2016). I still have new species candidates such as CV172, CV50, C4II258, C4I174, CV96, CV58, CV39, CV97, C4III282, C121, C4II189 and C4V358 (Figure 4.2). Coral reef ecosystems are considerable potential sources of novel *Vibrionaceae* family members.

All these findings with my results here, regarding the potential source of novel *Vibrio* species also suggest that reefs and reef oriented seawater are important potential habitats to study the microevolution of vibrios. The adaptability and dispersal of vibrios through water columns

may play vital roles in their persistence in the environment particularly in the case of reef ecosystems, *Vibrio* dispersal might be enhanced via the shedding of coral itself (Garren and Azam, 2012). *Vibrionaceae* is a leading taxon in dynamic microbial taxonomy (Sawabe et al., 2007; Thompson et al., 2009; Sawabe et al., 2013). The capability of vibrios to adapt to versatile and changing environments may be due to the presence of flexible gene pools (Hunt et al., 2008). So called HGT-mechanism (horizontal gene transfer) is the highlighted mechanism that greatly enhances bacterial evolution, diversity and adaptability towards new and altered ecological niches (Wiedenbeck and Cohan, 2011). Moreover, *Vibrio* genomes are also flexible and their genome architecture is easily manipulated due to the presence of mobile genetic elements with integrating conjugative elements, superintegron like gene cassettes, insertion sequences and transposes genes that may together make the vibrios cosmopolitan as well as being the fittest for survival in diversified habitats (Heidelberg et al., 2000; Makino et al., 2003; Cheng et al., 2003; Hasan et al., 2010; Chen et al., 2011; Yu et al., 2013).

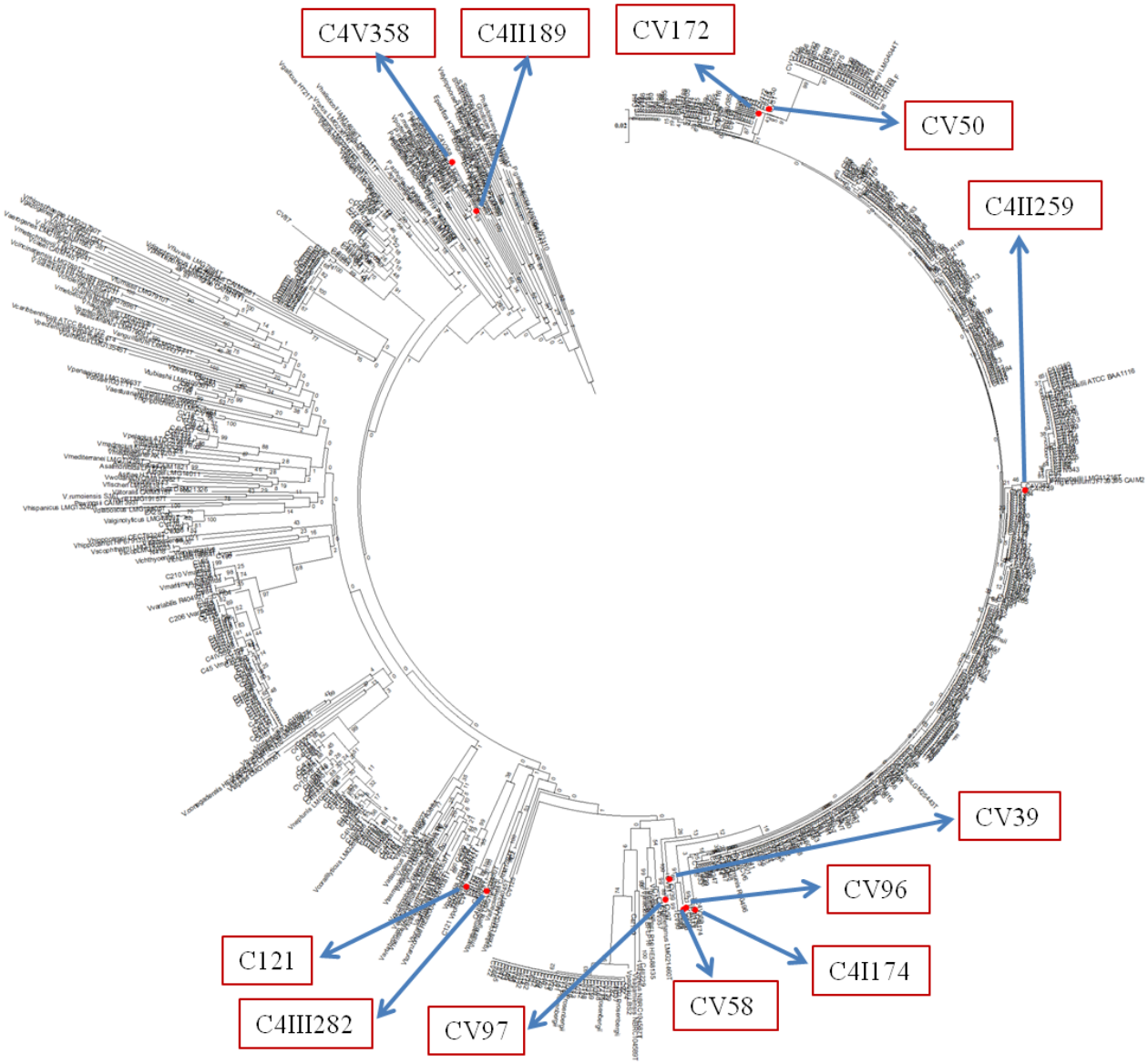

Figure 4.2. Potential new species candidates (*n*=12) from Ishigaki coral reef seawater.

Conclusion and future prospects

Knowledge on microbial diversity including adaptation as well as ecological processes that generate and maintain the biodiversity of microbiota is both interesting and essential. We have famous quotes relating to eco-evolutionary dynamics: nothing in biology makes sense except in the light of evolution (Dobzhansky), nothing in evolutionary biology makes sense except in the light of ecology (Grant), nothing in evolution or ecology makes sense except in the light of the other (Pelletier). Now it is the actual time to define 'other', in particular in studying complex ecological interactions.

Among microbes, vibrios represent one of the best studied models for the ecology and evolution of bactarial populations in nature, where *Vibrio* correlations assessment based on environmental and taxonomic scales are essential to better understand their dynamics as the learning of spatiotemporal dynamics of vibrios and the detection of relevant environmental drivers are crucial in predicting the future behavior of vibrios in stressed coral reef ecosystems. The curated data set of *Vibrionaceae pyrH* gene sequences of this study enabled the performing of the first characterization of *Vibrio* diversity and assessment of spatiotemporal diversity dynamics of *Vibrio* populations both at community and population levels along with their relationship to environmental determinants from Ishigaki coral reef seawater. The determined *Vibrio* community of seawater directly connected to Ishigaki coral holobionts included at least 22 known described species and 12 as yet undescribed species, which are more diverse compared to other reported tropical and temperate reef vibrios. The *Vibrio* diversity fluctuations were not related to site and year. This study also disclosed several unique vibrios from reef systems along with a proposed novel genus (*Thaumasiovibrio*) consisted by two novel species (*T. occultus* and *T. subtropicus*). The discovered unique vibrios were *V. hyugaensis*, *V. ishigakensis*, *V. astriarenae*, *V. nigripulchritudo*, *V. alfacsensis*, *V. azureus*, *P. aphoticum*, and *P. damselae* subsp. *piscicida*.

Several vibrios such as, *V. campbellii*, *V. owensii* and the C6 group of *V. hyugaensis* abundances seem to correlate with seawater temperature. *V. owensii* and C6 group of *V. hyugaensis* show significant negative correlations with increasing seawater temperature but *V. campbellii* is positively significant in this regard. This study also demonstrated the presence of most of the globally recognized opportunistic potential coral-pathogenic vibrios (*V. owensii, V. harveyi, V. alginolyticus*) including the notorious known coral pathogen, *V. coralliilyticus*. As diversity and dynamics of vibrios are considered as the early indicator of environmental stress and deteriorated coral health, so this pioneering study of Ishigaki coral reef seawater *Vibrionaceae* diversity dynamics and associated environmental variables will be an important gateway for further deep research relating to the monitoring, conservation and other necessary actions to stop reef decline.

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Acknowledgements

I would like to express my heartfelt honor, deepest sense of gratitude, and profound indebtedness to my academic advisor Prof. Dr. Tomoo Sawabe, Laboratory of Microbiology,

Faculty of Fisheries Sciences, Hokkaido University, Japan, for his scholastic guidance, teaching of scientific techniques, constructive criticism, constant encouragement and untiring cordial help throughout my doctoral course and during the writing up of this thesis.

I feel proud in expressing my profound regard to Prof. Dr. Takao Ojima, Laboratory of Marine Biotechnology and Microbiology, Faculty of Fisheries Sciences, Hokkaido University, Japan, for his cordial cooperation and helpful encouragement in all spheres of this study.

I owe a debt of gratitude to Dr. Sayaka Mino, Assistant Professor, Laboratory of Microbiology, Faculty of Fisheries Sciences, Hokkaido University, Japan, for her cordial help and valuable suggestions until the completion of my study.

I wish to take this opportunity to express my sincere and deep sense of appreciation and gratitude to respectable teachers Prof. Dr. Arai Katsutoshi, Prof. Dr. Takahashi Koretaro, Prof. Dr. Miyashita Kazuo, and Prof. Dr. Konno Kuniniko for their endless encouragements and affectionate feelings through out my study perion in Japan.

I am also greatful to Dr. Toko Sawabe, Department of Nutrition, Hakodate Junior College, Hakodate, Japan, for her useful encouragement and cooperation during the course of this study.

My special thanks to Prof. Dr. Fabiano Thompson and Dr. Pedro Milet Meirelles, Institute of Biology, SAGE-COPPE, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, for their valuable cooperations.

I am also grateful to all of my laboratory members, especially Feng Gao, Yohei Yamazaki, Nurhidayu Al-saari, and Mami Tanaka for their active cooperations at the important stage of this study.

I also would like to acknowledge the Japanese Ministry of Science, Education, Culture and Sports for scholarship support through the entire study period.

Last of all, it is my great pleasure to covey my heartfelt gratitude to my beloved parents, wife and other relatives, whose endless sacrifice, encouragement and patience enabled me to pursue my doctoral study. I also express my special thanks to all of my friends and well wishers in Japan and Bangladesh for their encouragement throughout the study period.

Appendix 1. Details strain list (*n*=685) isolated from Ishigaki coral reef sea water

strain	year	sampling site	species
C1	2012	Taketomi	*C1*
C2	2012	Taketomi	*V. alfacsensis*
C3	2012	Taketomi	*V. owensii*
C5	2012	Taketomi	*C1*
C6	2012	Taketomi	*C6*
C7	2012	Taketomi	*V. astrearenae*
C8	2012	Taketomi	*V. corallilyticus/neputunis*
C9	2012	Taketomi	*V. owensii*
C10	2012	Taketomi	*V. corallilyticus/neputunis*
C11	2012	Taketomi	*V. corallilyticus/neputunis*
C12	2012	Taketomi	*P. rosenbergii*
C13	2012	Taketomi	*V. corallilyticus/neputunis*
C14	2012	Taketomi	*V. corallilyticus/neputunis*
C15	2012	Taketomi	*V. pelagius*
C16	2012	Taketomi	*C16*
C17	2012	Taketomi	*C1*
C18	2012	Taketomi	*V. rotiferianus*
C20	2012	Taketomi	*V. astrearenae*
C21	2012	Taketomi	*V. astrearenae*
C22	2012	Taketomi	*C1*
C23	2012	Taketomi	*V. corallilyticus/neputunis*
C24	2012	Taketomi	*V. owensii*
C26	2012	Taketomi	*V. owensii*
C27	2012	Taketomi	*V. owensii*
C28	2012	Taketomi	*V. owensii*
C29	2012	Taketomi	*V. owensii*
C30	2012	Taketomi	*V. owensii*
C31	2012	Taketomi	*V. owensii*
C32	2012	Taketomi	*V. owensii*
C33	2012	Taketomi	*V. owensii*
C34	2012	Taketomi	*V. owensii*
C35	2012	Taketomi	*V. owensii*
C36	2012	Taketomi	*C6*
C37	2012	Taketomi	*V. owensii*
C38	2012	Taketomi	*V. corallilyticus/neputunis*
C39	2012	Taketomi	*V. corallilyticus/neputunis*
C40	2012	Taketomi	*P. rosenbergii*
C41	2012	Taketomi	*V. owensii*
C42	2012	Taketomi	*V. corallilyticus/neputunis*
C43	2012	Taketomi	*V. owensii*
C44	2012	Taketomi	*V. corallilyticus/neputunis*
C45	2012	Taketomi	*V. variabilis/maritimus*
C46	2012	Taketomi	*C46*
C47	2012	Taketomi	*V. variabilis/maritimus*
C48	2012	Taketomi	*V. owensii*
C49	2012	Taketomi	*C49*
C50	2012	Taketomi	*C49*
C51	2012	Taketomi	*V. variabilis/maritimus*
C52	2012	Taketomi	*C6*

Continued			
C53	2012	Taketomi	*C6*
C54	2012	Taketomi	*C6*
C55	2012	Taketomi	*C49*
C56	2012	Taketomi	*C49*
C57	2012	Taketomi	*P. rosenbergii*
C58	2012	Miyara	*C58*
C59	2012	Miyara	*C58*
C60	2012	Miyara	*C58*
C61	2012	Miyara	*V. owensii*
C62	2012	Miyara	*P. rosenbergii*
C63	2012	Miyara	*C58*
C64	2012	Miyara	*C1*
C65	2012	Miyara	*C1*
C66	2012	Miyara	*C58*
C67	2012	Miyara	*C58*
C68	2012	Miyara	*C68*
C69	2012	Miyara	*C6*
C70	2012	Miyara	*V. owensii*
C71	2012	Miyara	*C71*
C72	2012	Miyara	*C1*
C73	2012	Miyara	*V. owensii*
C74	2012	Miyara	*V. owensii*
C75	2012	Miyara	*C49*
C76	2012	Miyara	*V. harveyi*
C77	2012	Miyara	*P. rosenbergii*
C79	2012	Miyara	*V. owensii*
C80	2013	Miyara	*V. owensii*
C81	2014	Miyara	*V. owensii*
C82	2012	Miyara	*C6*
C126	2012	Miyara	*V. owensii*
C127	2012	Miyara	*C68*
C128	2012	Miyara	*V. corallilyticus/neputunis*
C129	2012	Miyara	*P. rosenbergii*
C130	2012	Miyara	*C6*
C131	2012	Miyara	*C6*
C132	2012	Miyara	*C6*
C133	2012	Miyara	*V. owensii*
C134	2012	Miyara	*V. owensii*
C96	2012	Osaki	*V. owensii*
C97	2012	Osaki	*V. harveyi*
C98	2012	Osaki	*V. pelagius*
C99	2012	Osaki	*C6*
C100	2012	Osaki	*P. rosenbergii*
C101	2012	Osaki	*P. rosenbergii*
C102	2012	Osaki	*P. rosenbergii*
C103	2012	Osaki	*P. rosenbergii*
C104	2012	Osaki	*P. rosenbergii*
C105	2012	Osaki	*P. rosenbergii*
C106	2012	Osaki	*V. tubiashii*

Continued			
C107	2012	Osaki	*C1*
C108	2012	Osaki	*V. variabilis/maritimus*
C109	2012	Osaki	*P. rosenbergii*
C110	2012	Osaki	*C6*
C111	2012	Osaki	*V. owensii*
C112	2012	Osaki	*V. owensii*
C113	2012	Osaki	*P. rosenbergii*
C114	2012	Osaki	*V. owensii*
C117	2012	Osaki	*V. harveyi*
C119	2012	Osaki	*P. aphoticum*
C120	2012	Osaki	*P. rosenbergii*
C121	2012	Osaki	*C121*
C122	2012	Osaki	*V. corallilyticus/neputunis*
C123	2012	Osaki	*P. rosenbergii*
C124	2012	Osaki	*V. campbellii*
C125	2012	Osaki	*C49*
C137	2012	Osaki	*V. variabilis/maritimus*
C138	2012	Osaki	*V. harveyi*
C139	2012	Osaki	*V. rotiferianus*
C140	2012	Osaki	*C46*
C142	2012	Osaki	*C71*
C143	2012	Osaki	*V. harveyi*
C144	2012	Osaki	*C6*
C145	2012	Osaki	*C6*
C146	2012	Osaki	*C16*
C147	2012	Osaki	*C6*
C148	2012	Osaki	*V. alginolyticus*
C149	2012	Osaki	*C68*
C151	2012	Osaki	*V. owensii*
C152	2012	Osaki	*V. owensii*
C153	2012	Osaki	*C46*
C154	2012	Osaki	*C6*
C83	2012	Sekisei	*C49*
C84	2012	Sekisei	*C46*
C85	2012	Sekisei	*V. variabilis/maritimus*
C87	2012	Sekisei	*V. owensii*
C88	2012	Sekisei	*V. owensii*
C89	2012	Sekisei	*V. corallilyticus/neputunis*
C90	2012	Sekisei	*V. alginolyticus*
C91	2012	Sekisei	*C68*
C92	2012	Sekisei	*P. rosenbergii*
C93	2012	Sekisei	*C68*
C94	2012	Sekisei	*C1*
C95	2012	Sekisei	*C6*
C156	2012	Sekisei	*C156*
C157	2012	Sekisei	*C46*
C158	2012	Sekisei	*C46*
C159	2012	Sekisei	*C68*
C160	2012	Sekisei	*C68*

Continue			
C161	2012	Sekisei	*V. owensii*
C162	2012	Sekisei	*C46*
C163	2012	Sekisei	*C46*
C164	2012	Sekisei	*C164*
C165	2012	Sekisei	*V. owensii*
C166	2012	Sekisei	*V. owensii*
C167	2012	Sekisei	*V. owensii*
C168	2012	Sekisei	*V. owensii*
C169	2012	Sekisei	*C46*
C170	2012	Sekisei	*V. owensii*
C171	2012	Sekisei	*C46*
C172	2012	Sekisei	*V. owensii*
C173	2012	Sekisei	*C58*
C174	2012	Sekisei	*C6*
C175	2012	Sekisei	*C68*
C176	2012	Sekisei	*C6*
C177	2012	Sekisei	*C68*
C178	2012	Sekisei	*V. owensii*
C179	2012	Sekisei	*C46*
C180	2012	Sekisei	*V. owensii*
C182	2012	Sekisei	*P. rosenbergii*
C183	2012	Sekisei	*C46*
C184	2012	Sekisei	*V. owensii*
C185	2012	Sekisei	*C6*
C187	2012	Sekisei	*V. owensii*
C188	2012	Sekisei	*C6*
C189	2012	Sekisei	*V. owensii*
C190	2012	Sekisei	*V. owensii*
C191	2012	Sekisei	*V. owensii*
C192	2012	Sekisei	*V. orientalis*
C193	2012	Sekisei	*C6*
C195	2012	Sekisei	*C16*
C196	2012	Sekisei	*C46*
C197	2012	Sekisei	*V. variabilis/maritimus*
C198	2012	Sekisei	*C46*
C199	2012	Sekisei	*C164*
C200	2012	Sekisei	*V. owensii*
C202	2012	Sekisei	*P. rosenbergii*
C204	2012	Sekisei	*C46*
C206	2012	Sekisei	*V. variabilis/maritimus*
C207	2012	Sekisei	*V. variabilis/maritimus*
C208	2012	Sekisei	*V. owensii*
C209	2012	Sekisei	*V. variabilis/maritimus*
C210	2012	Sekisei	*V. variabilis/maritimus*
C212	2012	Sekisei	*C1*
C213	2012	Sekisei	*V. variabilis/maritimus*
C214	2012	Sekisei	*V. variabilis/maritimus*
C215	2012	Sekisei	*V. variabilis/maritimus*
C216	2012	Sekisei	*C1*

Continue			
C217	2012	Sekisei	*V. ponticus*
C219	2012	Sekisei	*V. variabilis/maritimus*
C221	2012	Sekisei	*V. owensii*
C222	2012	Sekisei	*C6*
C223	2012	Sekisei	*V. astrearenae*
C224	2012	Sekisei	*V. owensii*
CV1	2013	Sekisei	*V. campbellii*
CV2	2013	Sekisei	*V. campbellii*
CV3	2013	Sekisei	*C6*
CV6	2013	Sekisei	*V. owensii*
CV7	2013	Sekisei	*V. owensii*
CV8	2013	Sekisei	*V. corallilyticus/neputunis*
CV9	2013	Sekisei	*C1*
CV121	2013	Sekisei	*C46*
CV122	2013	Sekisei	*V. owensii*
CV123	2013	Sekisei	*C16*
CV124	2013	Sekisei	*C164*
CV125	2013	Sekisei	*CV125*
CV126	2013	Sekisei	*V. variabilis/maritimus*
CV127	2013	Sekisei	*V. harveyi*
CV128	2013	Sekisei	*C46*
CV129	2013	Sekisei	*C46*
CV130	2013	Sekisei	*C71*
CV131	2013	Sekisei	*C71*
CV132	2013	Sekisei	*C6*
CV133	2013	Sekisei	*C6*
CV134	2013	Sekisei	*C16*
CV135	2013	Sekisei	*C16*
CV136	2013	Sekisei	*V. alginolyticus*
CV137	2013	Sekisei	*V. pelagius*
CV138	2013	Sekisei	*C6*
CV139	2013	Sekisei	*P. rosenbergii*
CV140	2013	Sekisei	*P. rosenbergii*
CV141	2013	Sekisei	*P. rosenbergii*
CV142	2013	Sekisei	*P. rosenbergii*
CV143	2013	Sekisei	*P. rosenbergii*
CV144	2013	Sekisei	*P. rosenbergii*
CV145	2013	Sekisei	*V. tubiashii*
CV146	2013	Sekisei	*C1*
CV147	2013	Sekisei	*V. variabilis/maritimus*
CV148	2013	Sekisei	*P. rosenbergii*
CV149	2013	Sekisei	*C6*
CV150	2013	Sekisei	*V. owensii*
CV151	2013	Sekisei	*V. owensii*
CV152	2013	Sekisei	*P. rosenbergii*
CV153	2013	Sekisei	*V. owensii*
CV154	2013	Sekisei	*V. owensii*
CV155	2013	Sekisei	*P. rosenbergii*
CV156	2013	Sekisei	*V. harveyi*

Continue			
CV157	2013	Sekisei	*V. owensii*
CV158	2013	Sekisei	*P. aphoticum*
CV159	2013	Sekisei	*P. rosenbergii*
CV10	2013	Miyara	*V. corallilyticus/neputunis*
CV11	2013	Miyara	*V. campbellii*
CV12	2013	Miyara	*V. campbellii*
CV13	2013	Miyara	*V. harveyi*
CV14	2013	Miyara	*V. harveyi*
CV15	2013	Miyara	*V. corallilyticus/neputunis*
CV16	2013	Miyara	*V. corallilyticus/neputunis*
CV17	2013	Miyara	*V. nigripulchritudo*
CV18	2013	Miyara	*V. pelagius*
CV19	2013	Miyara	*V. campbellii*
CV20	2013	Miyara	*V. harveyi*
CV21	2013	Miyara	*V. harveyi*
CV22	2013	Miyara	*P. rosenbergii*
CV23	2013	Miyara	*C58*
CV24	2013	Miyara	*C58*
CV25	2013	Miyara	*C58*
CV26	2013	Miyara	*C16*
CV27	2013	Miyara	*C16*
CV28	2013	Miyara	*C16*
CV102	2013	Miyara	*C46*
CV103	2013	Miyara	*V. owensii*
CV104	2013	Miyara	*V. variabilis/maritimus*
CV105	2013	Miyara	*C6*
CV106	2013	Miyara	*C6*
CV107	2013	Miyara	*C58*
CV108	2013	Miyara	*V. owensii*
CV109	2013	Miyara	*C58*
CV110	2013	Miyara	*C58*
CV111	2013	Miyara	*C58*
CV112	2013	Miyara	*V. owensii*
CV113	2013	Miyara	*V. owensii*
CV114	2013	Miyara	*CV50*
CV115	2013	Miyara	*CV50*
CV116	2013	Miyara	*C46*
CV117	2013	Miyara	*C46*
CV118	2013	Miyara	*C71*
CV119	2013	Miyara	*C71*
CV120	2013	Miyara	*C58*
CV29	2013	Taketomi	*V. alginolyticus*
CV30	2013	Taketomi	*C6*
CV31	2013	Taketomi	*P. rosenbergii*
CV32	2013	Taketomi	*P. rosenbergii*
CV33	2013	Taketomi	*P. rosenbergii*
CV34	2013	Taketomi	*P. aphoticum*
CV35	2013	Taketomi	*C49*
CV36	2013	Taketomi	*C46*

Continue			
CV37	2013	Taketomi	*V. owensii*
CV38	2013	Taketomi	*CV38*
CV39	2013	Taketomi	*CV39*
CV40	2013	Taketomi	*V. owensii*
CV41	2013	Taketomi	*V. owensii*
CV42	2013	Taketomi	*V. owensii*
CV43	2013	Taketomi	*V. owensii*
CV44	2013	Taketomi	*P. rosenbergii*
CV45	2013	Taketomi	*V. owensii*
CV46	2013	Taketomi	*V. owensii*
CV47	2013	Taketomi	*P. rosenbergii*
CV48	2013	Taketomi	*V. harveyi*
CV49	2013	Taketomi	*V. owensii*
CV90	2013	Taketomi	*CV38*
CV91	2013	Taketomi	*CV39*
CV92	2013	Taketomi	*V. owensii*
CV93	2013	Taketomi	*V. owensii*
CV94	2013	Taketomi	*V. variabilis/maritimus*
CV95	2013	Taketomi	*V. owensii*
CV96	2013	Taketomi	*CV96*
CV97	2013	Taketomi	*CV97*
CV98	2013	Taketomi	*V. owensii*
CV99	2013	Taketomi	*V. owensii*
CV100	2013	Taketomi	*C16*
CV101	2013	Taketomi	*C6*
CV50	2013	Osaki	*CV50*
CV51	2013	Osaki	*CV50*
CV52	2013	Osaki	*C46*
CV53	2013	Osaki	*V. owensii*
CV54	2013	Osaki	*V. variabilis/maritimus*
CV55	2013	Osaki	*V. variabilis/maritimus*
CV56	2013	Osaki	*V. ponticus*
CV57	2013	Osaki	*V. variabilis/maritimus*
CV58	2013	Osaki	*CV58*
CV59	2013	Osaki	*V. variabilis/maritimus*
CV60	2013	Osaki	*C164*
CV61	2013	Osaki	*V. owensii*
CV62	2013	Osaki	*V. owensii*
CV63	2013	Osaki	*V. variabilis/maritimus*
CV64	2013	Osaki	*V. variabilis/maritimus*
CV65	2013	Osaki	*V. variabilis/maritimus*
CV66	2013	Osaki	*P. rosenbergii*
CV67	2013	Osaki	*C1*
CV68	2013	Osaki	*C6*
CV69	2013	Osaki	*C1*
CV70	2013	Osaki	*V. astrearenae*
CV71	2013	Osaki	*C1*
CV72	2013	Osaki	*V. variabilis/maritimus*
CV73	2013	Osaki	*V. variabilis/maritimus*

Continue			
CV74	2013	Osaki	*P. rosenbergii*
CV75	2013	Osaki	*C164*
CV76	2013	Osaki	*V. harveyi*
CV77	2013	Osaki	*C6*
CV78	2013	Osaki	*V. owensii*
CV79	2013	Osaki	*P. rosenbergii*
CV80	2013	Osaki	*V. owensii*
CV81	2013	Osaki	*C1*
CV82	2013	Osaki	*C1*
CV83	2013	Osaki	*V. variabilis/maritimus*
CV84	2013	Osaki	*V. variabilis/maritimus*
CV85	2013	Osaki	*C1*
CV86	2013	Osaki	*V. mediterranei*
CV87	2013	Osaki	*C1*
CV88	2013	Osaki	*CV38*
CV89	2013	Osaki	*V. owensii*
CV160	2013	Osaki	*C121*
CV161	2013	Osaki	*V. corallilyticus/neputunis*
CV162	2013	Osaki	*P. rosenbergii*
CV163	2013	Osaki	*V. campbellii*
CV164	2013	Osaki	*C49*
CV165	2013	Osaki	*V. owensii*
CV166	2013	Osaki	*C68*
CV167	2013	Osaki	*V. corallilyticus/neputunis*
CV168	2013	Osaki	*P. rosenbergii*
CV169	2013	Osaki	*C6*
CV170	2013	Osaki	*V. alginolyticus*
CV171	2013	Osaki	*C68*
CV172	2013	Osaki	*CV172*
CV173	2013	Osaki	*V. owensii*
CV174	2013	Osaki	*V. owensii*
CV175	2013	Osaki	*C46*
CV176	2013	Osaki	*C6*
CV177	2013	Osaki	*C68*
CV178	2013	Osaki	*C46*
CV179	2013	Osaki	*C46*
CV180	2013	Osaki	*C68*
CV181	2013	Osaki	*C68*
CV182	2013	Osaki	*V. owensii*
CV183	2013	Osaki	*C46*
CV184	2013	Osaki	*C46*
CV185	2013	Osaki	*V. owensii*
CV186	2013	Osaki	*V. owensii*
CV187	2013	Osaki	*V. owensii*
CV188	2013	Osaki	*V. owensii*
CV189	2013	Osaki	*V. owensii*
CV190	2013	Osaki	*C46*
CV191	2013	Osaki	*V. owensii*
CV192	2013	Osaki	*C46*

Continue			
CV193	2013	Osaki	*V. owensii*
CV194	2013	Osaki	*C6*
CV195	2013	Osaki	*C6*
CV196	2013	Osaki	*C68*
CV197	2013	Osaki	*C6*
CV198	2013	Osaki	*C68*
CV199	2013	Osaki	*V. owensii*
CV200	2013	Osaki	*C46*
CV201	2013	Osaki	*V. owensii*
CV202	2013	Osaki	*V. owensii*
CV203	2013	Osaki	*C6*
C4I1	2014	Miyara	*V. variabilis/maritimus*
C4I10	2014	Miyara	*C58*
C4I100	2014	Miyara	*C4I100*
C4I101	2014	Miyara	*V. owensii*
C4I106	2014	Miyara	*V. astrearenae*
C4I107	2014	Miyara	*V. owensii*
C4I108	2014	Miyara	*V. variabilis/maritimus*
C4I109	2014	Miyara	*C6*
C4I11	2014	Miyara	*V. astrearenae*
C4I112	2014	Miyara	*C6*
C4I113	2014	Miyara	*C6*
C4I114	2014	Miyara	*C68*
C4I115	2014	Miyara	*C68*
C4I116	2014	Miyara	*C68*
C4I122	2014	Miyara	*C6*
C4I123	2014	Miyara	*V. variabilis/maritimus*
C4I124	2014	Miyara	*C68*
C4I125	2014	Miyara	*C68*
C4I126	2014	Miyara	*V. corallilyticus/neputunis*
C4I127	2014	Miyara	*V. corallilyticus/neputunis*
C4I129	2014	Miyara	*V. variabilis/maritimus*
C4I13	2014	Miyara	*V. communis*
C4I130	2014	Miyara	*V. variabilis/maritimus*
C4I131	2014	Miyara	*V. variabilis/maritimus*
C4I132	2014	Miyara	*V. owensii*
C4I133	2014	Miyara	*C164*
C4I135	2014	Miyara	*V. owensii*
C4I136	2014	Miyara	*V. variabilis/maritimus*
C4I137	2014	Miyara	*P. rosenbergii*
C4I138	2014	Miyara	*C58*
C4I14	2014	Miyara	*V. communis*
C4I140	2014	Miyara	*C46*
C4I141	2014	Miyara	*V. owensii*
C4I142	2014	Miyara	*V. owensii*
C4I143	2014	Miyara	*C121*
C4I144	2014	Miyara	*V. campbellii*
C4I145	2014	Miyara	*V. campbellii*
C4I146	2014	Miyara	*V. corallilyticus/neputunis*

Continue			
C4I147	2014	Miyara	*V. owensii*
C4I148	2014	Miyara	*C121*
C4I149	2014	Miyara	*C6*
C4I15	2014	Miyara	*V. owensii*
C4I150	2014	Miyara	*V. campbellii*
C4I151	2014	Miyara	*V. owensii*
C4I152	2014	Miyara	*V. owensii*
C4I153	2014	Miyara	*V. owensii*
C4I154	2014	Miyara	*C121*
C4I155	2014	Miyara	*V. owensii*
C4I156	2014	Miyara	*V. owensii*
C4I157	2014	Miyara	*V. corallilyticus/neputunis*
C4I158	2014	Miyara	*V. corallilyticus/neputunis*
C4I159	2014	Miyara	*V. owensii*
C4I160	2014	Miyara	*V. owensii*
C4I161	2014	Miyara	*V. owensii*
C4I162	2014	Miyara	*V. corallilyticus/neputunis*
C4I163	2014	Miyara	*V. owensii*
C4I164	2014	Miyara	*V. owensii*
C4I165	2014	Miyara	*V. owensii*
C4I166	2014	Miyara	*V. owensii*
C4I167	2014	Miyara	*V. owensii*
C4I168	2014	Miyara	*V. owensii*
C4I169	2014	Miyara	*V. owensii*
C4I17	2014	Miyara	*V. owensii*
C4I170	2014	Miyara	*V. owensii*
C4I171	2014	Miyara	*V. owensii*
C4I172	2014	Miyara	*V. owensii*
C4I173	2014	Miyara	*V. owensii*
C4I174	2014	Miyara	*C4I174*
C4I175	2014	Miyara	*V. owensii*
C4I176	2014	Miyara	*C4I174*
C4I177	2014	Miyara	*C4I174*
C4I178	2014	Miyara	*V. owensii*
C4I179	2014	Miyara	*V. harveyi*
C4I180	2014	Miyara	*V. owensii*
C4I181	2014	Miyara	*V. tubiashii*
C4I182	2014	Miyara	*V. corallilyticus/neputunis*
C4I183	2014	Miyara	*V. owensii*
C4I184	2014	Miyara	*V. harveyi*
C4I185	2014	Miyara	*V. owensii*
C4I186	2014	Miyara	*V. owensii*
C4I19	2014	Miyara	*V. astrearenae*
C4I2	2014	Miyara	*V. tubiashii*
C4I20	2014	Miyara	*V. astrearenae*
C4I21	2014	Miyara	*C58*
C4I22	2014	Miyara	*C58*
C4I23	2014	Miyara	*C58*
C4I24	2014	Miyara	*C58*

Continue			
C4I25	2014	Miyara	*V. campbellii*
C4I26	2014	Miyara	*C49*
C4I27	2014	Miyara	*V. corallilyticus/neputunis*
C4I28	2014	Miyara	*V. astrearenae*
C4I29	2014	Miyara	*C6*
C4I3	2014	Miyara	*V. harveyi*
C4I30	2014	Miyara	*C49*
C4I31	2014	Miyara	*V. astrearenae*
C4I32	2014	Miyara	*V. owensii*
C4I33	2014	Miyara	*V. owensii*
C4I34	2014	Miyara	*V. owensii*
C4I35	2014	Miyara	*C58*
C4I36	2014	Miyara	*V. variabilis/maritimus*
C4I37	2014	Miyara	*V. corallilyticus/neputunis*
C4I38	2014	Miyara	*V. astrearenae*
C4I39	2014	Miyara	*V. owensii*
C4I4	2014	Miyara	*C68*
C4I40	2014	Miyara	*V. astrearenae*
C4I41	2014	Miyara	*V. owensii*
C4I42	2014	Miyara	*V. pelagius*
C4I45	2014	Miyara	*V. variabilis/maritimus*
C4I46	2014	Miyara	*V. variabilis/maritimus*
C4I47	2014	Miyara	*V. variabilis/maritimus*
C4I48	2014	Miyara	*V. variabilis/maritimus*
C4I49	2014	Miyara	*V. owensii*
C4I50	2014	Miyara	*V. tubiashii*
C4I51	2014	Miyara	*V. owensii*
C4I54	2014	Miyara	*V. harveyi*
C4I55	2014	Miyara	*V. campbellii*
C4I56	2014	Miyara	*V. campbellii*
C4I58	2014	Miyara	*V. variabilis/maritimus*
C4I59	2014	Miyara	*C6*
C4I6	2014	Miyara	*V. variabilis/maritimus*
C4I60	2014	Miyara	*C6*
C4I62	2014	Miyara	*C6*
C4I63	2014	Miyara	*C68*
C4I64	2014	Miyara	*C68*
C4I65	2014	Miyara	*C68*
C4I66	2014	Miyara	*C6*
C4I67	2014	Miyara	*V. variabilis/maritimus*
C4I68	2014	Miyara	*C68*
C4I69	2014	Miyara	*C68*
C4I7	2014	Miyara	*V. variabilis/maritimus*
C4I70	2014	Miyara	*V. corallilyticus/neputunis*
C4I71	2014	Miyara	*V. corallilyticus/neputunis*
C4I72	2014	Miyara	*V. variabilis/maritimus*
C4I73	2014	Miyara	*V. variabilis/maritimus*
C4I75	2014	Miyara	*V. variabilis/maritimus*
C4I76	2014	Miyara	*V. variabilis/maritimus*

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C4I77	2014	Miyara	*V. variabilis/maritimus*
C4I78	2014	Miyara	*C68*
C4I79	2014	Miyara	*C1*
C4I8	2014	Miyara	*V. communis*
C4I92	2014	Miyara	*C1*
C4I98	2014	Miyara	*C1*
C4I99	2014	Miyara	*V. ponticus*
C4II187	2014	Osaki	*V. owensii*
C4II188	2014	Osaki	*V. corallilyticus/neputunis*
C4II189	2014	Osaki	*C4II189*
C4II191	2014	Osaki	*V. campbellii*
C4II193	2014	Osaki	*V. campbellii*
C4II194	2014	Osaki	*V. corallilyticus/neputunis*
C4II196	2014	Osaki	*V. corallilyticus/neputunis*
C4II198	2014	Osaki	*C49*
C4II199	2014	Osaki	*V. corallilyticus/neputunis*
C4II200	2014	Osaki	*V. corallilyticus/neputunis*
C4II201	2014	Osaki	*V. campbellii*
C4II204	2014	Osaki	*V. campbellii*
C4II206	2014	Osaki	*V. campbellii*
C4II209	2014	Osaki	*V. campbellii*
C4II210	2014	Osaki	*V. variabilis/maritimus*
C4II211	2014	Osaki	*V. pelagius*
C4II212	2014	Osaki	*V. variabilis/maritimus*
C4II213	2014	Osaki	*C16*
C4II215	2014	Osaki	*V. variabilis/maritimus*
C4II216	2014	Osaki	*V. variabilis/maritimus*
C4II217	2014	Osaki	*C16*
C4II218	2014	Osaki	*C16*
C4II220	2014	Osaki	*V. campbellii*
C4II221	2014	Osaki	*V. campbellii*
C4II229	2014	Osaki	*V. azureus*
C4II230	2014	Osaki	*C46*
C4II231	2014	Osaki	*C46*
C4II232	2014	Osaki	*C46*
C4II233	2014	Osaki	*V. campbellii*
C4II234	2014	Osaki	*C46*
C4II235	2014	Osaki	*C46*
C4II236	2014	Osaki	*V. variabilis/maritimus*
C4II237	2014	Osaki	*V. variabilis/maritimus*
C4II238	2014	Osaki	*V. variabilis/maritimus*
C4II239	2014	Osaki	*C164*
C4II247	2014	Osaki	*C164*
C4II248	2014	Osaki	*C164*
C4II251	2014	Osaki	*C49*
C4II252	2014	Osaki	*C1*
C4II253	2014	Osaki	*C1*
C4II254	2014	Osaki	*V. campbellii*
C4II255	2014	Osaki	*V. owensii*

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C4II256	2014	Osaki	*C46*
C4II257	2014	Osaki	*C6*
C4II258	2014	Osaki	*V. corallilyticus/neputunis*
C4II259	2014	Osaki	*C4II259*
C4III260	2014	Taketomi	*V. owensii*
C4III263	2014	Taketomi	*C6*
C4III264	2014	Taketomi	*C71*
C4III265	2014	Taketomi	*C46*
C4III266	2014	Taketomi	*C71*
C4III267	2014	Taketomi	*C71*
C4III269	2014	Taketomi	*V. campbellii*
C4III271	2014	Taketomi	*C71*
C4III272	2014	Taketomi	*C71*
C4III273	2014	Taketomi	*C71*
C4III274	2014	Taketomi	*C71*
C4III275	2014	Taketomi	*V. corallilyticus/neputunis*
C4III276	2014	Taketomi	*V. owensii*
C4III278	2014	Taketomi	*V. owensii*
C4III280	2014	Taketomi	*V. corallilyticus/neputunis*
C4III281	2014	Taketomi	*V. corallilyticus/neputunis*
C4III282	2014	Taketomi	*C4III282*
C4III284	2014	Taketomi	*C58*
C4III285	2014	Taketomi	*C58*
C4III286	2014	Taketomi	*C58*
C4III287	2014	Taketomi	*V. owensii*
C4III288	2014	Taketomi	*C68*
C4III289	2014	Taketomi	*V. harveyi*
C4III290	2014	Taketomi	*V. harveyi*
C4III291	2014	Taketomi	*C4III282*
C4III292	2014	Taketomi	*V. harveyi*
C4III293	2014	Taketomi	*V. harveyi*
C4III294	2014	Taketomi	*V. corallilyticus/neputunis*
C4III295	2014	Taketomi	*V. harveyi*
C4III296	2014	Taketomi	*C1*
C4III297	2014	Taketomi	*V. astrearenae*
C4III298	2014	Taketomi	*V. astrearenae*
C4III299	2014	Taketomi	*V. astrearenae*
C4III300	2014	Taketomi	*V. astrearenae*
C4III301	2014	Taketomi	*V. owensii*
C4III304	2014	Taketomi	*V. astrearenae*
C4III305	2014	Taketomi	*C68*
C4III306	2014	Taketomi	*V. harveyi*
C4III307	2014	Taketomi	*V. harveyi*
C4III308	2014	Taketomi	*C4III282*
C4III309	2014	Taketomi	*V. harveyi*
C4III310	2014	Taketomi	*V. harveyi*
C4III311	2014	Taketomi	*V. corallilyticus/neputunis*
C4III314	2014	Taketomi	*V. harveyi*
C4III315	2014	Taketomi	*C1*

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C4III316	2014	Taketomi	*V. astrearenae*
C4III318	2014	Taketomi	*V. astrearenae*
C4III319	2014	Taketomi	*V. astrearenae*
C4III320	2014	Taketomi	*V. astrearenae*
C4IV323	2014	Sekisei	*C1*
C4IV324	2014	Sekisei	*V. astrearenae*
C4IV327	2014	Sekisei	*V. campbellii*
C4IV326	2014	Sekisei	*V. campbellii*
C4IV325	2014	Sekisei	*V. campbellii*
C4IV329	2014	Sekisei	*V. corallilyticus/neputunis*
C4IV330	2014	Sekisei	*V. corallilyticus/neputunis*
C4IV331	2014	Sekisei	*V. corallilyticus/neputunis*
C4IV332	2014	Sekisei	*V. corallilyticus/neputunis*
C4IV333	2014	Sekisei	*V. corallilyticus/neputunis*
C4IV334	2014	Sekisei	*V. pelagius*
C4IV335	2014	Sekisei	*V. pelagius*
C4IV336	2014	Sekisei	*V. corallilyticus/neputunis*
C4IV337	2014	Sekisei	*V. corallilyticus/neputunis*
C4IV338	2014	Sekisei	*V. corallilyticus/neputunis*
C4IV339	2014	Sekisei	*V. harveyi*
C4IV340	2014	Sekisei	*V. harveyi*
C4IV343	2014	Sekisei	*V. campbellii*
C4IV346	2014	Sekisei	*V. harveyi*
C4IV347	2014	Sekisei	*V. communis*
C4IV348	2014	Sekisei	*V. communis*
C4IV350	2014	Sekisei	*V. variabilis/maritimus*
C4IV351	2014	Sekisei	*V. owensii*
C4IV352	2014	Sekisei	*V. campbellii*
C4IV353	2014	Sekisei	*V. campbellii*
C4V355	2014	Iriomote	*C68*
C4V357	2014	Iriomote	*V. rotiferianus*
C4V358	2014	Iriomote	*C4V358*
C4V359	2014	Iriomote	*C4I174*
C4V360	2014	Iriomote	*V. variabilis/maritimus*
C4V361	2014	Iriomote	*C1*
C4V365	2014	Iriomote	*V. owensii*
C4V368	2014	Iriomote	*V. campbellii*
C4V371	2014	Iriomote	*V. campbellii*
C4V372	2014	Iriomote	*V. campbellii*
C4V374	2014	Iriomote	*V. campbellii*
C4V375	2014	Iriomote	*V. harveyi*
C4V377	2014	Iriomote	*V. pelagius*
C4V379	2014	Iriomote	*C46*
C4V380	2014	Iriomote	*V. campbellii*
C4V382	2014	Iriomote	*V. campbellii*
C4V383	2014	Iriomote	*C4II259*
C4V384	2014	Iriomote	*C46*
C4V385	2014	Iriomote	*C71*