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Microdiversity and evidence for high dispersal rates in the marine actinomycete '*Salinispora pacifica*'

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Summary

In July of 2006 and January of 2008, a total of 671 marine sediment samples were collected at depths from 5 to 2012 m throughout the Fijian islands and selectively processed for the cultivation of marine actinomycetes belonging to the genus *Salinispora*. The primary objectives were to assess the diversity, distribution and phylogeny of '*S. pacifica*', the least well studied of the three species in the genus. Employing a sequential screening method based on antibiotic sensitivity, RFLP patterns, and 16S rRNA and ITS sequence analyses, 42 of 750 isolates with *Salinispora*-like features were identified as '*S. pacifica*'. These strains represent the first report of '*S. pacifica*' from Fiji and include 15 representatives of 4 new '*S. pacifica*' 16S rRNA sequence types. Among the '*S. pacifica*' strains isolated, little evidence for geographical isolation emerged based on 16S, ITS or secondary metabolite biosynthetic gene fingerprinting. The inclusion of isolates from additional collection sites and other *Salinispora* spp. revealed a high degree of dispersal among '*S. pacifica*' populations and phylogenetic support for the delineation of this lineage as a third species.

Introduction

The biogeographical distributions of closely related microbial populations remain an important topic in microbial ecology. Recent studies indicate that the geographical distributions of marine planktonic diatoms do not appear to be limited by dispersal (Cermeno and Falkowski, 2009), that a stable supply of spore forming thermophilic bacteria is continually introduced into Arctic marine sediments (Hubert *et al*., 2009), and that dispersal limitation does not appear to be a dominant force structuring arctic microbial communities (Chu *et al*., 2010). Evidence also exists for sufficiently high cross-continent migration rates in *Bacillus* species to prevent the accumulation of neutral mutations due to geographical isolation (Roberts and Cohan, 1995). In contrast, numerous examples of geographically isolated populations have been documented in extremophiles (Cho and Tiedje, 2000; Papke *et al*., 2003; Whitaker *et al*., 2003). Further evidence of geographical isolation comes from a recent study in which it was reported that dispersal limitation led to the establishment of endemic, microdiverse populations of *Prochlorococcus* (Martiny *et al*., 2009). It has also recently been shown that most bacteria demonstrate a limited distribution within specific habitat types, with abundant bacteria being largely confined to individual samples (Nemergut *et al*., 2011). Thus, as might be expected, there is growing evidence that biogeographical patterning varies among taxa and is closely linked to the ecology and physiology of the organisms being studied.

Genes involved in secondary metabolism have been used to assess biogeographical patterns among bacteria (Wawrik *et al*., 2007). These genes are responsible for the biosynthesis of small molecules that mediate important functional traits such as allelopathy, chemical communication and iron acquisition. Type I polyketide synthase (PKSI) genes are responsible for the production of many important secondary metabolites including the antibiotic erythromycin (Cortes *et al*., 1990) and the anticancer agent epothilone (Cortes *et al*., 1990; Bollag *et al*., 1995). Bacteria can maintain complex assemblies of PKS genes (Fischbach and Walsh, 2006), many of which are not expressed under normal laboratory conditions (Bentley *et al*., 2002). Recently, it was shown that HGT plays an important role in the evolution of PKSI genes (Ginolhac *et al*., 2005) and that ketosynthase (KS) domains within polyketide synthase genes are phylogenetically informative in terms of making predictions about the structures of the secondary metabolites produced by complex biosynthetic pathways (Ginolhac *et al*., 2005; Gontang *et al*., 2010). Additionally, terminal restriction fragment length polymorphism (T-RFLP) has been used to demonstrate that subpopulations of bacteria cluster together based on collection site (Edlund *et al*., 2011) providing further evidence for endemism associated with secondary metabolism (Wawrik *et al*., 2007).

Salinispora is an obligate marine genus within the order Actinomycetales. It displays a pan-tropical distribution in

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near-shore marine sediments (Mincer *et al*., 2002). To date, three species have been recognized, of which *S. arenicola* and *S. tropica* have been formally described (Maldonado *et al*., 2005) while '*S. pacifica*' has been proposed (Jensen and Mafnas, 2006). The distributions of the three species vary, with *S. arenicola* being reported from the broadest geographical range while *S. tropica* has only been reported from the Caribbean. '*S. pacifica*' has been isolated from multiple locations with the exception of the Caribbean (Jensen and Mafnas, 2006), and thus there is currently no culture-based or culture-independent (Mincer *et al*., 2005) evidence for the co-occurrence of *S. tropica* and '*S. pacifica*'. This is in contrast to *S. arenicola*, which co-occurs with both species. Interestingly, secondary metabolism represents one of the major phenotypic differences observed among *Salinispora* species (Jensen *et al*., 2007). This observation was supported in a comparative genomic analysis, which revealed that secondary metabolism is the largest class of functionally annotated, species-specific genes (Penn *et al*., 2009). These results were used to suggest that secondary metabolism plays a role in ecological diversification among *Salinispora* species.

This study was undertaken to examine the diversity, distribution and phylogeny of '*S. pacifica*', the least well studied of the three *Salinispora* species. A variety of phylogenetic markers were used to examine the coherence of this lineage and to test for biogeographical patterns over a range of geographical scales. The results reveal new diversity within '*S. pacifica*' and evidence that dispersal rates are greater than the rates of sequence divergence, resulting in populations that are broadly distributed over large geographical ranges.

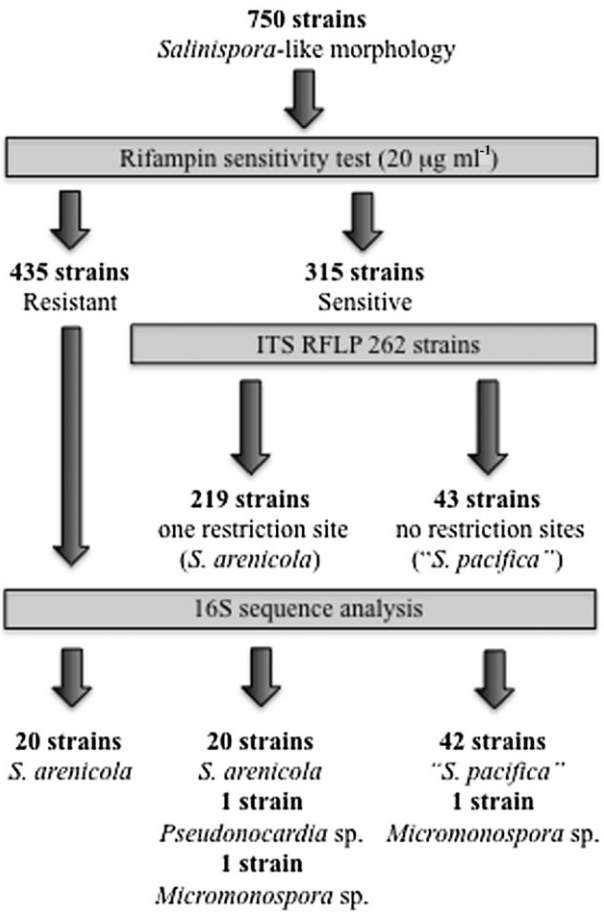

Fig. 1. Work flow. Strains with *Salinispora*-like morphologies were tested for sensitivity to rifampin and sensitive strains further screened by RFLP to distinguish *S. arenicola* from other *Salinispora* species. 16S rRNA gene sequence analyses were used to test the accuracy of this approach and to confirm the identity of a subset of the strains.

Results

Actinomycete isolation and antibiotic sensitivity testing

A total of 671 marine sediment samples collected throughout the Fijian islands were processed for the selective cultivation of actinomycetes related to the genus *Salinispora*. These samples yielded 750 isolates with *Salinispora*-like morphological features including orange pigmentation and the production of spores that blackened the colony surface. Preliminary antibiotic sensitivity tests revealed that *S. arenicola*, which is known to produce compounds in the rifamycin class (Kim *et al*., 2006; Jensen *et al*., 2007), grew well on media containing 20 μg ml^{-1} rifampin while the growth of '*S. pacifica*' and *S. tropica*, which are not known to produce compounds in this class, was inhibited (data not shown). As a rapid method to distinguish *S. arenicola* from other *Salinispora* species, the 750 candidate *Salinispora* isolates were tested for sensitivity to rifampin (Fig. 1). In total, 315 strains were sensitive to this antibiotic, suggesting they may belong to species other than *S. arenicola*. These strains consisted of 75 for which growth was completely inhibited and 240 for which growth was visibly reduced resulting in a total of 315 sensitive strains. Growth of the remaining 435 strains was unaffected by rifampin suggesting they are *S. arenicola*.

ITS RFLP and sequence analyses

Partial 16S rRNA sequences were obtained for 20 of the rifampin resistant strains and all were confirmed as previously identified *S. arenicola* sequence types (Fig. 1). To facilitate the identification of the large number of rifampin-sensitive isolates, 262 of the 315 strains were subjected to an ITS RFLP screen using the endonuclease BanI (Fig. 1). This enzyme has one restriction site in the *S. arenicola* ITS region but none in '*S. pacifica*' or

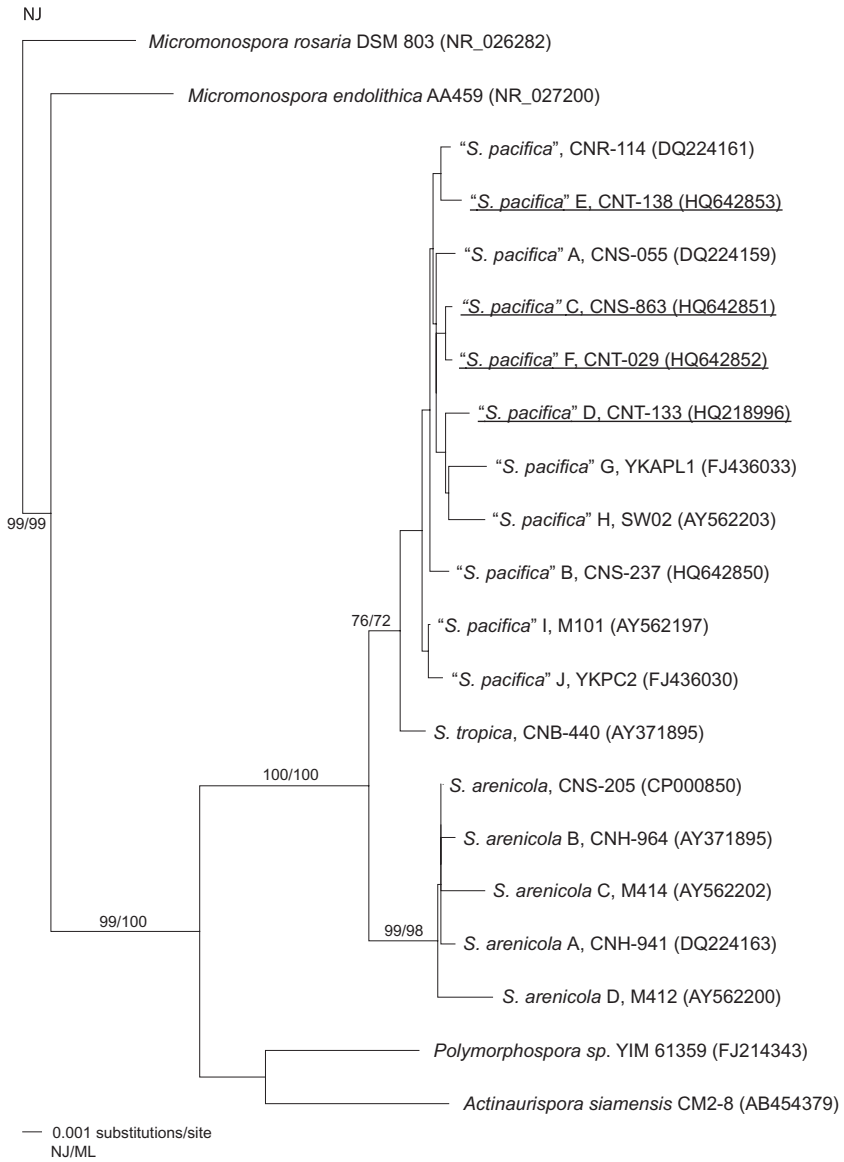

Fig. 2. Neighbour-joining phylogenetic tree created from nearly complete 16S rRNA gene sequences (1316 bp) of all *Salinispora* sequence types identified to date. Species names are followed by sequence type (letter, except in the case of the first sequence type discovered for each species which was not assigned a letter), strain name and accession number (in parentheses). New '*S. pacifica*' sequence types identified in this study (C–F) are underlined. Bootstrap values above 50%, calculated from 1000 resamplings, are shown for NJ and ML methods on the respective nodes.

S. tropica (Fig. S1). Following digestion, 219 of the PCR-amplified ITS products generated banding patterns consistent with *S. arenicola* while the remaining 43 were not digested suggesting the strains were *S. tropica*, '*S. pacifica*' or potentially new *Salinispora* spp. Partial 16S rRNA sequences of these 43 strains identified 42 as '*S. pacifica*' while one proved to be a *Micromonospora* sp. (Fig. 1). Of the 219 strains that displayed one ITS BanI restriction site, 22 were sequenced and 20 of these were identified as *S. arenicola*, one as a *Pseudonocardia* sp., and one as a *Micromonospora* sp. Thus the ITS RFLP proved effective at distinguishing between *S. arenicola and 'S. pacifica*' among the rifampin sensitive strains.

Although no new *Salinispora* species-level diversity was cultured based on partial 16S sequencing, a total of 42 strains from Fiji were identified as '*S. pacifica*'. These strains include seven different 16S rRNA gene sequence types of which four had not previously been observed (Fig. 2). In addition, seven strains from Hawaii were identified as previously reported '*S. pacifica*' sequence types. *Salinispora arenicola* strains were also recovered from the Hawaiian samples. These represent the first reports of *Salinispora* spp. from Hawaii.

A careful analysis of sequence data deposited in GenBank was performed to compile other reports of this genus. All *Salinispora* sequence types were evaluated to confirm that nucleotide changes occurred in sites that are conserved in $\leq 90\%$ of all bacteria (Cannone *et al*., 2002). Sequences that did not meet this criterion and were reported only once were excluded from the analysis to help eliminate PCR and sequencing errors (Table 1). GenBank deposits include the first reports of *S. arenicola*

Table 1. *Salinispora* spp. variable nucleotide positions.

Species	Sequence type	Strain number	Accession number	Base pair position relative to *E. coli* J01695 (% conservation of position)											
				139 (<80%)	145 (<80%)	179 (<80%)	184 (<80%)	187 (<80%)	188 (<80%)	189 (<80%)	193 (<80%)	201 (<80%)	204 (<80%)	215 (<80%)	221* (<80%)
'*S. pacifica*'	~	CNR-114	(DQ224161)	T	T	G	G	T	G	G	C	T	T	A	C
'*S. pacifica*'	A	CNS-055	(DQ224159)	C	T	G	T	T	G	G	C	T	T	A	C
'*S. pacifica*'	B	CNS-237	(HQ642850)	C	T	G	G	T	G	G	C	T	T	A	C
'*S. pacifica*'	C	CNS-863	(HQ642851)	C	T	G	G	T	G	G	C	T	T	A	C
'*S. pacifica*'	D	CNT-133	(HQ218996)	C	T	G	G	T	G	G	C	T	T	A	C
'*S. pacifica*'	E	CNT-138	(HQ642853)	T	T	A	G	T	G	G	C	T	T	A	C
'*S. pacifica*'	F	CNT-029	(HQ642852)	C	T	G	G	T	G	G	C	T	T	A	C
'*S. pacifica*'	G	YKAPL1	(FJ436033)	C	T	A	G	T	G	G	C	C	T	(–)	C
'*S. pacifica*'	H	SW02	(AY562203)	C	T	G	G	T	G	G	C	T	T	A	C
'*S. pacifica*'	I	M101	(AY562197)	C	T	G	G	T	G	G	C	T	T	A	C
'*S. pacifica*'	J	YKPC2	(FJ436030)	C	T	G	G	C	G	G	C	T	T	(–)	C
S. arenicola	~	CNS-205	(AY371897)	C	T	G	G	C	A	T	T	T	G	A	T
S. arenicola	A	CNH-941	(DQ224163)	C	T	G	G	C	A	T	T	T	G	A	T
S. arenicola	B	CNH-964	(AY040623)	C	T	G	G	C	A	T	T	T	G	A	T
S. arenicola	C	M414	(AY562202)	C	T	G	G	C	A	T	T	T	G	A	T
S. arenicola	D	M412	(AY562200)	T	C	G	G	C	A	T	T	T	G	A	T
S. tropica	~	CNB-440	(AY040617)	C	T	G	G	T	G	G	C	T	T	A	T

234* (80–90%)	264* (90–98%)	373 (<80%)	549 (<80%)	616 (<80%)	628 (<80%)	640 (<80%)	653 (<80%)	842 (<80%)	843 (<80%)	1018 (<80%)	1260 (<80%)	1276 (<80%)	1311 (<80%)	Reference	Source
A	A	A	G	C	G	T	G	G	G	T	A	T	T	1	Sediment
A	A	A	G	C	G	T	G	G	G	T	A	T	T	1	Sediment
A	A	A	A	C	G	T	G	G	A	T	A	T	T	4	Sediment
A	A	A	G	C	T	T	G	G	G	T	A	T	T	This study	Sediment
A	A	A	G	C	G	T	G	G	G	C	A	C	T	This study	Sediment
A	A	A	A	C	G	T	G	G	G	T	A	T	T	This study	Sediment
A	A	A	A	C	T	T	G	G	G	T	A	T	T	This study	Sediment
A	A	A	A	C	G	T	G	A	G	C	A	T	T	GenBank	Sponge
A	A	A	G	C	T	T	G	A	G	C	A	T	T	2	Sponge
A	A	A	A	C	G	T	G	A	G	T	A	T	T	2	Sponge
A	A	A	A	C	G	T	G	A	G	T	A	T	T	GenBank	Sponge
G	G	A	A	C	G	T	G	G	G	T	G	T	T	This study	Sediment
G	G	A	A	C	G	G	G	G	G	T	G	T	T	1	Sediment
G	G	A	A	T	G	T	G	G	G	T	G	T	T	3	Sediment
G	G	G	A	C	G	T	A	G	G	T	G	T	T	2	Sponge
G	G	A	A	C	G	T	G	G	G	T	G	T	C	2	Sponge
G	G	A	A	C	G	T	G	A	G	T	A	T	T	3	Sediment

Underlined positions are variable in '*S. pacifica*'. The asterisk denotes '*S. pacifica*' specific nucleotides. ~: original sequence type (not assigned a letter); (–): gap. References: 1, Jensen and Mafnas (2006); 2, Kim *et al.* (2005); 3, Mincer *et al.* (2002); 4, Jensen *et al.* (2007).

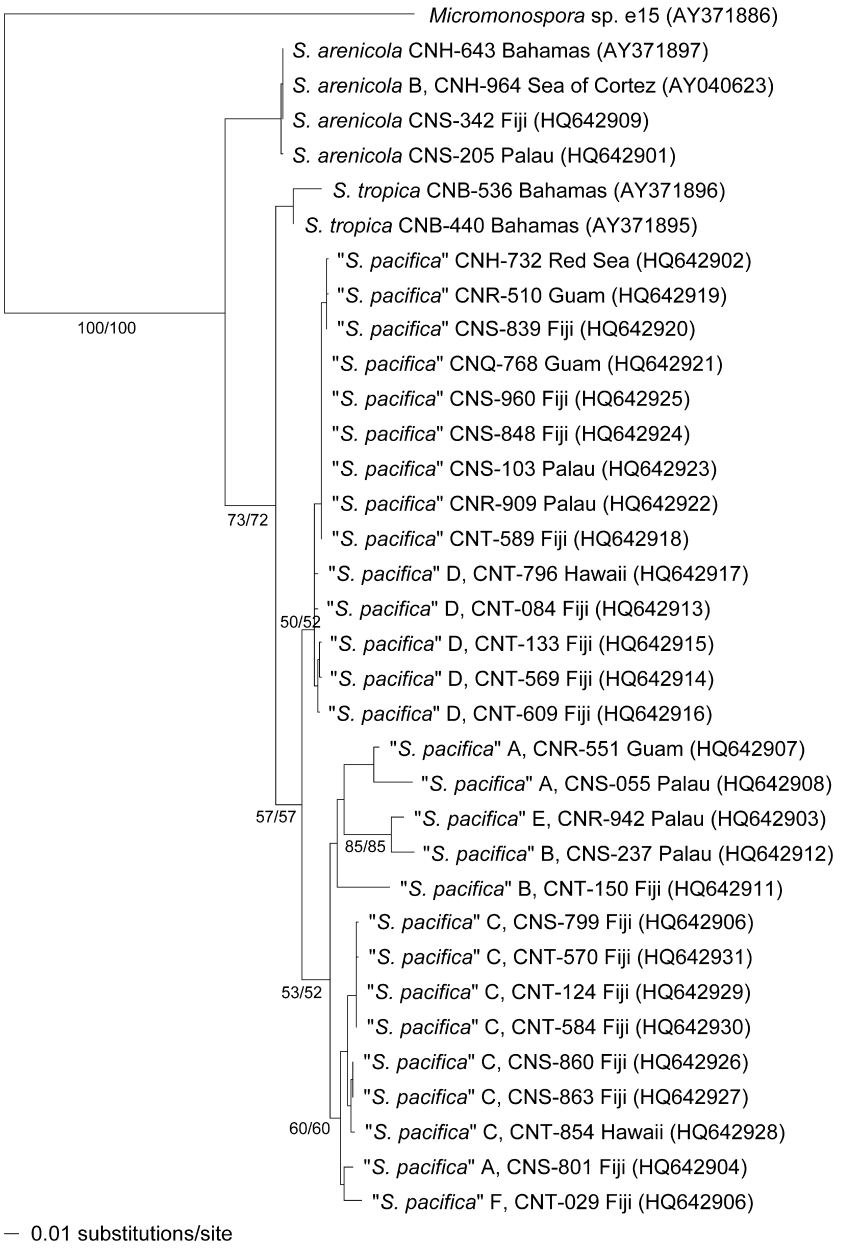

Fig. 3. Neighbour-joining phylogenetic tree generated from 16S-23S ITS sequences (291 bp). Species names are followed by 16S rRNA gene sequence type (letter, except in the case of the first sequence type discovered for each species which was not assigned a letter), strain name, location and accession number (in parentheses). Bootstrap values above 50%, calculated from 1000 resamplings, are shown for NJ and ML methods on the respective nodes.

from the South China Sea (FJ232412) and off the Pacific coast of Costa Rica (Solano *et al*., 2009). Interestingly, marine sponges collected from the Great Barrier Reef, Australia were the only source of *S. arenicola* sequence types C and D (Kim *et al*., 2005), which raises the total number of sequence types for this species to five (Table 1). Marine sponges were also the only source of four additional '*S. pacifica*' sequence types (G–J). This raises the total number of '*S. pacifica*' sequence types to 11, which is in stark contrast to one and five reported to date for *S. tropica* and *S. arenicola* respectively.

The 16S rRNA tree provides clear resolution of the genus *Salinispora* and the separation of *S. arenicola* from the other two species but only weak support for the delineation of *S. tropica* and '*S. pacifica*' (Fig. 2). The 11 '*S. pacifica*' sequence types (original, A–J) are clearly visualized in this tree; however, their relationship to each other is unresolved. A phylogenetic analysis of *Salinispora* spp. ITS sequences reveals the same species-level relationships with improved yet still inconclusive support for the delineation of *S. tropica* and '*S. pacifica*' (Fig. 3). For this reason, sequence data were also acquired for *recA*, a housekeeping gene widely used in phylogenetic studies. These sequences again show the same species-level relationships and, when concatenated with the 16S data, yielded a highly supported tree that clearly supports *S. tropica* and

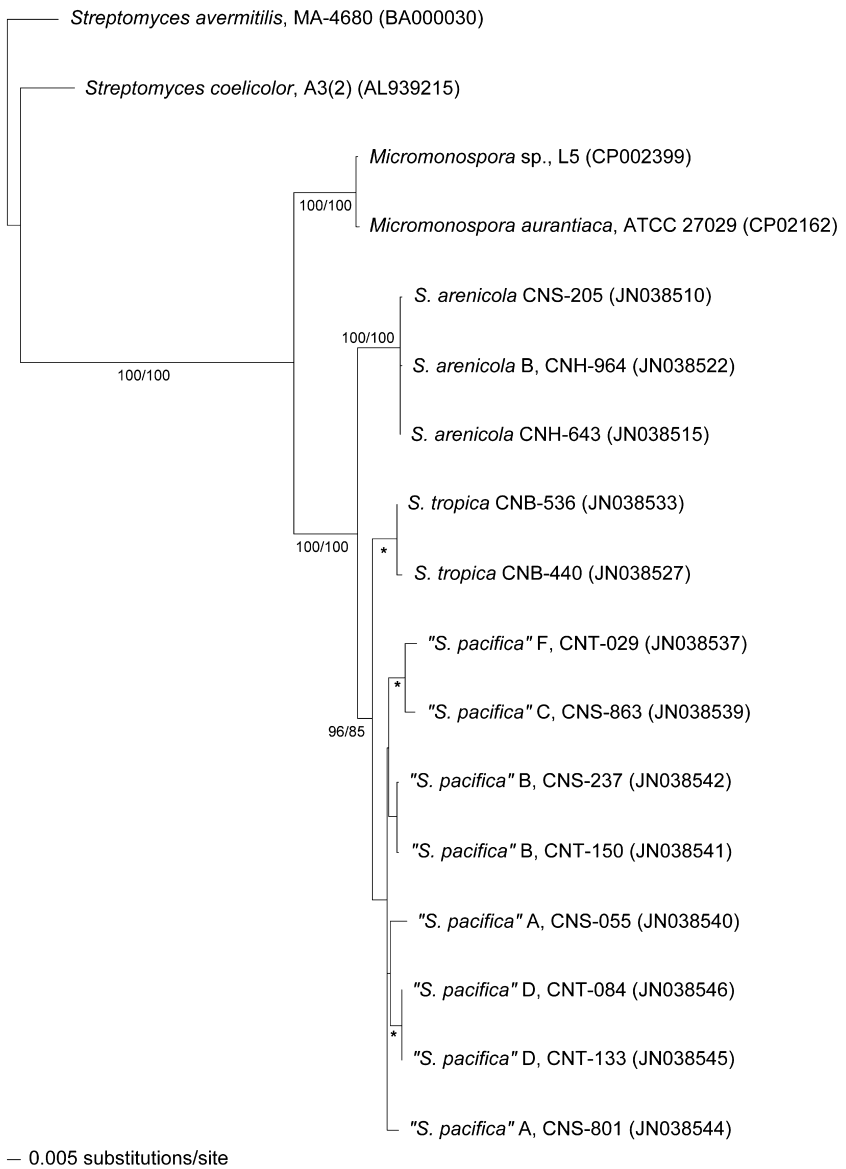

Fig. 4. Neighbour-joining phylogenetic tree generated from concatenated *recA* and 16S nucleotide sequences (1220 bp total). Strains include five of the seven '*S. pacifica*' sequence types isolated in this study. Species names are followed by 16S rRNA gene sequence type (letter, except in the case of the first sequence type discovered for each species which was not assigned a letter), strain name and accession number (in parentheses). Bootstrap values ≥ 85%, calculated from 1000 resamplings, are shown for NJ and ML methods on the respective nodes. The asterisk indicates additional nodes with ≥ 99% bootstrap support.

'*S. pacifica*' as distinct clades and sister taxa that share a common ancestor with *S. arenicola* (Fig. 4).

Interestingly, 13 of the strains that demonstrated reduced growth on media prepared with rifampin were identified as *S. arenicola*. This was surprising considering that *S. arenicola* is known to produce compounds in the related rifamycin class and thus would be expected to maintain resistance to this antibiotic. To determine if the rifampin-sensitive strains possessed the genetic potential to produce this class of compounds despite being sensitive to them, seven strains were probed for the *rifK* gene. This gene is involved in the production of the amino hydroxy-benzoic acid starter unit in rifamycin biosynthesis (Floss and Yu, 2005). Sequence verified PCR products confirming the presence of the *rifK* gene were obtained from all of these rifampin-sensitive *S. arenicola* strains, suggesting they possess the pathway associated with rifamycin production. In general, normal growth or complete growth inhibition on rifampin proved effective predictors of *S. arenicola* and '*S. pacifica*' respectively, among strains that possessed *Salinispora*-like morphologies. However, strains that exhibited partial growth inhibition at the test concentration could not be effectively identified using this approach.

Biogeographical analyses

A 16S rRNA allelic distribution map (Fig. 5) reveals that the original '*S. pacifica*' sequence type has been reported from all locations from which this species has been cultured. While some '*S. pacifica*' sequence types are broadly distributed, it cannot be determined if the limited

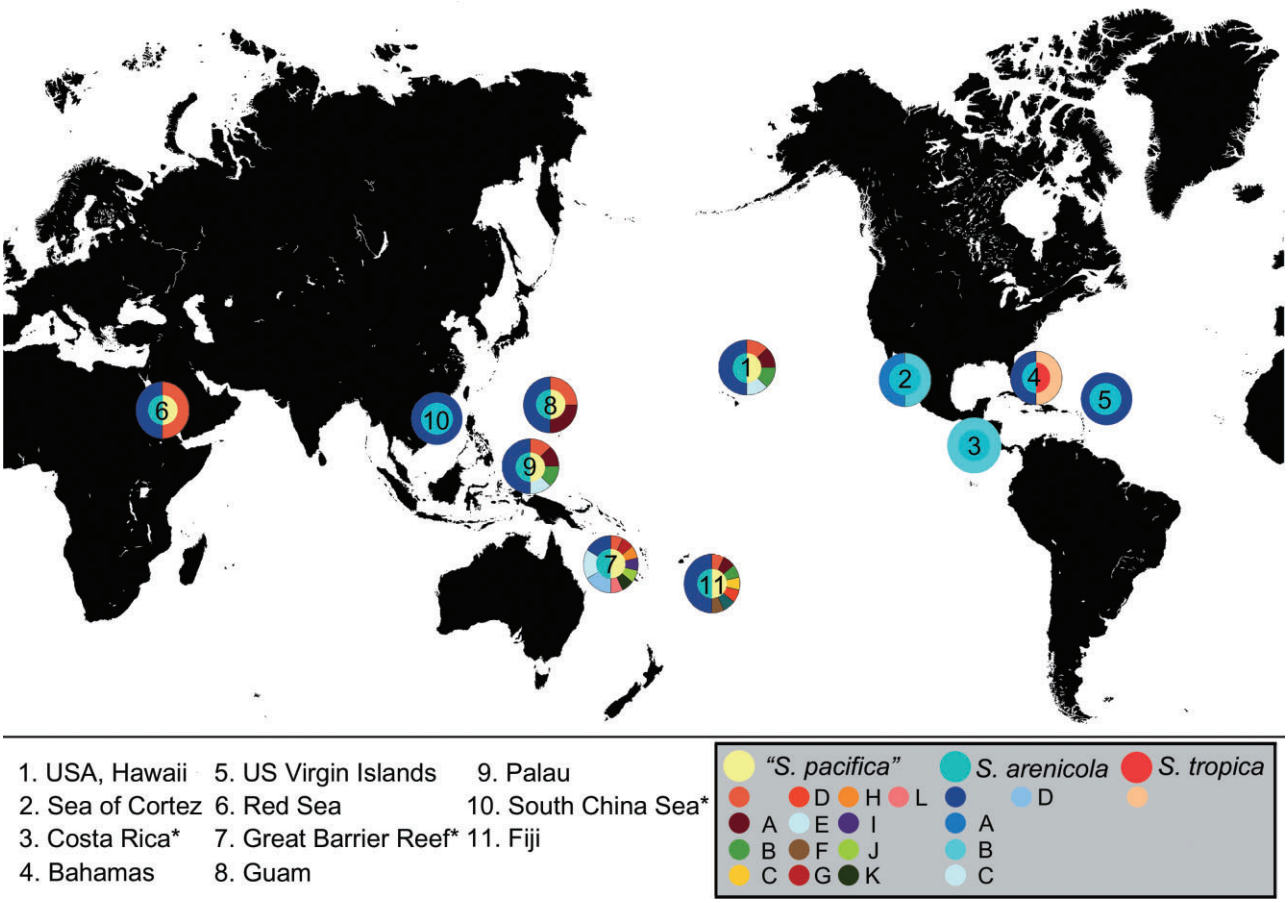

Fig. 5. Allelic distribution map of *Salinispora* spp. 16S rRNA gene sequence types (the first sequence type discovered for each species was not assigned a letter). Numbers indicate collection site, inner circle colours indicate which species where recovered, and outer circle colours indicate 16S alleles (sequence types). Asterisks indicate sequences obtained from GenBank.

distribution of others is an artefact of culturing effort. In the case of *S. arenicola*, the original 16S rRNA sequence type has been reported from all locations except the eastern Pacific where sequence types A and B are observed. Independent studies have confirmed the isolation of *S. arenicola* sequence types A and B from the Sea of Cortez (Jensen and Mafnas, 2006) and off the Pacific coast of Costa Rica (Solano *et al.*, 2009) with no evidence for the occurrence of the original sequence type at these locations. The failure to identify *S. tropica* among the strains cultured from Fiji or among any GenBank deposits outside of the Caribbean adds future support for the relatively limited distribution reported for this species (Jensen and Mafnas, 2006).

The more highly resolved ITS tree includes 45 '*S. pacifica*' strains derived from diverse locations including Fiji, Hawaii, the Red Sea, Guam and Palau. The detection of '*S. pacifica*' strains that are clonal at this locus (Fig. 3) despite being isolated from locations as distant as Guam (CNQ-768) and Fiji (CNS-960) provides evidence that dispersal rates can exceed the rates of ITS sequence evolution. Although some evidence of endemism was observed (e.g. sequence type F in Fiji), this may be an artefact of relative culturing effort and should be interpreted with caution (Fig. 3). To test for a more formal relationship between geographical origin and genetic divergence, isolation by distance (IBD) was performed on ITS sequences from representatives of each ITS sequence type observed at each of the three main sampling locations in Fiji. This analysis revealed that across a distance of 250 km the within-population genetic diversity was equal to that among populations ($F_{st} < 0.05$ between all populations). To test for patterns on a larger geographical scale, '*S. pacifica*' strains from all six global collection sites were analysed. Even across areas larger than 8000 km, no significant correlation was found between geographical and genetic distances ($P > 0.05$). A final IBD analysis included strains representing all sequence types, locations and *Salinispora* species. The results of a Mantel test revealed a weak visual but statistically insignificant relationship ($r = 0.35$, $P = 0.84$) between genetic similarity and geographical distance. The program RAMI, which uses

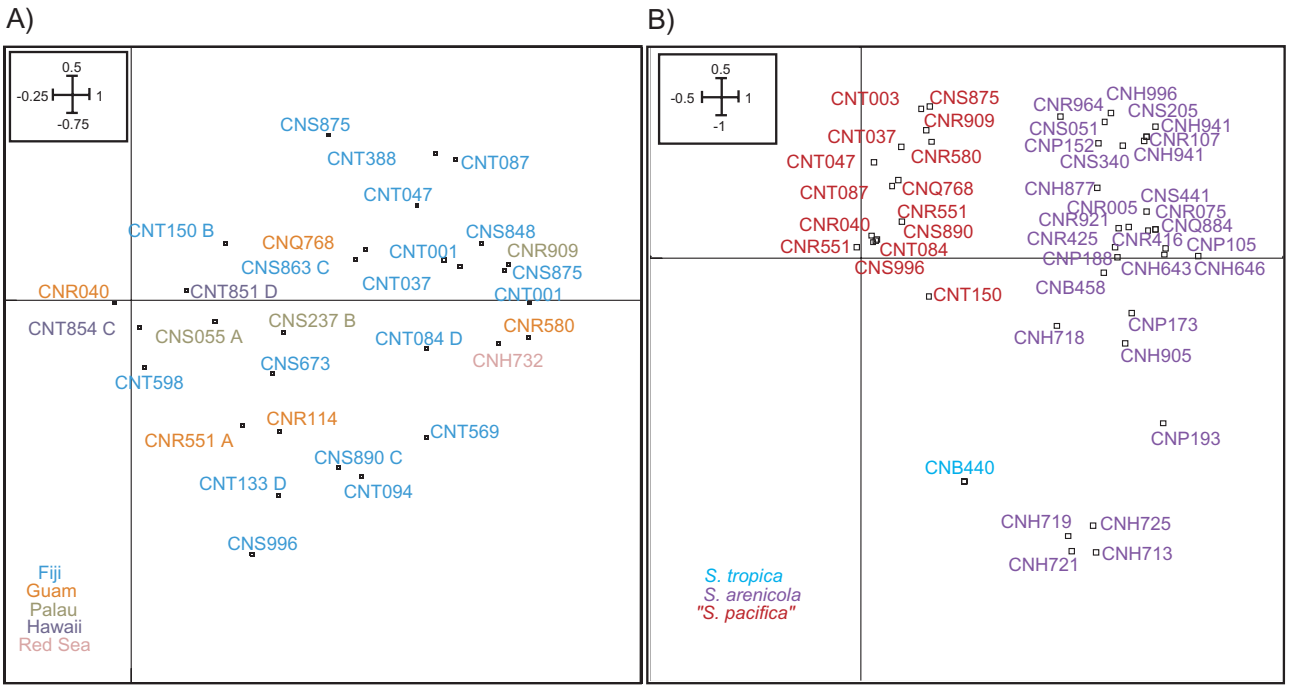

Fig. 6. Principal component analysis of ketosynthase (KS) T-RFLP data.
A. '*S. pacifica*' isolates representing different 16S sequence types derived from diverse locations. Locations are colour-coded while 16S sequence types follow strain name. Eigenvalues for the first and second ordination axes were 23.6% and 5.8%.
B. *Salinispora* strains representing all three species (colour-coded) showing clear taxonomy-based clustering. Eigenvalues for the first and second ordination axes were 23.4% and 8.5%.

patristic distances to analyse closely related clusters of sequences, was also used to analyse the data (Pommier *et al.*, 2009). When the analysis was run using the ITS, 16S and concatenated data sets with a patristic distance set at 0.01, no significant correlations between sequence clustering and geographical location were observed. Overall, these results indicate that *Salinispora* dispersal rates are generally greater than the rates of ITS evolution.

To determine if the secondary metabolite biosynthetic genes maintained by individual '*S. pacifica*' strains differed depending upon the location from which they were recovered, as has been observed for *S. arenicola* (Edlund *et al.*, 2011) and other actinomycetes (Wawrik *et al.*, 2007), KS domains from PKS genes were PCR-amplified and subjected to T-RFLP analysis. The results for 30 '*S. pacifica*' strains derived from five geographically distant locations were subjected to a principal component analysis (PCA), which revealed no visual evidence of clustering based on geographical origin or 16S sequence type (Fig. 6A). Eigenvalues of 23.6% and 5.8% for the first and second ordination axes respectively captured only 29.4% of the variability in the data set further supporting a lack of clustering among the '*S. pacifica*' strains analysed. A similar analysis using data from 46 strains representing all three *Salinispora* species (Fig. 6B) revealed visual evidence of species-specific clustering; however, the eigenvalues of 23.4% and 8.5% for the first and second ordination axes, respectively, also captured only a small fraction of the variability in the data set. However, when comparing '*S. pacifica*' and *S. arenicola*, the PCA coordinates were significantly different along the first ordination axis (*t*-test, $P < 0.001$) (Fig. 6B), supporting the concept that secondary metabolism is a functional trait that differentiates *Salinispora* species (Penn *et al.*, 2009). Although only one *S. tropica* strain was analysed, it appears well separated from '*S. pacifica*' providing preliminary evidence that these two species maintain distinct sets of biosynthetic genes.

Discussion

The biogeographical distributions of bacteria remain largely unknown. In some cases, the majority of taxa appear restricted to individual samples (Nemergut *et al.*, 2011) or their distributions are limited by dispersal (Whitaker *et al.*, 2003; Martiny *et al.*, 2009). However, there is a growing body of literature in which dispersal limitation does not appear to be a dominant force structuring microbial communities (Hubert *et al.*, 2009; van Gremberghe *et al.*, 2011). Resolving bacterial biogeographical distributions is complicated by uncertainties over how to assign species-level units of diversity. In the absence of a unified,

theory-based species concept, bacterial species are generally recognized at the molecular phylogenetic level as clusters of related gene sequences (Papke *et al*., 2011). It has been proposed that sequence clusters can be used to resolve ecologically distinct populations (Cohan, 1996), and this concept has been successfully tested in a limited number of cases (Ferris *et al*., 2003; Johnson *et al*., 2006; Koeppel *et al*., 2008). However, linking fine-scale phylogeny to sets of functional traits that define bacterial ecotypes remains a fundamental challenge in microbial ecology (Hunt *et al*., 2008). Although it is widely recognized that the high degree of conservation of the 16S rRNA gene provides poor species-level resolution (Konstantinidis and Tiedje, 2007), this marker provided preliminary evidence for the delineation of '*S. pacifica*' as a third *Salinispora* species. Results from the present study provide new support for the delineation of *S. tropica* and '*S. pacifica*' as sister taxa, additional evidence for the geographical isolation of these two species, and little evidence for the role of geographical isolation in the generation of the diversity observed within '*S. pacifica*'.

Although no new species-level *Salinispora* diversity was cultured, four new 16S rRNA sequence types were identified within '*S. pacifica*'. When considering data deposited in GenBank, this raises the total for this species to 11 sequence types relative to five for *S. arenicola* and one for *S. tropica* (Fig. 2). The comparatively high level of intraspecific diversity in '*S. pacifica*' is surprising given that both the 16S rRNA, ITS and concatenated 16S/*recA* phylogenies support the divergence of *S. arenicola* prior to the speciation of *S. tropica* and '*S. pacifica*' (Figs 2–4). The highly diversified '*S. pacifica*' clade could be explained by a number of evolutionary events including niche expansion, geographical isolation or the corresponding loss of diversity in *S. arenicola* and *S. tropica* due to periodic selection. However, neither F_{st} nor IBD revealed evidence for allopatry in '*S. pacifica*' suggesting that geographical isolation is not linked to the diversity observed. It remains possible, however, that the dissimilar levels of diversity observed in *S. arenicola* and '*S. pacifica*' is an artefact of culturing effort. This possibility is not supported by the results of '*S. pacifica*' 16S BLAST queries, which yield more than twice the number of *S. arenicola* hits relative to '*S. pacifica*' and include data from geographically distant samples collected by multiple research groups using different cultivation techniques. While the limited diversity observed in *S. tropica* may be due to a small population size, the events responsible for the relatively high levels of diversity observed in *'S. pacifca*' remain unresolved. In the future, it will be interesting to determine if this diversity is neutral or linked to ecological divergence.

Despite the lack of evidence for allopatric diversification in '*S. pacifica*', there is preliminary evidence for endemism among the *S. arenicola* sequence types. While the standard sequence type is broadly distributed, recent data from strains cultured off the coast of Costa Rica (Solano *et al*., 2009) and from the Sea of Cortez (Maldonado *et al*., 2009) complement an earlier study (Jensen and Mafnas, 2006) that first documented the existence of *S. arenicola* sequence types A and B in the eastern Pacific. At present, there is no evidence for the co-occurrence of the original *S. arenicola* sequence type with sequence types A and B; however, further studies are needed to establish these patterns. In general, however, intraspecific population genetic structures in *Salinispora* spp. do not show strong evidence of endemism as has been reported more broadly for soil actinomycetes (Wawrik *et al*., 2007). These differences may be due to increased rates of dispersal in seawater relative to soils, the sensitivity of the methods applied, or the level of taxonomic diversity encompassed by different studies.

Salinispora tropica was not detected among the strains cultured from Fiji nor has it been reported from any locations outside of the Caribbean. Likewise, '*S. pacifica*' has yet to be reported from the Caribbean and thus there is no cultivation-based evidence for the co-occurrence of these two species at any sampling locations (Fig. 5). These results are supported for the Caribbean when culture-independent methods were applied (Mincer *et al*., 2005). The non-overlapping distributions of *S. tropica* and '*S. pacifica*' suggest a potential role for allopatry in the generation of these two lineages. However, it remains possible that these species co-occurred at the time of divergence or that they remain undetected at some sites due to differences in relative population sizes. In the present study, none of the 82 sequence-verified *Salinispora* strains from Fiji were identified as *S. tropica*. Thus it can be estimated that if *S. tropica* did occur at this location, its relative abundance ($< 1{:}82$) is below the detection limits of the methods employed. The application of deep sequencing techniques to detect rare community members (Sogin *et al*., 2006) will help determine if species are rare or absent at specific sites. Ultimately, it remains a challenge to ascribe biogeographical patterns to historical contingencies such as geographical isolation (Martiny *et al*., 2006), especially given the difficulties in demonstrating the absence of a taxon at a given location. The weak evidence of species-level geographical isolation detected in the IBD analysis, despite the apparent lack of overlap in the distributions of *S. tropica* and '*S. pacifica*', may in part be due to the high level of sequence similarity among *Salinispora* spp.

Secondary metabolites are linked to an organism's fitness and therefore represent an emerging marker to study population structure and function. This class of functional genes is known to be susceptible to HGT yet can reveal biogeographical patterns (Wawrik *et al*., 2007) sug-

gesting they may be indicators of localized adaptation or recent speciation events. In support of this, taxonomically meaningful patterns of secondary metabolite production have been detected in bacteria (Jensen *et al*., 2007) and fungi (Larsen *et al*., 2005). To explore this trait in '*S. pacifica*', polyketide biosynthetic gene diversity was assessed by T-RFLP. One goal of this analysis was to test for evidence of biogeographical clustering that may have remained undetected using more conserved phylogenetic markers. The absence of clustering in the '*S. pacifica*' T-RFLP data (Fig. 6A) provides further support for high rates of dispersal and no evidence that intraspecific 16S sequence types are linked to the maintenance of different secondary metabolite profiles. Therefore, there is no evidence for the presence of cryptic species or nascent speciation events in '*S. pacifica*' based on this trait. When a second analysis that included all three species was performed, significant differences were observed between *S. arenicola* and '*S. pacifica*' along the first ordination axis (Fig. 6B), providing further support that secondary metabolism represents a functional trait that can be used to delineate *Salinispora* species (Penn *et al*., 2009) and an additional line of support for the description of *S. tropica* and '*S. pacifica*' as distinct species.

The genus *Salinispora* has proven to be an interesting model with which to study bacterial biogeography and species concepts. The present study provides new data on the diversity and distribution of '*S. pacifica*', additional support for the delineation of this lineage as a new species, and a rapid antibiotic sensitivity/RFLP screen that can be used for preliminary taxonomic assignments within the genus. The broad distribution of '*S. pacifica*' suggests that dispersal rates generally exceed the rates of 16S and ITS evolution. This has been observed for other spore-forming bacteria (Roberts and Cohan, 1995) and adds to a growing body of literature in which dispersal limitation does not appear to be a dominant force structuring microbial communities as defined by 16S or ITS sequencing. The designation of the three lineages as distinct species occurs at the finest level of phylogenetic resolution that can be supported with the markers analysed and supports the concept that bacterial species diversity, as we understand it today, remains dramatically underestimated.

Experimental procedures

Sediment collection, processing and bacterial isolation

Sediment samples were collected from 7 to 24 July 2006 and 18 January to 14 February 2008 from three main collection sites in the Yasawa islands, Beqa lagoon and the Kadavu islands, Fiji. Additionally, 37 sediment samples were collected in December 2008 around the island of Maui, Hawaii. Samples were collected from shore, by skin or SCUBA diving, or with autonomous or tethered, surface-deployed sediment samplers, to depths of 2040 m and placed into sterile 50 ml Whirl-Pak bags (NASCO, Modesto, CA). All Fijian sediment samples were processed for actinomycete isolation at the University of the South Pacific (USP) in Suva, Fiji within 3 days of collection. When possible, Fijian samples were frozen prior to processing; otherwise they were kept refrigerated at 4°C. The Hawaiian samples were frozen within 5 h of collection. Frozen samples from the 2008 Fijian collection were further processed, while all Hawaiian samples were processed for the first time, at the Scripps Institution of Oceanography (SIO), La Jolla, CA in February 2008 and January 2009 respectively.

Processing consisted of air-drying sediments in a laminar flow hood followed by stamping onto medium M1 (1 g of starch, 0.4 g of yeast extract, 0.2 g of peptone, 18 g of agar, 1 l of seawater) and SWA (18 g of agar, 1 l of seawater) using previously described methods (Gontang *et al*., 2007). Additionally, the Hawaiian samples were diluted 1:5 in sterile seawater, vortex mixed, heat-shocked at 55°C for 9 min, and 75 μl of supernatant was spread with a sterile glass rod onto medium A1 (4 g of yeast extract, 2 g of peptone, 10 g of starch, 18 g of agar, 1 l of seawater) or SWA. All primary isolation plates contained 100 μg l^{-1} cycloheximide (final concentration) to inhibit fungal growth.

All plates were incubated at room temperature and monitored for up to 16 weeks. Colonies with morphological features characteristic of the genus *Salinispora* (Maldonado *et al*., 2005) were removed from the primary isolation plates using sterilized toothpicks and repeatedly transferred onto medium A1 until pure cultures were obtained as evidenced by uniform colony morphology. Pure cultures were then cryopreserved at –80°C in medium A1 (without agar) with 10% glycerol added as a cryoprotectant.

Antibiotic sensitivity screen

Representatives of the three *Salinispora* species were plated on medium A1 containing 20 μg ml^{-1} rifampin (Sigma-Aldrich, St Louis, MO). *Salinispora arenicola*, which produces compounds in the rifamycin class (Kim *et al*., 2006; Jensen *et al*., 2007), was observed to grow well at this concentration while '*S. pacifica*' and *S. tropica* did not. As a rapid method to distinguish *S. arenicola* from other *Salinispora* species, all strains isolated from the Fiji collections with morphological features characteristic of *Salinispora* spp. were tested for growth on medium A1 containing 20 μg ml^{-1} rifampin. The effectiveness of this method was tested using sequence-based approaches as described below.

Nucleic acid extraction, PCR amplification and RFLP analysis

Genomic DNA was extracted according to the DNeasy protocol (QIAGEN, Valencia, CA) with previously described changes (Gontang *et al*., 2007). Purified genomic DNA was used immediately or stored at –20°C. 16S rRNA gene sequences were PCR-amplified using the primers FC27 (5′-CCGCGGCTGCTGGCACGTA-3′) and RC1492 (5′–GTGCGGGCCCCCGTCAATT-3′) as previously described

(Gontang *et al*., 2007). The ITS region of the rRNA operon (507 bp) was PCR-amplified using the previously described primers L1 (5′-CAAGGCATCCACCGT-3′) and G1 (5′-GAAGTCGTAACAAGG) and program (Jensen *et al*., 1993). The PCR conditions are presented as Appendix S1. *RecA* gene sequences were amplified using the primers recA_AF (5′-TTGCTCTCGCTCAGATCGACAAACAGTTC-3′) and recA_AR (5′-GCCACGTCCGGGTTCTCCCGAAGGAACTC GCG-3′). PCR conditions and clean-up were the same as those listed for the 16S rRNA amplification with the exception that the annealing temperature was set to 70°C.

In silico analysis of previously published *S. arenicola*, *S. tropica* and '*S. pacifica*' ITS sequences revealed a BanI endonuclease restriction site in *S. arenicola* that was not present in the other two species. Based on this, ITS PCR products were digested with two units of BanI restriction enzyme (New England BioLabs) at 37°C for 2 h followed by 15 min at 65°C. After digestion, the product was run on a 2% agarose gel for approximately 1 h at 120 volts and a gel image taken with a Kodak Gel Logic 100 gel imager.

16S rRNA, ITS and recA *gene sequencing and analysis*

Partial 16S rRNA gene sequences were obtained using the FC27 primer while nearly full sequences were obtained with this and other previously described primers (Mincer *et al*., 2002). Partial ITS sequences (507 bp) were obtained using primer L1 (described above), of which 291 bp were used for the phylogenetic analyses. Partial *recA* sequences were obtained using the primer recA_AF of which 710 bp were used in the phylogenetic analyses. DNA sequencing was performed by SeqXcel, San Diego, CA, the University of Hawaii, Manoa, and the UCSD Cancer Center. *Salinispora* 16S rRNA sequences reported in previous studies (Kim *et al*., 2006; Solano *et al*., 2009) or downloaded from GenBank were also included in select analyses. Variable 16S rRNA nucleotide positions were only included if they occurred in regions that possessed ≤ 90% conservation (Cannone *et al*., 2002) or were observed in multiple strains.

All sequences were edited using the Sequencher software package (version 4.5; Gene Codes Co., Ann Arbor, MI) and aligned using MacClade (version 4.07, Sinauer Associates, Sunderland, MA) or ClustalX, version 2.0.11 (Chenna *et al*., 2003). The sequence data were independently analysed using jModelTest (Posada, 2008) and best fit likelihood models chosen. Maximum likelihood trees were drawn using PAUP (Swofford, 2003) and Phyml (Guindon and Gascuel, 2003). Neighbour-joining trees were also constructed using PAUP. Bootstraps were preformed with 1000 replicates, resampling all sites.

rifK *PCR protocol*

Salinispora arenicola isolates that demonstrated inhibited growth on media containing 20 μg ml^{-1} rifampicin were probed for the presence of the *rifK* gene, which is involved in rifamycin biosynthesis (Floss and Yu, 2005). The specific primers Rif_1247f (5′-GCGGCAGGGTGAGTGTTC-3′) and Rif_1247r (5′-CACCGTGCTGTCCGAAGG-3′) (Edlund *et al*., 2011) were used to amplify the rif1247B locus for this experiment. The PCR conditions are presented as Appendix S1. Isolates that did not yield a product were screened again with the previously published primers 4F (5′-CCTGCGTGGCCGCAGTACGACGAC-3′) and 5R (5′-CTGCGC GCGCAGCACGGACGC-3′) (Kim *et al*., 1998). *RifK* PCR products were sequence verified for six of the strains.

Isolation by distance and RAMI *analysis*

Isolation by distance analysis was run using three sets of *Salinispora* ITS sequence data, each of which included one representative from each 16S rRNA gene sequence type observed at each location. The first set was comprised of '*S. pacifica*' strains from each of the three collection sites in Fiji (Beqa Lagoon, Kadavu and the Yasawa islands), the second set included '*S. pacifica*' strains from four different locations throughout the South Pacific, while the third set included strains representing all three *Salinispora* spp. and six global locations. A similar set of analyses was also run using 16S rRNA sequence data from the same strains. The Isolation by Distance Web Service Version 3.16 (Jensen *et al*., 2005) was used to calculate F_{st} values and to perform a Mantel test for IBD (10 000 randomizations). The locations used in the analyses included Palau (7°30′N, 134°30′E), Guam (13°28′N, 144°47′E), the Bahamas (24°15′N, 76°00′W), the Red Sea (27°40′N, 34°09′E), Hawaii (21°N, 157°W) and Fiji (18°00′S, 175°00′E). Biogeographical patterning and fine-scale phylogenetic diversity within '*S. pacifica*' was examined using the program RAMI to cluster groups based on patristic distances (Pommier *et al*., 2009).

Secondary metabolite gene fingerprinting

Ketosynthase domains associated with PKSI genes were analysed by T-RFLP using previously described methods (Edlund *et al*., 2011). The strains targeted were derived from various locations and included all '*S. pacifica*' 16S rRNA gene sequence types. The PCR conditions are presented as Appendix S1. Following PCR amplification, duplicate 75 μl reactions were combined, ethanol precipitated, diluted in 30 μl of OmniPur water, and run on a 1% agarose gel for 45 min at 90 volts with a 100 bp ladder (Invitrogen Corporation, Carlsbad, CA). Bands c. 600 bp in size were excised and purified using the MinElute gel extraction kit (QIAGEN). Eluted PCR products (10 μl) were digested at 37°C for 2 h followed by 15 min at 65°C in 5 μl of Buffer 4 (New England BioLabs), 0.5 μl of 10X BSA, 10 U HhaI and OmniPur water (EMD Chemicals, Carlsbad, CA) added to a volume of 50 μl. Digested PCR products were ethanol precipitated, diluted in 5 μl of OmniPur water, and 2 μl added to 8 μl of master mix containing 8.2 μl of 0.1% TWEEN and 0.13 μl MegaBACE™ ET550-R size standard. Samples were then incubated at 95°C for 1 min and placed on ice. Fluorescently labelled terminal restriction fragments (T-RFs) were separated and detected using a MegaBace 1000 capillary sequencer (GE Healthcare, Sunnyvale, CA) equipped with three arrays of 16 capillaries with an interior diameter of approximately 100 μm. Samples were injected electrokinetically at 3 kV and separated with an applied voltage of 5 kV for 179 min at a run

temperature of 44°C. Additionally, some samples were analysed at Genewiz (Genewiz, South Plainfield, NJ). The lengths of the fluorescent T-RFs were determined using the Gene Marker software (SoftGenetics, LLC State College, PA) and Molecular Dynamics Genetic Profiler software packages (GE Healthcare, Piscataway, NJ).

Statistical analysis of T-RFLP profiles

Terminal restriction fragment length polymorphism analyses were performed on one to three independent replicates for each strain. Only peaks with fluorescence intensities over 50 units were counted. The results were recorded in a presence (+) or absence (–) matrix that was sorted according to T-RF size in base pair. T-RFs with sizes ±1.0 bp were assigned to the median or most common number of base pairs. PCAs were performed on the data matrix using the multivariate analysis software ADE-4 (http://pbil.univ-lyon1.fr/ADE-4) as previously described (Edlund *et al*., 2008). Significant clustering patterns in the PCA plot were determined by a two-tailed *t*-test (Mann–Whitney *U*-test) of '*S. pacifica*' and *S. arenicola* coordinates from the first ordination axis using the software package Prism 5 (GraphPad Software, La Jolla, CA).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. ITS RFLP. BanI restriction digestion of 507 bp PCR amplified *Salinispora* spp. 16S-23S ITS region. Lane 1: 500 bp ladder; lanes 2–5: '*S. pacifica*' ITS sequences that lack a BanI restriction site; lane 6: *S. arenicola* ITS sequence, which has one BanI restriction site resulting in two bands equal to c. 200 and 300 bp. Faint bands may be non-specific products.

Appendix S1. PCR and T-RFLP conditions.

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