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PHYLOGENETIC ANALYSIS FOR DETECTION OF MULTIPLE FOULING EVENTS: A PILOT STUDY OF BARNACLES AT MOOREA ISLAND (FRENCH POLYNESIA)

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ABSTRACT

Sequencing the cytochrome oxidase I gene and 16rRNA gene as DNA Barcodes as a phylogenetic methodology, we identified the origin of two invasive barnacles sampled from Vai'are Bay (Moorea Island) in 2011: *Chthamalus proteus* and *Amphibalanus amphitrite*. Reconstructed phylogenies strongly support multiple introductions of *Chthamalus proteus*: from a Brazilian lineage first identified at the island in 2004 and from a lineage located in Hawaii that same year. The unique *Amphibalanus amphitrite* haplotype clustered with lineages distributed from Japan to Malaysia. The results demonstrate multiple introduction events from different origins, that may enhance invasion processes in South Pacific islands.

Key words. — Barnacles, Moorea, phylogenetic analysis, biofouling, COI, 16S

RÉSUMÉ

En utilisant le séquençage du gène de la cytochrome oxydase I et du gène de l'ARNr 16 par la méthode des code-barres d'ADN, nous avons déterminé l'origine phylogénétique de deux balanes invasives récoltées dans la Baie de Vai'are (île de Moorea) en 2011 : *Chthamalus proteus* et *Amphibalanus amphitrite*. La reconstruction phylogénétique soutien fortement les introductions multiples de *Chthamalus proteus* : d'une première lignée brésilienne identifiée dans l'île en 2004, et d'un lignée localisée à Hawaï la même année. L'unique haplotype *Amphibalanus amphitrite* se regroupe avec des lignées distribuées du Japon à la Malaisie. Les résultats démontrent des introductions multiples de différentes origines, qui pourraient favoriser les processus d'invasion dans les îles du Pacifique sud.

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INTRODUCTION

There are different gateways for introduction of exotic species into a habitat. Shipping is believed to be one of the most important pathways for transfer of non-indigenous species across marine regions (Leppakoski et al., 2002). This pathway involves several potential vectors-transport of organisms in ballast water and ballast tank sediments, and fouling of hull, sea chests, anchors and anchor chains, etc. (Hewitt et al., 2009), depending on adult or larval biology status of the species (Farrapeira et al., 2007; Yamaguchi et al., 2009; Tøttrup et al., 2010). Ballast water (BW) is recognized as the most significant of these vectors (Molnar et al., 2008). Approximately 2.2 to 12 billion tonnes of BW are transported across the world oceans annually (Endresen et al., 2004), transferring some 7000 species daily in the process (Gollasch & David, 2011).

Maritime transport is not only a source of invaders but also a paradigmatic vector of long-distance dispersal with relatively predictable patterns if the main routes are known (e.g., Wonham et al., 2001; Occhipinti-Ambrogi & Galil, 2010). The spread can be predicted when the vectors are known and also when the natural population structure in native locations, an indicator of the natural dispersal capacity, is known (Gaither et al., 2013). The widespread use of maritime highways for commercial and recreational interchanges provides opportunities for repeated waves of invasions (Galil et al., 2007). Multiple invasion hits can be traced employing phylogenetic methodology when geographically separated lineages can be identified from DNA sequences. An example is the barnacle *Chthamalus proteus* Dando & Southward, 1980, native to tropical Atlantic waters. The origin of its invasion in the Pacific Ocean has been demonstrated to be diverse because lineages from Brazil, Panama and Caribbean have been unambiguously identified in different Pacific regions using the Barcoding cytochrome oxidase gene COI (Zardus & Hadfield, 2005).

In other cases, the fouling behaviour of the species can increase the gene flow among distant populations and, thus, homogenize genetic structuring making the origin determination of the invasion difficult. An example is the barnacle *Amphibalanus amphitrite* (Darwin, 1854), which is distributed in warm and temperate waters worldwide, suggesting that it is dispersed through human-mediated activities via ballast water for larvae and via vessels fouling for adults in long distances routes (Chen et al., 2014). It is believed that the expansion of global trade in the 20th century has led to transport of *A. amphitrite* from Indo-Pacific waters to those of Europe and even to North and South America (Carlton et al., 2011).

In the present study we developed a phylogenetic approach for determining the most likely origin of two fouling barnacles (*C. proteus* and *A. amphitrite*) settled

in the main port of the Moorea Island: Vaiare (French Polynesia). This island is part of a biodiversity hotspot (Roberts et al., 2002) and barnacle invasions can seriously compromise the integrity of coral reef ecosystem (e.g., Coles & Eldredge, 2002). *Chthamalus proteus* of Brazilian origin was detected there in 2004 (Zardus & Hadfield, 2005). In the present study the origin of barnacles sampled in 2011 was traced employing the same methodology to test the possibility of multiple invasion hits, from the same or different origins, occurring in the same places.

MATERIAL AND METHODS

Sampling

Samples were taken from 16 points located in different ecosystems within the coral reefs that surround the Moorea Island (Richard, 1985): from water channels in Papetoai, Maharepa and Temae; from deep lagoons in Vaiare, Farehau and Afareaitu; from fringing reefs in Tiahura, Maatea, Atiha, Vaianae, Haapiti, Tiki and Hauru; and finally from bay environments in Opunohu, Paopao and Entre-Deux-Baies (Ardura et al., 2015). Exotic barnacles were only detected in Vaiare port area.

Exotic barnacles (the Caribbean barnacle *Chthamalus proteus* and the striped acorn barnacle *Amphibalanus amphitrite*) were visually identified and manually sampled ($N = 20$ of each species) randomly from approximately 200 m^2 in the intertidal habitat of the rocky shore close to the marina of Vaiare, approximately at 500 m from the Moorea Ferry Terminal, in August 2011. The exact coordinates of the sampling point are 17.304°S 149.549°W.

Genetic analysis

Total DNA was extracted from the samples using the E.Z.N.A. Mollusc DNA kit (IOMEGA, bio-tek), following manufacturer's instructions. The tubes were stored at 4°C for immediate DNA analysis, and aliquots were frozen at −20°C for long-time preservation.

A fragment of the partial COI gene was amplified by polymerase chain reaction (PCR), employing the primers described by Geller et al. (2013). The amplification reaction was performed in a total volume of 40 μl, consisting of 1× Promega (Madison, WI) buffer, 2.5 mM $MgCl_2$, 0.25 mMdNTPs, 20 pmol of each primer, approximately 20 ng of template DNA and 1 U of DNA Taq polymerase (Promega), and the following PCR conditions: initial denaturing at 95°C for 5 minutes, 35 cycles of denaturing at 95°C for 1 minute, annealing at 48°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 5 minutes.

Additional sequencing of the mitochondrial 16S rRNA gene with the primers described by Palumbi (1996) was carried out for both species in order to confirm the taxonomic identification with a second marker. The amplification reaction was performed in a total volume of 40 μl with the same conditions described above for the COI gene and the following PCR conditions: initial denaturing at 95°C for 5 minutes, 30 cycles of denaturing at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes and final extension at 72°C for 7 minutes.

PCR products were visualised in 2% agarose gels with 3 μl of 10 mg/ml ethidium bromide. Sequencing was performed at Macrogen Europe (The Netherlands).

Sequence edition and phylogenetic analysis

Sequences were visualized and edited employing the BioEdit Sequence Alignment Editor software (Hall, 1999) and they were aligned with the ClustalW application (Thompson et al., 1994) included in BioEdit. The sequences obtained were compared with international databases employing the program BLAST within NCBI (http://www.ncbi.nlm.nih.gov/) and BOLD system (http://www.boldsystems.org/) for identifying the species.

Sequences obtained in this work were used together with GenBank *A. Amphitrite* (KC138445, KM211362-KM211497) and *C. proteus* (AY822889, AY822950, AY822955, AY822956, AY822959, AY822961, AY822963, AY822964, AY822975, AY822984, AY822998, AY823005, AY823008 and AY823026) sequences from Chen et al. (2014) and Zardus & Hadfield (2005), respectively, for subsequent phylogenetic analysis.

The phylogenetic analysis was performed with the software MEGA 6.0 (Tamura et al., 2013). Phylogenetic trees containing the reference and presently sequenced COI fragments were reconstructed using this software and selecting the Maximum Likelihood (ML) algorithm. The molecular substitution model was chosen using the software jModeltest (Posada, 2008), using a corrected Akaike information criterion (AICc), to determine the best suited model of sequence evolution and accompanying evolutionary parameter values for the data. Robustness of the ML topology in this case was assessed using 1000 bootstrap replicates.

Genetic differentiation between populations was measured with FSTs statistics, which is based on differences in nucleotide diversity between haplotypes. The software Arlequin (Excoffier et al., 2005) was employed for estimates of FSTs values and their statistical significance between samples pairs, i.e., the significance of population differentiation, with 10 000 permutations for significance.

A median-joining network (Bandelt et al., 1999) was constructed to represent visually the intra-specific genealogy of the dataset of haplotypes and their relative frequencies in the sampled populations using the software network 5.0 (avail-

able online at http://www.fluxus-technology.com), with default settings. The haplotypes table was made with the same software.

RESULTS

The 20 COI sequences obtained from Moorea Island barnacle samples (10 per species), 615 nucleotides long, were unambiguously assigned in both BOLD and GenBank to two species: *Chthamalus proteus* and *Amphibalanus amphitrite*. The results were confirmed with 16S rRNA genes, with the same number of sequences (20, 10 per species), 485 nucleotides long, that also gave the closest match with these species in the GenBank database (Accession numbers KJ663820 and KJ663821 for the 16S rDNA haplotypes found for the *C. proteus* and *A. amphitrite* samples in this study, respectively).

The COI sequences of *Chthamalus proteus* corresponded to two different haplotypes, each of them found in 50% of the cases. They were submitted to GenBank under accession numbers KJ663817 and KJ663818. The dataset containing these two haplotypes and the reference sequences with >97% identity (table I) best fitted the mutation model of Tamura & Nei (1993), with a proportion of invariant sites (TrN + I); which was employed in the settings for reconstructing the ML phylogenetic tree. In the phylogenetic tree obtained from these sequences (fig. 1a), both haplotypes clustered with the Brazilian and Hawaiian lineage with a small bootstrap, which could suggest a secondary introduction from Hawaii in some cases. The haplotype network obtained (fig. 1b) supports this suggestion, since both haplotypes (H14 and H15) clustered with Brazilian and Hawaiian ones (H1, H2 and H5) but not in strong way.

The COI sequences matching with *Amphibalanus* corresponded to one unique haplotype which was submitted to GenBank under Accession number KJ663819. The ML phylogenetic tree (fig. 2) was constructed using the mutation model of Tamura & Nei (1993), with a proportion of invariant sites (TrN + I) and includes 16 other *Amphibalanus* sequences, representing each clade described by Chen et al. (2014) from GenBank (table I), confirming the species assignment of *A. amphitrite* obtained from BLAST. From the ML phylogenetic tree the origin area of the individuals found in Moorea Island was closer to clade 1 described by Chen et al. (2014), because our haplotype clustered together with this clade and separated from the branch that contained the other two clades. Besides, the genetic distances calculated (FSTs), together with the haplotype network analysed, clarify the closest population of Moorea Island in the same direction (table II, fig. 3). These genetic distances were calculated based on the nucleotides composition of each sequence; in table III we can see the differences inside clade 1 with the representative haplotypes described by Chen et al. (2014) together with Moorea haplotype found in this study.

TABLE I

Geographic origin and GenBank accession numbers (A.N.) of the COI sequences used in this study

Species	Identification in this study	Sampling location	A.N.	Reference
Amphibalanus amphitrite	Moorea haplotype	Vaiare Bay (Moorea)	KJ663819	This study
Amphibalanus amphitrite	KM211477-Singapore (Clade 1)	Singapore	KM211477	Chen et al. (2014)
Amphibalanus amphitrite	KM211389-Taiwan (Clade 1)	Taiwan	KM211389	Chen et al. (2014)
Amphibalanus amphitrite	KM211469-U.S.A. (Clade 1)	U.S.A.	KM211469	Chen et al. (2014)
Amphibalanus amphitrite	KM211432-Hong Kong (Clade 1)	Hong Kong	KM211432	Chen et al. (2014)
Amphibalanus amphitrite	KM211490-Singapore (Clade 1)	Singapore	KM211490	Chen et al. (2014)
Amphibalanus amphitrite	KM211497-U.S.A. (Clade 1)	U.S.A.	KM211497	Chen et al. (2014)
Amphibalanus amphitrite	KM211441-Hong Kong (Clade 1)	Hong Kong	KM211441	Chen et al. (2014)
Amphibalanus amphitrite	KM211482-Singapore (Clade 2)	Singapore	KM211482	Chen et al. (2014)
Amphibalanus amphitrite	KM211416-Newport (Clade 2)	Newport, RI, U.S.A.	KM211416	Chen et al. (2014)
Amphibalanus amphitrite	KM211459-Malaysia (Clade 3)	Malaysia	KM211459	Chen et al. (2014)
Amphibalanus amphitrite	KM211447-Malaysia (Clade 3)	Malaysia	KM211447	Chen et al. (2014)
Amphibalanus amphitrite	KM211396-Taiwan (Clade 3)	Taiwan	KM211396	Chen et al. (2014)
Amphibalanus amphitrite	KM211456-Malaysia (Clade 3)	Malaysia	KM211456	Chen et al. (2014)
Amphibalanus amphitrite	KM211465-Thailand (Clade 3)	Thailand	KM211465	Chen et al. (2014)
Amphibalanus amphitrite	KM211460-Malaysia (Clade 3)	Malaysia	KM211460	Chen et al. (2014)
Chtamalus proteus	Haplotype-Cht1	Vaiare Bay (Moorea)	KJ663817	This study
Chtamalus proteus	Haplotype-Cht2	Vaiare Bay (Moorea)	KJ663818	This study
Chtamalus proteus	Hawaiian Islands	Hawaiian Islands	AY822889	Zardus & Hadfield (2005)
Chtamalus proteus	Mariana Islands	Mariana Islands	AY822950	Zardus & Hadfield (2005)
Chtamalus proteus	Caroline Islands	Caroline Islands	AY823026	Zardus & Hadfield (2005)
Chtamalus proteus	Moorea1	Opunohu Bay (Moorea)	AY822955	Zardus & Hadfield (2005)
Chtamalus proteus	Moorea2	Opunohu Bay (Moorea)	AY822956	Zardus & Hadfield (2005)
Chtamalus proteus	Moorea3	Opunohu Bay (Moorea)	AY822959	Zardus & Hadfield (2005)
Chtamalus proteus	Moorea4	Opunohu Bay (Moorea)	AY822961	Zardus & Hadfield (2005)
Chtamalus proteus	Moorea5	Opunohu Bay (Moorea)	AY822963	Zardus & Hadfield (2005)
Chtamalus proteus	Moorea6	Opunohu Bay (Moorea)	AY822964	Zardus & Hadfield (2005)
Chtamalus proteus	Panama	C. America (Portobello, Panama)	AY822975	Zardus & Hadfield (2005)
Chtamalus proteus	Lesser Antilles	Lasser Antilles (St Joris Bay)	AY822984	Zardus & Hadfield (2005)
Chtamalus proteus	Greater Antilles	Greater Antilles (Brewers Bay, Range Cay)	AY822998	Zardus & Hadfield (2005)
Chtamalus proteus	Brazil1	S. America (Brazil)	AY823005	Zardus & Hadfield (2005)
Chtamalus proteus	Brazil2	S. America (Brazil)	AY823008	Zardus & Hadfield (2005)

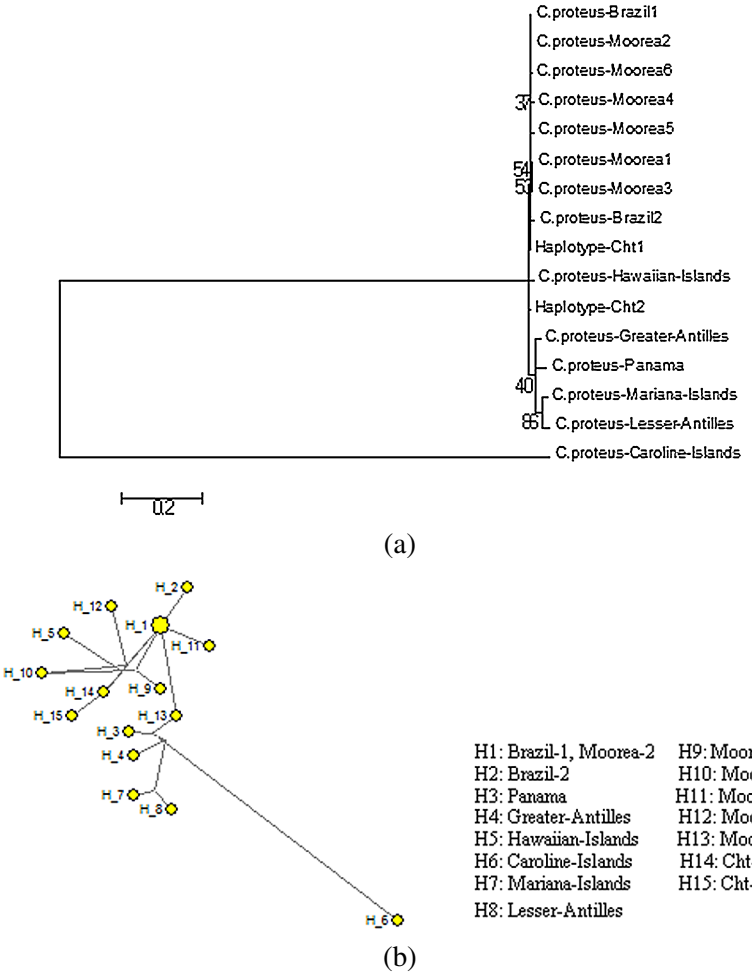

Fig. 1. (a) Maximum Likelihood phylogenetic tree constructed based on COI gene fragment containing the two haplotypes found in this study from Moorea and other *Chtamalus proteus* sequences from the GenBank. Bootstrap values presented in percent. (b) Median-joining network showing the relationships among the same sequences described in the ML phylogenetic tree defined by COI sequence variation. This figure is published in colour in the online version of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/15685403.

DISCUSSION

Our results indicate multiple fouling hits introducing invasive barnacle species to small islands such as Moorea. *Chthamalus proteus* was described in another bay of the island for the first time in 2004. Seven years later it has been localised in a different bay, and in addition we identified two haplotypes; one from Brazil (GB-AN AY823005 and AY823008) (as the previously described lineage introduced in Moorea) and another one, probably from Hawaii (GB-AN

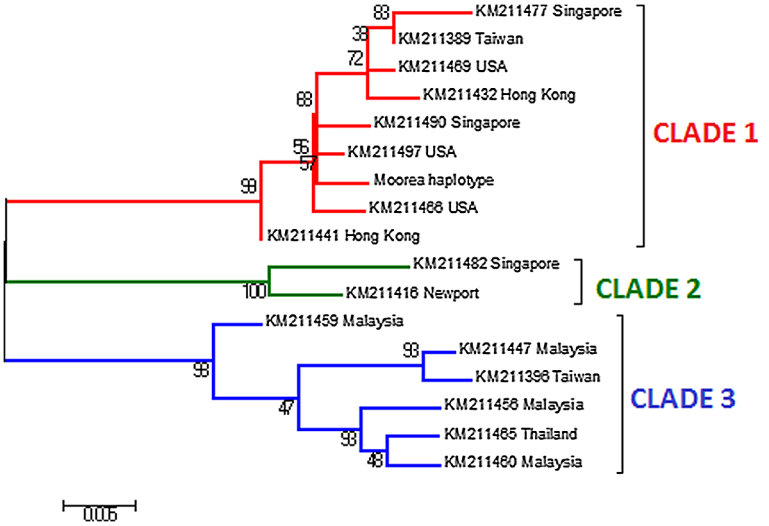

Fig. 2. Maximum Likelihood phylogenetic tree constructed based on COI gene fragment containing the haplotype found in this study from Moorea and other *Amphibalanus* sequences from the GenBank. Bootstrap values presented in percent. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/15685403.

KM211389, KM211432, KM211441, KM211477 and KM211490), not previously described. This second one is likely to originate from a later introduction from Hawaii, most likely via maritime transport since it was sampled near the main marina of Moorea. The main way of expansion of *C. proteus* is likely to be secondary transfers by fouling rather than natural dispersion. This is inferred from a lot of shorter continuous extents of spread or CES (geographical distance over which there is suitable habitat and no gaps in distribution of greater than 100 km), which is more probable than maximum extent of spread in its introduced range in Hawaii (Gaither et al., 2013). However the CES reported for this species in Hawaii was 529 km, which exceeds by far the distance between Opunohu Bay (where

TABLE II

Test for significance differentation between populations based on pairwise genetic distances (FSTs) with COI sequences for *A. amphitrite*

	Clade 1	Clade 2	Clade 3	Moorea Island
Clade 1	–			
Clade 2	0.80784*	–		
Clade 3	0.80067*	0.70884*	–	
Moorea Island	0.40409*	0.98245*	0.83931*	–

* $p<0.05$.

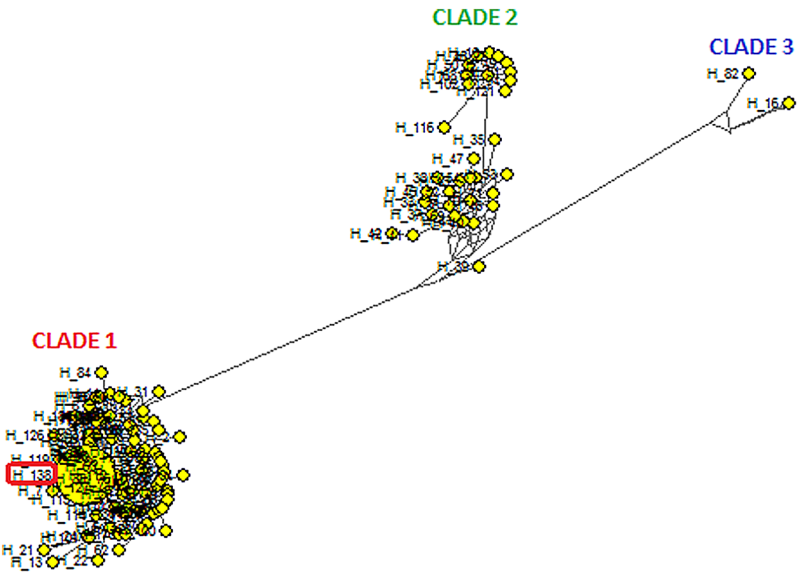

Fig. 3. Median-joining network showing the relationships among the Moorea *Amphibalanus* haplotype (H-138) and the 3 clades described by Chen et al. (2014), defined by COI sequence variation. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/15685403.

the species was reported in 2004, coordinates 17.504°S 149.856°W) and Vaiare, roughly 25 km of coastline. The possibility of natural dispersion from Opunohu Bay cannot be ruled out; moreover, the close proximity of our Haplotype 1 with that described from Moorea Island in 2004 seems to support the hypothesis of a natural expansion along the Moorea's coastline.

For *Amphibalanus amphitrite* the closer reference sequence was obtained from acorn barnacles from the region from Japan to Malaysia, correspond to the clade 1 described by Chen et al. (2014) (figs. 2 and 3). Additional populations are found in Australia, India and Saudí Arabia, but whether *A. amphitrite* is native or introduced to these regions is yet unknown (Chen et al., 2014). Besides, this clade is also found in Hawaii, California and North Carolina, all regions where it has long been recognised as a non-native species (Carlton & Eldredge, 2009; Carlton et al., 2011). Despite of the ambiguity about the native-non-native distribution of the species, its transport on fouled ships' hulls and possibly, more recently, as larvae in ballast water has doubtlessly contributed to the global distribution of different clades (figs. 2 and 3). Therefore, the way of introduction to Moorea Island was likely maritime (e.g., Mineur et al., 2012), as in the case of *Chthamalus*. However, the previous presence of this species in Moorea Island cannot be discarded, and the conditions for primary or secondary transfer cannot be deduced from the present data. *Amphibalanus amphitrite* has been described in so many places that it could

TABLE III

Nucleotide differences inside reference haplotypes of clade 1 and Moorea haplotype

Position	33	145	156	180	249	261	273	282	303	314	327	356	357	393	395	396	408	411	426	462	501	518	526	528	541	543
H1	T	T	G	C	A	G	G	T	C	C	T	C	G	G	C	T	C	A	G	A	A	C	C	A	T	A
H2	C	C	G	C	G	G	A	T	C	T	T	G	T	G	C	T	C	A	G	A	A	A	C	C	C	G
H3	T	C	G	C	G	G	A	T	C	T	T	C	G	A	C	T	C	G	G	A	A	A	C	A	C	G
H4	T	C	A	C	A	A	A	C	C	C	T	C	G	G	C	T	C	A	G	A	A	C	C	A	C	A
H5	C	C	G	C	G	G	A	T	T	C	T	C	T	G	C	T	T	A	G	A	A	C	C	A	C	G
H6	T	T	G	T	A	G	A	T	C	C	T	C	G	A	C	T	C	A	G	A	A	C	C	A	C	A
H7	C	C	G	C	G	G	A	T	C	T	T	C	A	G	C	T	C	A	G	A	A	A	C	A	C	G
H8	T	C	G	C	A	G	A	T	C	C	T	C	G	G	C	T	C	A	A	A	G	C	T	A	C	A
H9	T	C	G	C	A	G	A	T	C	C	G	C	A	G	T	T	C	A	G	A	A	C	C	A	C	A
H10	T	C	G	C	A	G	A	T	C	C	T	C	G	G	C	C	C	A	G	G	A	C	C	A	C	A

H1, KM211491; H2, KM211485; H3, KM211477; H4, KM211467; H5, KM211436; H6, KM211414; H7, KM211383; H8, KM211379; H9, KM211372; H10, Moorea haplotype (KJ663819).

be considered a cosmopolitan species (Galil et al., 2011), and it is very difficult to trace the real origin of the invasion. According to the data, *A. amphitrite* seems to have been introduced just once or at least we cannot conclude the introduction by multiple hits in this area. Therefore, a deeper analysis including many other locations around the island and reference specimens from other geographical areas would be advisable.

This work shows the return to the idea that the development of the economic world leaves no area protected from alien introduction and that even small, very isolated islands, such as the Polynesian islands, are facing introduction, making the evaluation of the real impact on ecosystems difficult.

While economic development is needed, the type of tools displayed in this study can help to control and prevent the entry of invasive species and, finally, it would allow the economic development of the area together with environmental conservation of this pretty area which attracts a lot of tourism.

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