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Research Article

Molecular investigation of the invasive sponge *Hymeniacidon sinapium* (de Laubenfels, 1930) in Elkhorn Slough, California

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Abstract

A large number of invasive marine invertebrates are recognized from Elkhorn Slough (ES), California. One of these species in the slough is treated as *Hymeniacidon sinapium* (Family Halichondriidae) but its species identity is in doubt pending molecular confirmation. The purpose of this investigation was to confirm the presence of *H. sinapium* in ES, determine its distribution in the slough, and compare its genetic diversity to others in California and worldwide. To address these goals, 23 specimens of *Hymeniacidon* were analyzed using DNA sequences of the nuclear rDNA internal transcribed spacers (ITS1 + ITS2) and the 5.8S exon. The sequences were compared against those of *H. sinapium* from: San Diego and Tomales Bay in California; Japan; and South Korea. All ES sequences were found to be nearly identical to the other *H. sinapium* sequences, differing by only 1-3 nucleotides. ES specimens displayed five unique genotypes: three showed intragenomic polymorphisms (IGPs) in the ITS1 region (positions 155, 181, and 195). These data conclusively document the presence of *H. sinapium* in ES as well as define the species to a relatively narrow portion of its eastern shores (~4 km long). Since the genetic diversity of *H. sinapium* in ES is higher than that reported worldwide, its presence in ES is likely the result of multiple introductions. One of the IGPs in ES was found to be the most abundant and was widely distributed in the slough: an indication that it may be spreading.

Key words: *Hymeniacidon sinapium*; Halichondriidae; ITS; Elkhorn Slough; invasive

Introduction

Elkhorn Slough (ES) is an 8,000 year old, transform-margin estuary that feeds the Monterey Bay in central California (Schwartz et al. 1986). The estuary is protected primarily by the California Department of Fish and Game (Van Dyke and Wasson 2005; NERRS 2012) and is a highly-studied estuary with a wealth of historical data (Van Dyke and Wasson 2005). ES is a U.S. National Estuarine Research Reserve wetland and is classified as sensitive habitat because of the many fishes, birds, and mammals that use it for reproduction or refuge (Yoklavich et al. 1991; Emmett et al. 2000; ESNERR 2006). Due to early anthropogenic activities, a number of changes to the biodiversity in ES have occurred (Van Dyke and Wasson 2005). One of these activities is the brief mariculture of the Atlantic oyster (*Crassostrea virginica* Gmelin, 1791) during the 1920s and, during the 1930s, the more active cultivation of the Japanese oyster (*C. gigas* Thunberg, 1793) (Barrett 1963). The latter is implicated in the introduction of many invasive invertebrates to ES (Wasson et al. 2001). A second activity, diking, eliminated a significant area of marshland, resulting in the transformation of approximately 2/3 of the habitat in ES into fresh and brackish water marshland (Van Dyke and Wasson 2005). Attempts to reclaim the marsh habitat by intentional levee breaches were somewhat successful; however, locations like Parsons Slough and South Marsh that together compose the Parsons Slough Complex (Figure 1A), had become mudflats and have yet to recover to fully restored marshland (Van Dyke and Wasson 2005).

A recent survey of invasive fauna in ES uncovered 56 non-native species, one of which was a sponge that aggressively dominates large portions of the small amount of intertidal substrate that remains in ES (Wasson et al. 2001). The sponge is characterized by its bright-

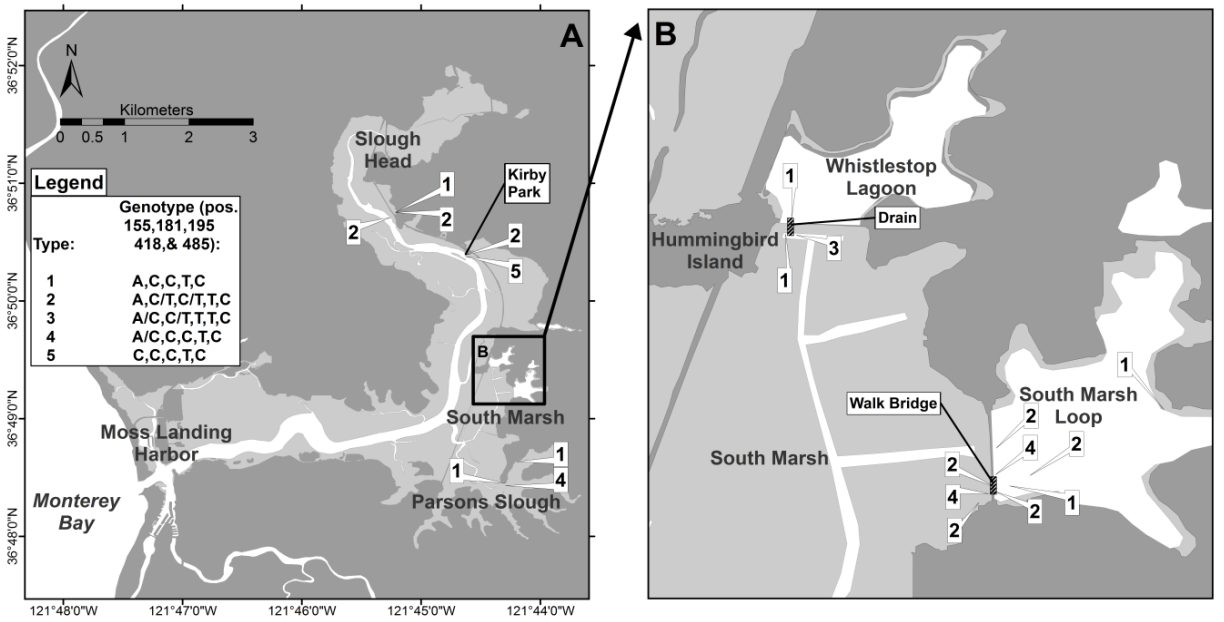

Figure 1. Map of ES indicating collection sites and genotypes for *Hymeniacidon sinapium* characterized by ITS1+ ITS2 polymorphisms (A), inset map of South Marsh displaying genetic diversity with connectivity maintained by a drain and a small channel under a walk bridge (B) (maps supplied by Elkhorn Slough National Estuarine Research Reserve—ESNERR: http://www.elkhornslough.org/gis/index.htm).

orange color, and it forms long, thin, tube-like and furrowed projections from its soft masses with megasclere style spicules throughout the body tissue (Hoshino et al. 2008). This sponge was considered similar in appearance to *Hymeniacidon sinapium* (de Laubenfels, 1930), a species that is native to Japan and Korea but was originally reported by de Laubenfels from specimens found in Newport Bay, California. In this portion of southern California it was said to be common along rocky, surf-beaten portions of the coast and to be most abundant on oyster beds in quiet, very warm water (de Laubenfels 1932). Wasson et al. (2001), who treated the *Hymeniacidon* as an invader to ES, postulated that it was introduced via oysters based on the observed co-occurrence. Another sponge of similar habit occurs in the northern California estuary, Tomales Bay, and may also be *H. sinapium* (Lee et al. 2007). Once established in these marine habitats, *Hymeniacidon* can influence community composition by its vigorous filter-feeding and by providing a solid substrate in an otherwise soft-bottom habitat (Ruiz et al. 1999; Wasson et al. 2001). Because of the similarity in morphology of the ES *Hymeniacidon* sp. to *H. sinapium*, Lee et al. (2007) identified it as *H. sinapium*. Some authors (e.g., Wasson et al. 2001; Lee et al. 2007) have questioned its identity as *H. sinapium*. The lack of a definitive designation for the ES sponge is likely due to the absence of precise anatomical indicators (Park et al. 2007). Phenotypic traits do not always assure accurate identification in sponges because appearance often varies within a species (Neigel and Schmahl 1984; Bell and Barnes 2001).

Using molecular techniques to identify putative exotic species and to trace the origin of an invader is necessary to understanding its potential impacts on a community (Marston and Villalard-Bohnsack 2002; Johnson et al. 2011; Stefaniak et al. 2012). Genetic analyses can also sometimes determine if multiple introductions occurred during colonization (Genton et al. 2005; Facon et al. 2008). Previous research employing molecular methods have identified populations of *Hymeniacidon* in its native Southeast Asian ranges and non-native extents in California (Park et al. 2007; Hoshino et al. 2008). These investigators targeted nuclear rDNA sequence variation found in the internal transcribed spacers (ITS1 + ITS2) and 5.8S

regions. This study analyzed the ITS and 5.8S regions of *Hymeniacidon* from ES and Tomales Bay to: 1) conclusively establish the identity of the sponge in both estuaries; 2) define its geographic distribution in ES; and 3) investigate its genetic relatedness to other populations around the world.

Methods

During the spring and summer months in 2010, field work consisted of documenting the occurrences of *Hymeniacidon* and sampling the sponge from the shoreline of the ES main channel and the Parsons Slough complex (Figure 1A). Twenty-three specimens were harvested by hand from muddy intertidal habitat in the eastern portion of ES along the main channel and southeastern marsh embayments. This sampling represents the current intertidal distribution of *Hymeniacidon* in ES. A single specimen was also taken from Tomales Bay, Marshall, California in July 2011 (Figure 1); otherwise, no sampling occurred outside of ES because of project constraints and the inability to find suitable *H. sinapium* habitats in or near Monterey Bay. Harvested specimens were approximately 2 cm × 2 cm in size and were stored on ice in slough water until processed at Hartnell College, Salinas, California. At the laboratory, 15 mg of sponge were rinsed with deionized water to remove attached microorganisms and inserted into microcentrifuge tubes. The remaining portion of the sample was preserved as a voucher. DNA extractions followed the DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA) protocol with the following modifications: 1) Digestions were performed using 25 µl of proteinase K and 225 µl of ATL tissue lysis buffer at 56 ºC for 30-60 min; 2) After digestion, the cellular debris was pelleted by centrifugation at 8,000 rpm for 1 min, then 200 µl of supernatant was removed. The resulting extract was processed using the manufacturer's instructions (Qiagen). Negative controls were processed in parallel to monitor for contamination.

Primer sequences were those reported by Park et al. (2007). The PCR protocol followed the Qiagen TopTaq™ PCR handbook, using 6 µl of diluted DNA (10 water:1 DNA extract) as template for 50 µl reactions that included 10 µl of Q-solution (Qiagen). Samples were amplified with a Perkin Elmer Cetus DNA Thermal Cycler (Waltham, Massachusetts, USA) using a 3 min denaturation step, followed by 35 cycles with 45 s at 55˚C, 1 min at 72˚C, and 25 s at 92˚C. The positive reactions were purified using the QIAquick PCR Purification Kit (Qiagen), labeled with Big Dye Terminators 3.1, and electrophoresed on a ABI Prism 310 (AB PE Applied Biosystems, Foster City, California). The data were inspected using Chromas Lite Version 2.01 (Technelysium Pty Ltd, Queensland, Australia) software and visually assessed against published sequences using nucleotide BLAST online searches hosted by the National Center for Biotechnology Information and the National Library of Medicine. DNA sequences generated in this analysis are deposited in GenBank (JQ658450 – JQ658473). The ITS sequences collected from Korea and the United States by Park et al. (2007, EF217355 – EF217361) as well as collections from Japan (Hoshino et al. 2008, AB373166 – AB373169) were included as part of the molecular analysis (Table 1).

Results

Twenty-four sequences of the ITS1, ITS2, and 5.8S exon were determined in this study: 23 from ES and 1 from Tomales Bay (Table 1). All sequences were matched greater than 98% to sequences of *H. sinapium* published in GenBank by Park et al. (2007) and Hoshino et al. (2008) with 22 of the sequences matching greater than 99%. From the 24 sequences, five genotypes were identified from ES (Figure 1, Table 1), and a sixth from Tomales Bay (Figure 2). The five genotypes from ES differed by 1-3 nucleotides from previously published sequences of *H. sinapium*. Analysis of individuals from ES revealed intragenomic polymorphisms (IGPs) in the ITS1 sequence at positions 155, 181, and 195 (Table 1). Of the 23 specimens from ES, 13 yielded heterogenetic forms, represented by three heterogenetic types: Type 1—181 (C/T) + 195 (C/T); Type 2—155 (A/C) + 181 (C/T); and Type 3—155 (A/C). Nine of the ES collections displayed genotype 1, which differed from the San Diego population by 1 polymorphism (position 155), from southwestern Japanese types by 2 nucleotides (positions 143 and 418), and from South Korean specimens by 1 nucleotide (position 485). Nine collections were represented in the slough by genotype 2. This type in part contained sequences (heterogenetic for positions 155 and 181) matching specimens from genotype

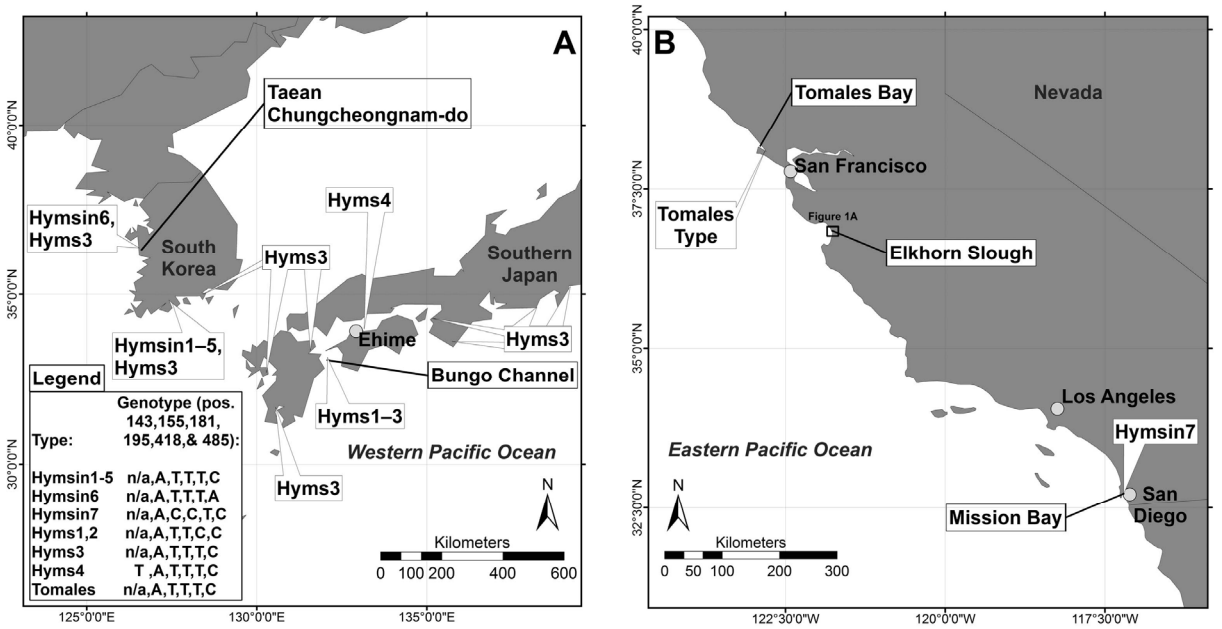

Figure 2. Sampling for *H. sinapium* in South Korea and Southwestern Japan (A) (Hoshino et al. 2008; Park et al. 2007), California coastline sampling outside of Elkhorn Slough consisted of Tomales Bay and Mission Bay with sequence types explained in Table 1 (B) (maps supplied by Environmental Systems Research Institute, Inc. http://www.esri.com).

Table 1. Comparison of variable ITS1, ITS2, and 5.8S sites for *H. sinapium*. Only sites where polymorphisms occur are shown. Korean and San Diego (Mission Bay) sequences were obtained from Park et al. (2007). Japanese sequences are those reported by Hoshino et al. (2008). Frequencies of samplings for the Central California genotypes are as follows: type 1- 9 collections, type 2- 9 collections, type 3- 1 collection, type 4- 3 collections, type 5- 1 collection, and Tomales Type- 1 collection.

Seq. Type	Accession No.	Location	143	155	181	195	418	485
Genotype 1	JQ658455, JQ658456, JQ658458, JQ658460–JQ658462, JQ658466, JQ658467, JQ658471	Throughout eastern ES	N/A	A	C	C	T	C
Genotype 2	JQ658450, JQ658451, JQ658453, JQ658454, JQ658457, JQ658463, JQ658468–JQ658470	Throughout northeastern ES	N/A	A	C/T	C/T	T	C
Genotype 3	JQ658464	Whistlestop Lagoon, ES	N/A	A/C	C/T	C	T	C
Genotype 4	JQ658452, JQ658459, JQ658465	South Marsh Loop, ES	N/A	A/C	C	C	T	C
Genotype 5	JQ658472	Kirby Park, ES	N/A	C	C	C	T	C
Tomales Type	JQ658473	Tomales Bay, Marshall California, USA	N/A	A	T	T	T	C
Hymsin7	EF217361	Mission Bay, San Diego California, USA	N/A	C	C	C	T	C
Hyms3	AB373167, AB373168	Southwestern Japan	N/A	A	T	T	T	C
Hymsin1–5	EF217355–EF217359	Southern Korea	N/A	A	T	T	T	C
Hymsin6	EF217360	Taean Chungcheongnam-do Korea	N/A	A	T	T	T	A
Hyms4	ABI373169	Southwestern Japan by Ehime	T	A	T	T	T	C
Hyms1, 2	AB373166	Southwestern Japan by Bungo Channel	N/A	A	T	T	C	C

1 in ES, Tomales Bay, San Diego, southwest Japan, and South Korea. The other three sequence types were nearly identical to the sponge samples from San Diego, differing by 1 polymorphism. The sequence of *H. sinapium* from Tomales Bay was identical to sequences of *H. sinapium* from southwestern Japan and southern Korea (Table 1).

The distribution of *H. sinapium* in ES primarily occupies the eastern shores where it occurs from an undefined region in the northern wetland portion in the main channel (36º50′49″N, 121º45′28″W) and extends to Parsons Slough to the south (36º48′26″N, 121º44′17″W) (Figure 1). Qualitative field observation indicates this sponge to be most prevalent in the marshland in the southeast corner of ES known as the South Marsh. This region also contains the majority of the genetic diversity. In the South Marsh, the sponges were concentrated into two small lagoons that—despite the small geographic range—expressed 4 of the 5 genotypes (Table 1, Figure 1B). The two lagoons, Whistlestop Lagoon and South Marsh Loop, are respectively connected to the larger South Marsh in ES by a tidal drainpipe and a small channel under a walk bridge (Figure 1B). Sponges were very common at the tidal drain and under the walk bridge. At these two points the sampling yielded every genotype except type 5 (Table 1). The two most abundant genotypes in the slough are genotypes 1 and 2 (each represented by 9 individuals), and they were also the most widespread (Figure 1). Genotype 2, unlike genotype 1, contains a heterogenetic signature for two of the polymorphic sites (Table 1).

Discussion

The DNA sequences of *H. sinapium* from ES are consistent with sequences of *H. sinapium* reported by Park et al. (2007) and Hoshino et al. (2008). The population of *Hymeniacidon* in Tomales Bay was listed in Light's manual (Lee et al. 2007) as *Hymeniacidon sp.*; but as Lee et al. (2007) suggested, it may be conspecific with *H. sinapium*. These molecular findings confirm that *Hymeniacidon* in Tomales Bay is assignable to *H. sinapium*. The only discrepancies were three IGPs in the ES sponges (Table 1). Intragenomic polymorphisms are common in sponges and some contain as many as ten genotypes within the ITS region of a single individual (Wörheide et al. 2004). Hoshino et al. (2008) noted the presence of IGPs in *H. sinapium* from Asia, but not from the specimen they analyzed from San Diego, California. In this study, samples showing IGPs were reanalyzed, and they produced consistent results. Continuity within and among the samples indicated that the ITS regions and 5.8S gene are stable markers for this scale of a study in *Hymeniacidon*.

Genetic heterogeneity is well documented in sponges (Duran et al. 2004; Wörheide et al. 2004; Hoshino et al. 2008). Although the comprehensive studies of Park et al. (2007) and Hoshino et al. (2008) analyzed a combined 264 specimens of *H. sinapium* from populations in the Pacific (Figure 1, 2) and Atlantic Oceans, they only reported six genotypes. In contrast, the population of *H. sinapium* from ES exhibited five genotypes. This large number of intragenomic variations in ES for such a relatively small distribution might be explained by introgressive hybridizations that started between the introduced San Diego and Japanese populations (Rhymer and Simberloff 1996; LaJeunesse and Pinzón 2007). The population from San Diego is represented by an ITS1 that exhibits three cytosines at positions 155, 181, and 195, whereas the Japanese population has an adenine and two thymines, respectively. Recent genetic exchanges between these two populations may account for an IGP of *H. sinapium* as displayed by its ITS1 region having combined genetic characters from both San Diego and Japan, with what appears to be a small hybrid swarm of other similar heterogenetic types (Table 1) (Rhymer and Simberloff 1996). Notably the Japanese genotype was not found in ES. If not the result of a sampling bias, then this observation suggests either the Japanese genotypes were never introduced to ES or they were suppressed by the other ES genotypes implying potential survival vigor. Even apparent with this low number of samples, the number of heterogenetic types was nearly equal with those not exhibiting heterogeneity (Figure 1); thus indicating that this heterogenetic form is prevalent in the Slough and might be competing with non-heterogenetic types. More extensive sampling could elucidate the true number of heterogenetic types compared to non-heterogenetic types and address questions of further non-sampled genotypes such as the Japanese sequence or hybrid vigor.

The "hotspot" for *H. sinapium* in ES is South Marsh (Figure 1). This is noteworthy because the marsh was reclaimed wetland until 1980 (Van Dyke and Wasson 2005); consequently, the sponges in this location probably represent the

most recently colonized individuals in ES. It is interesting that these newer *Hymeniacidon* individuals in the South Marsh exhibit greater sponge densities and higher genetic diversity than potentially older colonizations along the main channel. It is likely that the various species in the South Marsh mudflats do not compete with *H. sinapium* for recruitment space as much as the species along the ES main channel; thus the *Hymeniacidon* in the South Marsh could colonize to a greater extent due lower competitive pressures (Shea and Chesson 2002).

The high degree of genotypic diversity suggests that the *H. sinapium* population in ES is the result of multiple introductory events to the slough from at least two separate populations and, based on the presence of three different IGPs, the population is experiencing high rates of gene flow. This multiple-event conclusion is supported by historical records which indicate the slough was repeatedly planted with various species of oyster from the early 1900s to 1980 (Conte 1996; Wasson et al. 2001). A review by Roman and Darling (2007) found many cases where multiple introductions contributed to invasive populations exhibiting greater genetic diversity than each of their native populations. Additionally, from an examination of five molecular studies regarding species introduced by shellfish importation, they cite three studies that showed an increase or no difference in genetic diversity when compared to their endemic populations – further supporting the conclusion that the genetic diversity exhibited by *H. sinapium* in ES is likely a result of multiple introductions.

With regards to its vector, Wasson et al. (2001) hypothesized that *H. sinapium* was introduced to ES by oyster cultivation. Starting in 1929, ES was seeded with Atlantic and Japanese oysters (Bonnot 1935). Considering that *C. gigas* seed was sourced from parts of Japan in which *H. sinapium* is distributed (Park et al. 2007; Hoshino et al. 2008), it is reasonable to conclude that Japan is the geographic origin for this sponge in ES. An alternative hypothesis is that *H. sinapium* (*H. heliophila* Parker, 1910, Hoshino et al. 2003) was introduced to California by means of the Atlantic oyster. There is no support for this because the sequences exhibited by *H. sinapium* in the Atlantic are not found in San Diego Bay, Tomales Bay, or ES in California. The Californian genotypes however, are found in Asia. This raises the hypothesis proposed by Hoshino et al. (2008), that because of its low genetic diversity in Japan, *H. sinapium* is possibly an invader to Asia. This too is unlikely considering that ES is a small estuary with no international shipping, and to our knowledge neither ES nor any of the other sites in California have served as the source of any seed for any invertebrate having mariculture purposes in Japan or elsewhere (Barrett 1963; Fujiya 1970). Hence the population in ES must be introduced from Asia.

The data confirm the putative invader's identity in ES as *H. sinapium*, support its Japanese origins, and quantify its genetic diversity. All of these aspects are important to conservation management. Genetic techniques, through accurate identification, can help find the endemic range for an exotic (Stefaniak et al. 2012). Once identified, the endemic populations can be used as a comparative resource against the invading populations to isolate mechanisms contributing to the invaders' successful colonization (Zambrano et al. 2006; Edelist et al. 2012). Molecular techniques could also infer vectors for invasions so conservation efforts could institute regulation that constricts or prevents species invasions (Goldstien et al. 2010). Additionally, increased genetic diversity and gene flow has been shown to aide species invasiveness (Ellstrand and Schierenbeck 2000; Roman 2006). As a result, some researchers advocate hampering gene flow as a form of invasive species remediation (Dlugosch and Parker 2007). With these molecular tools, aggressive species are recorded and their threat is quantified so as to mitigate their ecological impacts.

Elkhorn Slough is the second largest estuary in California and was the United States' first estuarine sanctuary (Schwartz et al. 1986). From an ecological, geographical, and historical perspective, it is important to understand that the community make-up in this estuary represents the long-term effects of initially significant anthropogenic disruption and later as the product of exhaustive conservation efforts. Scientists examining the inhabitants of this wetland not only can analyze the impacts from historical disturbance, they can determine the efficiency of past and current conservation measures. Genetic tools can aid this analysis of past influences and current conservation. This paper demonstrated that information from molecular investigations can surpass simply confirming a species' identification, it can consider the exotic status of a alleged invader based on its genetic diversity and trace its genetic origins.

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Supplementary material

The following supplementary material is available for this article.

Appendix 1. Records of *Hymeniacidon sinapium* from Elkhorn Slough, and Pacific Ocean coastlines.

This material is available as part of online article from:
http://www.aquaticinvasions.net/2013/Supplements/AI_2013_Fuller_Hughey_Supplement.pdf