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**Research Article**

# Genetic variation in native and introduced populations of the red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Crustacea, Decapoda, Cambaridae) in Mexico and Costa Rica

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## Abstract

The genetic variation among nine populations of the red swamp crayfish *Procambarus clarkii* was examined using partial sequences of the mitochondrial COI gene. Three populations (Illinois and Louisiana, United States and northern Coahuila, Mexico) represented the native range and six populations came from areas where the species has been introduced (central Coahuila, southern Nuevo León, Durango, Chihuahua and Chiapas, Mexico, and Cartago, Costa Rica). A 689 bp fragment was amplified from 37 samples. Uncorrected genetic distances among sequences were p = 0 to 0.02031 and 12 haplotypes were found. A phylogenetic reconstruction shows that the three populations from the native range remain very similar to each other and some introduced populations can be directly associated to one of them. The populations from Nuevo León, central Coahuila and Costa Rica were the most divergent ones. Overall the genetic variation found in *P. clarkii* in both native and introduced populations is low.

**Key words:** *Procambarus clarkii*, COI, mtDNA, introduced species

## Introduction

The impact of introduced crayfish on ecosystems, communities and native species has been widely recognized, with the loss of biodiversity being one of the most important effects (Harper et al. 2002; Nystrom 2002; Rodríguez et al. 2005). Many of the documented introductions were planned to initiate production programs (Lowery and Mendes 1977; Barbaresi and Gherardi 2000). Three species of crayfish have been reported as introduced and having now established viable wild populations in Mexico: the red swamp crayfish *Procambarus clarkii* (Girard, 1852), the northern crayfish *Orconectes virilis* (Hagen, 1870) and the Australian red claw crayfish *Cherax quadricarinatus* (von Martens, 1868) (Campos and Contreras-Balderas 1985; Campos and Rodríguez-Almaraz 1992; Bortolini et al. 2007). The red swamp crayfish *P. clarkii* is one the most highly introduced species of freshwater invertebrates around the world. Native to northern Mexico, in northern Tamaulipas and Nuevo Leon, and the southern United States, from Texas, Louisiana and Florida to Ohio and southern Illinois (Hobbs 1972; Rodríguez-Almaraz and Muñiz 2008), it has been reported from over 20 countries (Souty-Grosset et al. 2006; Foster and Harper 2007). Although native to northern Mexico, *Procambarus clarkii* has also been introduced into other regions within Mexico in Durango, Sonora, Baja California and Chiapas (Hernández et al. 2008; Alvarez et al. 2011).

Regarding the genetic variation of introduced populations, investigations have focused on two main questions: first, which is the source for the introduced population, as their origin is often unknown and traits from the source population can be useful to understand the ecological context that allows the invaders to become successfully established (Holway et al. 1998; Figueroa et al. 2010); and second, from an evolutionary standpoint, how can an introduction often consisting of a few individuals, with a presumed limited genetic variation, generate enormous populations (Tsutsui et al. 2000)?

In the case of *P. clarkii* both questions are especially relevant because so many introduction events have occurred and even subsequent reintroductions into the same areas apparently have occurred several times (Barbaresi et al. 2003). A population of an invasive species in the introduced range that originated from multiple sources can show through admixtures with above-normal levels of genetic variation (Kolbe et al. 2007). Studies on genetic variation can be viewed as a first step to understand how a species can become a successful invader. However, studies comparing behavioral or ecological traits of individuals from the native and introduced ranges are needed to elucidate more precisely what key traits are contributing to their success.

In this study we compare the genetic variation in the mitochondrial gene COI of *P. clarkii* among samples from the native range in Mexico and the United States and from introduced populations in western and southern Mexico and Costa Rica. The goals of the comparison are to explore the identity of the source populations of the introduced populations and the degree of variation among them.

## Materials and methods

The samples of *Procambarus clarkii* analyzed in this study came from six introduced populations in Mexico: Chihuahua, Coahuila (Coahuila-central), Nuevo Leon, Durango and Chiapas; and Cachí Dam, Province of Cartago, Costa Rica, (Table 1, Figure 1). From the native range we included samples from northern Coahuila (Coahuila-north), Mexico, and Louisiana and a sequence from a population in Illinois, in the United States, from Genbank (Table 1, Figure 1).

### *Analysis of mitochondrial DNA*

The total DNA of the freshwater crayfish was extracted following the phenol-chloroform procedure suspending it in 100 µl of distilled water and stored at -20°C as the last step (Hillis et al. 1996). Sequences of a fragment of the mitochondrial gene COI of approximately 700 pb were obtained with primers LCO 1490 and HCO 2198 (Folmer et al. 1994). Polymerase chain reaction (Saiki et al. 1988) was performed using a Bio Rad MJ Mini Personal Thermal Cycler. The 25 µL reaction volume consisted of 1.5 µL of each primer, 2.5 µL of buffer, 2.5 µL of dNTP´s, 1.25 µL of $MgCl_2$, 0.125 µL of Taq polymerase and 1 µL of DNA, the rest was $ddH_2O$. Thermal cycling conditions were: 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec and extension at 72°C for 1.3 min, followed by a final extension at 72°C for 1.3 min. Agarose gel electrophoresis was used and the products obtained were visualized in a UV transilluminator. The products that were positive for amplification were purified using MontageTM PCR columns (Millipore, Bedford, U.S.A.) and the purified product was re-suspended in 20 µl of ddH2O. The following step was the cyclical sequencing in both directions (Hillis et al. 1996) using the BigDye Terminator kit (Applied Inc. Biosystems) and the same primers. Sequenced products were purified using Sephadex and Centri Sep Spin Columns (Princeton Separations, Adelphia, N.J.). The products were processed in a 3100 Genetic Analyzer (16 capillaries) ABI Prism automated sequencer.

### *Analysis of DNA sequences*

The obtained sequences were visualized with the software Chromas 2 and visually aligned with BioEdit 7 (Hall 1999). jModelTest 0.1.1 (Posada and Crandall 1998) was used to determine the appropriate model that better fit the nucleotide substitution in the obtained sequences. Nucleotide diversity "$\pi$" was estimated with the DnaSP v5 (Librado and Rozas 2009) program to determine the degree of variation between the different sequences (Avise 1999), base frequencies were obtained and tested with chi square ($\chi^2$) for significant differences among sequences and the pairwise nucleotide sequence divergence was calculated using p distances, the former estimates were obtained using PAUP (Swofford 2002). The different haplotypes were identified and its diversity "HD" was obtained with the DnaSP v5 program (Librado and Rozas 2009). An uncorrected distance phenogram was elaborated using PAUP (Swofford 2002) with the nearest neighbor (NJ) method and the model suggested by jModelTest 0.1.1 (Posada and Crandall 1998). In order to test the branch support a bootstrap routine with 1000 pseudo replicates was carried out. Phylogenetic analysis of the COI data set employed both maximum parsimony (MP) and maximum likelihood (ML) optimality criteria. The ML analysis was run in PAUP (Swofford 2002) using a heuristic search with tree bisection - reconnection branch

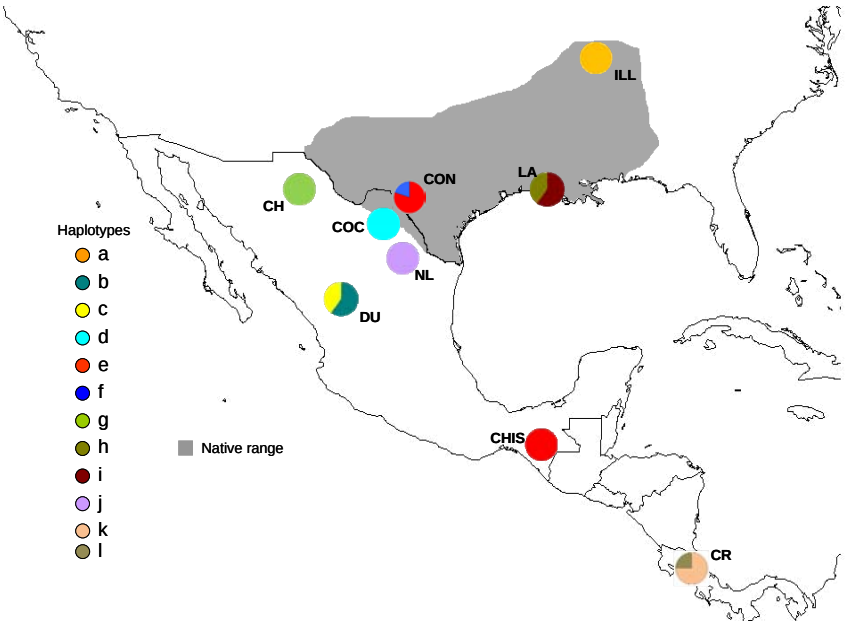


**Figure 1.** Geographic distribution of of haplotypes of *Procambarus clarkii*, within the native range (in grey) and those introduced, included in this study: ILL, Illinois, U.S.A.; LA, Louisiana, U.S.A.; CH, Las Varas, Chihuahua, Mexico; CON, Río Jimenez, Coahuila, Mexico; COC, Río Sabinas, Coahuila, Mexico, NL, Sabinas Hidalgo, Nuevo Leon, Mexico; DU, El Arenal, Durango, Mexico; CHIS, Teopisca, Chiapas, Mexico; CR, Cachí Dam, Costa Rica.

**Table 1.** Localities of the analyzed native (bold) and introduced populations of *Procambarus clarkii* in Mexico, United States and Costa Rica.

| Country | State | Locality | Geographic coordinates | | No. of organisms |
|---|---|---|---|---|---|
| United States | Illinois | **Horseshoe Lake (ILL)** | 37°08'18.8″ | -89°20'33.5″ | 1 |
| | Louisiana | **Kaplan (LA)** | 29°59'29.5″ | -92°15'36.8″ | 5 |
| Mexico | Chihuahua | Las Varas (CH) | 29°47'49.8″ | -106°41'33.2″ | 3 |
| | Coahuila | **Río Jiménez (CON)** | 29°09'16.3″ | -100°45'51.5″ | 5 |
| | | Río Sabinas (COC) | 27°58'09.7″ | -101°34'54.2″ | 5 |
| | Nuevo León | Sabinas Hidalgo (NL) | 26°29'00.8″ | -100°13'15.2″ | 4 |
| | Durango | El Arenal (DU) | 24°02'35.6″ | -104°25'42.0″ | 5 |
| | Chiapas | Teopisca (CHIS) | 16°33'13.8″ | -92°28'34.4″ | 5 |
| Costa Rica | Cartago | Cachí Dam (CR) | 09°49'30.6″ | -83°49'15.9″ | 4 |

**Table 2.** Uncorrected (p) distance matrix estimated from COI haplotypes. Capital letters identify the population (Figure 1), in bold those from the native range, the haplotype appears in lower case (Figure 2). Coahuila-north "e" haplotype is the same as that from Chiapas.

| | **ILL** a | DU b | DU c | COC d | **CON** e | **CON** f | CH g | **LA** h | **LA** i | NL j | CR k | CR l |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ILL** a | | | | | | | | | | | | |
| DU b | 0.009 | | | | | | | | | | | |
| DU c | 0.007 | 0.007 | | | | | | | | | | |
| COC d | 0.010 | 0.015 | 0.013 | | | | | | | | | |
| **CON** e | 0.003 | 0.006 | 0.004 | 0.009 | | | | | | | | |
| **CON** f | 0.006 | 0.009 | 0.007 | 0.012 | 0.003 | | | | | | | |
| CH g | 0.007 | 0.010 | 0.009 | 0.013 | 0.004 | 0.007 | | | | | | |
| **LA** h | 0.007 | 0.007 | 0.003 | 0.013 | 0.004 | 0.007 | 0.009 | | | | | |
| **LA** i | 0.009 | 0.009 | 0.001 | 0.012 | 0.006 | 0.009 | 0.010 | 0.004 | | | | |
| NL j | 0.007 | 0.010 | 0.009 | 0.004 | 0.004 | 0.007 | 0.009 | 0.009 | 0.010 | | | |
| CR k | 0.016 | 0.020 | 0.019 | 0.015 | 0.015 | 0.017 | 0.019 | 0.019 | 0.017 | 0.019 | | |
| CR l | 0.017 | 0.020 | 0.020 | 0.016 | 0.016 | 0.019 | 0.020 | 0.020 | 0.019 | 0.020 | 0.001 | |

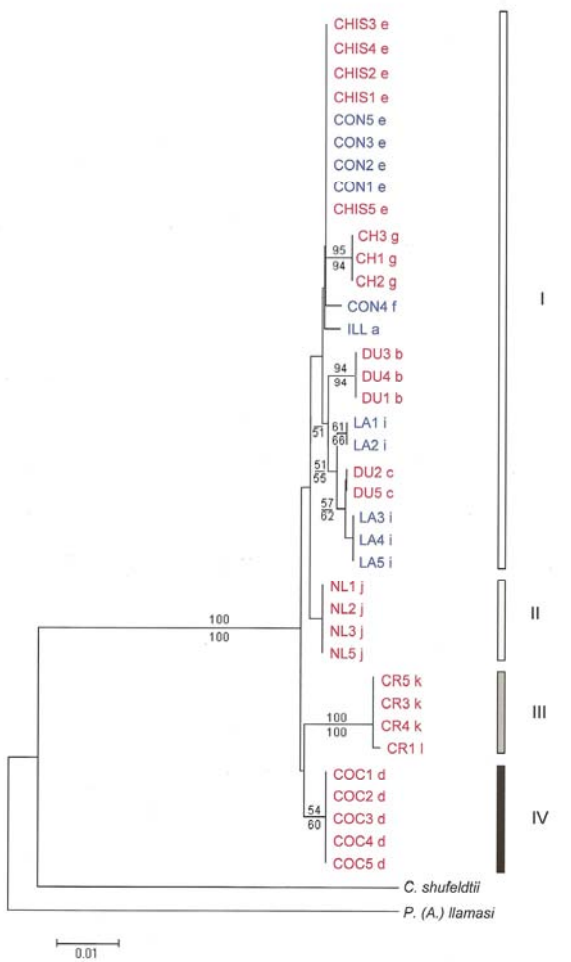


**Figure 2.** Distance tree (NJ) based on 37 sequences with population, haplotype and outgroup, based on a GTR nucleotidic model. Bootstrap values above the branches correspond to ML analysis and to MP below the branches. In green: localities from the native range, in red: the introduced ones.

swapping. Prior to the maximum likelihood analysis, jModelTest 0.1.1 (Posada and Crandall 1998) was used to determine an appropriate model of sequence evolution. A MP heuristic search was conducted in PAUP with tree bisection-reconnection branch swapping. Transitions and transversions were weighted equally. For both, MP and ML trees, bootstrap support values were computed based on 500 replicates. Sequences of *Procambarus* (*A.*) *llamasi* obtained in Cacao, Quintana Roo, Mexico (access number JN000909) and of *Cambarellus shufeldtii* from the GenBank (access number EU921149) were used as outgroups in all cases. Finally a haplotype network was obtained using statistical parsimony with TCS v1.21 (Clement et al. 2000), to explore the relationships among populations.

## Results

A total of 37 sequences were obtained from 9 populations, including one from GenBank. The length of the amplified region of the COI gene was 689 bp that correspond to the interval between sites 28 and 716 of the complete gene (1534 pb) taken from the blue crab *Callinectes sapidus* (GenBank access number AY682075). Nucleotide diversity was $\pi$ = 0.0084. The frequency of nucleotides in the alignment was of A= 0.2706, C= 0.1307, G= 0.194, T= 0.4047, no significant differences among nucleotide composition were found. ($\chi^2$ = 2.357, df= 108, p=1). Uncorrected genetic distances among sequences were p = 0 to 0.02031, which is equivalent to 0 to 14 variable sites (Table 2). Among sequences 26 variable sites were observed of which 23 were parsimony informative. The changes between nucleotides, taking the first codon position were: 18 transitions, 4 transversions and 4 sites had both in different populations. Twelve haplotypes were found (Tables 2, 3) with a total diversity of HD = 0.901. Two haplotypes were found for Coahuila-north, Costa Rica, Durango and Louisiana; Coahuila-north and Chiapas shared the same haplotype; Chihuahua, Coahuila-central, Illinois and Nuevo Leon had a single unique haplotype each (Figure 2).

The three methods used (NJ, MP, ML) for the phylogenetic reconstruction showed the same result. The tree shows four main groups (Figure 3). Group I includes all the native populations (Coahuila-north, Louisiana and Illinois) and three introduced populations (Chiapas, Chihuahua and Durango). The differences among these populations are very small, Coahuila-north haplotype “e” differs in two nucleotide changes from Chihuahua and one from Illinois, being identical to Chiapas. Louisiana and Durango with two unique haplotypes each mix together, with haplotype “b” from Durango being slightly divergent. Group II consists of four identical and unique sequences from the introduced population in Nuevo Leon. Group III, represented by the population from Costa Rica, also introduced, has

**Table 3.** Haplotypes and GenBank access numbers for the mitochondrial gene COI of the studied *Procambarus clarkii* populations.

| Population and specimen | Haplotype | Site | | | | | | | | | | | | | | | | | | | | | | | | | GenBank access no |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 6 | 17 | 39 | 72 | 81 | 141 | 195 | 207 | 333 | 342 | 453 | 485 | 510 | 513 | 573 | 610 | 639 | 671 | 677 | 681 | 683 | 684 | 685 | 686 | |
| Illinois | a | A | T | G | C | T | G | G | T | T | T | T | A | A | C | A | G | G | C | A | T | T | T | G | A | C | A | AY701195 |
| Durango | b (3) | • | • | A | • | • | • | • | • | G | C | • | • | • | T | • | • | A | • | • | • | • | • | • | T | • | • | JN000898 |
| | c (2) | • | • | A | • | • | • | • | • | • | • | C | • | • | • | • | • | A | T | • | • | • | • | • | T | • | • | JN000899 |
| Coahuila central | d (5) | • | • | A | • | C | • | A | • | • | • | • | • | • | • | • | A | • | • | • | • | • | G | T | • | • | C | JN000900 |
| Coahuila north | e (4) | • | • | A | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | T | • | • | JN000901 |
| | f (1) | • | • | A | T | • | A | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | T | • | • | JN000902 |
| Chiapas | e (5) | • | • | A | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | T | • | • | JN000901 |
| Chihuahua | g (3) | • | • | A | • | • | • | • | • | • | • | • | G | • | • | G | • | • | • | G | • | • | • | • | T | • | • | JN000903 |
| Louisiana | h (2) | • | • | A | • | • | • | • | • | • | • | C | • | G | • | • | • | A | • | • | • | • | • | • | T | • | • | JN000904 |
| | i (3) | • | • | A | • | • | • | • | • | • | • | C | • | • | • | • | • | A | T | • | • | • | • | • | T | • | C | JN000905 |
| Nuevo León | j (4) | • | • | A | • | C | • | A | • | • | • | • | • | • | • | • | A | • | • | • | • | • | • | • | T | • | • | JN000906 |
| Costa Rica | k (3) | C | A | A | • | • | • | • | C | • | • | • | • | • | • | • | • | • | • | • | C | C | G | A | C | A | C | JN000907 |
| | l (1) | C | A | A | • | • | • | • | C | C | • | • | • | • | • | • | • | • | • | • | C | C | G | A | C | A | C | JN000908 |

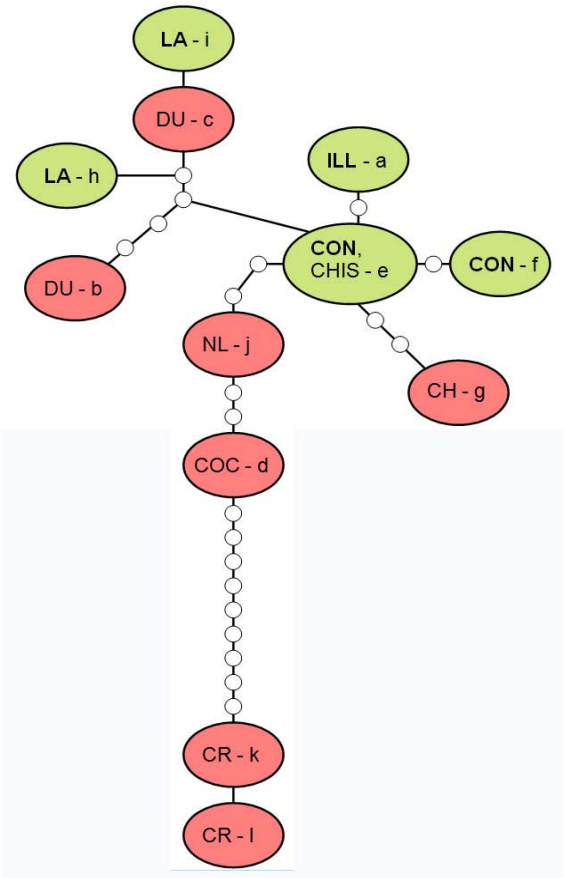


**Figure 3.** Haplotype network constructed using the haplotypes of the COI gene of *Procambarus clarkii*. Small circles represent intermediate haplotypes not obtained in the analysis. Letters a-l refer to the different haplotypes, other abbreviations correspond to the localities, in green haplotypes from the native range, in red the introduced ones.

the two more divergent haplotypes, a fact that is reflected in the estimated p distances (Table 2). Group IV contains five identical sequences from the introduced Coahuila-central population.

The obtained haplotype network adds interesting hypotheses showing the possible relationships of haplotype “e”, shared by the Coahuila-north and Chiapas populations, with other four populations (Illinois, Coahuila-north haplotype “f”, Chihuahua, and Nuevo León) and with a group formed by haplotypes from Durango and Louisiana (Figure 4). The network shows a close relationship of all the haplotypes from the native range with those from Durango, Nuevo León and Chihuahua. The haplotypes from Coahuila-central and Costa Rica are the most divergent ones.

## Discussion

The obtained results suggest that the three populations from the native range remain very similar to each other and some introduced populations can be directly associated to one of them. Chiapas could derive from Coahuila-north as both populations share the same haplotype; similarly Durango could derive from Louisiana as the haplotypes from both are very similar and mingle in the same clade. The haplotype from Chihuahua appears to be equally similar to Illinois and to Coahuila-north. The Nuevo Leon and Coahuila-central populations could have originated from Coahuila-north, which is both geographically and genetically close. The most

divergent population was that from Costa Rica, where *P. clarkii* was first introduced around 1966 (Huner 1977). Thus, in regards to the question of origin of the introduced populations, our results show that the resolution level given by mtDNA can be used to trace some direct relationships.

Several hypotheses have been proposed to explain the genetic variation found in introduced populations of *P. clarkii*. Although the different molecular techniques used in crayfish detect genetic variability at scales that may not be directly comparable, for example the use of mtDNA sequences versus allozyme variability, microsatellites or RAPD's (Barbaresi et al. 2003, 2007; Yue et al. 2010), some general issues can be addressed. One of the first studies to describe the genetic variability of *P. clarkii* is that of Busack (1988) who studied nine populations within the native range of the species in the southeastern United States, from Illinois to Texas, using enzyme electrophoresis. His results showed very little variation among populations (low heterozygosities and genetic distances) that was attributed to a probable recent origin of the species in the late Miocene, when most of the current native range became emergent. Yue et al. (2010) using microsatellites obtained a "significant heterozygote deficit" in six populations of *P. clarkii* studied in China, including one that probably represents the first population introduced to China in 1929. The authors hypothesized on the occurrence of recent bottlenecks as the factor that has reduced the genetic variability of these populations. Barbaresi et al. (2007) obtained sequences of the 16S and COI mitochondrial genes of *P. clarkii* of 12 populations in Italy, Spain, France, Portugal and Switzerland. No variation was found in the 16S sequences (one haplotype) and with COI, 6 haplotypes were identified with one of them present in 10 of the 12 populations; however, in the latter case all haplotypes were very similar differing by one or two nucleotide substitutions.

In contrast with the results described above, Barbaresi et al. (2003), using RAPD markers, found an "unexpectedly high" degree of genetic variation in *P. clarkii* populations from Italy and Portugal, associating this pattern to repeated introductions into the same areas. RAPD techniques have a finer resolution, thus making these results difficult to compare with the aforementioned studies.

These commented studies as well as the one presented herein agree in that both, within the native range and throughout very diverse introduced ranges, *P. clarkii* exhibits reduced genetic variation. In this study and that of Busack (1988) populations from the native range were genetically very similar, suggesting that independent from the number of organisms introduced little genetic variation is to be found. Regarding introduced *P. clarkii* populations, it is interesting to note that very little differentiation has occurred with respect to native populations, even after 80 or more years in the case of populations from east-central China (Yue et al. 2010) or more than 35 years in the case of European populations (Barbaresi et al. 2007). However, due to the low genetic variation found, microsatellite analyses might be more helpful.

Based on the low genetic variation found for *P. clarkii* in this and other studies, the question remains as to how such a species can become a successful invader? A recent review of the available evidence suggests that many introduced species have undergone genetic bottlenecks and still have been able to become established and prosper in the introduced range (Dlugosch and Parker 2008). Baker (1965) proposed the idea of a "general-purpose genotype" which would produce through plasticity different phenotypes to cope with a range of environmental conditions. This is certainly a model that fits what has been observed for *P. clarkii* across the world in different introduction events (Barbaresi et al. 2007; Yue et al. 2008, 2010). However, further comparisons of key attributes between native and introduced populations are needed to determine if it is plasticity alone or rapid adaptation what has made *P. clarkii* a successful invader.

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