A history of invasion: *COI* phylogeny of Manila clam *Ruditapes philippinarum* in Europe

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Abstract

The Manila clam *Ruditapes philippinarum* - synonym *Venerupis philippinarum* (Adams and Reeve, 1850) is now one of the top 5 most commercially valuable bivalve species worldwide. Originally from the Indo-Pacific region, it has been introduced in many countries for fisheries and aquaculture, including estuarine environments along Atlantic and Mediterranean European coasts. Yet despite its commercial value and widespread distribution, the precise origins of stocks remain speculative and the genetic diversity of introduced populations is poorly known. Thus, the aim of this work was to collect mtDNA *COI (Cytochrome oxidase I)* gene sequences from 5 European countries with Manila clam stocks and compare them with native Asian populations to evaluate their genetic diversity and identify possible routes of invasion. The *COI* gene sequencing supported a strong founder effect in the European populations with 3 main haplotypes occurring at high frequencies, derived from Japan. However, high haplotype diversity was also observed due to the occurrence of 10 rare haplotypes. This supports hypotheses (i) there have been additional, previous unrecorded, introductions as previously hypothesized by analysis of *16S* rDNA, and (ii) there has been a limited loss of genetic diversity in introduced populations, as previously suggested by microsatellite data. This is the first genetic comparison of Manila clam populations introduced in to Europe with native clams. Genetic data herein presented are fundamentally important for the traceability of clam products and stock management programmes and will also inform discussion on the potential resilience of exploited Manila clam populations.

Key words

Manila clam, *COI*, genetic diversity, Europe, Non-indigenous species

1. Introduction

Among commercially exploited bivalves, the Manila clam *Ruditapes philippinarum* - synonym *Venerupis philippinarum* (Adams and Reeve, 1850) is of considerable international importance and considered among the top 5 most commercially valuable bivalve species worldwide (over 250,000 tons for year) (Astorga, 2014). Originally distributed in the Indo-Pacific region it has been introduced in many countries for fisheries and aquaculture (Gosling, 2003), including European Atlantic and Mediterranean coastal waters (Gosling, 2003). As reported by Flassch and Laborgne (1992), until the 1990s the main European stocks originated from a small pool of organisms introduced from North America (see Table 1 for a summary of initial introductions in Europe). Following the available data on licensed introductions, the first introductions in Europe dates back to 1972-1974 in Arcachon Bay, France by IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer). Flassch and Leborgne (1992) reported that a total of 500,000 spat, and 1,000 adults from Puget Sound (South Western Canada, Pacific coast) were introduced into the Arcachon Bay, roughly representing a total biomass of 70 kg. The same population from Puget Sound was used for the first introduction of Manila clam in the UK in 1980, at the MAFF (Ministry of Agriculture, Fisheries and Food) Fisheries Laboratory, Conwy-North Wales (Humphreys et al., 2015). The near-by Menai Strait was identified as the location of the first introduction into UK coastal waters in 1983 (Humphreys et al., 2015). In the same year, the first introduction in the Northern Adriatic Sea also occurred, conducted by the Co.S.PA.V (Consorzio per lo Sviluppo della Pesca e dell'Acquacoltura del Veneto) in the Venice lagoon using seed from Great Britain (Breber, 1985). In a short period of time Manila clam was introduced in other Adriatic coastal lagoons, namely Marano, Caleri, Scardovari, Goro (Pellizzato, 1990). All these first introductions were conducted with clams coming from SeaSalter Shellfish Company (M. Pellizzato, pers. Comm.) which operated from hatcheries in south-east and north-west England, and the company was established with clams from Conwy (Humphreys et al., 2015). In Spain, Manila clam was already occurring in the mid '80s (Perez-Camacho and Cuna, 1985) in many different coastal

areas (Galicia, Cantabria, Andalusia, and Cataluña). The first report of Manila clam in Portugal dates back to 1984 in Ria Formosa (Algarve) probably originated from Spain (Ruano and Sobral, 2000), even if no information about the status of the Spanish "source" population (hatchery or naturalised) is available. The species is not yet licensed in Portugal (Chainho et al., 2015) even if aquaculture was the most likely vector of introduction (Chainho et al., 2015). However, since the '80s, naturalised Manila clam populations have been reported in many estuarine systems all over the country (Gaspar, 2010; Chainho, 2014; Chainho et al., 2015; Velez et al., 2015a; b). Today Manila clam is considered the dominant bivalve species in the Tagus estuary and is one of the most abundant clams in the Ria de Aveiro and Sado estuary (Chainho, 2014; Velez et al., 2015a). Nowadays, the production of Manila clam in Europe derives mainly from fisheries of naturalised populations, established after human-mediated introductions. This is the case in France, specifically Arcachon Bay, where the whole production derives from the original introduced and naturalised population (Bald et al., 2009; Sanchez et al., 2014), and also England (Humphreys et al., 2015), Spain (e.g. the Bay of Santander- Bidegain and Juanes, 2013) and Portugal (Chainho et al., 2015). Detailed literature data are available for UK, where the first reported naturalized Manila clam population was observed in Poole Harbour (Jensen et al., 2004), where the first licensed introduction dates back to 1988 from seeds originated from Conwy hatchery, and wild clams appeared about two years latter (Humphreys et al., 2015). Between 1980 and 2010 the Manila clam has become naturalized in 11 British estuaries. The most extensive newly established wild populations is in Southampton Water, which lies about 48 km east of Poole Harbour, and where Manila clam likely arrived in 2002 (Humphreys et al., 2015). It is possible that this has originated via natural larval dispersal from Poole Harbour (Herbert et al., 2012) or anthropogenic means (Humphreys et al., 2015).

Aquaculture facilities have been also successfully established for Manila clam in UK, Italy (Northern Adriatic Sea) and in Spain, especially in Galicia (Robert et al., 2013). In Spain, hatcheries

mainly provide seeds for local associations of producers. Most of the production takes place in private parks (concessions for a period of years) and on beaches that are managed by local associations (Robert et al., 2013). In Italy, Manila clam spread occurred rapidly, and quickly populations became naturalised (Pellizzato, 1990) thus its exploitation became the most economically important fishing activity, especially in the Venice Lagoon (see Boscolo Brusà et al., 2013 for a complete list of references). However, due to the initial lack of reliable regulation and unsustainable exploitation of fisheries resources, there has been a constant decrease in clam production (Boscolo Brusà et al., 2013), which determinated a recent transition from clam fishing to clam farming activities, and to the rational management of natural spat (Boscolo Brusà et al., 2013). Currently, in the Venice lagoon most clam harvesting is carried out in licensed areas directly managed by farmers (Boscolo Brusà et al., 2013), using seeds derived from natural spat. This system has been already established in other Northern Adriatic Sea lagoons, like Goro lagoon, where the production remained stable for almost 3 decades (Bartoli et al., in press). In general, the problem for Manila clam cultivation in Europe is the same as global shellfish aquaculture: high quality seed availability (Robert et al., 2013). Although efforts have been made to improve the hatchery production, clam farming of Manila clam still depends on natural seeds (Robert et al., 2013).

As underlined in previous paragraphs, Manila clam is a valuable economic resource for some European countries. However, as pointed out by Astorga (2014), although aspects of the species biology have been studied genetic resources are still largely unknown. For several fisheries and aquaculture commercial species, especially fish, biotechnology and genetic research have developed significantly in the last decade (Astorga, 2014); however similar applications for valuable molluscs have been minimal (Astorga, 2008; 2014) and for Manila clam in particular. In fact, whole genome reference sequences, high-density SNP genotyping arrays or genotyping-by-sequencing have been developed especially for fish (Yáñez et al., 2015). As for Manila clam, few studies have been

devoted to the genetic diversity and structure of populations in its native range (see as examples Sekine et al., 2006; Vargas et al., 2008; Liu et al., 2007; Mao et al., 2011; An et al., 2012; Kitada et al., 2013, Nie et al., 2015) and in introduced ecosystems (Chiesa et al., 2011; 2014; 2016; Mura et al., 2012; Hurtado et al., 2012). Yet a comparative study of native and introduced populations has not previously been undertaken and no genetic information is available concerning the differences occurring among productive stocks worldwide, or potential invasion pathways that might compromise the ability to perform predictions of genetic diversity and population structure of non-indigenous taxa (Holland, 2000).

The genetic structure of an invasive population depends on several factors, including the effective population size at the time of introduction and the genetic diversity of the source population (Holland, 2000). If an introduction occurs as a single event, starting from a limited number of founders, population genetic theory predicts that alleles will be fixed and lost at an accelerated rate relative to the source population (Mayr, 1963; Hartl and Clark, 1997; Holland, 2000). The gene pool of the introduced population is expected to be limited, as a result of the stochastic process of the introduction mechanism (Holland, 2000). However, if the introduction involves a large genetically diverse assortment of individuals, it is expected to have little or no reduction in heterozygosity and allelic diversity relative to the gene pool of the source population (Holland, 2000). In fact, a founding population which derives from numerous previously isolated populations has the potential to produce a genetically highly diverse assortment of offspring. It has been already proposed by Roman and Darling (2007) that invasions from multiple discrete source populations, or admixture, may be the standard rather than the exception in invasion biology and that the co-occurrence of mitochondrial lineages, geographically separated in the native range, could be considered an evidence of multiple introductions events (Taylor & Keller, 2007).

Furthermore, genetic data on Manila clams is fundamental for studies associated with clam traceability and safety, preventing fraud and supporting management programmes of exploited

populations. This is particularly important for a highly exploited resource like Manila clam, both for fisheries and aquaculture. In fact, the erosion of the genetic diversity determinates a high risk of introgression and a reduction of fitness of the exploited populations, and also their resilience capability as relict populations (Frankham et al., 2010). In the Venice lagoon, an overexploitation of Manila clam that has occurred in the last decades has resulted in a huge reduction of the naturalised population (Boscolo Papo et al., 2013) with possible consequences for genetic diversity and demographic structure.

Previously, studies conducted on Manila clam populations from the Northern Adriatic Sea, Portugal (Ria de Aveiro) and Spain (Galician coast) demonstrated a strong founder effect by *16S*rDNA gene sequencing, but also enhanced haplotype diversity occurring in introduced populations (Chiesa et al., 2011, 2014). Moreover, microsatellite genotyping in the same populations showed a limited loss of genetic diversity, and even though several loci were affected by null alleles, globally the number of alleles was comparable to those observed in native Asian populations (Chiesa et al., 2011, 2016; Chiesa et al., in press).

Considering that previous studies on Asian populations were also conducted with *COI* gene fragment sequencing (Sekine et al., 2006; Mao et al., 2011; Kitada et al., 2013), the present work aimed to collect mtDNA *COI* gene sequences also from 5 European countries hosting Manila clam aquaculture and fishing activities, and for the first time to compare genetic diversity between these introduced stocks and native Asian populations. This is the first genetic study to investigate invasion routes of Manila clams in Europe and the genetic diversity of commercial stocks, which will contribute to the basic knowledge in the field of invasion biology, and support management programmes of this valuable economic resource in European countries.

2. Methods

2.*1 Sampling procedures*

Manila clam was collected from introduced naturalised populations in the Northern Adriatic Sea (N= 111), and along the Atlantic coast in Portugal (Ria de Aveiro lagoon, Óbidos lagoon and Ria Formosa, Tagus and Sado estuaries, N = 71), North Western Spain (Galicia, N = 10), South Western France (Arcachon, N = 15) and Southern UK (Poole Harbour and Southampton, N =16). A total of 223 samples were analyzed. Details on sampling locations are provided in Fig.1 and Table 2. Haplotypes previously identified by *16S* rDNA (Chiesa et al., 2014) were resubmitted for *COI* genotyping.

2.2 DNA extraction and purification

High molecular weight genomic DNAs were extracted and purified from ethanol-fixed mantle and foot tissue stored at -20 °C using the Wizard genomic DNA Purification kit (Promega) following a standardized protocol (Chiesa et al., 2011; 2014). Ethanol-fixed mantle and foot tissue stored at -20 °C were selected for the extraction to avoid the interferences of the DUI – the Doubly Uniparental Inheritance (Plazzi and Passamonti, 2010). This phenomena was already described in bivalves like Manila clam (Passamonti and Scali, 2001) and blue mussel (Zouros et al., 1994), implying the existence of two mtDNAs in adult males, the so called "F – type" mitochondrial genome which prevails in somatic tissues, while the so called "M-type" mitochondrial genome is strongly predominant in gonads (Cao et al., 2004). Sperm carry only M-type mtDNAs, which nucleotide sequence can diverge from the F-type mtDNA up to the 30%. For this reason, for phylogenetics and biogeographic analyses the F-type DNA should be selected, due to its maternal inheritance. To avoid the co-extraction and amplification of M-type mtDNA, specific tissues should be selected for DNA extraction, as they carry a very little quantity of M-type mtDNA, even in males. Generally mantle and foot tissues are selected for clams (see as examples Kappner and Bieler, 2006; Plazzi and Passamonti, 2010; Chiesa et al., 2011).

2.3 Mitochondrial DNA analyses

Amplification of a *COI* gene fragment was achieved with a multiple set of primers: *COI* universal primers *LCO1490*: 5'-GGTCAACAAATCATAAAGATATTGG-3' and *HCO219*8: 5-'TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al., 1994); degenerated *COIF-ALT*: 5'-ACAAATCAYAARGAYATYGG-3' and *COIR-ALT:* 5'-TTCAGGRTGNCCRAARAAYCA-3' designed for Veneridae family (Kappner and Bieler, 2006; Mikkensen et al., 2006) and specific Manila clam primers designed by PRIMER 3 (Rozen and Skaletsky, 1998) named *COI ALT LIV FW:* 5'-AACMAATCATAAAGATATTGG-3' and *COI ALT LIV RV:* 5'-AACTTCRGGRTGACCAAAAA-3' amplifying 704 bp of the *COI* gene fragment.

For those samples not amplifying with a single PCR, a nested approach was used with internal primers designed by PRIMER 3 (Rozen and Skaletsky, 1998) named *COI FIL INT FW*: 5'-TTTTTCAWTTTGGGCTGGTY-3' and *COI FIL INT RV* 5'-CTCCCAACCCTATTGGRTCR-3', amplifying a 618 bp *COI* gene fragment.

A reaction volume of 50 µl containing 1 U of GoTaq Polymerase (Promega, Madison, WI, USA), Mg2+ 1.5 mM and dNTPs 0.2 mM, and10 pmol of each primer was used for each reaction. PCR – touch down profile was set as follows for *LCO1490/HCO2198* and *COIF-ALT/ COIR-ALT*: 40 cycles of 30 s at 95°C, 45 s at 45°C, and 60 s at 72°C; after an initial 10 min denaturation step at 95°C and a final extension at 72°C for 10 min (Chiesa et al., 2011). For newly designed *COI ALT LIV FW/RV* primers the following profile was performed: 35 cycles of 30 s at 94°C, 55 s at 48°C, and 45 s at 72°C; after an initial 3 min denaturation step at 94°C and a final extension at 72°C for 10 min. For newly designed *COI FIL INT FW/RV* primers the nested profile was performed as: 35 cycles of 30 s at 94°C, 50 s at 52°C, and 40 s at 72°C; after an initial 3 min denaturation step at 94°C and a final extension at 72°C for 5 min.

Fragment sequencing was performed by MACROGEN Europe service (Amsterdam, the Netherlands). Multiple alignments of sense and antisense sequences were conducted using MEGA 6.06 (Tamura et al., 2013) and Sequencer 4.2 (Gene Code Corporation). The experimental

199 sequences were aligned and compared with those of *R. philippinarum* obtained by GenBank from
200 native Asian populations, and other species of the same genus including the Asian *Ruditapes*
201 *variegatus* (synonym *R. variegata*, Sowerby 1852) and *Ruditapes decussatus* (Linnaeus,1758), the
202 latter is the native species of southern and western England, the Iberian Peninsula and the
203 Mediterranean (Poppe and Goto, 1991). When obtaining sequences from GenBank, we followed the
204 recommendations from Plazzi and Passamonti (2010) namely in retrieving female specimen data
205 only due to the DUI, whenever this information was available. See Supplementary Table 1 for
206 detailed Accession numbers and original sources.
207 Haplotype network analysis was performed through TCS v1.21 (Clement et al., 2000), with
208 confidence threshold at 95% for *Ruditapes* genus sequences to test whether *R. philippinarum*
209 haplotypes formed a single network separate to congeneric species (Hart and Sunday, 2007;
210 Lucentini et al., 2011). Data were converted into a rdf file using DNA-alignment software and then
211 a median-joining network (Bandelt et al., 1999) was constructed using Network 4.611 (both from
212 Fluxus-Engineering: http://www.fluxus-engineering.com) for *R. philippinarum* haplotypes and
213 outgroups.
214 The identification of variable and parsimony informative sites, the translation of nucleotide
215 sequences, the pairwise genetic distances, the nucleotide base composition and the
216 transition/transversion ratios were calculated using MEGA 6.06 (Tamura et al., 2013).
217 Spatial or demographic expansion was estimated through Tajima's D neutrality test (Tajima, 1989)
218 performed using DNAsp 5.0, assessing significance with 1000 permutations (Rossetti and Remis,
219 2012) and testing data for 4 different subsets: at large scale for the entire *R. philippinarum* pool, for
220 the European pool, and separately for the Atlantic and for the Adriatic pools.
221 Statistical selection of best-fit models of nucleotide substitution was performed by means of
222 jModelTest (Guindon and Gascuel, 2003; Darriba et al., 2012). This selection was based on 203
223 substitution schemes including scheme frequency, I and G rate variation, testing a total of 1624

models. On the basis of these results, the Jukes-Cantor model was used to assess the evolutionary history among *R. philippinarum*, *R. decussatus* and other outgroups; Maximum Likelihood and Neighbour Joining methods were inferred in MEGA6.06 estimating standard error by a bootstrap procedure (1000 replicates). In particular, for the Maximum likelihood method a discrete Gamma distribution was used to model evolutionary rate differences among sites (G categories = 4).

3. Results

Cytochrome oxidase I gene fragments were successfully sequenced and aligned unambiguously with those of GenBank for 491 bp. The final dataset comprised 465 sequences, 223 from this work. The overall number of mutations within the whole *R. philippinarum* dataset was 105 including both original and reference samples, and no insertion or deletion was observed. Among the European *R. philippinarum* sequences, 11 point mutations, 9 transitions (at positions 57, 96, 102, 126, 158, 321, 386, 426, 487) and 2 transversions (positions 6 and 330) were identified.

174 haplotypes were identified including outgroups and 166 considering the whole *R. philippinarum* dataset (not shown).

The European *R. philippinarum* samples belonged to 13 haplotypes (*RpCOI*1-*RpCOI*13) whose GenBank Accession numbers are reported in Table 3. These haplotypes are closely related and grouped into a single network that is the only haplogroup emerging from these data (Fig. 2). The 13 haplotypes were differently represented on the whole dataset, *RpCOI*1, *RpCOI*2 and *RpCOI*3 those showing the highest haplotype probability among European *R. philippinarum* haplotypes, respectively equal to 0.178 (*RpCOI*1), 0.150 (*RpCOI*2) and 0.229 (*RpCOI*3) (Fig. 2, Table 4). The other haplotypes, mainly those newly described, had a lower probability and were represented by only 1 or a few sequences, showing, consequently, lower weight values (Table 4). These differences in "consistency" of the *COI* haplotypes reflect their geographical distribution among the European countries. Observing haplotypes distribution, in fact, clearly emerged a complex pattern (Fig. 3,

Supplementary Table 2), as 3 of them (*RpCOI*1, *RpCOI*2 and *RpCOI*4), were shared among Atlantic (UK, Spain, Portugal, France) and Adriatic populations (Italy). The remaining ones were identified only in the Atlantic (*RpCOI*3, *RpCOI*6, *RpCOI*7, *RpCOI*8) or in the Adriatic (*RpCOI*5, *RpCOI* 9, *RpCOI*10, *RpCOI*11, *RpCOI*12, *RpCOI*13) group.

The Tajima's Neutrality Test performed on the whole *R. philippinarum* sequences showed the occurrence of 105 segregating sites and a Tajima statistics test D value of -1.898. Considering only European samples, Tajima's D value was 0.255 with 11 segregating sites. Restringing to fine scale, i.e. to either Atlantic or Adriatic samples, Tajima's D value was 0.965 (8 segregating sites) and -0.929 (10 segregating sites), respectively.

JModelTest identified JC as the best model (-lnL = 320042.66). Bootstrap ML (Fig. 4) and NJ (not shown) phenograms performed with this model showed almost the same topology. Among *R. philippinarum* haplotypes, 3 main clusters can be identified as showed in Fig. 4. Cluster A (in blue) included mainly the Japanese, European and some Chinese haplotypes from both Genbank and from this work; clusters B (in green) and C (in red) included the majority of the Chinese haplotypes obtained from Genbank (Fig. 4).

All the 13 haplotypes identified in European populations grouped within the cluster A among different sub clusters (Fig. 5).

4. Discussion

The 13 *COI* haplotypes observed in the 20 European sampling sites were characterized by 3 common haplotypes (*RpCOI*1, 2, 3) connected to 10 derived and rare haplotypes (*RpCOI*4-13). Interestingly, haplotypes *RpCOI*1 and *RpCOI*2 were the most frequent and comprised almost 70% of the analyzed sequences, both from Atlantic and Adriatic populations. A similar pattern was previously observed for Portuguese, Spanish and Italian introduced populations by the direct sequencing of a *16S*rDNA fragment (Chiesa et al., 2011; 2014). Moreover, the relatively high

haplotype diversity observed in introduced populations reflect the genetic structure that has already been described for natural Chinese and Japanese populations (Mao et al., 2011; Kitada et al., 2013). A limited loss of genetic diversity in introduced populations was also indicated by microsatellite (Chiesa et al., 2011; 2016) and allozyme (Moraga, 1986) data.

The most common haplotypes identified in European samples *(RpCOI*1-3) have been previously observed in native populations. Specifically, *RpCOI*1 corresponded to the haplotype *h6* (Kitada et al., 2013) from Japan; *RpCOI*2 to the haplotype *h21* (Kitada et al., 2013) from China Sea and Japan, and included also the samples of Qingdao, Nanao Bay, Rushan, Tianjin, Kagawa, Mikawa Bay, Tokyo Bay, Ariake Bay (Mao et al., 2011). The *RpCOI*3 corresponded to the haplotype *h32* (Kitada et al., 2013), from East China Sea and Japan, and also included the samples from Qingdao, Tianjin, Kagawa, Akkeshi, Mikawa Bay, Tokyo Bay, Ariake Bay, Notsuke Bay from the paper of Mao et al. (2011). The *RpCOI*5 corresponded to the haplotype *h53* from Mikawa Bay and *RpCOI*8 to the haplotype *h58,* already identified in Japan (Kitada et al., 2013). The other 8 haplotypes were newly described, considering all the *R. philippinarum* sequences previously collected and registered in GenBank.

The D value of Tajima test calculated for the entire *R. philippinarum* dataset (D <0) showed the occurrence of many polymorphic sites (>100) and many haplotypes with low frequencies, indicating a population expansion mainly in the natural range of distribution. Yet when the Tajima test was performed only on European samples (D > 0) it indicated the occurrence of multiple alleles, some at low (<25%), but others at high frequencies (>70%). This situation is frequently observed when a sudden population contraction or a founder effect occurs (Tajima, 1989). Data from Atlantic populations (positive D value) are consistent with balanced selection following the first Manila clam introduction in Europe. As for Adriatic populations, the negative value is consistent with a founder effect and additional introductions. This interpretation is also reinforced

298 by the frequency data of different haplotypes in Atlantic and Adriatic areas obtained within this
299 research.

300 The Maximum Likelihood radial tree performed on the whole Manila clam dataset showed the
301 occurrence of 3 main clusters, as already described for the North-West Pacific Ocean by Mao et al.
302 (2011): the lineage A included most of the Japanese populations, and some Chinese populations
303 (specifically those from Kiaochow Bay, Rushan and Laizhou) whilst the lineages B and C were
304 composed mainly of Chinese populations. As shown by the condensed tree in Fig. 5, all the 13
305 haplotypes observed in European populations belonged to cluster A and were distributed within 9
306 sub-clusters. The haplotype position in the radial tree does not support a recent evolution of the
307 European haplotypes, including those newly described, which supports the hypothesis of an ancient
308 evolution of the *COI* haplotypes of Manila clam. The occurrence of new haplotypes in the
309 introduced populations, not previously described in native regions, may be due to a sampling bias
310 among native and invaded communities. It is noteworthy that the most common haplotypes in
311 European populations could be clearly identified within cluster A, mainly composed of Japanese,
312 but also some Chinese haplotypes. Thus the *COI* data suggest the hypothesis that European
313 populations of Manila clam could derive from Japanese and Chinese populations of the lineage A.

314 Reconstructing the routes of invasion within the European countries it is interesting to note that the
315 3 haplotypes with the highest probability- *RpCOI*1, *RpCOI*2 and *RpCOI*3 - were occurring in all
316 European populations, except for those of southern UK. These results confirm the hypothesis of a
317 major human mediated introduction event commencing from a common pool within European
318 countries. Portuguese (Ria de Aveiro, Óbidos and Ria Formosa lagoons, and Tagus and Sado
319 estuaries) and Spanish (Galician coast) populations herein analyzed shared their haplotypes with
320 France, Italy and UK, supporting the hypothesis of a strong founder effect also in the Iberian
321 peninsula. However, especially in Portuguese populations, rare haplotypes with limited geographic

distribution were observed, supporting the hypothesis of additional introduction events, probably intentionally and conducted by fishermen. The two English populations shared the same frequent haplotype - *RpCOI*4 (also observed in Spain and Northern Adriatic Sea with low frequencies) but the common European haplotypes (*RpCOI*1, 2, 3) are missing in these samples. This result may be explained by a bottleneck effect in the British populations. The naturalised British population is at the northern extremity of the species range which may not represent an optimal environment, even though Poole Harbour is shallow, warm and has lagonal characteristics (Humphreys, 2005). This hypothesis is also consistent with reported population densities which are significantly lower than those recorded in southern European sites such as on the Italian Adriatic coast (Breber, 2002; Humphreys et al., 2007; 2015). Isolated individuals as relics of otherwise unsuccessful spatfalls have also been observed in southern England (Humphreys et al., 2015). Together this could have determined the reduced haplotype diversity of naturalised populations. Although the samples from southern England were small, both Poole and Southampton populations were genetically similar, indicating that differences with southern populations are likely to be valid. As reported in the introduction section, the established population in Poole Harbour dates back to 1990 (Humphreys et al., 2015), whilst the naturalised Southampton population appeared later. Both natural dispersal from Poole (Herbert et al., 2012) and human-mediated introductions (Humphreys et al., 2015) are equally valid mechanisms for population establishment.

Finally, considering both genetic and informations from the literature, probable invasion routes for European populations of Manila clams can be formulated (Fig. 6). These routes are mainly human mediated, although for Southampton water a natural expansion cannot be excluded, as reported above. As described in literature, a major introduction event in Europe occurred from North America (Flassch and Leborgne, 1992), where Manila clam was previously introduced from Japan and placed overboard in Ladysmith Harbour (Canada) (Flassch and Leborgne, 1992). As also

reported by Humphreys et al. (2015), Japanese clams were taken to the Hawaiian Islands (Bryan, 1919; Yap, 1977), then other Japanese clams reached the North American Pacific coast in the 1930s as an accidental introduction with stocks of Pacific oyster (Quayle, 1949). Clams from the Puget Sound were then separately introduced into France (1972-74) and then in UK (Conwy, Wales) (1980); from southern England the same pool was introduced in Northern Adriatic Sea (1983). In the early 1990s, clams from Northern Adriatic were frequently transported to Spain (M. Pellizzato, pers. comm.), and most probably from Spain to Portugal. Genetic data from this work confirmed the occurrence of a main founder effect in European populations. Moreover, the phylogenetic analysis confirmed that among the 5 haplotypes occurring in Europe and already described in the natural range of distribution, 3 are deriving from Japan (*RpCOI*1, *RpCOI*5, *RpCOI*8) and 2 of them were already described both in Japan and China (*RpCOI*2, *RpCOI*3). The possible Japanese origin of Manila clam European populations is supported also by literature data on *Perkinsus olseni* and *P. chesapeaki* infections in European populations of *R. philippinarum,* as recently reviewed by Ruano et al. (2015). However, the occurrence of a high number of rare haplotypes with limited geographic distribution suggests additional introduction events not recorded previously. These introductions could have occurred intentionally for commercial exploitation without registration. In the Northern Adriatic Sea, for the first 2 years after introduction clam seeds for aquaculture activities were imported from England (Turolla, 2008) but then from Spain (TINAMENOR aquaculture facilities) and USA (California) during the middle and late 1990s (M. Pellizzato, pers. comm.). It is well known that over the period 1987 to 1991, Manila clam seed produced in Norway from a Scottish stock were massively exported for cultivation in Spain (Mortensen and Strand, 2000). Multiple introduction events could have occurred also due to the existence of mixed source populations, since Manila clams in Europe have been introduced from non-native populations, already manipulated for commercial purposes.

370 Finally, as the European Atlantic coast is subjected to introduction of oyster seed for culture
371 (mainly *Crassostrea gigas*), both from European and non-European countries, accidental species
372 introduction is possible (Wolff and Reise, 2002) as previously documented for Manila clams in
373 North America (Quayle, 1949).
374
375 ***4. 1. Conclusions***
376 This paper provides the first genetic comparison of Manila clam populations introduced in to
377 Europe with native clams. The direct sequencing of a *COI* gene fragment has provided data
378 supporting a strong founder effect of European populations, with 3 main haplotypes occurring at
379 high frequencies.
380 However, high haplotype diversity due to the occurrence of 10 rare haplotypes, suggests (i)
381 additional introductions –probably intentionally conducted- following the main event, and (ii) a
382 limited loss of genetic diversity in introduced populations.
383 Establishing geographic origins and the diversity and structure of exploited populations has
384 significant implications for the management and traceability of clam stocks. The occurrence of
385 illegal clam exploitation in moderate and highly polluted environments could represent a serious
386 risk for human consumption. Thus, knowledge of geographic origin is fundamental to product
387 traceability within the clam market. The genetic profile of clam populations could be a useful tool
388 to trace origin of stocks, preventing fraud concerning clam products and avoiding mislabeling in
389 European countries. Moreover, the genetic data can help to understand the structure of exploited
390 populations, especially in terms of their variability and resilience to exploitation and selection
391 driven by aquaculture activities. The maintenance of high genetic diversity in exploited clam
392 populations is necessary to ensure the survival of the resource over time and the preservation of
393 population's fitness. In fact, the high reproductive capability, growth rate and the capacity to
394 respond to environmental changes are strongly influenced by levels of genetic diversity.

In conclusion, the genetic resources of Manila clam in Europe should be furtherly investigated and monitored to ensure its sustainable exploitation.

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611 **Table 1**

612 First introductions (authorized or unauthorized) of Manila clam *R. philippinarum* in Europe.

Country	Year	Reference source
France	1972 (authorized)	Robert and Deltreil, 1990; Flassch and Laborgne, 1992
United Kingdom	1980 (authorized)	Humphreys et al., 2015
Italy	1983 (authorized)	Breber, 1985
Spain	1983-85 (unauthorized)	Perez-Camacho and Cuna, 1985
Portugal	1984 (unauthorized)	Ruano and Sobral, 2000

613

614

615 **Table 2**

616 Manila clam sampling sites. Estuarine environments herein analyzed are provided with Country,
617 Estuarine system, Site, Acronyms and number of analyzed specimen. The * symbol indicates the
618 populations already analyzed by *16S* rDNA (see Chiesa et al., 2014).

619

Country	Estuarine System	Site	Acronym	N
Italy	Marano Lagoon*	Site 1	UD	16
Italy	Marano Lagoon*	Site 2	GR	17
Italy	Venice Lagoon*	Busa	AV	10
Italy	Venice Lagoon*	Palude di Monte	BV	16
Italy	Venice Lagoon*	Fusina	FV	9
Italy	Po River Delta*	Marinetta	MA	10
Italy	Po River Delta*	Caleri	CA	11
Italy	Sacca degli Scardovari*	Scardovari	SC	11
Italy	Sacca di Goro	Goro	GO	11
Portugal	Ria de Aveiro Lagoon*	Murtosa	MU	7
Portugal	Ria de Aveiro Lagoon*	Esteiro Rio Boco	ST	8
Portugal	Ria de Aveiro Lagoon*	Costa Nova	CN	7
Portugal	Óbidos lagoon	Obidos lagoon	OB	20
Portugal	Ria Formosa	Ria Formosa	AL	2
Portugal	Tagus estuary	Tagus estuary	TA	11
Portugal	Sado estuary	Sado estuary	SD	16
Spain	Galician coast*	La Coruna	ES	10
France	Arcachon Bay	Arcachon Bay	AR	15
UK	Poole Harbour	Poole Harbour	PH	6
UK	Southampton	Southampton	SH	10

620

Table 3

COI haplotypes of *R. philippinarum* deposited in GenBank. Haplotype acronym and Accession numbers are provided.

Haplotype Acronym	**Genbank A.N.**
*RpCOI*1	KU252867
*RpCOI*2	KU252866
*RpCOI*3	KU252868
*RpCOI*4	KU252869
*RpCOI*5	KU252870
*RpCOI*6	KU252871
*RpCOI*7	KU252872
*RpCOI*8	KU252873
*RpCOI*9	KU252874
*RpCOI*10	KU252875
*RpCOI*11	KU252876
*RpCOI*12	KU252877
*RpCOI*13	KU252878

626 **Table 4**

627 Results of Minimum Spanning Network analysis. The probability weight is shown for each
628 haplotype.

Haplotype Acronym	**Haplogroup**	**Weight**
*RpCOI*1	H1	0.178
*RpCOI*2	H1	0.150
*RpCOI*3	H1	0.229
*RpCOI*4	H1	0.024
*RpCOI*5	H1	0.001
*RpCOI*6	H1	0.137
*RpCOI*7	H1	0.004
*RpCOI*8	H1	0.001
*RpCOI*9	H1	0.001
*RpCOI*10	H1	0.003
*RpCOI*11	H1	0.001
*RpCOI*12	H1	0.134
*RpCOI*13	H1	0.134

629

Figure Captions:

Fig. 1. Collection sites of Manila clam in Atlantic and Adriatic European coastlines (Modified from d-maps.com)

Fig. 2. Median-joining network of the 13 *COI* haplotypes for European samples of *R. philippinarum.* The most representative haplotype of the unique haplogroup is reported in a square instead the other haplotypes are reported in oval. The size of the ovals is proportional to the consistency of haplotypes. Small dots report base substitutions (see as example Lucentini et al., 2011).

Fig. 3. Geographic distribution of the 13 *R. philippinarum* haplotypes for each analyzed population. The underlined haplotypes *RpCOI*1, *RpCOI*2, *RpCOI*4 are shared among Atlantic and Adriatic populations.

Fig. 4. Maximum Likelihood (ML) radial tree of *R. philippinarum COI* haplotypes. Japanese Cluster A (blue) and Chinese clusters B (green) and C (red) are shown.

Fig. 5. Maximum Likelihood (ML) radial condensed tree of *COI* cluster A. European haplotypes are indicated by black circles. Japanese Cluster A (blue) and Chinese clusters B (green) and C (red) are shown.

Fig. 6. Manila clam routes of invasion in Europe, inferred from bibliographic information, expert opinion and *COI* data.

650 **Supplementary Table 1**

651 Selected *COI* gene sequences used in the phylogenetic analyses. For each species the GenBank
652 accession number and the original sources are reported.

species	GenBank A.N.	Original source
R. philippinarum	AB694757-AB694884	Kitada et al., 2013
	JN054502–JN544632	Mao et al., 2011
	AB244374-AB244412	Sekine et al., 2006
	HQ703306-HQ703311	Chen et al., 2011
R. variegatus	AB694885-AB694891	Kitada et al., 2013
R. decussata	DQ458492	Kappner and Bieler, 2006

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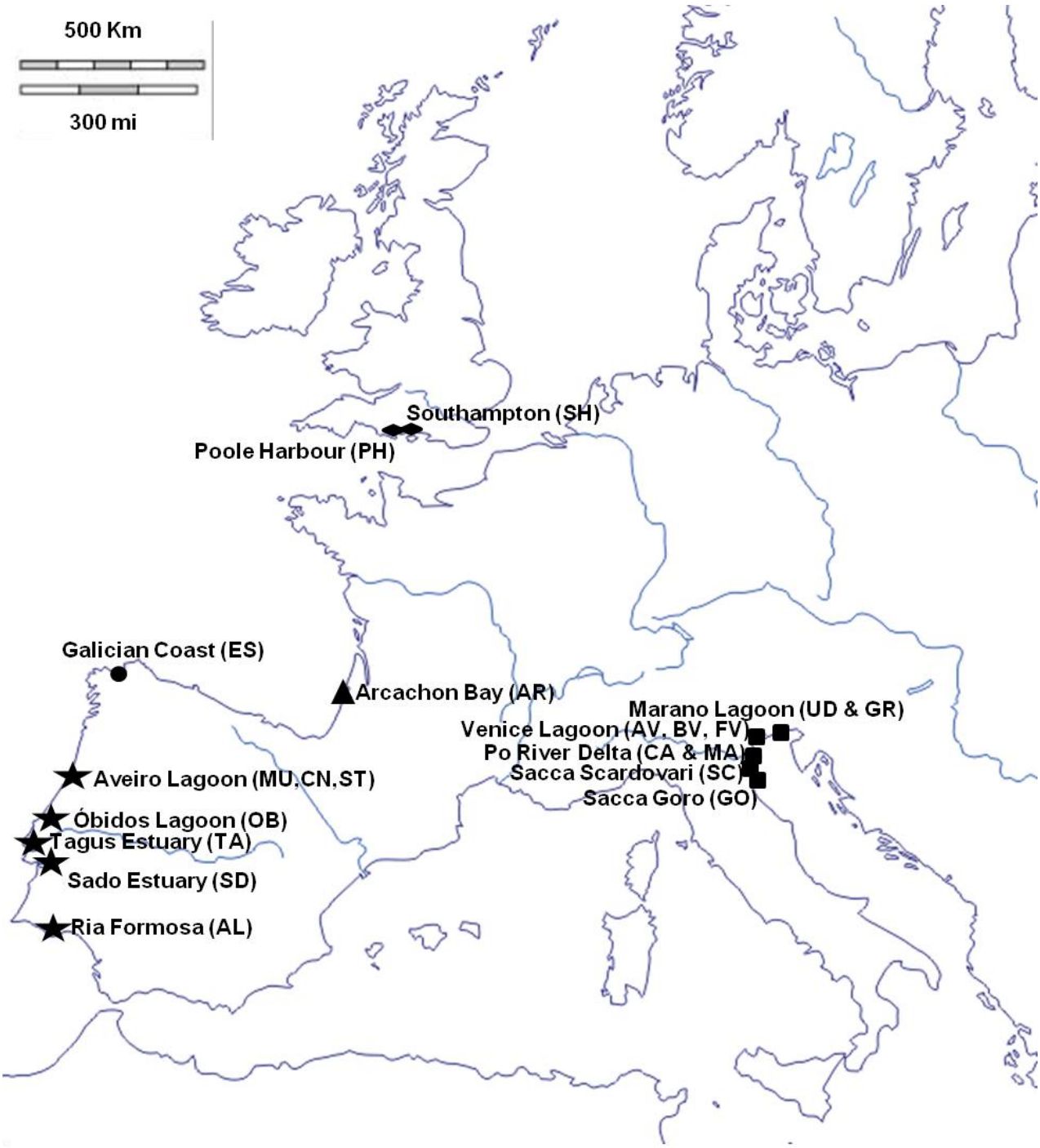
654

655 **Figure 1**



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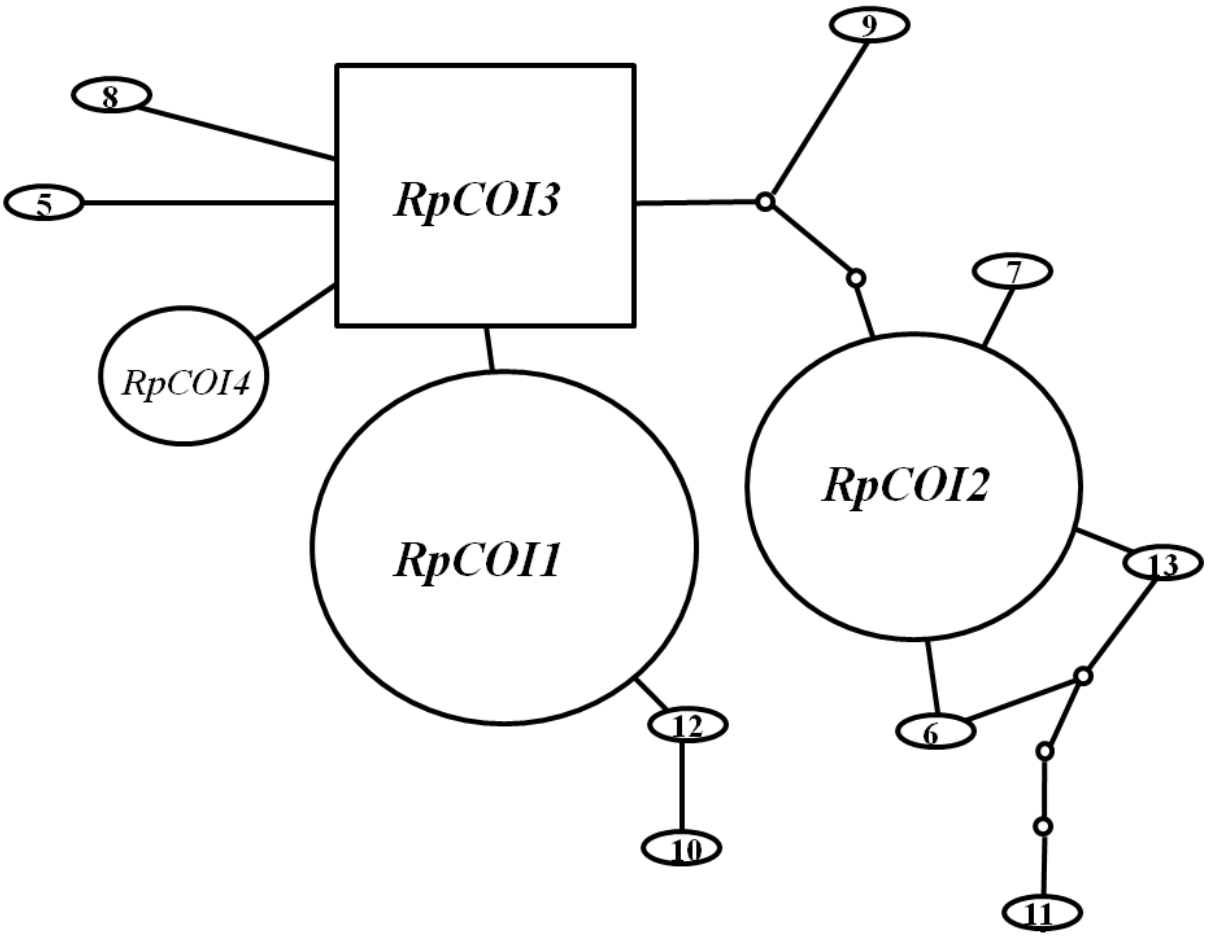
658 **Figure 2**



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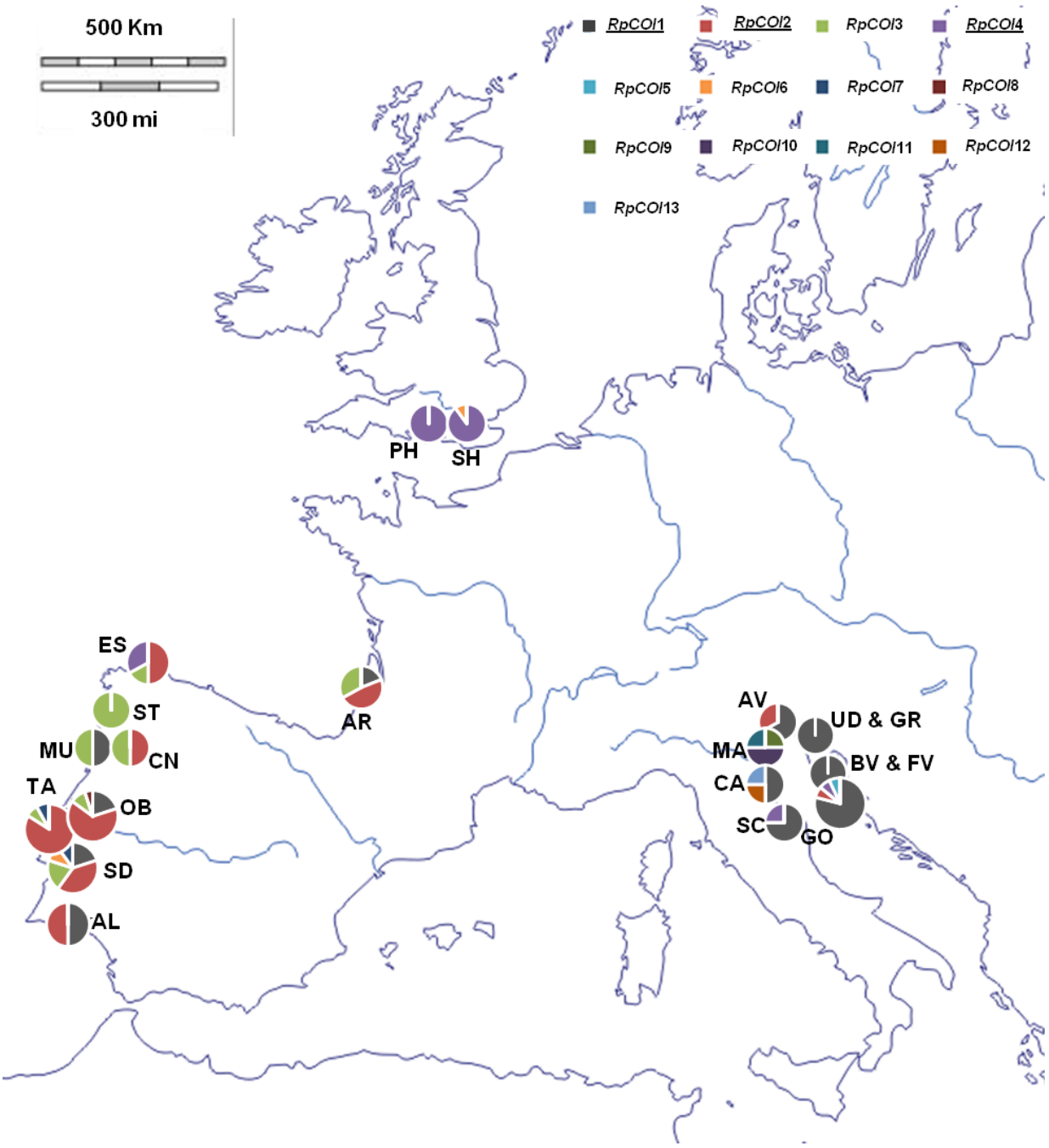
661 **Figure 3**



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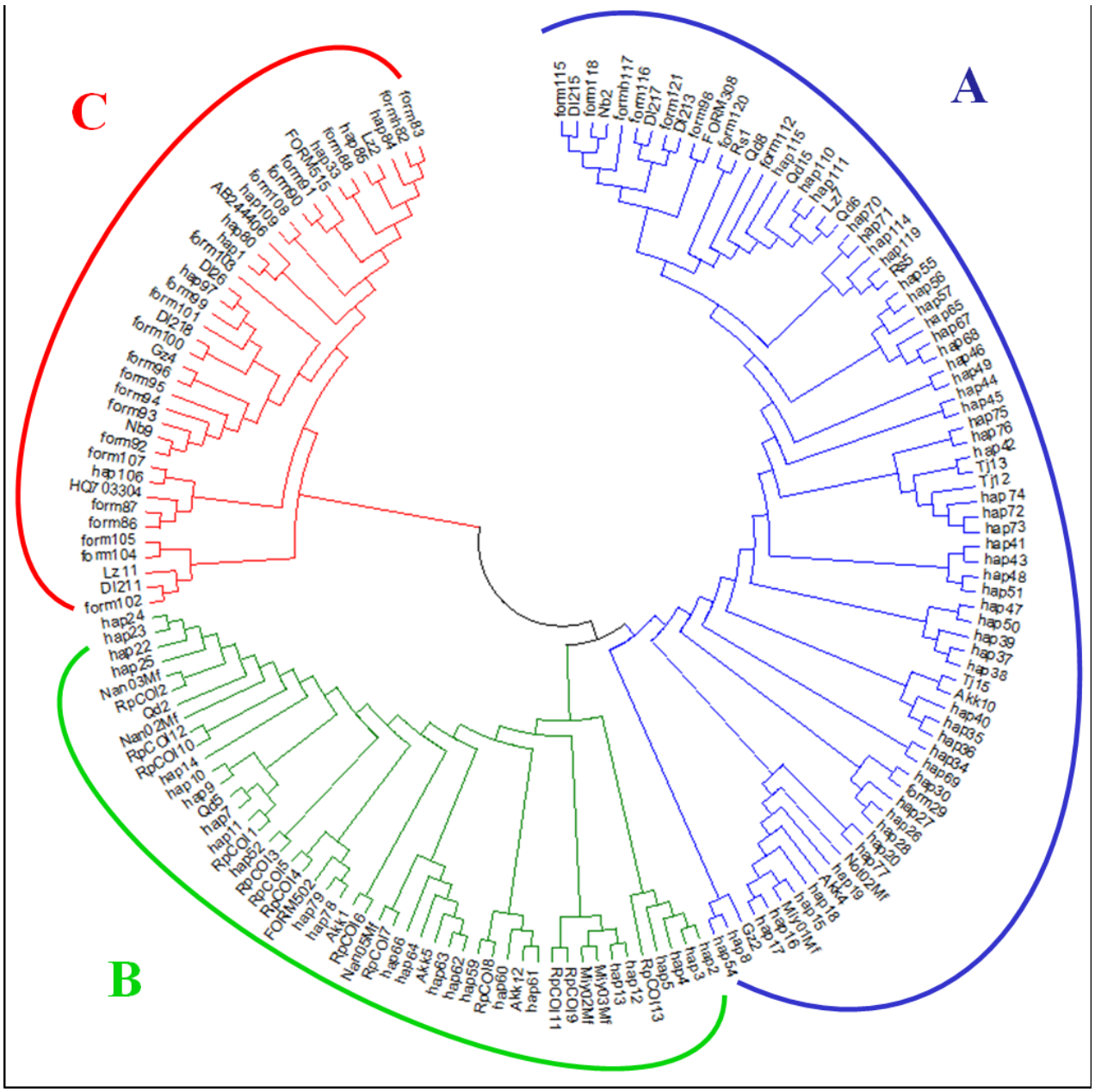
664 **Figure 4**



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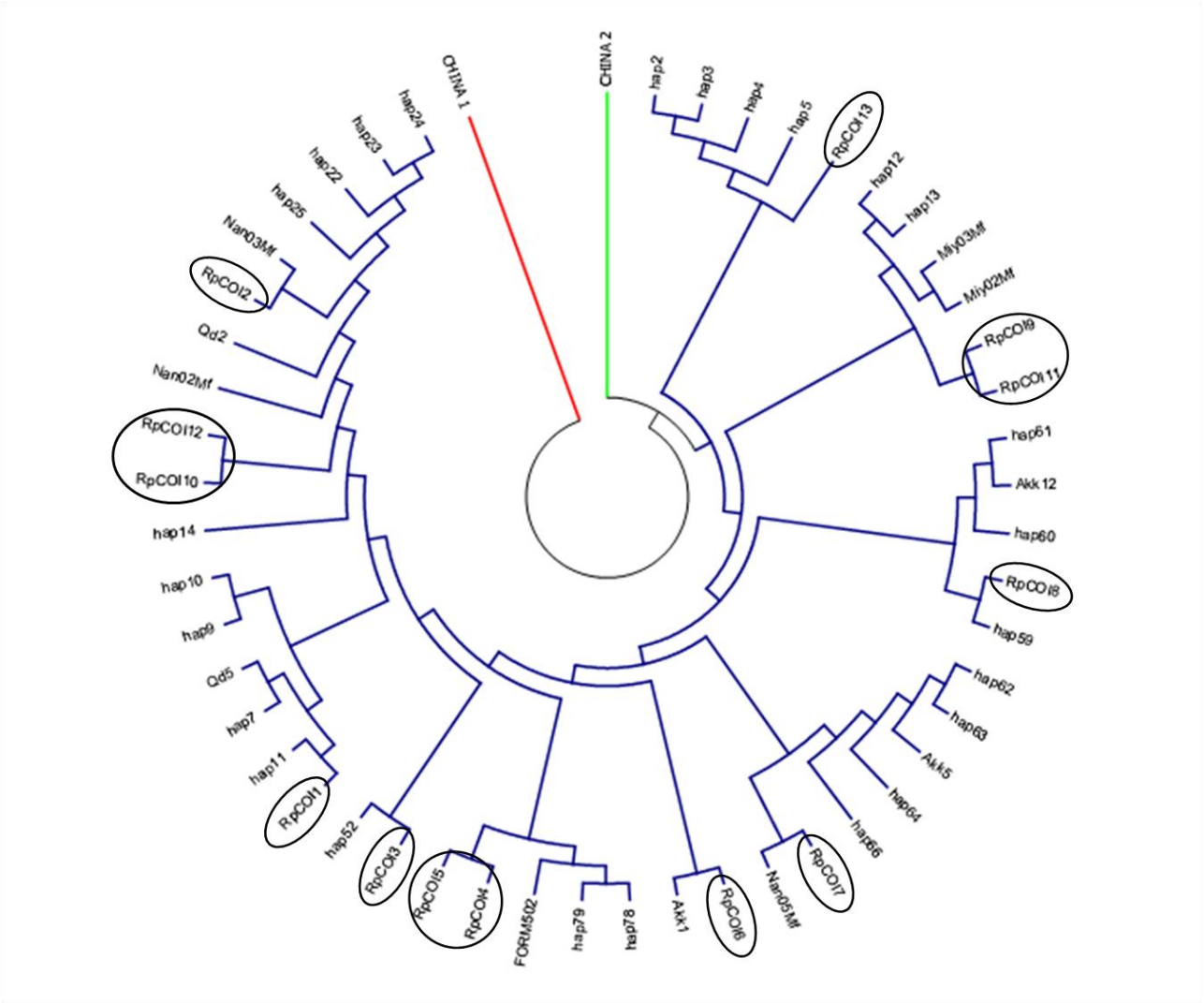
667 **Figure 5**



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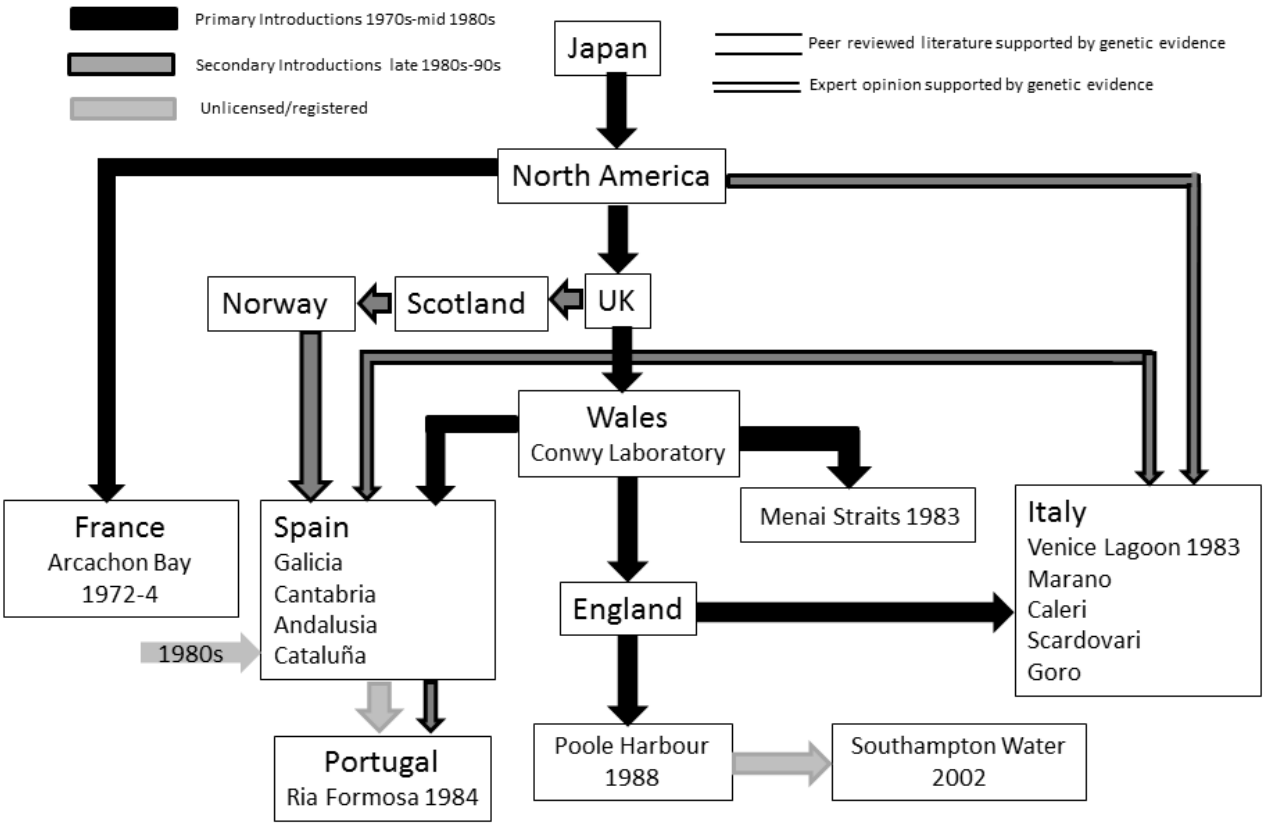
670 **Figure 6**



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