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Genetic diversity and demographic history of two endemic shrimp species

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled ‘**Genetic diversity and demographic history of two endemic shrimp species’** independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

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22.04.2022
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Abstract

Dugustella valentina and *Palaemon zariquieyi* are shrimp species endemic to the Valencia region, Spain. Using a mitochondrial Cytochrome c oxidase subunit-I (COI) marker sequenced from individuals of both species collected at multiple localities the demographic patterns of these species were evaluated by determining the nucleotide diversity, haplotype diversity, haplotype networking and Bayesian Coalescence inference. Also, the connectivity between populations from different localities was assessed using landscape genetic tools and the level of genetic diversity was compared with the samples from previous years. The results suggest the level of genetic diversity is fairly high but appears to be reduced over years for *D. valentina* while *P. zariquieyi* have similar patterns with the reference populations. In both the species the gene flow and connectivity between localities is substantial, possibly mediated by irrigation channels or translocation by floods, birds or humans. Additionally, the existence of a distant haplotype in *D. valentina* was subjected to phylogenetic analysis based on 16s rRNA and COI markers revealing occurrence of two distinct lineages due to secondary contact or possibly by mitochondrial capture from unrecognized or extinct lineage. Such evaluations on these understudied species can help us understand the population structure and anthropogenic pressure on them to implement appropriate management plans for their conservation.

Key words: *Dugustella valentina, Palaemon zariquieyi*, phylogeny, genetic diversity, landscape genetics, COI, 16s rRNA.

Contents

List of tables

List of figures

List of the abbreviations used in the thesis

AIS - Alleles in Space

BLAST- Basic Local Alignment Search Tool

bp - Base pairs

CBD - Convention on Biological Diversity

COI - Cytochrome c oxidase subunit-I (COI)

DNA – Deoxyribonucleic acid

ESS – Effective Sample Size

H3 – Histone 3

IUCN – International Union for Conservation of Nature

JRBD – Jucar River basin District

Mya – Million years ago

NCBI – National Center for Biotechnology Information

PCR – Polymerase chain reaction

rRNA – Ribosomal ribonucleic acid

1. Introduction and Literature Review

1.1. Introduction

Biological diversity is the essential for ecosystem functioning. International Union for Conservation of Nature (IUCN) recommends conservation of biodiversity at three important levels; genetic diversity, species diversity and ecosystem diversity. Decline in these diversity levels directly influence the decrease in functioning of ecosystem (Frankham 1995; Cardinale 2012). Anthropogenic factors such as urbanization, chemical pollution, overpopulation, over-exploitation of resources, hunting and fishing, etc have caused population decline in many species. Assessment from IUCN states more than 40,000 species have been threatened with extinction many are unassessed (IUCN Red List version 2021-3). Consequently, making our ecosystems less efficient in maintaining its balance (Frankham 1995; Pievani 2014). This has made it essential for human intervention to save them from extinction so as to prevent biodiversity loss (Scott et al. 2005).

Of particular interest in this context is the Iberian Peninsula. It has high ecological value due to its rich biodiversity and endemism (Medail & Quezel 1997). Human activities like aquiculture, building of artificial basins, introduced alien species in the inland water have severe impact on the local biodiversity (Hirsch et al. 2020). The rivers of the Iberian Peninsula tend to produce sudden floods during autumn and droughts during summer due to the regions diverse land forms and climatic conditions resulting in irregular hydrology (Mario Vargas et al. 1998). These irregular flows and drought periods with water scarcity led to manipulation of the hydraulic systems with construction of more than 1000 reservoirs, canals and dams with a long history of modifications by the Arabs and Romans (Sabater et al. 2009). In the recent times in these basins the natural flow and physical-chemical properties of the waters are subjected to alterations (Sabater & Tockner 2009) by the presence of artificial structures like reservoirs, dams and hydroelectric plants. Additionally, climate change has increased frequency of drought and floods events in the Mediterranean basins (Giorgi & Lionello 2008). All these factors tamper the natural functioning of the river systems ensuing adverse effects on the aquatic flora and fauna.

The consequences of these anthropogenic threats are, disruption of connectivity of biota, artificial dispersion and transfer of alien species which can lead to hybridisation and loss of genetic diversity, threatening these ecosystems (Robles et al. 2002).

1.2. Study area

1.2.1. Geological history

The Iberian Peninsula is biogeographically distinguished into three regions namely, the Mediterranean, Cantabrian and the Atlantic based on freshwater ichthyofaunal distribution (Hernando & -Soriguer n.d.). This region remained favourable, almost ice-free during the climatic oscillations in the Quaternary period (Hewitt 1996). As distribution of species tends to largely depend on climatic factors, it served as a refugia for many species, resulting in high levels of endemism (Hewitt 1996; Sousa-Guedes et al. 2020). As the region is stationed between the Southwestern Europe and North Africa while surrounded by the Atlantic Ocean and the Mediterranean Sea resulted in a heterogenous physiography holding a variety of species that require different ecological conditions (Hernando & Soriguer 1992; Mario Vargas et al. 1998a). The strait of Gibraltar acts as a barrier between the region and Africa. It was formed 5.33 million years ago (mya) and it is connecting the Atlantic and the Mediterranean waters after the isolation of Mediterranean Sea from rest of the oceans during the Messinian salinity crisis (5.6 mya) (Ruggieri 1967; Garcia-Castellanos et al. 2009). The Pyrenees separate Iberia from Europe. They originated in the Lower Eocene and formed their present configuration in the Upper Oligocene to Lowe Miocene (Mario Vargas et al. 1998). Their geological history contributes to the formation of a distinctive biogeographic entity comprising 11 major fluvial basins (John & Birks 1987) that date to the Quaternary period before which only endorheic basins were present in the region (Doadrio 1988). The run-offs and precipitation which causes the floods are influenced by the external boundaries and the mountains (Mario Vargas et al. 1998).

The Mediterranean subsector is composed of the levantine basins with Jucar, Turia, Mijares, Palancia and Serpis rivers. Out of these, the Jucar and Turia rivers were historically associated constantly, compared to the rest (Doadrio, 1988). The Segura basin originated during the formation of the Strait of Gibraltar in the upper Miocene-

Pliocene and the Jucar basin formed in the Pliocene when the southern sub-plateau opened into the Mediterranean (Mario Vargas et al. 1998a).

4.3.1 Human impacts

The study area (Figure 1) in the eastern Mediterranean basin focussed on the Jucar River basin district's (JRBD) aquatic habitats are comprised of diverse topographies such as the mountainous system, coastal plain and a continental plateau. It constitutes the major rivers Jucar, Turia, Mijares, Vinalopo, Serpis and Cenia, also all discharge into the Mediterranean Sea (Estrela et al. 2004).

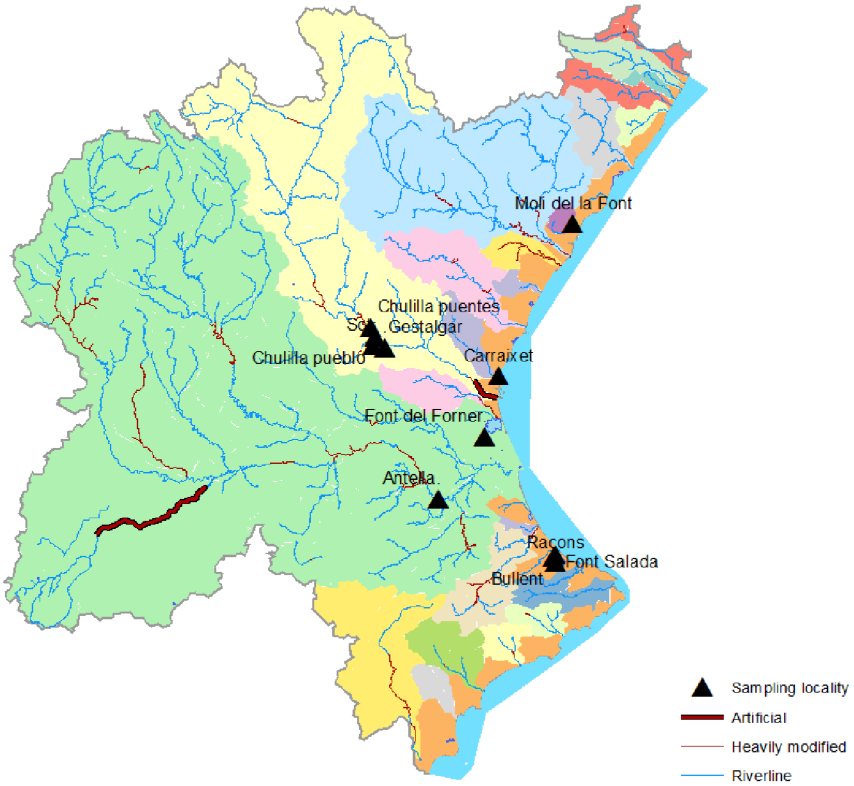

Figure 1. Map of rivers in the study area-Valencia, Spain with sampling localities created in ArcGIS Desktop V 10.5.

This region, covered with several inland and coastal wetlands, is not an exception to anthropogenic effects, it also suffers from hydrological alterations, drainage systems, salinity changes, pollution and mainly eutrophication due to agriculture and other exploitations by humans (Poquet et al. 2008a). The Jucar River basin district which is surrounded by dense population and agricultural lands have high water demand mainly for irrigation that depends on many dams along the network and reservoirs (Figure 2) to prevent recurrent floods caused due to intense rainfall in summer (Sabater et al. 2009). Noticeably, the alterations in the river Turia by creation of channels for agriculture dates back to the Middle Ages during the Moorish period and is known for the frequent flooding events followed by a major flooding in 1957 led to diversion of water from the river bed which was later converted into a park of 8.5 km in 1981 (River Turia Park / Park nad rzeka Turia) after drying for decades (Galan 2011). For example, the irrigation channel Acequia Real del Jucar which reaches to the fields in last end of River Jucar dates back to 8th centuary (Estrela et al. 2004). Another main channel is the Canal Jucar-Turia which supplies the irrigation and public water demand connects the rivers Turia and Jucar (Figure 3) (Estrela et al. 2004).

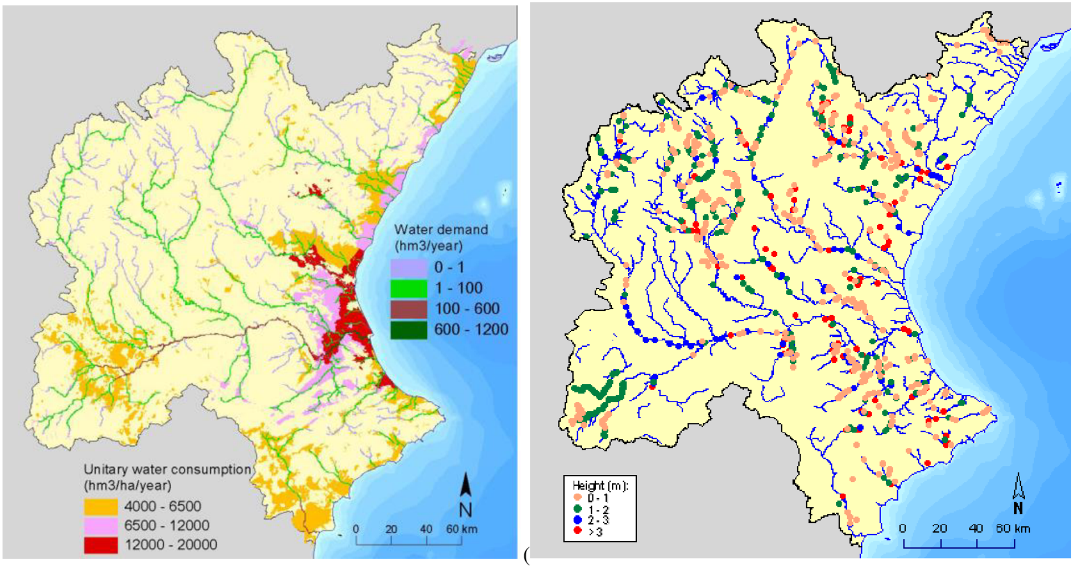

Figure 2. Water abstraction for agricultural use and location of retention structures (Estrela et al., 2004).

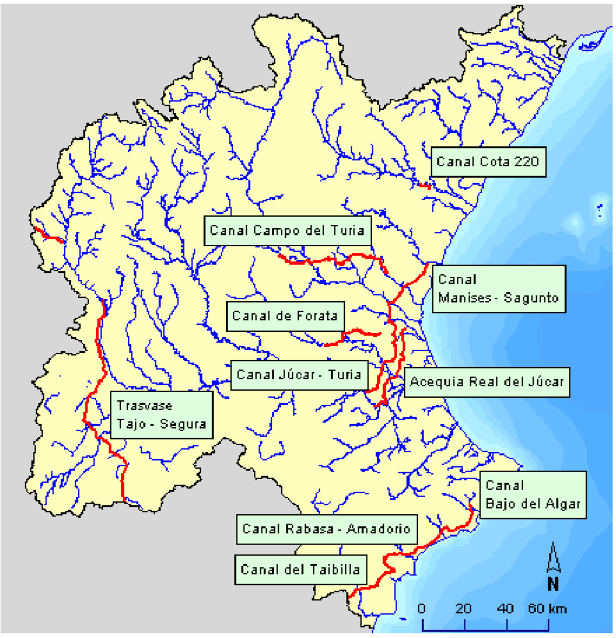

Figure 3. Location of main channels (Estrela et al., 2004).

Presence of retention structures and artificial channels alters the natural movement, abundance and distribution of riverine species (Washington 1999). Many freshwater species have its lifecycles synchronized with the seasonality of the hydrograph. For example, sluggish flow in the flood plains and the high organic matter provided refugium for rearing and breeding in many freshwater fishes like the Amazonian fish species *Colossoma macropomum* that migrates every rainy season to the forest and back to rivers via flood waters for food resources (Place 1998). Primarily, the mouth to middle stretches of the rivers Jucar, Turia, Mijares and Cabriel have the highest impact on the flow regime by the regulation structures like Dams and reservoirs compared to short watercourses and the upper parts (Figure 2). These alterations have resulted in severe modifications of the catchment and connectivity between the river basins impairing the ecosystem. All this threatens the biological community in this area which supports many autochthonous and endemic floral and faunal species favoured by the geological history of this region (Estrela et al. 2004). Many endemic species such as the Cyprinoid fishes, *Valencia hispanica* and *Lebias ibera* and decapods shrimps, *Dugastella valentina* and *Palaemon zariquieyi* were common in this region but now disappearing in many localities (Sollaud 1938; Sanz & Gómez 1984; Poquet et al. 2008a). To maintain species richness, conservation of such endemic species is important since they are found in only small populations and few regions making them more vulnerable to extinctions (Lamoreux et al. 2006).

1.3. Classification and review of study species

In the crustacean class Malacostraca, the order Decapoda consists diverse groups (Figure 4) distributed worldwide which encompasses shrimps, prawns, crabs, crayfish and lobsters having given importance as a food source. They can be found in a variety of habitats such as, fresh water, marine, and also semi-terrestrial. They can be surface dwellers, ground water dwellers or burrowers. And consequently, the evolution of their behaviour and morphology reflects the habitat type (Hobbs & Lodge 2010; Wolfe et al. 2019). Latest studies represent 2,725 genera with 14,756 species of which 46% are represented by the infraorder Brachyura (crabs) and still they are many undefined species (de Grave et al. 2009). In decapods when we consider higher classification the phylogenetic relationships are ambiguous and need further studies. For the classification of shrimp, like decapods, a most recent study of Wolfe et al. (2019) suggests formation of a clade consisting Stenopodidae (boxer shrimps), Dendrobranchiata (prawns), Procarididea and Caridea (true shrimps). The range of adaptation and biological diversity within the infraorder Caridea is remarkable among the decapod crustaceans. Within Caridea is the two main divisions, one with the class Atyidae which consists of fresh water shrimps and rest of the classes group together (Wolfe et al. 2019). This study was conducted in two fresh water shrimp species restricted to Valencian waters in Spain; *Dugastella valentina* from the class Atyidae and *Palaemon zariqueiyi* from the class Palaemonidae.

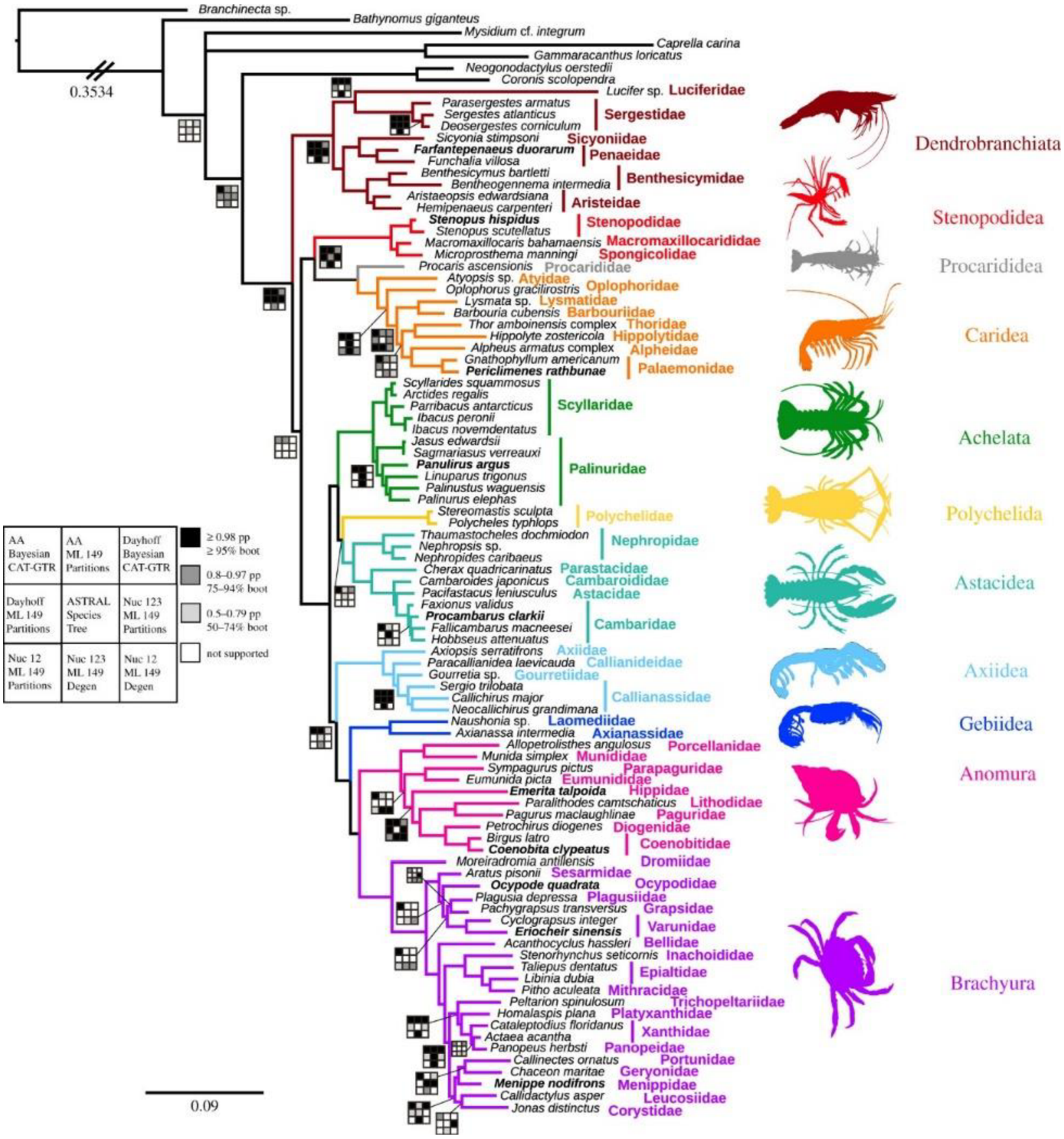

Figure 4: Phylogenetic classification of Decapoda (Wolfe et al. 2019).

1.3.1. Dugustella valentina

The Kangaroo shrimp, *Dugastella valentina (D. valentina)* (Ferrer Galdiano 1924) (Figure 5) is a Atyid epigean shrimp (Anderson & Cabana 2007) species endemic to the Gulf of Valencia (Figure 6), in the Iberian Peninsula (Sanz & Gómez 1984). It is categorised as 'Near Threatened' in IUCN assessment conducted in 2012 (IUCN Red List of Threatened Species. Version 2021-3*)*. Distribution study by Sanz and Gomez (1984) describes its presence in many freshwater habitats; rivers, lagoons, canals, estuaries and springs and it is in the tropic position <3 feeding on algae and detritus in these waters

(Anderson & Cabana 2007). Although it was found to inhabit slightly brackish waters in Almenera, Cullera and Denia and in the locality Alcoy the species has disappeared while it was recognized in this locality before 1968 and this study states that it could be because of high chemical contamination in the river Serpis due to agricultural and industrial activities. In many localities it inhabits it coexists with other shrimp species like *Atyaephyra desmaresti* (Atyidae), *Palaemon zariquieyi*, *Palaemon longirostris* and *Palaemon elegans* (Palaemonidae), cyprinodontid fish and gastropods of *Theodoxus* and *Melanopsis* (Sanz & Gómez 1984).

Figure 5: Specimen of *D. valentina* with larvae in the brooding pouch, collected in 2018.

The shrimps grow throughout their life. Females reach ovigerous stage between March to June with recruitment in May and June. Males exhibit sexual character when they reach a size of 12mm. In autumn and winter all individuals including the adults and young grow, reaching maximum size in June and by April all acquire sexual traits with no juveniles in the population. By August to September all individuals of the older generation disappear. Females are bigger than males and disappear in February which is very earlier to males of the same generation who disappear only in May and this could be because of the extra reproduction cost in females as they are in bigger so as to carry eggs hence more susceptible to predation (Santos & Brau 1994).

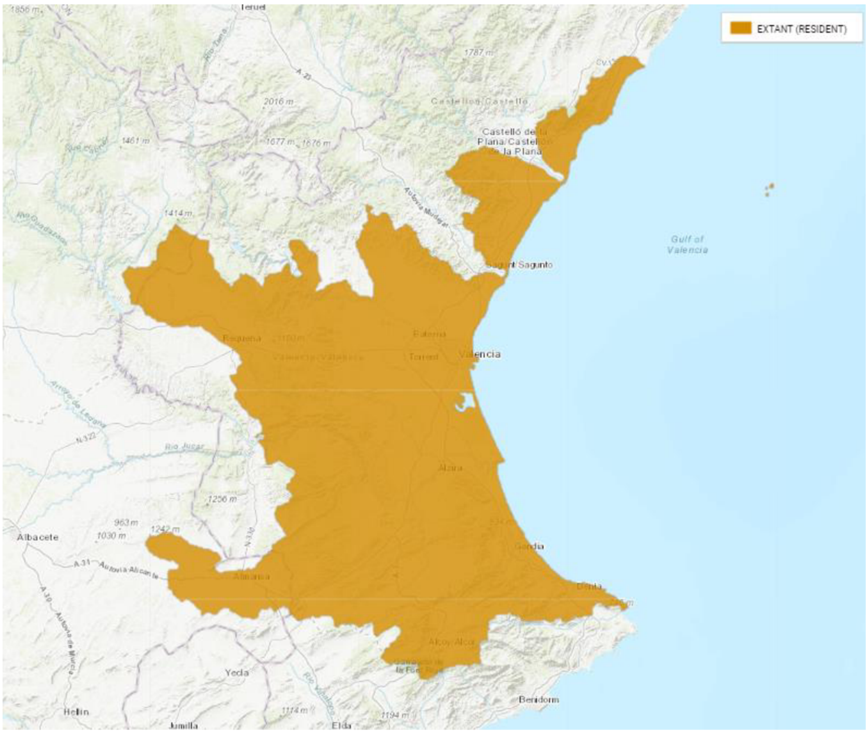

Figure 6: Distribution of *D. valentina*. (IUCN, 2013. The IUCN Red List of Threatened Species. Version 2021-3).

D. valentina shows abbreviated development with three lecithotrophic i.e., non-feeding stages (retains large amount of yolk as nutrient source) of larval development, consisting two zoeae (immobile) and a decapodid stage (do not swim but walk using pereipods). These shrimps show peculiar behaviour in parental care of carrying the juveniles after hatching in a brooding pouch hence having the common name “kangaroo shrimp” and this behaviour is exhibited solely in the genus *Dugastella* which consists of one other shrimp species *Dugastella marocana* (Rodriguez & Cuesta 2011) as it is a shift of freshwater invertebrates from r to K strategy (Vogt 2013)

1.3.2. Palaemon zariquieyi

Palaemon zariquieyi (*P. zariquieyi*) (Sollaud 1938) is an endemic Palaemonid species in the Gulf of Valencia (Figure 7 and Figure 8) inhabiting fresh to oligohaline brackish waters in the lagoons, canals and pools (Guerao 1993). It is an omnivore feeding on algae, organic detritus and benthic community predominantly having a carnivorous diet feeding on oligochaetes and chironomids and also includes insect larvae (Pérez-Bote 2006).

Figure 7: *P.zariquieyi*, specimen collected in 2018.

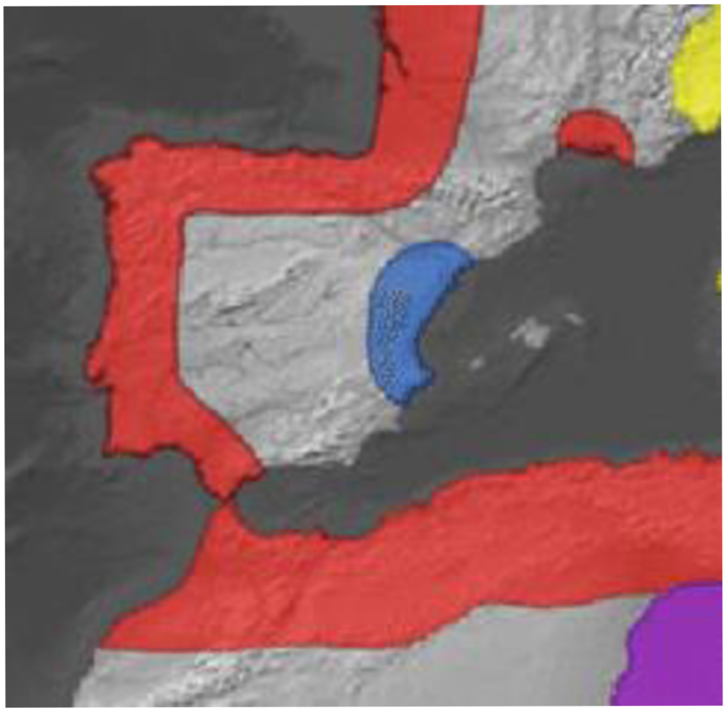

Figure 8. Distribution range of P. zariqyuieyi represented in blue (Christodoulou et al. 2016).

P. zariqyuieyi larvae undergoes and abbreviated development with three larval stages before metamorphosis that are lecithotrophic and only begins to feed in the juvenile stage. After hatching the larva lack free-swimming state and exists in benthic region (Guerao 1993). Compared to other *Palaemon* species with similar states of larval development, *P. zariquieyi* shows reduced morphological features of larvae while this kind of development is observed in Palaemonid species in amazon in the areas with poor planktonic growth (Magalhães & Walker 1988). Adding to this Palaemonids have a habit of changing their diet according to the stage of their life cycle in relation to their size. But, in case of *P. zariquieyi* this behaviour is not observed as the larval stage doesn't drastically vary from the adults (Pérez-Bote 2006) which could be an adaptation of this endemic species inhabiting landlocked freshwater, where the growth of plankton is limited (Urzúa et al. 2013).

1.4. Importance of measuring Genetic Diversity

Variations in the genetic composition called polymorphisms between individuals result in genetic diversity of a species. Genetic diversity is the key aspect in evolution of species to cope up with changes in selection pressure (Frankham 1995). It is important for survival of populations and to predict the degree of their vulnerability to extinction. In an ecosystem each species plays a different ecological role forming a cyclic system where one is dependent on other contributing to the functioning of the ecosystem. Loss of some species or populations at certain levels will interrupt this cycle thus affecting the whole ecosystem at different degrees depending on the ecological role of the lost species and number of other species. Hence, to conserve biodiversity, genetic diversity is important to consider at population levels as it determines the survival of the species and thus the species diversity (Booy et al. 2000; Verma 2017).

1.5. Gene flow in rivers and estuaries

Gene flow is important aspect in population genetics which significantly influence the genetic diversity and persistence of populations. In case of rivers, degree of dispersal of species and respectively the gene flow depends on structure of these streams, position of the inhabiting populations in this network and physiological traits of the species. In streams with unidirectional flow dispersal is only downstream resulting in less or no exchange in upstream populations. Also, another important factor to consider is the hydrological modifications (dams and other barriers) and habitat degradation constraining the connectivity of populations (Swanson et al. 1998; Alp et al. 2012). Hydrological connectivity among floodplains supports the persistence, dispersal, and recolonization of biota between these floodplains' habitats. These periodic increase in the productivity of these habitats due to the favourable flow regimes and availability of food supports the boom of aquatic as well the dependent terrestrial biota especially birds. Construction of dams and channelization flood affects this natural process. Construction of dams and channelization alters the connectivity and flow regimes disrupting this process resulting in loss of biotic communities (Swansonet al. 1998b; Jenkins & Boulton 2003; Leigh et al. 2010). Irrigation channels are major concern to freshwater biodiversity throughout the world as it facilitates artificial migration between naturally disconnected basins. This results in biotic homogenization. For example, Freshwater catfish (*Diplomystes* cf. *camposensis)* in Central Chile exhibits low genetic structure which is driven by artificial migration between river basins (Muñoz-Ramírez et al. 2015).

Water birds are also an important transporter who mediate the dispersal of aquatic organisms between isolated catchments and water habitats (Amezaga et al. 2002; Charalambidou & Santamaría 2002; de Meester et al. 2002). Habitat destruction of the migratory grounds and stop water habitat affects the occurrence as well as the dietary habits of these migratory and native birds (Amezaga et al. 2002; Santamaría & Klaassen 2002). Additionally, climate change has severe impact on Mediterranean wetlands resulting in population decline (Sutherland 1996). In the end decline if these birds that mediate influence aquatic organism dispersal impacts the diversity and local species richness of the biotic community.

In case of estuaries the spatial habitat patterns are discrete and the organisms inhabiting across have restricted dispersal or isolation due to physiological tolerance, physical barriers or geographic distance (Bilton et al. 2002). Therefore, it may restrict the gene flow which could promote in genetically unique taxa or cryptic speciation adapted to the local physiological conditions (Blaber 1991; Lee 2000; Bilton et al. 2002). Although, it depends on the migratory behaviour of the larvae. Some species migrate (amphidromy) (for example, *Macrobrachium moorei*) between sea and back (Export strategy) enabling gene flow, while the others (for example, *D. valentina*) restrain

(Retention strategy) to the estuary or with no dispersal to other estuaries through sea (Bilton et al. 2002; Bauer 2013).

1.6. Molecular studies in *D. valentina* and *P. zariquieyi*

González-Ortegón et al. (2016) evaluated the genetic structure of the shrimps, *D. valentina* and *P. zariquieyi*. COI marker was used for this purpose and the samples were collected between 2005 and 2008. This study outcomes suggests the shrimp *D. valentina* had low nucleotide diversity and high haplotype diversity. Haplotype network was well structured with frequencies of haplotypes being similar in different localities. In case of *P. zariquieyi* nucleotide and haplotype diversities were comparatively lower than any other species included in the study. Haplotype network was not well structured only few haplotypes were present in most localities and the rest were derived haplotypes that varied from the central haplotypes by singletons and found only in one or two individuals. Haplotype frequency clines were not observed in both species. When geographic and genetic distance were evaluated by Mantel test negative correlation was present in *D. valentina*. And *P. zariquieyi*, shows Isolation by distance pattern (IBD) which could be because of moderate salinity tolerance and lower dispersal ability. Limited gene flow was observed between closely located populations in *D. valentina* could be related to the brooding care behaviour lacking free larval stages. The study also suggests that in the southern distribution range the gene flow is asymmetric due to southwards flow of ocean current in this range (González-Ortegón et al. 2016).

To delineate phylogenetic relationships within Atyidae a nuclear Histone 3, nuclear 28S rRNA and mitochondrial 16S rRNA was used by (von Rintelen et al. 2012) as a concatenate marker combining both nuclear and mitochondrial genes, hence being more efficient in constructing phylogeny.

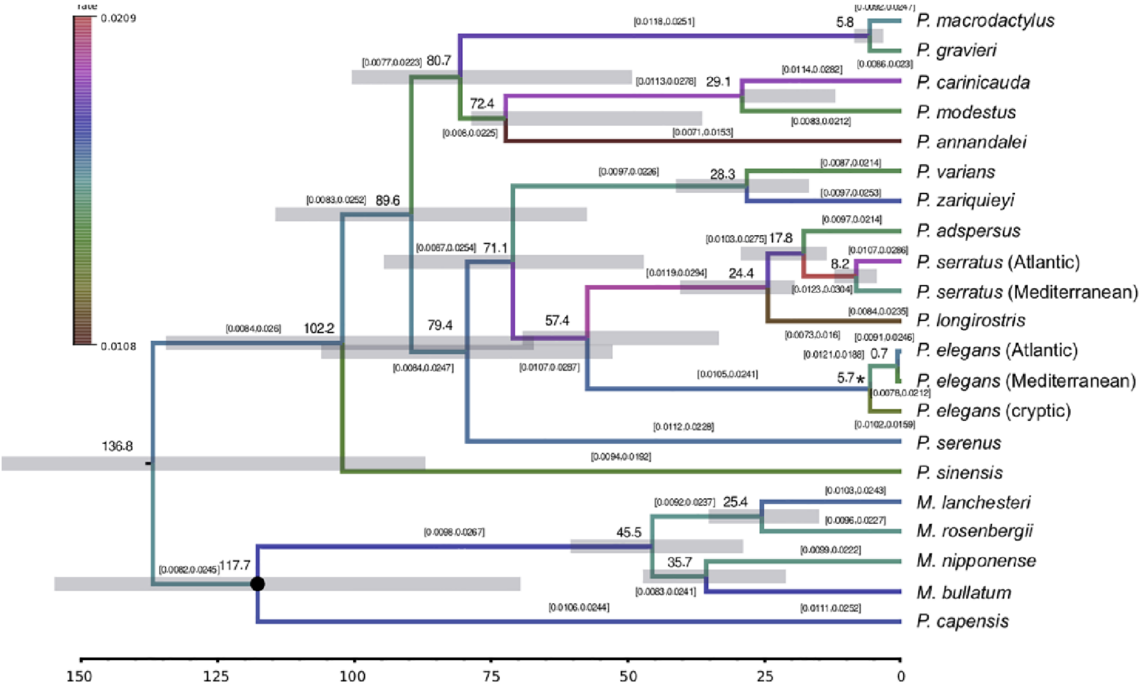

Figure 9: Phylogenetic tree with divergence time calibrated for *Palaemon* and *Macrobrachium* (González-Castellano et al. 2020)

In phylogeny, the molecular evolutionary clock is an important concept and states that at molecular level the rate of evolution is constant among species and through time because of neutrality of changes at molecular level assuming no selection acting on the chosen marker (Frankham 1995a; O'Reilly et al. 2015). This concept enables to use molecular data from DNA or Protein sequences and integrating also fossil records of species in estimation of geological ages at which species have diverged (Welch & Bromham 2005; O'Reilly et al. 2015). It is an important evolutionary biology tool used to estimate the geological time of events of speciation and extinctions (O'Reilly et al. 2015). To estimate molecular dating a calibration curve (assumption of constant change over time) of sequence divergence vs time drawn from the fossil record is created and the dates for events with known molecular divergence but unknown time can be read using this curve (Frankham 1995). González-Castellano et al. (2020) calibrated the molecular clock *Palaemon species* (Figure 9) by assuming the by constraining the most recent common ancestor (mrca) of *Palaemon elegans* to Messinian Salinity Crisis during late Miocene, 5.3-5.9 Mya

2. Aims of the Thesis

Dugustella valentina and *Palaemon zariquieyi* are shrimp species endemic to the Valencia region, Spain. We aim to evaluate genetic diversity, connectivity between populations and demographic patterns of these species based on a mitochondrial Cytochrome c oxidase subunit-I (COI) marker. Furthermore, to revaluate the phylogenetic relationship of one of the genetically distant groups, a nuclear Histone 3, nuclear 28S rRNA and mitochondrial 16S rRNA was also used.

3. Methods

3.1. Sample collection

Two shrimp species *D. valentina* (~200) and *P. zariquieyi (*~200) were morphologically recognised and collected in multiple localities (Table 1) (See Appendix 1 for sampling co-ordinates) from the river basins mainly in rivers Turia and Jucar in Valencia, Spain during the year 2018. The samples were fixed in 95 % ethanol for further analysis and stored at - 20 °C.

Table 1. List of sampling localities

Species	Place	Date of collection	No. of individuals collected	No. of individuals sequenced
P. zariquieyi	Ullal de la Font del Forner. Sollana	27/03/2018	8	6
D. valentina	Ullal de la Font del Forner. Sollana	27/03/2018	23	10
D. valentina	Rio Jucar. Antella	27/03/2018	29	6
P. zariquieyi	Rio Jucar. Antella	27/03/2018	22	6
D. valentina	Río Sot, Sot de Chera	03/03/2018	30	6
D. valentina	Rio Turia. Chullila Pueblo	03/04/2018	27	6
P. zariquieyi	Rio Turia. Chullila Pueblo	03/04/2018	12	6
P. zariquieyi	Molí de la Font. Castellón	06/07/2017	22	6
D. valentina	Rio Racons. Pego	04/04/2018	30	6
D. valentina	Río Bullent. Oliva	04/04/2018	25	5
P. zariquieyi	Carraixet. Alboraia	14/03/2018	50	4
D. valentina	Rio Turia	22/03/2018	30	6
P. zariquieyi	Río Turia. Gestalgar	03/04/2018	11	6
D. valentina	Río Turia. Gestalgar	03/04/2018	23	6
P. zariquieyi	Font Salda. Oliva	04/03/2018	22	6
Both species	Rio Turia. Chulilla Puentes	03/04/2018	21	18 (*D.v*), 3 (*P.z*)
P. zariquieyi	Prat de Cabanes	-	30	6

3.2. DNA isolation

A small piece of tissue from the abdominal region of the shrimps were cut dried and DNA was extracted from total 147 samples using DNeasy Blood&Tissue Kit (Qiagen) and stored at -20 °C. The quantity and quality of the extracted DNA was checked using Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer.

3.3. PCR

A cytochrome oxidase subunit 1 (COI) mitochondrial marker (~670 bp) was selected for further analysis and amplified by T 100 PCR thermal cycler (BIO_RAD) (For PCR conditions refer, Table 2 and Table 3) using the primers COH6 and COL6b (Refer Table 4 for the primer sequences) (Schubart & Huber 2006).

Table 2. PCR composition 1

Contents	Volume (µl)
Template DNA	1
COH6_ Forward primer (10µM/µl)	5
COL6B_Reverse primer (10µM/µl)	3
PPP mix (Top Bio)	12.5
Nuclease free water	3.5
Total volume	25

Table 3. Thermocycler conditions 1 [T 100 PCR thermal cycler (BIO_RAD)]

Step	Temperature °C	Time	No. of cycles
Initial denaturation	95	5 min	1
Denaturation	95	20 sec	40
Annealing	49	45 sec	

Extension	72	47 sec	
Final extension	72	5 min	1

Table 4: List of Primer sequences

Primer ID.	Sequence
COH6	TAD ACT TCD GGR TGD CCA AAR AAY CA
COL6b	ACA AAT CAT AAA GAT ATY GG
H3F	ATGGCTCGTACCAAGCAGACVGC
H3R	ATATCCTTRGGCATRATRGTGAC
28S-F-Cru	ACCCGCCTAATTTAAGCATAT
28S-R-Met	GCTATCCTGAGGGAAACTTC
16S-F-Car	TGCCTGTTTATCAAAAACATGTC
16S-R-Car1	GAAAGATAGAAACTAACCTGGCT

To construct phylogenetic tree to investigate the substantial genetic distance present in some sequences of *D. valentina* three markers were amplified (For PCR conditions refer, Table 5 and Table 6), a nuclear Histone 3 (328 bp), nuclear 28S rRNA (1090 bp) and mitochondrial 16S rRNA (540 bp) using the primers, (H3F and H3R), (28S-F-Cru and 28S-R-Met) and, (16S-F-Car and16S-R-Car1) adopted from von Rintelen et al. (2012) (Refer Table 4 for the primer sequences).

Table 5. PCR composition 2

Contents	Volume (µl)
Template DNA	1
Forward primer (10µM/µl)	2

Reverse primer (10µM/µl)	2
PPP mix (Top Bio)	12.5
Nuclease free water	7.5
Total volume	25

Table 6. Thermocycler conditions 2 [T 100 PCR thermal cycler (BIO_RAD)]

Step	Temperature °C	Time	No. of cycles
Initial denaturation	95	5 min	1
Denaturation	95	20 sec	40
Annealing	16S_ 50 H3_50 28S_60-52	16S_ 60 sec H3_60 sec 28S_120 sec	
Extension	72	47 sec	
Final extension	72	5 min	1

The PCR products were checked for amplification using 6x coral load dye, 100bp ladder on 1% agarose gel.

3.4. Sequencing

The amplified products were purified using Gel / PCR DNA fragment extraction kit (Geneaid) and sequenced by capillary electrophoresis in Genetic Analyzer (Applied Biosystems, USA) with forward primer at Biology Section, Faculty of Science, Charles University. Qualities of sequences were assessed, validated and aligned using Geneious v10.2.6 (Kearse et al. 2012) software.

3.5. Data analysis

3.5.1 Genetic differentiation

All haplotypes were compared with the existing reference sequences in NCBI database using BLAST tool and novel haplotypes were recognized.

Descriptive tests Tajima's *D* (Tajima 1989) and Fu's *Fs* (Fu 1997) for each species was calculated using DnaSP V6 software (Rozas et al. 2017) to estimate deviations from neutrality. $D < 0$ implies negative selection or expansion of population while $D > 0$ implies balanced selection or recent population bottleneck (Holsinger 2012). $Fs < 0$ implies the presence of surplus number of alleles followed by a recent expansion in the population or because of genetic hitchhiking while $Fs > 0$ implies allele deficiency as a consequence of recent population bottleneck or because of over dominant selection (Holsinger 2012).

Haplotype networks were created for each species using the software TCS V1.23 (Crandall 2000) (Connection limit = 95%) and the visualization of the networks were improved by tcsBU (Múrias Dos Santos et al. 2016).

3.5.2 Phylogeny in *D. valentina*

To assess the phylogenetic relationship between genetically distant (6.25%) sequences of *D. valentina* from three localities Gestalgar, Chulilla puentes and, Chulilla pueblo and the rest of haplotypes, three marker sequences (as mentioned in section 3.3) a nuclear Histone 3 (H3), nuclear 28S rRNA and mitochondrial 16S rRNA were to be concatenated to construct phylogenetic tree. No genetic variability between sequences were found in the nuclear markers hence only mitochondrial 16s rRNA and COI sequences were used to construct phylogenetic trees with *Atyaephyra desmaresti* as outgroup in MrBayes V3.2.7 (John & Fredrik 2001) using MCMC model (number of runs = 2, number of generations = 1000000, number of chains = 4) with HKY substitution model and the trees were visualized in FigTree V1.4.4 (Rambaut 2007). The appropriate mutation model was computed using JModeltest 2.1.10 (Posada 2008)

3.5.3 Demographic history and EPS

To evaluate the changes in the effective population sizes over time, Bayesian analysis of molecular sequences to generate Bayesian skyline plot (BSP) using MCMC model (chain length = 10,000,000 and burning period = 1,000,000) was performed in BEAST V2.6.6 (Rambaut et al. 2018) with HKY substitution model for both the species. The effective sample size (ESS) was calculated and visualised in Tracer 1.7.1 (Rambaut et al. 2018).

3.5.4 Landscape genetics

To analyse the possible presence of barriers to gene flow among populations Alleles in Space (AIS) (Miller 2005) was utilized in which the plot contains peaks, each spatially indicating a population according to sample coordinates and the height of the peak represents the genetic distance between individuals within that population based on genetic differentiation between the sequences. To estimate the gene flow between populations and asses the population structuring, Geneland V 4.0 .9 (Guillot et al. 2005) was used which clusters the populations according to spatial distribution of haplotypes based on posterior probability.

4. Results

4.1. Genetic differentiation

4.1.1 D. valentina

Out of 9 localities, from different river basins 69 sequences were obtained in *D. valentina*. A total 14 haplotypes were found to be present out of which 10 are newly identified. Analysis of descriptive statistics from DnaSp (Table 7) shows that the haplotype and nucleotide diversities are slightly lower than observations from reference samples (Table 8). Negative value of Tajima's *D* indicates recent population expansion after recent bottleneck or due to genetic hitchiking and Fu's *Fs* indicate recent bottleneck or over dominant selection. But of all the values only nH and Hd are significant.

Table 7: Descriptive statistics of *D. valentina* indicating the number of localities *(nL),* number of samples *(n),* number of haplotypes *(nH),* nucleotide diversity *(pi),* average number of pairwise differences *(k),* haplotype diversity *(Hd),* Tajima's *D (D),* Ramos-Onsins and Rozas's *R2 (R2),* Fu's *Fs (Fs)* and Raggedness, *r* (* represents significance P < 0.05).

nL	*n*	*nH*	*pi*	*k*	*Hd*	*D*	*R2*	*Fs*	*r*
9	69	14*	0.015	1.195	0.778*	-0.619	0.083	3.78	0.058

Table 8: *D. valentina* results from González-Ortegón et al. (2016). number of localities *(nL),* number of samples *(n),* nucleotide diversity *(pi),* haplotype diversity *(Hd),* Mantel test correlation r (Significance *P*) [M. r (P)]

nL	*n*	*pi*	*Hd*	M. r (P)
6	30	0.019	0.908	r = -0.0091 (P = 0.5335)

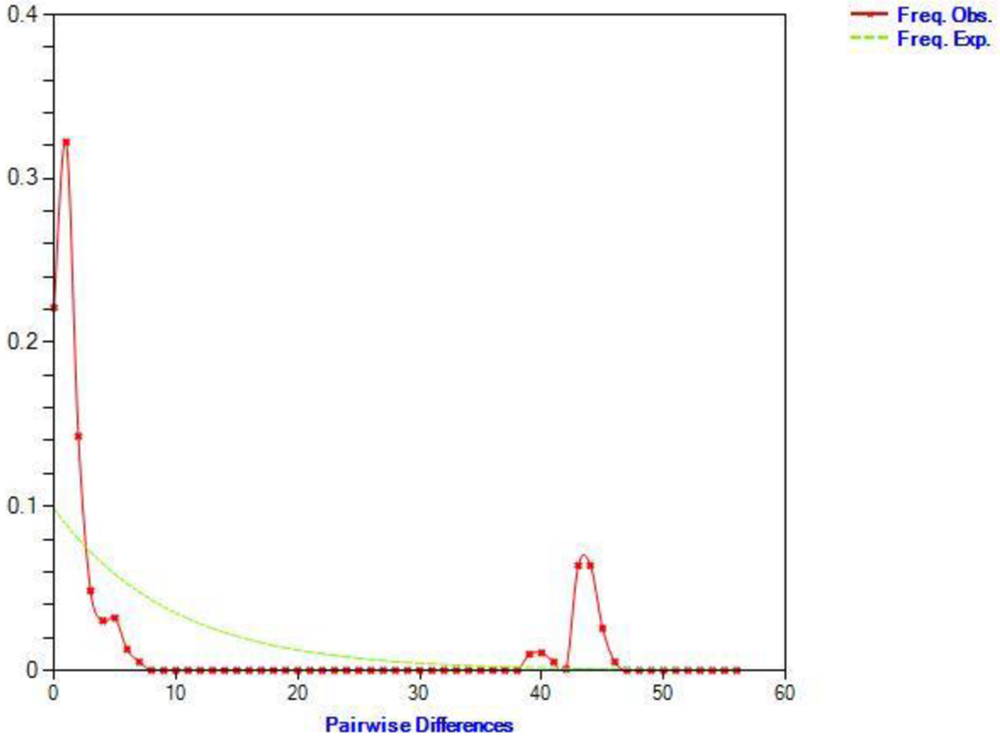

Figure 10. Mismatch distribution plotted in DnaSP V6

Mismatch distribution plot showing expected and observed differences between sequences have bimodal pattern of long period of isolation followed by secondary contact (Frankham et al. 2002) (Figure 10). When considered each peak belongs to different population/group they individually have unimodal distribution supporting expansion of populations.

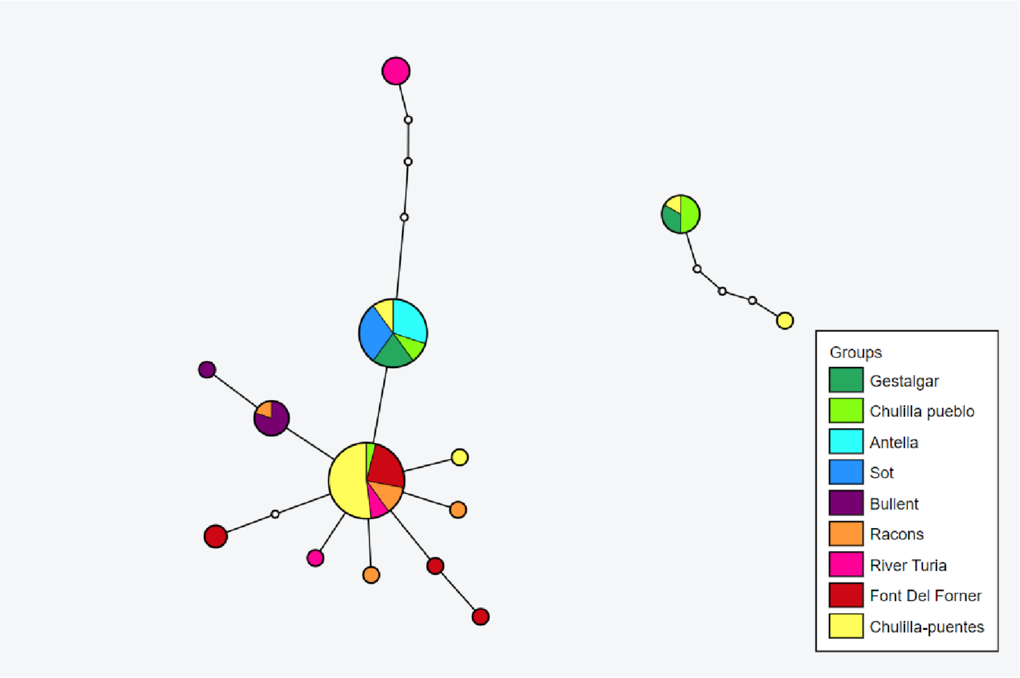

Figure 11. Haplotype network of *D. valentina* with groups representing localities constructed in TCS and visualized in tcsBeautifier.

Haplotype network (Figure 11) shows two widespread haplotypes and rest vary by 1-3 mutational steps found in one or two individuals. Two of the haplotypes represented by three localities (Gestalgar, Chulilla Puentes and, Chullila Pueblo) found to be genetically distant and doesn't form network with rest of the haplotypes majorly represented in the populations. Also, the distant haplotype is 100% similar to with haplotype 2 published in E. González-Ortegón et al. (2016) along with a similar haplotype (Figure.11). Nuclear markers (28S rRNA and H3) utilized to assess the phylogenetic relationship between these haplotypes did not represent the distance hence these markers were dismissed for further analysis but the mitochondrial 16S rRNA marker exhibited some genetic distance with other samples by 2 parsimonial sites. Phylogenetic tree from

Bayesian inference based on COI (Figure 13) supports the formation of different clade with a posterior probability of 100 %, and the tree based on 16S rRNA (Figure 12) with a posterior probability of 67%.

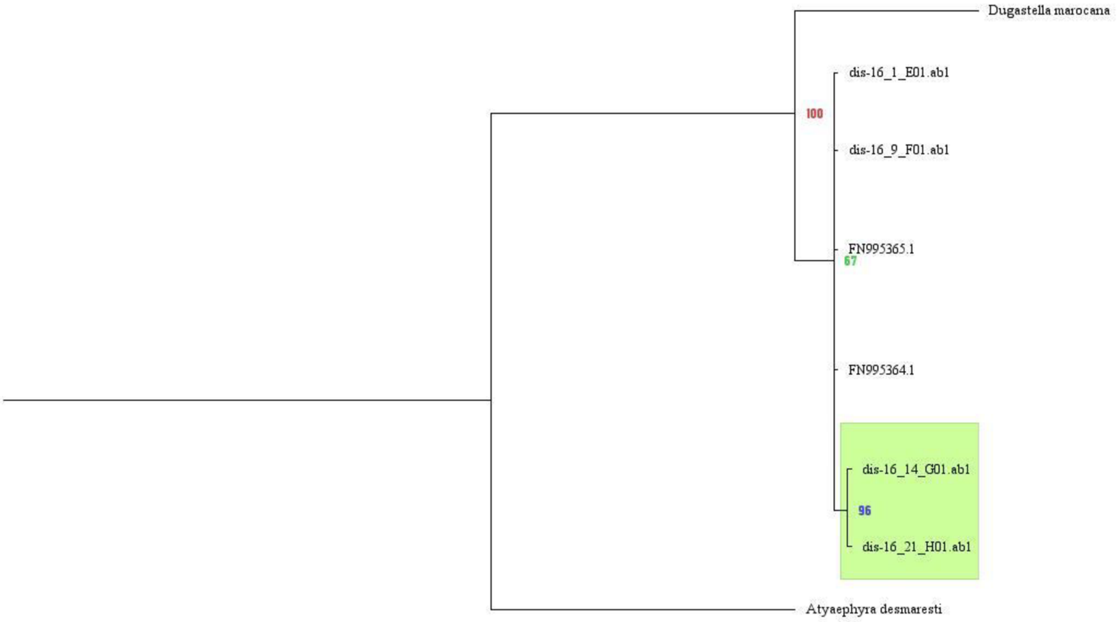

Figure 12. Phylogram based on 16S rRNA by bayesian inference MrBayes V3.2.7 and visualized in, numbers on the nodes represent the posterior probabilities. Clade highlighted in green represents Haplotype 02.

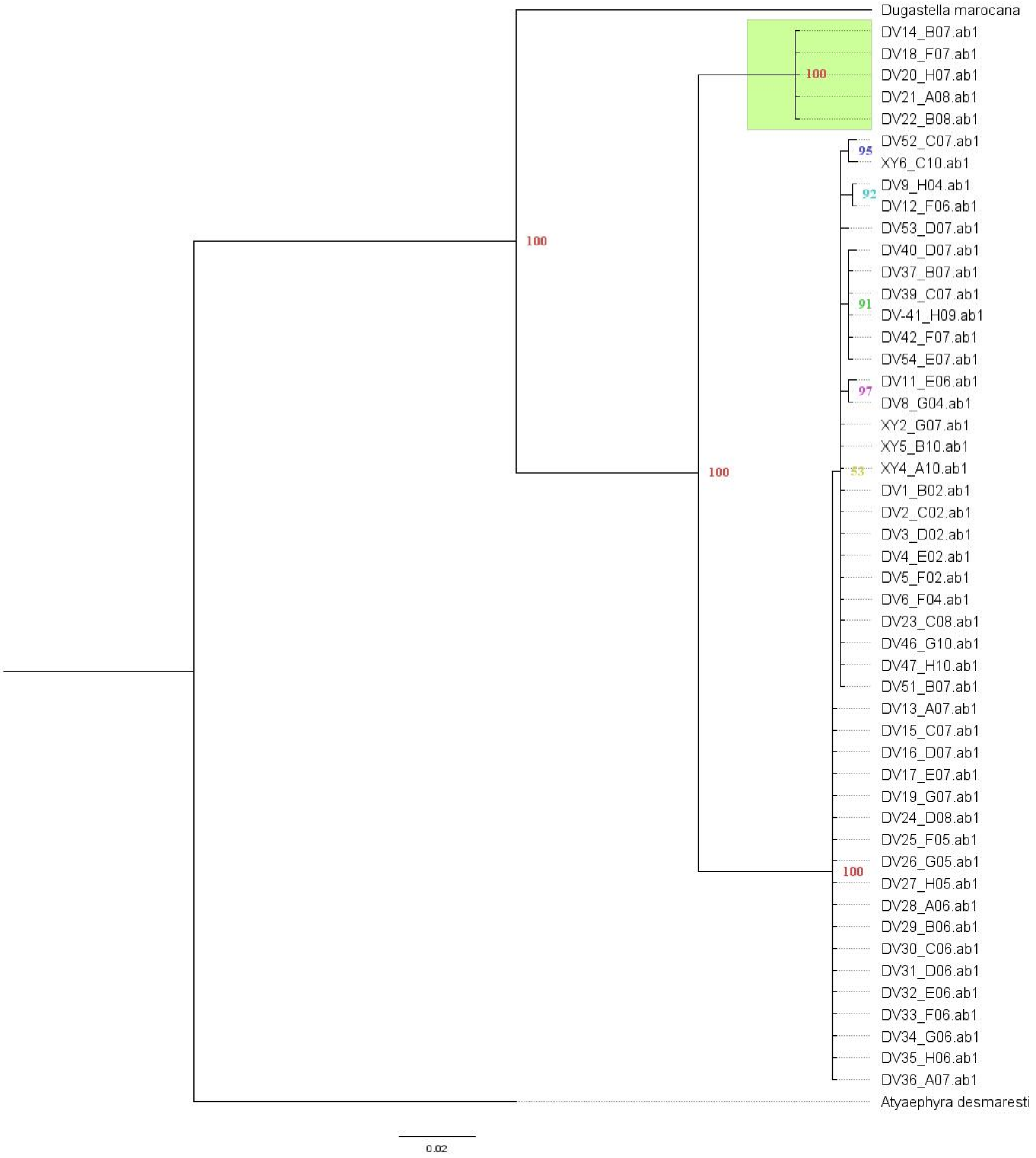

Figure 13. Phylogram based on COI by bayesian inference contructed in Mr.Bayes, numbers on the nodes represent the posterior probabilities. Clade highlighted in green represents haplotype 02.

4.1.2 P. zariquieyi

Out of 9 localities, from different river basins 50 sequences were obtained from *P. zariquieyi*. in A total of 6 haplotypes were found to be present in which 4 are newly identified. Analysis of descriptive statistics from DnaSp (Table 9) shows that the haplotype and nucleotide diversities are similar to reference samples (Table 10) and lower than *D. valentina* (Table 7). Positive value of Tajima's D indicates recent population decline and Fu's Fs indicate alelle deficiency because recent bottleneck or over dominant selection. But none of the values are significant.

Table 9: Descriptive statistics of *P. zariquieyi* indicating the number of localities (nL), number of samples *(n),* number of haplotypes *(nH),* nucleotide diversity *(pi),* average number of pairwise differences *(k)*, haplotype diversity (*Hd*), Tajima's D (*D*), Ramos-Onsins and Rozas's R2 (*R2*), Fu's Fs *(Fs)* and Raggedness, *r* (* represents significance P < 0.05).

nL	*n*	*nH*	*k*	*pi*	*Hd*	*D*	*R2*	*Fs*	*r*
9	50	5	1.195	0.002	0.612	0.171	0.118	0.387	0.246

Table 10: *P. zariquieyi* results from González-Ortegón et al. (2016). number of localities (nL), number of samples (n), nucleotide diversity (pi), haplotype diversity (Hd), Mantel test correlation r (Significance P) [M. r (P)].

nL	*n*	*pi*	*Hd*	M. r (P)
8	52	0.004	0.661	r = 0.5390 (P = 0.0043)

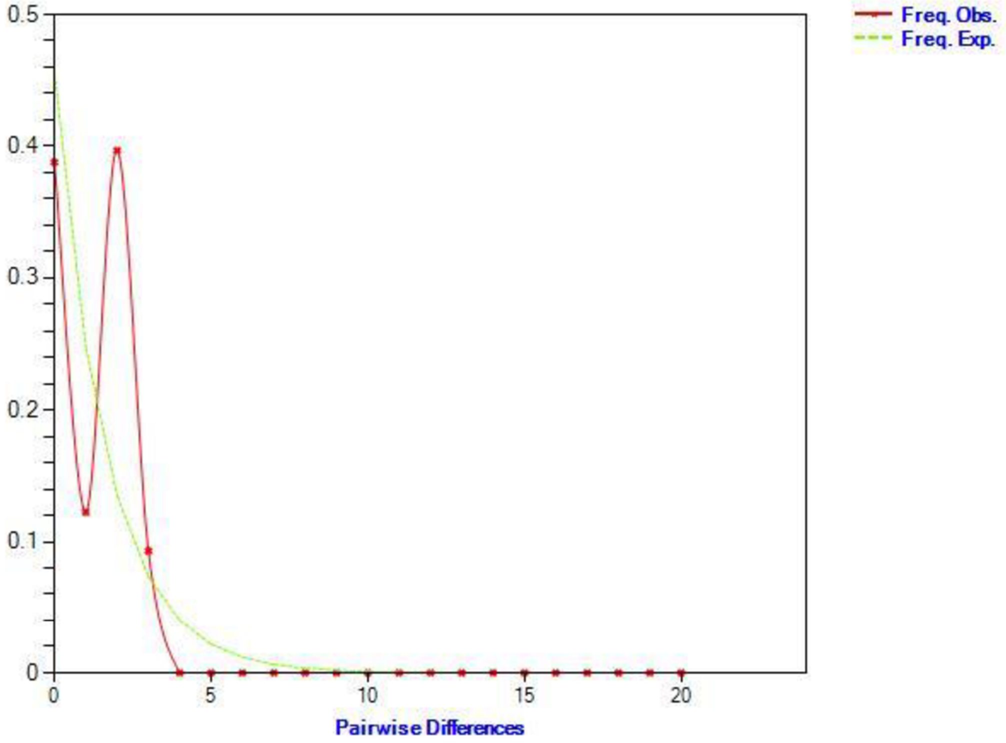

Figure 14. Mismatch distribution plotted in DnaSP V6.

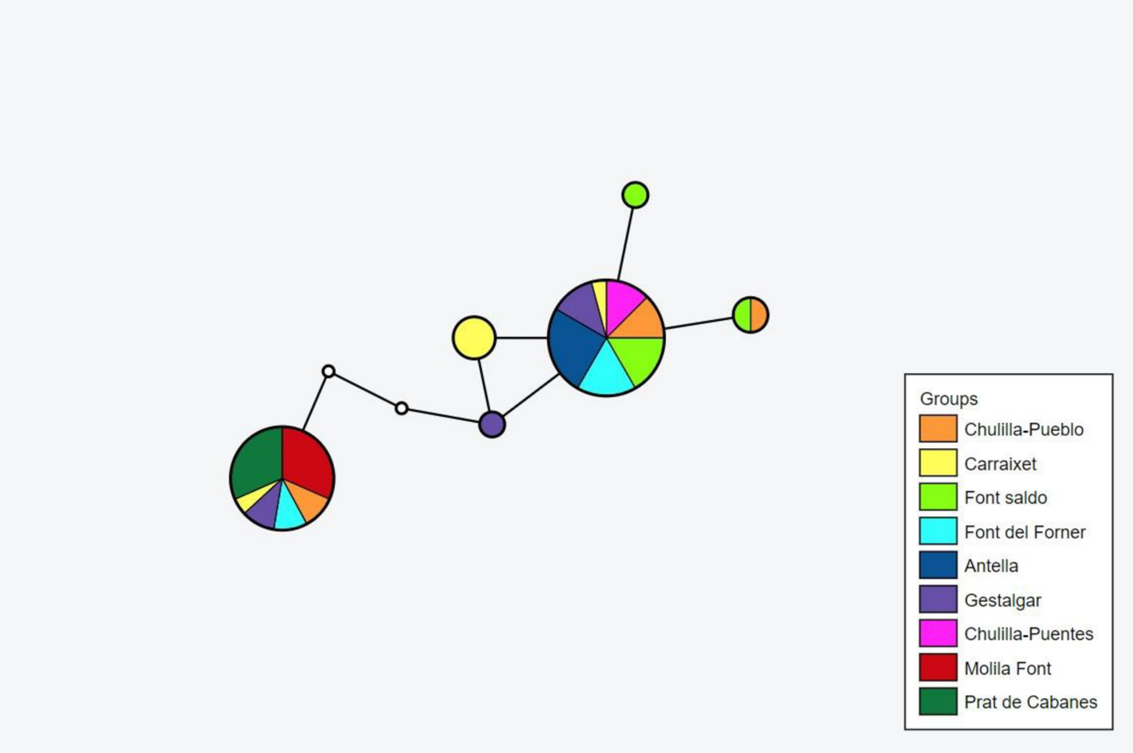

Figure 15. Haplotype network of *P. zariquieyi* with groups representing localities constructed in TCS and visualized in tcsBeautifier.

Mismatch distribution plot with bimodal distribution (Figure 14) with one peak close to zero suggests a pattern of reduced genetic diversity followed by bottleneck event (Frankham et al. 2002). Only two haplotypes are widespread across populations with four derived haplotypes differing from the widespread haplotypes by singletons. The derived haplotypes are represented by only few individuals with minimal structuring of the network (Figure 15).

4.2. Demographic history and effective population size

4.2.1 D. valentina

Bayesian skyline plot indicates long constant population size with recent bottleneck event. The most recent increase in population size visible on the plot has wide confidence interval thus no conclusion can be drawn (Figure 16). The assumption of ESS >200 is satisfied by ESS value of 827.4.

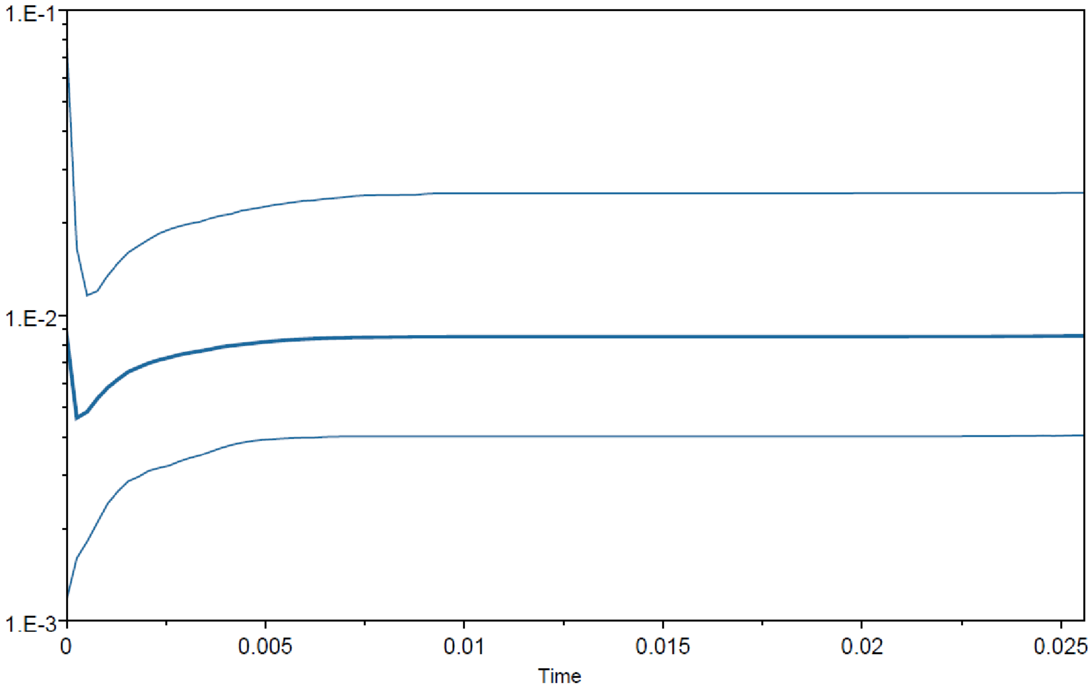

Figure 16. Bayesian skyline plot of *D. valentina*. The X axis represents the timeline with 0 representing the present, Y axis represents the effective population size (Ne), the central line shows median of Ne and the upper, lower lines shows 95% confidence interval.

4.2.2 *P. zariquieyi*

Bayesian skyline plot indicates long period of stable population size with recent increase. Latest trends in population size changes are inconclusive due to wide confidence interval from the median of ESS (Figure 17). The assumption of ESS >200 is satisfied by ESS value of 603.1.

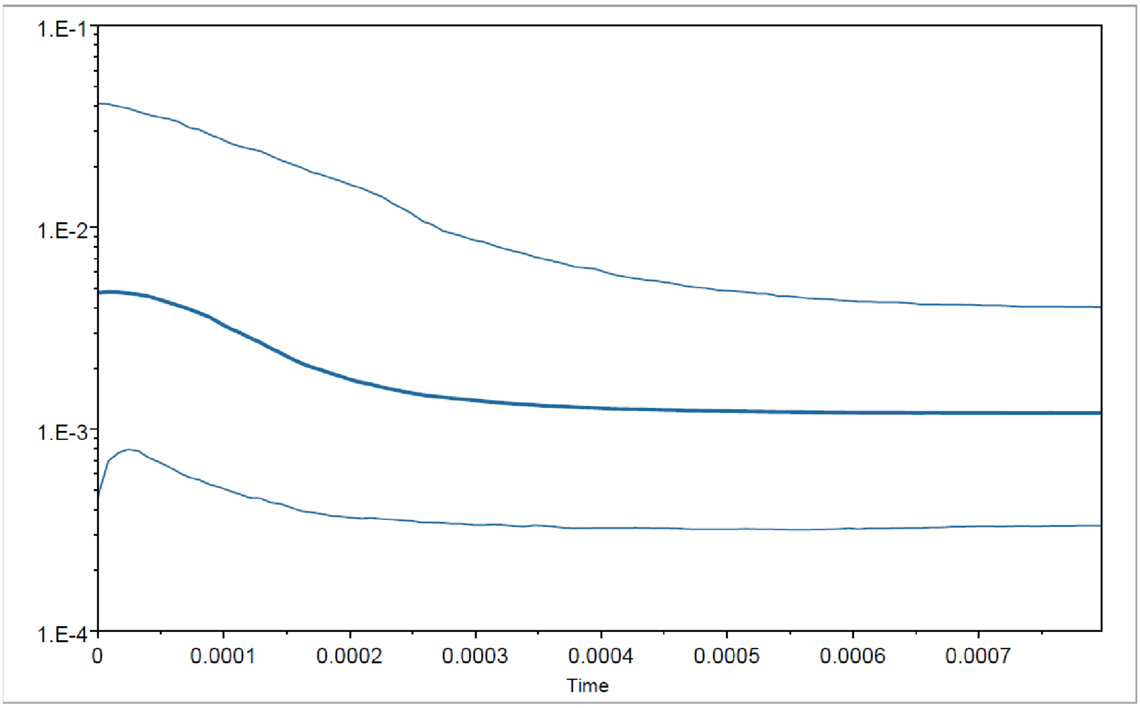

Figure 17. Bayesian skyline plot of *P. zariquieyi*. The X axis represents the timeline with 0 representing the present, Y axis represents the effective population size (Ne), the central line shows median of Ne and the upper, lower lines shows 95% confidence interval.

4.3. Landscape genetics

4.3.1 *D. valentina*

Based on the map of spatial dispersal of haplotypes the population is clustered into three (Figure 18). Two localities clusterred into a population (Figure 18.b _white cluster) which are located in the Bennituba ravine, Riaxol river (Racons) and the river Vedat (Bullent) that are disconnected from the Jucar and Turia river basin. Green cluster in the is shared between the localities present in Turia (Chulilla puentes) and Jucar (Font del forner) river basins while the yellow cluster is formed by rest of the localities that are also located the the Jucar and Turia river basins. Correspondingly, a barrier to gene flow is indicated between populations represented in yellow and green clusters.

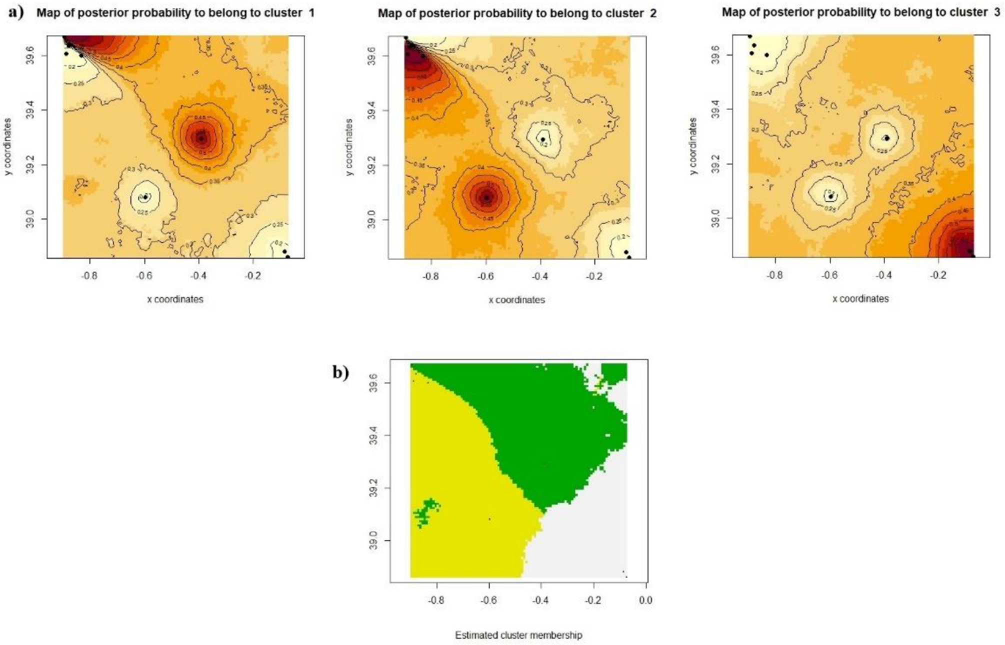

Figure 18. ***D. valentina*** **_a) Map of posterior probability of population membership & b) map of population clustering based on spatial distribution of haplotypes created in Geneland.**

Mantel test indicates negative correlation (r = -0.068, P = 0.111) between geographic and genetic distance but it is not significant (Figure 20) which is a similar observation to the reference samples (Table 8). Peaks in the landscape interpolation plot (Figure 19) shows high genetic distance within localities in the east in Chulilla puentes followed by the Gestalgar and Chulilla pueblo. Low genetic distances are shown in the locality Sot in east and in the west in Antella, Font del forner, Racons and Bullent.

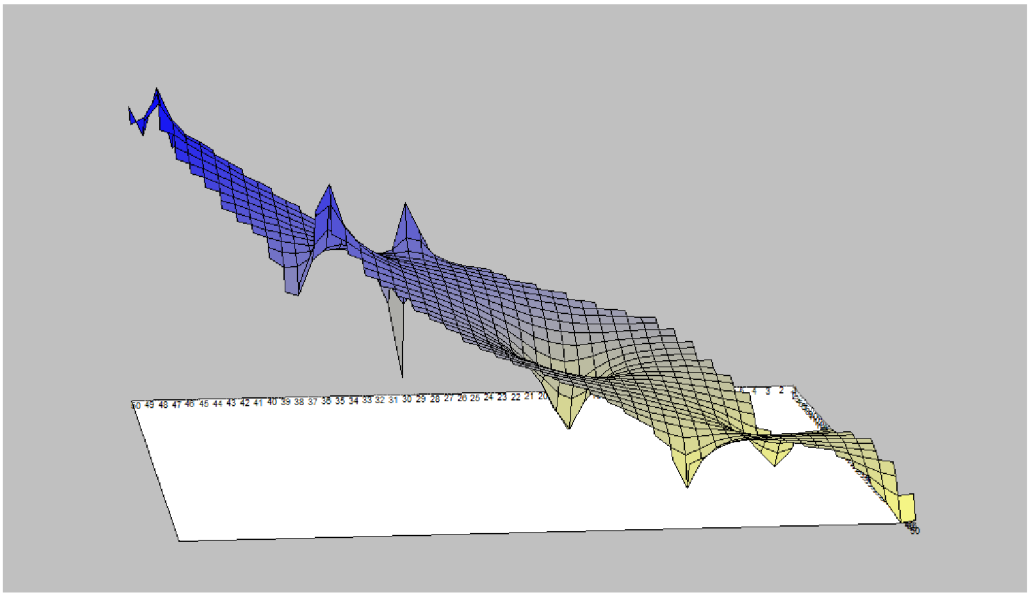

Figure 19. Landscape genetic interpolation plot of *D. valentina* created in Alleles in Space.

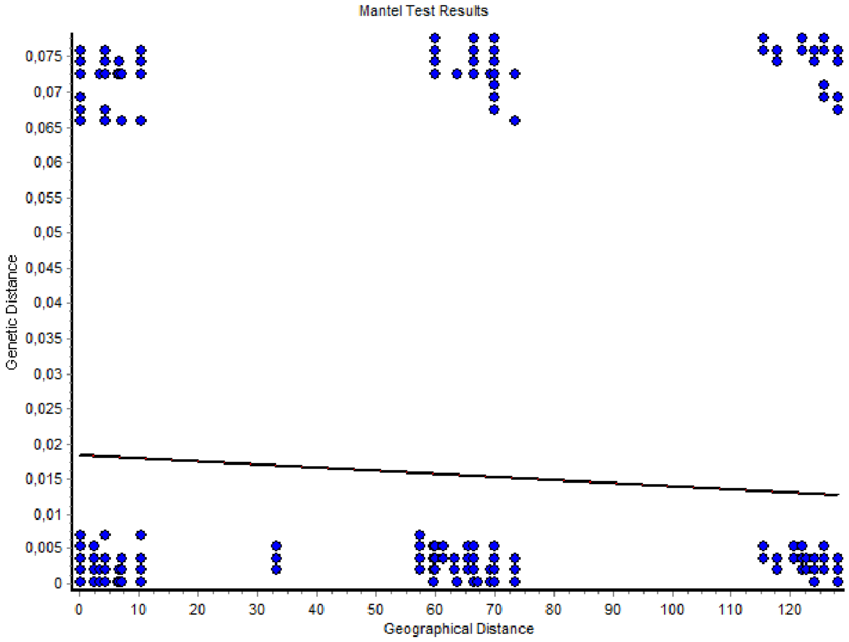

Figure 20. Mantel test indicating correlation between geographic and genetic distance in *D. valentina* created in Alleles in space.

4.3.2 P. zariquieyi

Based on the map of spatial dispersal of haplotypes the population is clustered into two with no strong barriers between the populations (Figure 21). Two localities (‘Moli de la font’ and ‘Carraixet’) clusterring into one population (Figure 13.b_Green cluster) located in diffrent courses disconnected from the Jucar and Turia basin while the rest of the localities clustering into a separate population (Figure 21.b_White cluster) which are present in the river basins Turia, Jucar and the river Vedat.

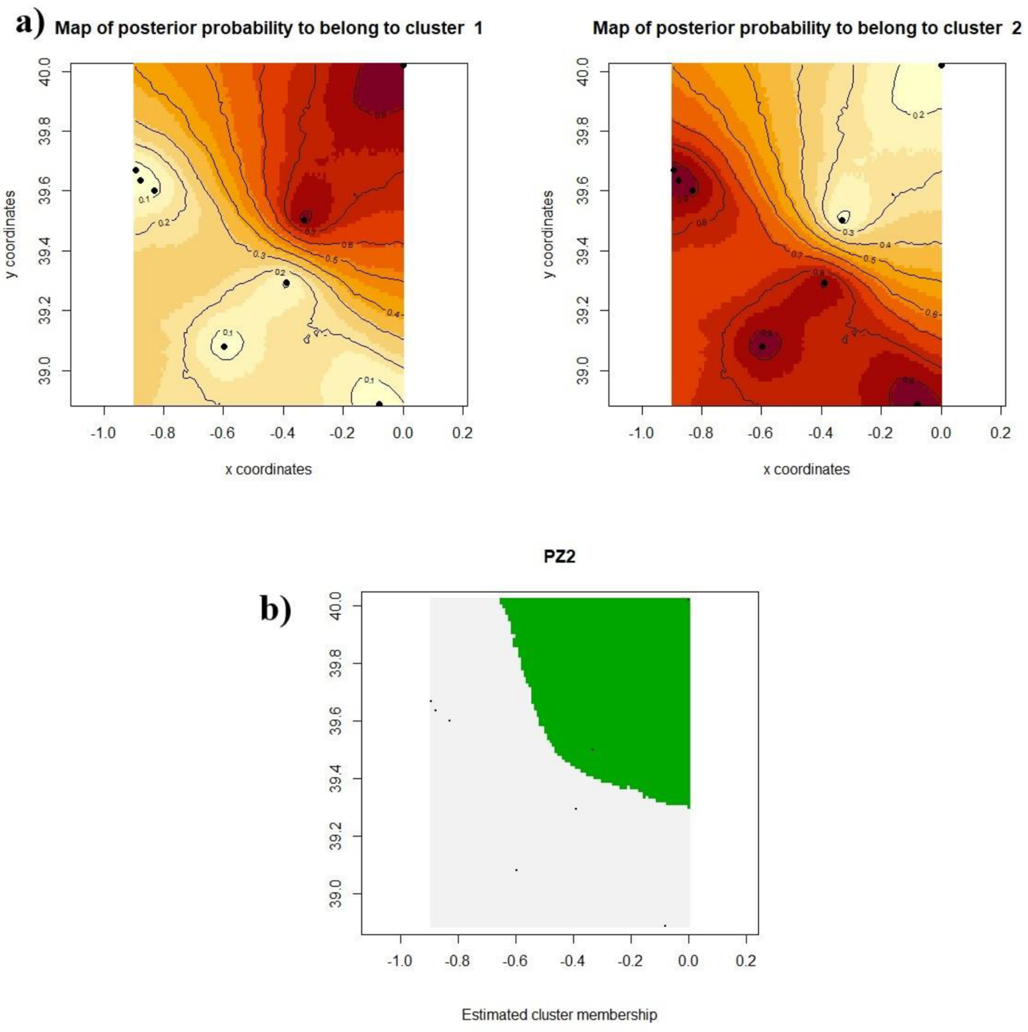

Figure 21. ***P.zariquieyi*_a) Map of posterior probability of population membership & b) map of population clustering based on spatial distribution of haplotypes created in Geneland.**

Mantel test indicates weak positive correlation (r= 0.085) between geographic and genetic distance which is typical patttern of Isolation by distance but it is not significant (p = 0.064) (Figure 23). IBD pattern was also observed in the reference samples (Table 10). Peaks in the landscape interpolation plot (Figure 22) shows high genetic distance within localities in the east in Chulilla puentes (highest), Gestalgar, and Chulila pueblo. Low genetic distances are shown in Moli del la font in the north and Font salada in the south of the sampling area.

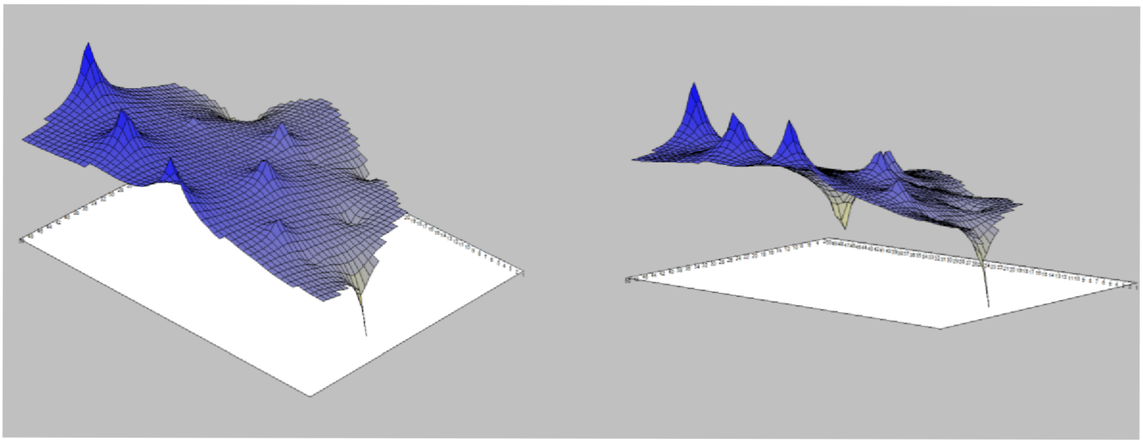

Figure 22. Landscape genetic interpolation plot of *P. zariquieyi* created in Alelles in Space

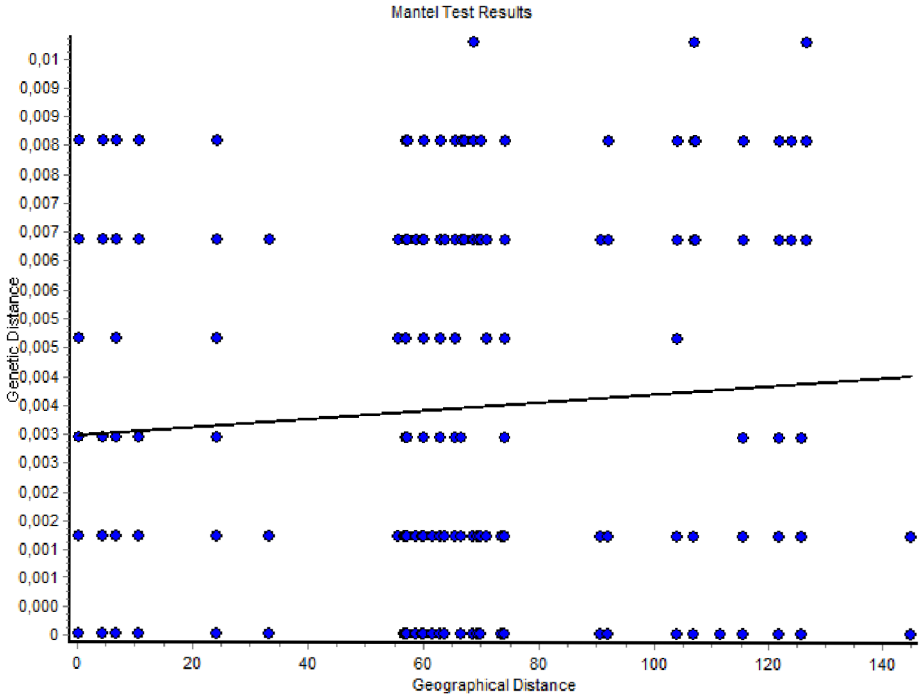

Figure 23. Mantel test indicating correlation between geographic and genetic distance in *P. zariquieyi* created in Alleles in space.

5. Discussion

In this study we have addressed the population genetic parameters using a short mitochondrial gene fragment of cytochrome oxidase I (COI) of the endemic shrimp species *D. valentina* and *P. zariquieyi* from samples collected in 2018. Our results were compared over a 10-year gap with the study from González-Ortegón et al. (2016) which used the populations sampled between 2005 to 2008 to understand the changes in the population structure and the possible explanations for it.

5.1. D. valentina

5.1.1 Dispersal, population connectivity and landscape genetics

The occurrence of numerous haplotypes within geographically close populations as well as many derived haplotypes presented in only few individuals within the same locality suggests population structuring in *D. valentina* despite of gene flow. When gene flow in considered within the populations/ localities Chulilla puentes followed by the Gestalgar and Chulilla pueblo in east exhibits highest genetic distance. While Sot in the east and rest of the localities situated in the south exibit low genetic distance. This could be influenced by spatial distribution of this fresh water species in the rivers situated in location having diverse geomorphic features like mountain systems (Estrela et al. 2004). This limits the dispersal of this species upstream. Another important factor to consider influencing gene flow is the abbreviated larval development having immobile stages constrained in the brooding pouch after which there is non-swimming but walking decapodid stage (Rodriguez & Cuesta 2011). This may limit the dispersal even within closely located populations contributing to high genetic variability. This pattern is a typical observation in freshwater Decapods which shows abbreviated development of plankton dependent larvae as an adaptation to freshwater habitats where planktonic food growth is limited (Vogt 2013).

Haplotypes commonly shared between the localities in Turia and Jucar basins suggests considerable gene flow even though they have separate river network channels. This correlates with the presence of the artificial channels like the irrigation channels ‘Canal Jucar-Turia’ and ‘Acequia real del Jucar’ connecting these river systems (Estrela et al.

2004). It is further is supported by the formation of separate population cluster from the localities in the Serpis river basin which is not connected to the other rivers by any artificial river structure (Estrela et al. 2004). Apart from geomorphological barriers presence of numerous regulation structures like weirs within these river networks could affect the migration of such species both biochemically and physiologically, as such structures result in siltation which reduces the dissolved oxygen concentration essential for supporting aquatic life and simultaneously act as physical barrier (Sabater & Tockner 2009).

Barriers portrayed within the close and same network between Chulilla puentes and the localities Gestalgar, Chulilla pueblo and Sot could be the result of differences in the local conditions where a canyon connecting the localities causes the reduced temperature and nutrient levels becoming an unfavorable condition. Other possibility is the difference in the sample ratio between Chullila puentes and the rest of localities. It is evident that there are moderate levels of geneflow even between the completely detached river systems that could be mediated through translocations either by flood events (not applicable to localities Chulilla puentes, Gestalgar, Chulilla pueblo and Sot in the east that are mountainous regions) which is typical in this region or through birds or fishermen (Banha & Anastácio, 2012; Stoffels et al., 2016).

5.1.2 Genetic diversity and demographic patterns

Haplotype diversity has been reduced from 0.908 to 0.778 and nucleotide diversity from 0.019 to 0.015 when compared the reference population sampled 10 years ago to the sampling done in this current research. This proposes that the populations of this endemic shrimp are suffering from decrease in population size or bottlenecks which might be caused by the artificial modifications and pollution in these rivers. The impact of the anthropogenic pressure in this region is supported for example, by the presence of smaller population size in the Mediterranean populations of the Atyid shrimp species *Atyaephyra. desmarestii* compared to the Atlantic populations (González-Ortegón et al. 2016). Pattern of long isolation period followed by secondary contact is indicated by bimodal distribution in mismatch distribution plot. The presence of distant haplotypes probably causes the plot to have bimodal pattern. When considered each peak belongs to different population/group they individually have unimodal distribution supporting expansion of populations. The first peak indicates the common haplotypes representing intra-clade pairwise differences (common haplotypes) and the second peak indicates the distant haplotype representing ancient interclade pairwise differences (Jenkins et al. 2018). Tajima's *D* statistics indicates population expansion followed after a recent bottleneck event, although it is non-significant likely because of low sample size. Bayesian skyline plot also support recent decline in effective population size followed by recent expansion in the population. This latest expansion cannot be confirmed as the confidence intervals of the plot are wide. Moreover, the presence of many derived haplotypes with star like haplotype network is another indicative of recent expansion of the population which was similarly observed for example in freshwater shrimp population of *P. sinensis* (Zhao et al., 2021).

5.1.3 Distant mitochondrial haplotypes

The presence of genetically distant (6.25%) haplotypes in the localities Chulilla pueblo, Gestalgar and Chulilla puentes which shares some variable sites in the mt-genome with the sister species, *D. marocana* can be result of mitochondrial capture from some unsampled species or extinct species/lineage. The bimodal pattern in mismatch distribution plot probably indicates the presence of two distinct lineages. This may be a result of secondary contact of two lineages that were separated for a long time followed by contact (Bremer et al. 2005; Jenkins et al. 2018). No variations were observed in the nuclear markers thus the possibility of occurrence of cryptic species is disregarded. Lack of nuclear variation between these lineages implies that the period of isolation was not sufficient to accumulate differences in the nuclear genome or that it became homogenized through recombination when they came in contact. In future, these distinct lineages should probably be analyzed separately as they represent different population it violates the assumptions of coalescent theory (Kingman 1982; Jenkins et al. 2018). Furthermore, Pigeon et al. (1998) explains the genetically (mitochondrial genome) distant groups in rainbow smelt (*Osmerus mordax*) with no spatiotemporal variations as sympatric occurrence of lineage originated from distinct glacial races that established partial reproductive barrier. But further evaluations are required to diagnose this haplotype forming separate clade.

5.2. *P. zariquieyi*

5.2.1 Genetic diversity

Low structuring of the haplotype network composed of only two central haplotypes with four derived haplotypes differing from the main ones by just one mutational step, occurring in very few individuals in *P. zariquieyi.* Thi*s* is evident from the observations of high haplotype diversity combined with low nucleotide diversity and the same pattern was observed in *P. zariquieyi* by E. González-Ortegón et al. (2016). This pattern is typical for populations that has expanded with low genetic diversity after recent bottleneck event (Grant & Bowen, 1998; de Jong et al., 2011; Zhao et al., 2021). This was reflected in this study with patterns of decrease in population size in the mismatch distribution plot, and Fu Fu's statistics due to low genetic diversity/ allele deficiency. Tajima's D neutrality test insist sudden population decline even though non-significant. The skyline plot shows long period of stable population size with recent increase. Latest trends in population size changes are inconclusive due to wide confidence interval from the median of ESS. This suggests that the number of samples should be increased to statistically support these conclusions.

5.2.2 Dispersal, population connectivity and landscape genetics

Although the overall genetic variation is low across the whole area, there is structuring of populations into two clusters. Samples from localities situated to the north of the sampling area, Moli de la Font in the Mijares river basin and Carraixet ravine, form a cluster, revealing substantial geneflow even though there are no natural or artificial connections between these water courses. Localities present to the south form another cluster between the localities in the Turia, Jucar and Serpis basins. The gene flow could be mediated through the irrigation channels that connect the river basins of Turia and Jucar, while in the river Serpis, despite having no connections to these rivers, migration is still possible.

Gene flow between these completely disconnected basins could be mediated through translocations by flooding events (not applicable to localities Chulilla puentes, Gestalgar, and Chulilla pueblo in the east that are mountainous regions), waterbirds, or inadvertently by fisherman (Banha & Anastácio 2012; Stoffels et al. 2016), as discussed in *D. valentina*. Alternatively, the observed pattern could be a result of north-to-south genetic gradient and isolation by distance (IBD) as identified by González-Ortegón et al. (2016) in this species.

Regardless of its statistical insignificance, IBD was observed using Mantel test. High genetic distances were identified within the eastern sampling localities Chulilla puentes (highest), Gestalgar, and Chulila pueblo similar to *D. valentina*. Lowest genetic distances were observed in the north-most and south-most sampling localities which are independent from rivers Turia and Jucar. This could be the result of this species' dispersal ability related to their abbreviated larval development with non-feeding stages lacking free swimming stage. The specifics of its life cycle could reduce its dispersal and thus gene flow as observed in freshwater decapods with abbreviated development (Vogt 2013). The larvae of *P. zariquieyi* are also more susceptible to predation compared to *D. valentina* larvae, which have increased protected time in the female brooding chamber because increased duration of parental care amplifies the chances of offspring survival (Shine 1978) . This may be the reason for better results of survival of *D. valentina* comparatively. Another factor to consider, apart from physiological barriers, is the osmoregulatory capacity that limits the dispersal of this species with a moderate tolerance to salinity (Guerao 1993). Similarly, as another euryhaline species, *P. varians* is associated with longer period of larval development and exhibits lower genetic variability across populations (González-Ortegón et al. (2016). As some of our observations suggest probable signs of population decline it can be proposed that these shrimps are suffering from human alterations like other ostrocad species (Poquet et al. 2008) and endemic Cyprinotoid fish *Valencia hispanica* (Oliva-Paterna et al. 2009) in these river systems that satisfy the high-water demand of the surrounding dense agricultural lands (Estrela et al. 2004). As high-water demand dries up some water channels, this increases the salt concentration of some waters (Sabater & Tockner, 2009), which ultimately is being detrimental to the survival of this species that could have once survived and dispersed in euryhaline waters but now unable to inhabit the same habitats that are being desiccated.

6. Conclusions

In this study, 10 new haplotypes were found out of 14 in *D. valentina* and 4 new haplotypes out of 6 in *P. zariquieyi* although many haplotypes previously presented were not found. This suggests that more intensive sampling is required. Possibly as a result of mitochondrial capture from unknown or extinct lineage or by secondary contact there exist a distant haplotype (6.25%) in *D. valentina* population in three localities. Gene flow patterns confirms the inability of both species to migrate through sea while dispersal to other rivers could be mediated by artificial structures or bird/ human/ flood mediated translocations. Genetic distance present within the individuals of same localities, seems to be higher in both species in Chulilla puentes, Gestalgar, and Chulila pueblo which are hilly regions in the east. Reduced genetic diversity in *D.valentina* and signs of population decline in *P. zarquieyi* along with Isolation by distance pattern shows the impact of water scarcity, pollution and modification of hydrological regime in both species. Recent population decline of *P. zariquieyi* based on demographic pattern indicates that higher attention needs to be paid these shrimps. Although some demographic patterns in *D. valentina* may indicate recent expansion of the population, the reduction in genetic diversity is an indicative that these shrimps are still vulnerable to such anthropogenic effects. Intense management and monitoring of these highly polluted and regulated waters as well as evaluation of the status of these shrimps and the fluvial ecosystem are necessary for effective protection of these endemic species.

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Appendices

List of the Appendices:

1. Data of Sampling localities
2. Image representation of level of polymorphic sites between the common and distant haplotypes in *D. Valentina* obtained from Geneious.

Appendix 1: Data of Sampling localities

Species	**Place**	**Latitude**	**Longitude**	**Date of collection**	**No. of individuals**
P. zariquieyi	Ullal de la Font del Forner. Sollana	39.2944455494226	-0.390108343345761	27/03/2018	8
D. valentina	Ullal de la Font del Forner. Sollana	39.2944455494226	-0.390108343345761	27/03/2018	23
D. valentina	Rio Jucar. Antella	39.0807110974123	-0.596549340539849	27/03/2018	29
P. zariquieyi	Rio Jucar. Antella	39.0807110974123	-0.596549340539849	27/03/2018	22
D. valentina	Río Sot, Sot de Chera	39.6070442374749	-0.887226516884973	03/03/2018	30
D. valentina	Rio Turia. Chullila pueblo	39.6351065188976	-0.878251337295047	03/04/2018	27
P. zariquieyi	Rio Turia. Chullila pueblo	39.6351065188976	-0.878251337295047	03/04/2018	12
P. zariquieyi	Molí de la Font. Castellón	40.020711	0.000560	06/07/2017	22
D. valentina	Rio Racons. Pego	38.86134906	-0.07508218	04/04/2018	30
D. valentina	Río Bullent. Oliva	38.88020277	-0.08507387	04/04/2018	25
P. zariquieyi	Carraixet. Alboraia	39.50228943	-0.33138914	14/03/2018	50
Both species	Rio Turia	-	-	22/03/2018	30
P. zariquieyi	Río Turia. Gestalgar	39.60057874	-0.83167362	03/04/2018	11
D. valentina	Río Turia. Gestalgar	39.60057874	-0.83167362	03/04/2018	23
P. zariquieyi	Font Salda. Oliva	38.88789059	-0.07943395	04/03/2018	22
Both species	Rio Turia. Chullila puentes	39.66858634	-0.89417713	03/04/2018	21
P. zariquieyi	Prat de Cabanes	-	-	-	30

Appendix 2: Image representation of level polymorphic sites between the common and distant haplotypes in *D. valentina* obtained from Geneious.