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How did this snail get here? Several dispersal vectors inferred for an aquatic invasive species

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SUMMARY

1. How species reach and persist in isolated habitats remains an open question in many cases, especially for rapidly spreading invasive species. This is particularly true for temporary freshwater ponds, which can be remote and may dry out annually, but may still harbour high biodiversity. Persistence in such habitats depends on recurrent colonisation or species survival capacity, and ponds therefore provide an ideal system to investigate dispersal and connectivity.
2. Here, we test the hypothesis that the wide distributions and invasive potential of aquatic snails is due to their ability to exploit several dispersal vectors in different landscapes. We explored the population structure of *Physa acuta* (recent synonyms: *Haitia acuta*, *Physella acuta*, Pulmonata: Gastropoda), an invasive aquatic snail originating from North America, but established in temporary ponds in Doñana National Park, southern Spain. In this area, snails face land barriers when attempting to colonise other suitable habitat.
3. Genetic analyses using six microsatellite loci from 271 snails in 21 sites indicated that (i) geographically and hydrologically isolated snail populations in the park were genetically similar to a large snail population in rice fields more than 15 km away; (ii) these isolated ponds showed an isolation-by-distance pattern. This pattern broke down, however, for those ponds visited frequently by large mammals such as cattle, deer and wild boar; (iii) snail populations were panmictic in flooded and hydrologically connected rice fields.
4. These results support the notion that aquatic snails disperse readily by direct water connections in the flooded rice fields, can be carried by waterbirds flying between the rice fields and the park and may disperse between ponds within the park by attaching to large mammals.
5. The potential for aquatic snails such as *Physa acuta* to exploit several dispersal vectors may contribute to their wide distribution on various continents and their success as invasive species.

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We suggest that the interaction between different dispersal vectors, their relation to specific habitats and consequences at different geographic scales should be considered both when attempting to control invasive freshwater species and when protecting endangered species.

Keywords: birds, flooding, large mammals, microsatellite, *Physa acuta*, temporary ponds

Introduction

Many freshwater species lack the ability to disperse across the landscape unaided, despite the importance of dispersal for organisms living in such discrete and, in many cases, temporary habitats (Hoffsten, 2004; Van de Meutter, De Meester & Stoks, 2007). Transport of aquatic species by vectors, that is, dispersal whereby organisms or their propagules are carried by more mobile species, is therefore an important aspect determining the biodiversity of wetlands and riverine systems (Bilton, Freeland & Okamura, 2001; Malmqvist, 2002) and the spread of aquatic invasive species (Van der Velde *et al.*, 2006; Van der Velde, Rajagopal & Bij de Vaate, 2010). Water (hydrochory; e.g. Malmqvist, 2002; Frisch & Threlkeld, 2005), wind (anemochory; e.g. Soons & Ozinga, 2005; Soons, 2006; Vanschoenwinkel *et al.*, 2008a), animals (zoochory; e.g. Figuerola, Green & Santamaría, 2003; Frisch, Green & Figuerola, 2007; Van Leeuwen *et al.*, 2012a) and more recent human activities can explain how aquatic species can persist in metacommunity structures (reviewed by Bilton *et al.*, 2001) and how aquatic species spread around the world.

The ability to be carried by more than one vector is likely to increase the dispersal success of species (Ozinga *et al.*, 2004) and may explain the invasive potential of alien species (e.g. Wilson, Naish & Boulding, 1999; Shurin & Havel, 2002). For aquatic invertebrates, however, the capacity to use several dispersal vectors has scarcely been explored (Vanschoenwinkel *et al.*, 2008b; Wilmer *et al.*, 2008). Here, we investigated the dispersal vectors used by a small invasive snail, *Physa* (*Costatella*) *acuta* Draparnaud, 1805 (recent synonyms: *Haitia acuta*, *Physella acuta*) (Gastropoda; Pulmonata; Physidae). Other synonyms for this species are *Physa heterostrophia* and *Physa integra*, as previous work has indicated that these all belong to a single widespread species (Dillon *et al.*, 2002). Described as *P. acuta* from Europe, it was discovered that this species originates from North America and was probably introduced via the aquarium trade or by the intentional introduction of water plants. This small snail can now be found on almost all continents and has been called the most cosmopolitan snail (Dillon *et al.*, 2002; Wethington & Lydeard, 2007). The question remains, however, of how this small snail managed to become so widespread after its initial introduction?

Physa acuta is capable of living in a broad range of freshwater habitats and shows tolerance to a wide range of environmental conditions (e.g. Kefford & Nuggeoda, 2005; Turner & Montgomery, 2009). It is known as a rapid (re)coloniser of freshwater systems where it is exposed to variable environmental conditions (Chlyeh *et al.*, 2006). It can efficiently disperse via water (Van de Meutter, Stoks & De Meester, 2006) and has been found to be carried in plant material on boats between lakes (Albrecht *et al.*, 2009). Anecdotal evidence suggests that it may also be dispersed by waterbirds (e.g. McAtee, 1914; Roscoe, 1955; Malone, 1965a). Its ability to self-fertilise can further promote rapid spread (Bousset *et al.*, 2004). Adult snails generally survive for one season in which they lay one clutch of 18–50 eggs (Gittenberger *et al.*, 2004). Both adult snails and eggs are surrounded by sticky mucus, by which eggs are attached to water plants or other substrata (Gittenberger *et al.*, 2004).

The aim of our study was to investigate the dispersal capabilities of this invasive freshwater snail in relation to landscape structure. Is its ability to use several dispersal vectors essential and effective for its spread as an invasive species? How does it cross various types of landscapes? Therefore, we studied the genetic structure of *P. acuta* in a system of geographically and hydrologically isolated ponds in Doñana National Park (SW Spain) in relation to the occurrence of different potential vectors: water, birds and large mammals. Population genetic structure has been successfully used to infer dispersal vectors in other aquatic invertebrates, because an increase in dispersal between habitats may result in higher genetic similarity between populations (e.g. Wilson *et al.*, 1999; Chlyeh *et al.*, 2006; Zickovich & Bohonak, 2007; Wilmer *et al.*, 2008). If used correctly, such genetic methods assess effective rather than potential dispersal (Bohonak & Jenkins, 2003; Marko & Hart, 2011). We used six highly polymorphic microsatellite markers to assess the genetic differentiation and population structure of *P. acuta* populations in isolated temporary ponds within the national park, and compared these to a more permanent population in rice fields nearby. By investigating the dispersal of *P. acuta* in Doñana National Park, we tested the hypothesis that invasive aquatic invertebrates use several vectors for their dispersal to disperse over various spatial scales.

Methods

Study area

Doñana National Park is a protected wetland in SW Spain (6°W, 37°N), bordered by the Guadalquivir River in the east and the Atlantic Ocean in the south and west (Fig. 1). The national park comprises marshlands, shallow streams, sand dunes and shrubland. Summers are hot and dry, whereas winters are cool and wet (see Serrano *et al.*, 2006 for wetland details). The sandy area of the park contains more than 3000 small ponds and is geologically young having formed only *c.* 6000 years ago. Of these ponds, *c.* 200 are man-made to provide cattle with drinking water during dry periods (these ponds are called 'zacallones'). In this study, we refer to the sandy area containing the ponds as 'the park'. A detailed description of the pond characteristics can be found in the study by Gómez-Rodríguez *et al.* (2009) and Florencio *et al.* (2011). The ponds vary in size, depth and altitude (mean area: *c.* 50 m²; mean depth: *c.* 1 m), but all are

hydrologically isolated. The mean altitude of the ponds in the park is 10.0 m (± 8.7 SD), ranging from 1.4 to 28.6 m above sea level. About half of the sampled ponds dry completely during summer. Occasionally, during years of heavy rainfall, the park may partially flood, causing lower-lying ponds to become hydrologically connected. Cattle (*Bos primigenius* Bojanus, 1827), fallow deer (*Dama dama* Linnaeus, 1758), red deer (*Cervus elaphus* Linnaeus, 1758), horses (*Equus ferus* Boddaert, 1785) and wild boar (*Sus scrofa* Linnaeus, 1758) are abundant in the park, feeding on natural marsh vegetation, mainly plants belonging to the Poaceae, Juncaceae and Cyperaceae (Venero, 1984).

The east of the park is formed by the remains of a large marsh. Rice fields start about 10–15 km east of the park, separated from it by areas cultivated with cotton, cereals or by uncultivated lands. Rice fields cover an area of about 360 km² on former marshlands that were converted during the twentieth century. The water level in the rice fields is regulated, and they are actively flooded every

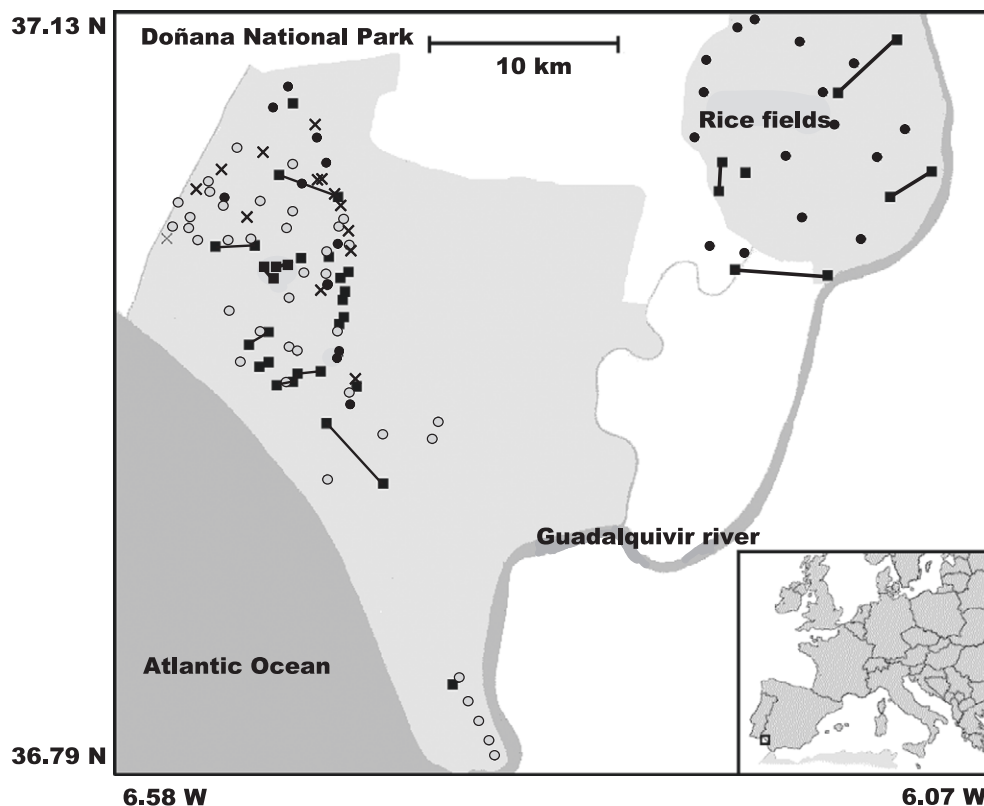


Fig. 1 Location of the studied area: Doñana National Park and the rice fields. Between the park and the rice fields are brackish fish ponds 'Veta la Palma', bordered by the Guadalquivir river. Filled circles denote ponds where *P. acuta* were present but not sampled; filled squares denote locations of sampling. At open circles, ponds contained water, but no snails were found. Crosses indicate ponds that were dry at the time of sampling. Ponds that were clustered for the analyses are either overlapping squares or are connected by lines, to form clusters with an average of 13 snails (see Methods for details). GPS locations are indicated in decimal degrees. The inset denotes the location of Doñana National Park in Europe.

year at the end of May with water pumped from the nearby Guadalquivir River. The fields remain flooded until the end of November or December, but this period may expand up until April in rainy winters (Toral *et al.*, 2011). We refer to this area as 'the rice fields'.

Field sampling

Samples of *Physa acuta* were collected between 10 and 20 November 2009, before the start of the autumn–winter rains. In the park, 69 of the 83 ponds visited contained water at the time of sampling, of which 40 (48%) contained *Physa acuta* (Fig. 1). Six concrete drinking troughs, made specially for cattle, also contained high densities of *P. acuta* and were mainly present in the north of the park. In the rice fields, all 25 locations visited contained numerous *P. acuta* ($>50 \text{ m}^{-2}$; Fig. 1), a much higher density than in other waterbodies close to the park. For each location, geographic position was recorded using a handheld GPS unit. After conversion to WGS1984 coordinates, pairwise geographic distances between sites were calculated using R for statistics (R-Development-Core-Team, 2011).

We sampled snails from 29 ponds in the park and at nine locations in the rice fields (Fig. 1), collecting between five and 20 snails per location (total $n = 271$). Snails collected from each site were immediately stored in containers with a CTAB–DMSO solution (1.5% cetyltrimethylammonium bromide–20% dimethyl sulphoxide in deionised water). Unfortunately, there were few snails at some sites, and we did not want to take all the snails. Since estimating allele frequencies based on only five individuals per pond was unrealistic, we pooled samples from ponds geographically close together and of similar altitude to increase sample sizes. Pooled samples were in Hardy–Weinberg equilibrium suggesting unlimited gene flow between pools or homogenous colonisation from the same source population, allowing comparative analysis of

pooled samples. Using identical methods in the park and rice fields, this resulted in 16 locations (hereafter referred to as sites) in the park and five in the rice fields (average 13 snails per site, see Fig. 1 for details of clustering). The average geographic distance between the sites was 7.3 km (± 5.2 SD), while the average distance between ponds within a site was 1.6 km (± 1.4 SD). The average difference in altitude within sites was 1.7 m (± 1.7 SD).

Potential vectors of dispersal in the park were large mammals, visiting ponds for drinking. To investigate their potential involvement in the transport of snails, we used data on the distribution of mammals in Doñana National Park from Soriguer *et al.* (2003). These authors estimated the density of deer, sheep, cows and horses by counting the densities of droppings in 422 transects covering 139 km in the park. Density ranged from 0 to over a 1000 droppings per hectare per mammal species. Sites in areas where over 500 droppings per hectare were found for one or more of the mammal species were considered as 'frequently visited' by large mammals. Sites with <500 droppings for all mammals were considered only occasionally visited. This resulted in eight sites in the park with low (<300) and eight sites in the park with high (>800) mammal density. Mean altitude of the more frequently visited ponds ($4.7 \text{ m} \pm 3.2$ SD) was lower than that of ponds not visited ($16.7 \text{ m} \pm 8.7$ SD, Student's *t*-test, $P < 0.001$).

Genetic analyses

Six microsatellite markers were used to assess the genetic diversity and differentiation within and between sites and habitats (Table 1). DNA was isolated using a Maxwell[®] 16 (Promega, Madison, WI, U.S.A.) with the accompanying Maxwell Tissue DNA purification kit. DNA was checked for quality by running 5 μL DNA on a 0.7% agarose gel and quantitatively by using a Qubit fluorometer (Invitrogen, Carlsbad, CA, U.S.A.). DNA concentration ranged from 50

Table 1 Microsatellite markers used. Number of alleles (*N*) is the number of alleles detected in all of the 271 individuals of *Physa acuta* collected from both Doñana National Park and the rice fields. Repeated motif and size range of alleles (in base pairs) are given. Accession No is the number on GenBank

Locus	<i>N</i>	Repeated motif	Size range	Publication	Accession No.
Pac1	3	(TG) ₂₂	100–118	(Sourrouille, Debain & Jarne, 2003)	AF532037
Pac2	5	(TATC) ₁₅	157–181	(Sourrouille <i>et al.</i> , 2003)	AF532038
Pac4	5	(TATC) ₂₄ (TGTC) ₉ (TG) ₄	209–257	(Sourrouille <i>et al.</i> , 2003)	AF532039
Pac5	4	(YGTC) ₂₀	241–265	(Sourrouille <i>et al.</i> , 2003)	AF532040
Pac7	10	(GT) ₇ (ATGT) ₂₁ (CTGT) ₂₂ (ATGT) ₃	302–432	(Sourrouille <i>et al.</i> , 2003)	AF532041
27	5	(TG) ₂₈	151	(Monsutti & Perrin, 1999)	AF108764
Average	5.1		214		

to 300 ng mL⁻¹ and was sufficient for subsequent PCR. PCRs were performed separately for each locus to be able to control product concentrations on the gel, but products from two loci with non-overlapping alleles were combined for genotyping analysis. PCRs were performed in volumes of 25 µL with a Biometra T Gradient Personal Thermal Cycler (Westburg, The Netherlands), containing the following components: 1 µL of template DNA, 2.0 µL purified bovine serum albumin (10 mg mL⁻¹, New England Biolabs, Inc), 1 µL of 5 pmol µL⁻¹ forward primer (Biolegio, Nijmegen, The Netherlands), 1 µL of 5 pmol µL⁻¹ IR800 dye (LI-COR Biosciences, Lincoln, NE, U.S.A.)-labelled reverse primer (Biolegio), 2.5 µL reaction buffer (Bioline, Luckenwalde, Germany), 0.2 µL Taq polymerase (BioTAQ 5 units µL⁻¹), 1.0 µL dNTP (5.0 mM) and 1.0 µL 50 mM MgCl. Cycling conditions were a hot start at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1.5 min, annealing at 60 °C for 1 minute and elongation at 72 °C for 1.5 min. After a final elongation step for 5 min at 72 °C, samples were stored at 4 °C until separation using a LICOR 4200 s2 within 24 h. Alleles were sized by comparison to a 50–350 Sizing Standard IRD800 ladder (LI-COR Biosciences).

Statistical analyses

Prior to all analyses, data were checked for the presence of null alleles, scoring errors due to stuttering and large allele dropout with Microchecker (2.2.3; Van Oosterhout *et al.*, 2004). There was no evidence for scoring errors due to stuttering or evidence for large allele dropouts. Null alleles were present only at one locus (pac7), for which observed heterozygosity was lower than expected. Since the frequency of null alleles in pac7 was on average only 7%, which is considered low enough to have little impact on F_{ST} estimates (Oddou-Muratorio *et al.*, 2009), we present the results including pac7.

Pairwise linkage disequilibrium was used to determine whether loci assorted independently using Arlequin 3.5 (Excoffier & Lischer, 2010) with sequential Bonferroni correction (Rice, 1989). Deviations from Hardy–Weinberg equilibrium were tested for all sites and deviations expressed as a fixation index, F (Wright, 1978), where positive and negative values represent a deficit and excess of heterozygotes, respectively. Chi-squared tests were used to determine whether the observed number of heterozygotes was significantly different from those expected under Hardy–Weinberg equilibrium using the genetics program Arlequin 3.5 (Excoffier & Lischer, 2010). The levels of population subdivision were quantified using hierarchical analysis of standardised genetic vari-

ance (F) statistics (Wright, 1969) performed in TFPGA 1.3 (Miller, 1997). We used F_{ST} to denote the total variation among all sites, F_{HT} for the variation among habitats (park versus rice fields) and F_{SH} for the variation among sites within each habitat. These parameters were calculated using the formulations of Weir & Cockerham (1984) with 95% CI determined by bootstrapping over loci with 10 000 replicates. Statistical significance of values of F was assumed when the 95% confidence intervals of the mean did not cross zero. In order to test for relative differences in gene flow between sites, the average number of effective migrants per generation was derived by calculating $N_{em} = (F_{ST}^{-1} - 1)/4$ (Wright, 1931).

The level of allelic differentiation between sites and habitats in relation to geographic distance (i.e. isolation by distance) was tested by comparing the pairwise $F_{ST}/(1 - F_{ST})$ matrix to the pairwise matrix of linear geographic distances in kilometres. Significance was assessed using a Mantel test with 10 000 permutations (Sokal, 1979) in TFPGA 1.3 (Miller, 1997).

In addition to these analyses to detect the average amount of gene flow between the sampled sites, we also ran STRUCTURE 2.2.3 (Pritchard, Stephens & Donnelly, 2000) to potentially identify individual migrants within our samples. Assignment tests were run for several selections of the data set, that is, for either all sites, all sites in the park or all sites in the rice fields. We ran the analysis with and without prior information on which individuals were sampled at each site. In all cases, we used an admixture model with 750 000 MCMC repeated permutations after a burn-in period of 500 000 permutations. The best value of K was calculated based on the second-order rate of change of the likelihood (delta K) (following Evanno, Regnaut & Goudet, 2005).

Results

Genetic variation among sites and habitats

The six microsatellite loci showed high levels of polymorphism (Table 1). The mean within-population diversity represented by the expected heterozygosity (Nei, 1973) was $0.48 \pm 0.30SD$ in both park and rice fields. The observed heterozygosities were also similar for park and rice fields, that is, $0.35 \pm 0.23SD$ and $0.34 \pm 0.21SD$, respectively. In 16 of the 21 sites, single-locus heterozygosity departed from Hardy–Weinberg equilibrium, involving 27 of the 126 estimates. However, only 13 of these remained significant after a sequential Bonferroni correction and all represented heterozygous deficit suggested deviations due to mating system (i.e. inbreed-

Table 2 Population structure of *Physa acuta* as indicated by hierarchical F -statistics; n denotes the number of populations sampled. Structure over all populations is indicated by F_{ST} values, between rice and park populations by F_{HT} values, and for the rice fields and the park in isolation by F_{SH} values (with for the park the distinction between only those ponds with high or only those with low density of droppings of large mammals). Standard deviations (SD) and confidence intervals (95% CI) are indicated. F_{IS} is the within-population structure and N_{em} the estimated number of migrants per population. Significant values are in boldface

	n	$F_{ST/HT/SH}$	SD	95% CI	F_{IS}	SD	95% CI	N_{em}
All populations (F_{ST})	21	0.075	0.016	0.096 to 0.040	0.24	0.13	0.42 to 0.026	3.1
Rice fields vs park (F_{HT})	2	0.024	0.010	0.039 to 0.0019				10.2
Rice fields only (F_{SH})	5	-0.0047	0.010	0.014 to -0.021	0.32	0.17	0.56 to -0.0019	>50
Park: high mammals (F_{SH})	8	0.077	0.030	0.13 to 0.027	0.19	0.14	0.37 to -0.058	3.0
Park: low mammals (F_{SH})	8	0.11	0.037	0.16 to 0.037	0.24	0.094	0.41 to 0.086	2.0

ing/self-fertilisation) rather than a Wahlund effect due to the pooling of samples from different populations. Tests for linkage disequilibrium over a total of 315 pairwise comparisons revealed 16 interlocus associations. However, none of these associations remained significant after the application of a sequential Bonferroni correction.

Population structure based on microsatellites

The hierarchical analysis of F -statistics revealed significant levels of population subdivision among all sites and habitats (Table 2). The mean F_{ST} (\pm SD) across six loci was 0.075 ± 0.016 . Approximately 32% of this variation could be attributed to variation among habitats ($F_{HT} = 0.024 \pm 0.010$). We detected no significant genetic subdivision among sites within the rice fields ($F_{SH} = -0.0047 \pm 0.010$), indicating substantial gene flow within this habitat. In contrast, we detected significant genetic differentiation among sites within the park, with ponds frequently visited by large mammals having lower genetic differentiation than less visited ponds (F_{SH} : high mammal = 0.077 ± 0.030 vs low mammal = 0.11 ± 0.037).

There was a large and significant heterozygote deficit across all sites ($F_{IS} = 0.24 \pm 0.13$; Table 2) suggesting substantial inbreeding including the extreme of self-fertilisation. This pattern was driven primarily by significant heterozygous deficits in isolated ponds in the park, receiving few or no visits from large mammals. In contrast, while heterozygous deficits were detected in other ponds in the park and sites within the rice fields, these were not significantly different from zero (Table 2).

Isolation by distance

When calculated over all ponds in the park, Mantel tests revealed significant isolation-by-distance patterns between geographic distance and pairwise F_{ST} estimates ($r = 0.60$, $P = 0.004$, $F_{ST}/(1-F_{ST}) = 0.0074(\text{GeoDist}) + 0.0$

16). However, for those ponds visited frequently by large mammals (cows, horses, wild boars and fallow and red deer), the pattern of isolation by distance broke down. When calculated only among those ponds visited less frequently by large mammals, Mantel test correlations indicated even stronger isolation by distance ($r = 0.76$, $P = 0.001$; $F_{SH}/(1-F_{SH}) = 0.0122(\text{GeoDist}) + 0.014$; Fig. 2), which was also true when calculated on the same spatial scale (of only 15 km between-pond distances) as the data for the frequently visited ponds ($r = 0.59$, $P = 0.001$). There was no correlation between altitudinal distance between ponds and genetic distance between ponds in the park (Mantel test, $r = 0.01$, $P = 0.54$). In the rice fields, no isolation by distance was detected suggesting a well-mixed population with little structuring (Mantel test, $r = 0.33$, $P = 0.15$).

Assignment tests

The assignment tests had limited power to identify individual dispersers, probably due to the strong pattern of isolation by distance present (which tends to give mixed memberships in several groups and reduces the biological meaning of the K -value (Pritchard *et al.*, 2000)) and possibly due to small sample sizes for some sites. Clear dispersal patterns and population origins could not be determined. Although the results should be interpreted with caution, running assignment tests for the park data only revealed two clusters ($K = 2$) when running with prior site information, and four clusters ($K = 4$) when running without prior site information. Overall, the population farthest to the south and to the north were most different from each other and from all core populations in the centre of the park. These two populations also had the highest average F_{SH} value compared to the core populations. Similarly, two clusters were found in the rice fields when running the test with prior site information, but four clusters were found when running without site information. In the rice fields, all populations

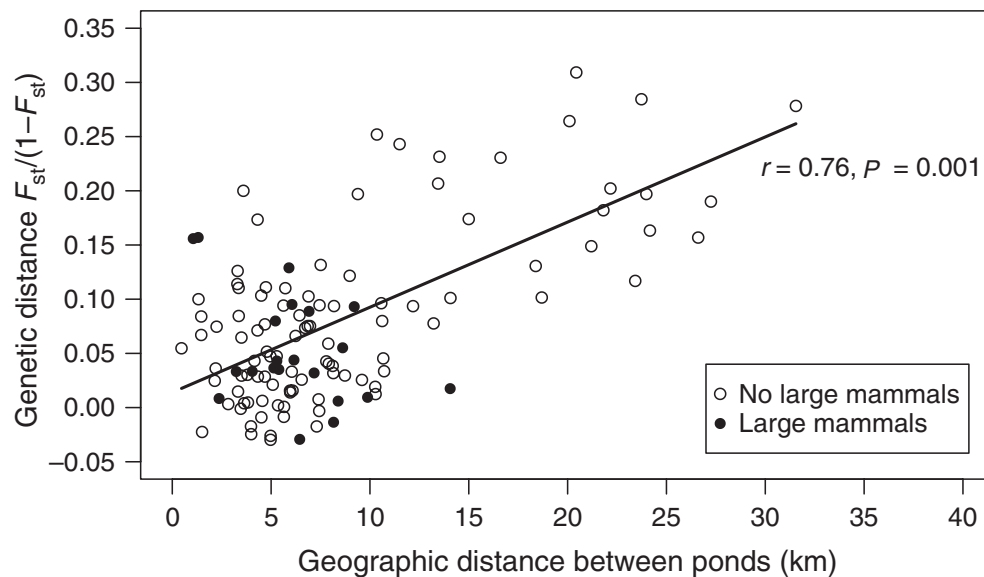


Fig. 2 Genetic distance between *Physa acuta* populations based on all six loci is plotted against geographic distance between ponds in Doñana National Park. Data include all sites in the park, but are separated by (i) either pairs of ponds where both ponds had high densities of large mammals (closed circles) or (ii) where one of the ponds had low densities of large mammals in case no transport was assumed possible (open circles).

consisted of a mixture of these clusters in all cases. Running the assignment tests for both park and rice field data together indicated three clusters ($K = 3$), both with and without site included as prior information.

Discussion

The degree of gene flow between aquatic snail populations in Doñana National Park was found to vary with habitat type and structure. Inferred population structures could not be attributed to a single passive dispersal vector, but instead showed the patterns consistent with dispersal by several dispersal vectors acting on different spatial scales. *Physa acuta* is known to be capable of dispersal by water connecting ponds (Van de Meutter *et al.*, 2006) and in irrigation systems (Chlyeh *et al.*, 2006) and has been found to be transported in macrophytes attached to boats (Albrecht *et al.*, 2009). Snails have been observed on the feathers of trapped waterbirds (e.g. McAtee, 1914; Roscoe, 1955), observations inferring dispersal by birds. These observations have more recently also received support from genetic analyses on snail populations (Wilmer *et al.*, 2008; Von Oheimb *et al.*, 2011), including populations of *P. acuta* (Dillon & Wethington, 1995). The occurrence of *P. acuta* in the isolated, temporary ponds of Doñana National Park was most consistent with combined dispersal by waterbirds, large mammals and water.

Dispersal potential by waterbirds

Allelic richness, heterozygosity and levels of genetic differentiation indicated that *P. acuta* in the park and the rice fields were very similar (F_{HT} ; Table 2), suggesting that occasional exchange of snails between (parts of) the park and the rice fields could occur. Although the rice fields and the marsh formerly formed a unique habitat with clear hydrologic connections, these connections were broken during the second half of the twentieth century. Since the establishment of these new habitats <70 years ago, a range of additional potential vectors is now likely to contribute to dispersal over differing spatial scales. The small ponds in the park that fluctuate in snail population size and may even completely dry out during part of the season were expected to show genetic structure associated with bottlenecks or founder effects (in the case of no dispersal) because of population sizes of sometimes only a few dozen snails. Instead, similarity with snails from the rice fields suggested that snails have either maintained stable populations over time since isolation (without effects of reduced population sizes when ponds dry), or might occasionally exchange individuals with the rice field population. Other water sources than the rice fields in close proximity to the park also contained *Physa*, but at much lower density than the rice fields, pointing towards the rice fields as most likely source population.

As many waterbirds visit both areas (Rendon *et al.*, 2008), we suggest that birds are the most likely vector for transport between habitats on this scale (tens of km). Since there are no longer any direct connections by water or humans between the rice fields and the park, these alternative vectors seem less likely. Zoochory has previously been suggested as the most probable transport mechanism on this scale for aquatic snails found on oceanic islands and ponds in the desert (Dillon & Wethington, 1995; Wilmer *et al.*, 2008). Many aquatic snails have the necessary prerequisites for successful external transport by birds (Van Leeuwen & Van der Velde, 2012). There are previous records of upland sandpipers (*Bartramia longicauda* Bechstein, 1812) and white-faced glossy ibis (*Plegadis chihi* Vieillot, 1817) with numerous *Physa* snails found between their feathers (McAtee, 1914; Roscoe, 1955). The sticky mucus that surrounds the eggs and adult snails (Gittenberger *et al.*, 2004) is further support for avian dispersal. Whereas external attachment and dispersal of eggs and/or adults is the most probable mode of transport (Malone, 1965b), egg capsules of *Physa anatina* Lea, 1864 might also be transported in the digestive system or the crop of killdeer (*Charadrius vociferous* Linnaeus, 1758) and mallards (*Anas platyrhynchos* Linnaeus, 1758) (Malone, 1965b). More recently, successful internal transport potential has been found for whole snails (Cadée, 2011; Gittenberger, 2012; Van Leeuwen *et al.*, 2012b; Wada, Kawakami & Chiba, 2012), although not for *Physa*. Dispersal by waterbirds between rice fields and the park, whether internal or external, may be sufficient to explain genetic similarity at this large spatial scale.

Dispersal potential by large mammals

Large mammals such as boar, elephants (*Loxodonta africana* Blumenback, 1797) and buffalos (*Syncerus caffer* Sparrman, 1779) have previously been found to carry invertebrates in drying mud (e.g. Vanschoenwinkel *et al.*, 2008c, 2011). The abundance of cattle, wild boar and fallow and red deer in the park provides a possible mechanism for snails to disperse between ponds by attaching to their legs or bodies, potentially assisted by drying mud. This idea is strengthened by the observation that all the drinking troughs for large mammals in the park contained high snail densities. To test the potential for this mode of dispersal in our system, we divided the ponds in the park into groups with either high or low mammalian visitation rates. If large mammals indeed disperse snails, the more frequent exchange of snails between ponds visited by mammals would reduce genetic

differentiation between these populations (Mader, van Vierssen & Schwenk, 1998; Wada *et al.*, 2012).

Over all ponds of the park, gene flow seemed to occur at low frequency at the scale of several kilometres. When the F_{SH} value of the park (0.11; Table 2) was considered in comparison with the expected heterozygosity (0.48, the maximum possible value of F_{SH}), the populations in the park were found to be relatively isolated (Charlesworth, 1998; Meirmans & Hedrick, 2011). Significant isolation by distance in the park (Fig. 2) indicated more exchange of snails between neighbouring ponds than between ponds further apart (e.g. a distinct pattern of isolation by distance).

However, after considering only ponds frequently visited by large mammals, the pattern of isolation by distance broke down (Fig. 2). The F_{SH} value calculated among these ponds was small and non-significant (0.077; Table 2), while still present in the ponds less visited by mammals when calculated on the smaller spatial scale of only 15 km between-pond distances. This observation was supported by the assignment tests that, despite their limited explanatory power, indicated relative isolation and high average F_{SH} values of the most northerly and southerly populations, where overall visitation rates of mammals were lowest. Therefore, our data support the idea that large mammals transport snails between some ponds inside the park.

The fact that the ponds frequently visited by mammals also had an average lower altitude does not rule out the possibility that flooding may occasionally connect these ponds. However, our sampling points lie outside the flooding area of the marsh (<http://mercurio.ebd.csic.es/imgs2/>) and, although nearby ponds might be connected during rainy winters (see also Serrano *et al.*, 2006), such connections are unlikely to occur at the large scale of our analyses. For these reasons, we clustered ponds separated by a few hundreds of metres to increase sample sizes for analyses, in which case dispersal by flooding would only connect ponds within sites. A Mantel test on isolation by altitude did not yield any correlation between altitude and genetic differentiation, thus favouring visits by large mammals as a predictor of the patterns on the smaller to intermediate scale between ponds inside the park.

Passive drifting in flowing water as means of dispersal for aquatic invertebrates

Passive drift has been claimed as the most important means of transport for many aquatic invertebrates (Bilton *et al.*, 2001). Currents and flooding events can increase gene flow (Kawata *et al.*, 2005), and an increase in water exchange between areas also increases the exchange of

invertebrates (Van de Meutter *et al.*, 2007; Siziba *et al.*, 2011). In our system, we attribute the higher gene flow at the small scale in the rice fields (supported by high values of N_{em} ; Table 2) to their frequent flooding. For several months of the year, the snails can freely move through the rice fields, or might be moved by the incoming water (Frisch & Threlkeld, 2005; Siziba *et al.*, 2011), particularly when they are adhered, upside down, to the water surface for grazing or breathing, or attach to floating plant material (Bickel, 1965).

Our analyses suggest that the ability to move freely through the flooded rice fields caused at least 15 times more exchange of individuals between sites compared than what was detected between sites in the park, where hydrologic isolation prevails and dispersal largely depends on alternative vectors. Assignment tests did not detect any structure in the rice fields. Although extrapolating genetic differentiation directly to dispersal rates can only be done with care (Whitlock & McCauley, 1999; Bohonak & Roderick, 2001), our relatively young populations of no more than 200 years old (the first record of the species from the Iberian Peninsula was in 1845 (Cobo *et al.*, 2010 and literature therein) were probably already in equilibrium, as indicated by the isolation by distance. For the gene flow estimates, we only compared populations relatively similar to one another, and thus, we can also only interpret the number of migrants per generation as a relative rather than an absolute number of snails (Bossart & Prowell, 1998). Only when no water connections were present in our system, other dispersal vectors were likely to be of significance for the dispersal of snails (see also Niggebrugge *et al.*, 2007). However, since many birds also fly between sites within the rice fields, this mode of dispersal may also contribute to the panmictic population in the rice fields, in addition to hydrochory.

Effects of dispersal on reproductive choice

Sixteen of the 21 sites investigated had single-locus heterozygosity departing from Hardy–Weinberg equilibrium, all due to heterozygote deficits. These heterozygote deficits could be the result of null alleles. However, deficits were not only concentrated around *pac7*, which was the locus that was mainly affected by null alleles. Alternatively, the heterozygote deficits could be the result of introductions of snails from waterbodies more distant from the park than from the rice fields. However, a more likely explanation is that they result from inbreeding, including the extreme of self-fertilisation. Self-fertilisation is frequently found in *Physa* and pulmonates in general (Dillon & Wethington, 1995; Jarne *et al.*, 2000). Inbreeding

involves combining similar genotypes, which typically increases the production of homozygous offspring. Individuals of *P. acuta* are known to prefer outcrossing over selfing in experimental set-ups, because selfing often reduces their reproductive success (Wethington & Dillon, 1997). Therefore, we expected F_{IS} to be greater in isolated ponds, where introduction by a small number of individuals has limited the opportunity for outcrossing and promoted selfing and inbreeding of subsequent generations. This expectation was supported by significant inbreeding coefficients in those ponds that were the most isolated and that were the least visited by large mammals (Table 2). In contrast, we detected no significant inbreeding in the rice fields or the ponds more frequently visited by large mammals, again suggesting that snails preferred outcrossing and had the opportunity to do this with introduced conspecifics. The advantages of sexual reproduction in such stochastic environments might be substantial. Although we cannot disentangle selfing from inbreeding in our research, snails seem highly suitable for isolated and ephemeral environments by being able to self-fertilise after initial colonisation, and subsequently reproduce sexually to allow adaptations in a stochastic environment and prohibit inbreeding.

Implications

The population structure of the invasive *P. acuta* occurring in Doñana was found to vary between habitats and spatial scales. Thus, our genetic data are consistent with snail dispersal by several vectors acting at different scales. At the largest scale, waterbirds were the most likely vector connecting the rice fields and the park, leading to only low levels of connectivity. Within the rice fields, the populations were panmictic, which we attribute to extensive dispersal by water at this intermediate scale. At the small to intermediate scales between hydrologically isolated ponds in the park, the connectivity was much lower than in the rice fields, but still present if large mammals were abundant.

Together, these findings suggest that various dispersal vectors, acting at different scales, facilitated the overall occurrence of *P. acuta* in Doñana. The capacity to disperse by several passive dispersal vectors might explain the cosmopolitan distribution of this small snail and its success as an invasive species. These findings for *P. acuta* suggest that aquatic invertebrates such as snails may often rely on several vectors for their dispersal, even within a single system. This suggestion has important implications for managing protected, as well as invasive, freshwater species. Whereas invaders with the ability to use several

dispersal vectors are likely to be the most successful, and may not be controlled by excluding only one vector, native species with this ability might form the most robust populations with resistance to habitat fragmentation and environmental changes (Kokko & Lopez-Sepulcre, 2006; Pearson, 2006).

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