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Conservation genetics of the endangered depressed river mussel, Pseudanodonta complanata, *using amplified fragment length polymorphism (AFLP) markers*

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ABSTRACT

1. Genetic analysis is increasingly recognized as a key tool for understanding demography, and is particularly useful for describing patterns of gene flow between putative populations. Most effort has been directed towards vertebrate systems, where any one study often benefits from marker development in related species. The greater diversity of invertebrate taxa presents a challenge, but amplified fragment length polymorphism (AFLP) markers offer a solution, yielding high levels of polymorphism and no prior knowledge of a species' genome.

2. AFLP markers have been used to analyse an unusual metapopulation of an invertebrate, the endangered freshwater mussel, *Pseudanodonta complanata*, sampled from river systems across the UK. This was done to assess the extent to which individual river systems were genetically isolated from one another.

3. The results show patterns of weak genetic differentiation across the UK, with one hydrologically isolated population in the south west showing clear genetic differentiation from the rest of the country. However, the UK population as a whole exhibits significant isolation by distance, particularly when one population subject to fish stocking is removed, this population probably being seeded with mussel glochidia larvae which use fish as vectors. Genetic estimates of inbreeding reveal a complicated pattern in which inbreeding peaks at intermediate densities. High-density populations may be genetically diverse due to their size, while the lowest density populations may represent transient groups of emigrants from other, larger populations.

4. The findings show that limited gene flow does exist between some but not all river systems. The isolation of the south-west population indicates that dispersal is variable and should not be assumed to be present. Waterways that remain hydrologically isolated may require special attention in conservation programmes as they can harbour genetically distinct populations. The balance between river management activities and conservation priorities therefore needs careful consideration.

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KEY WORDS: conservation; genetics; *Pseudanodonta complanata*; depressed river mussel; AFLP

INTRODUCTION

Freshwater mussels play an essential role in many freshwater ecosystems and are found throughout the world (Roe and Hoeh, 2003). They dominate the zoobenthic biomass of both lotic and lentic freshwater ecosystems (Mann, 1965). Mussels are filter feeders and are important because of their ability to purify water by removing suspended matter from the water column. One adult unionid mussel can filter approximately 40 L of water per day (Tankersley and Dimock, 1993) which has important consequences for fish, other invertebrates and aquatic macrophtyes. Freshwater mussels can be viewed as keystone species and are therefore important for setting conservation priorities (Aldridge *et al*., 2007).

Over the last few decades, freshwater mussels have declined throughout the world (Bogan, 1998). For example, in the USA there are 297 species of native mussel of which 60% are endangered or threatened and 12% are now considered extinct (Ricciardi *et al*., 1998). In Britain, there is evidence for decline in all the native species (Aldridge, 2004). On a national scale,

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the main causes of decline are pollution and habitat loss (Aldridge, 2004), the most important factors being weed cutting and dredging (Aldridge, 2000). The most endangered native British unionid mussel is the depressed river mussel, *Pseudanodonta complanata* (Rossmässler, 1835), one of Europe's rarest freshwater mussels. It is reported to be rare or threatened with extinction across most of its international range (Zettler, 1998) and appears on the IUCN Red List described as a 'near threatened' species (Baillie and Groombridge, 1996). It is included on the Priority List of species of particular concern in the Biodiversity Action Plan for the United Kingdom (Anon, 1995). Recent surveys suggest that *P. complanata* has been lost from approximately 30% of its UK sites over the last 100 years (Müller, 1999).

P. complanata is a relatively small mussel, reaching up to about 95 mm in length that lives inconspicuously buried within the sediment. In Britain it is restricted to rivers and streams. To understand better the population structure of *P. complanata* it is desirable to conduct genetic studies. Such studies can reveal patterns of importance to conservation, for instance by identifying genetically distinct populations and revealing evidence of inbreeding. For example, studies in North America on *Amblema plicata* revealed low levels of genetic structuring, attributed to the highly vagile fish hosts (Elderkin *et al*., 2007). In contrast, despite high levels of dispersal, the Australian intertidal gastropod *Nerita atramentosa* revealed a striking phylogeographic split (Waters *et al*., 2005). These studies emphasize the importance of genetic studies in quantifying the extent to which dispersal potential is realized.

Studies on *A. plicata* highlight the added complexity in species of freshwater mussels that depend on fish hosts for their larvae (Kat, 1984). Historical changes in fish abundance, interconnectivity of catchments, stocking of fish into new watercourses and specificity for host fishes are all likely to affect patterns in relatedness and gene flow among unionid populations. Previous work attempted to use mitochondrial DNA to examine *P. complanata* population structure in Britain but failed to reveal useful levels of intraspecific variability (Müller, 1999; Kallersjo *et al*., 2005). Microsatellites offer another possibility, and have been developed for several related species (Zanatta and Murphy, 2006; Gardestrom *et al*., 2008). However, a number of recent studies have emphasized the utility of amplified fragment length polymorphism (AFLP) markers for the analysis of species where other markers have yet to be developed (Dasmahapatra *et al*., 2008). The AFLP technique can rapidly reveal large numbers of polymorphic traits distributed across the genome, traits that can be used to assess population substructure, relatedness and relative levels of genetic diversity.

Here British *Pseudanodonta complanata* are studied using AFLP markers to determine the level of population structure present in relation to the different catchment areas.

METHODS

Sample collection and preservation

Eighty-two specimens of *Pseudanodonta complanata* were collected from 12 locations in the UK between and 2006 and 2008 (Table 1 and Figure 1). The UK distribution of *P. complanata* extends from the western Somerset Levels (South Drain) to East Anglia (River Waveney), and from Sussex in the south (River Arun) to North Yorkshire (River Derwent) (Kerney, 1999; Müller, 1999). The samples used in this study therefore provide good coverage of the known distribution. Up to 15 individuals were collected from each site (Table 1). All mussels had their abductor muscles excised and were preserved in 95% ethanol.

Table 1. Population locations, codes, OS grid references and number of *Pseudanodonta complanata* samples collected at each location

Location	Population code	River	OS grid reference	Number of samples collected
Old West River, Aldreth	1	Great Ouse	TL437722	2
River Arun	2	Arun	TQ021153	15
Waveney	3	Waveney	TM423920	15
Yalding	4	Medway	TQ687504	11
River Thames — Penton Hook to Bell Weir (a)	5	Thames	TQ040697	5
River Derwent — Howsham Bridge	6	Derwent	SE733625	6
River Thames — Penton Hook to Bell Weir (b)	7	Thames	TQ028717	8
Medway Ringlestone	8	Medway	TQ759554	2
Medway Barming	9	Medway	TQ724539	7
Great Ouse — Oakley, near Bedford	10	Great Ouse	TL008544	3
River Wye	11	Wye	SO518133	3
Somerset, South Drain	SD	South Drain (Brue)	ST385427	5
			Total	82

In a threatened species where one or more populations have been reduced to low levels for long periods a correlation between genetic diversity and population size is expected. In *P. complanata* the population sizes are unknown and difficult to assess directly. However, during sampling it was possible to estimate catch per unit effort (CPUE). Since the sampling sites differed greatly in ease of access and the efficiency with which an area could be surveyed, this is at best a rather crude method. Consequently, for this study CPUE was used as a measure of relative local abundance.

Morphological data

It is possible that *P. complanata* populations are sufficiently isolated to have developed appreciable variation in morphology that in turn might reflect genetic relatedness. To test this hypothesis the height, length, width, and hinge length of all individuals was recorded using callipers accurate to 0.1 mm. Since these measurements are highly correlated, a principal components analysis (PCA) was used to extract largely independent hypervariables using Minitab (release 15). The distance between two individuals or populations was then estimated as the Euclidean distance between the first two principal components.

Genetic data

The AFLP protocol used was similar to that of Vos *et al*. (1995) and is described in detail by Hoffman *et al*. (2009). DNA was extracted from approximately 5 mm^3 of tissue by incubation in 315 µL of extraction buffer (10 µL Proteinase-K, 5 µL RNAse, 285 µL TE and 15 µL 20% SDS) at 55°C

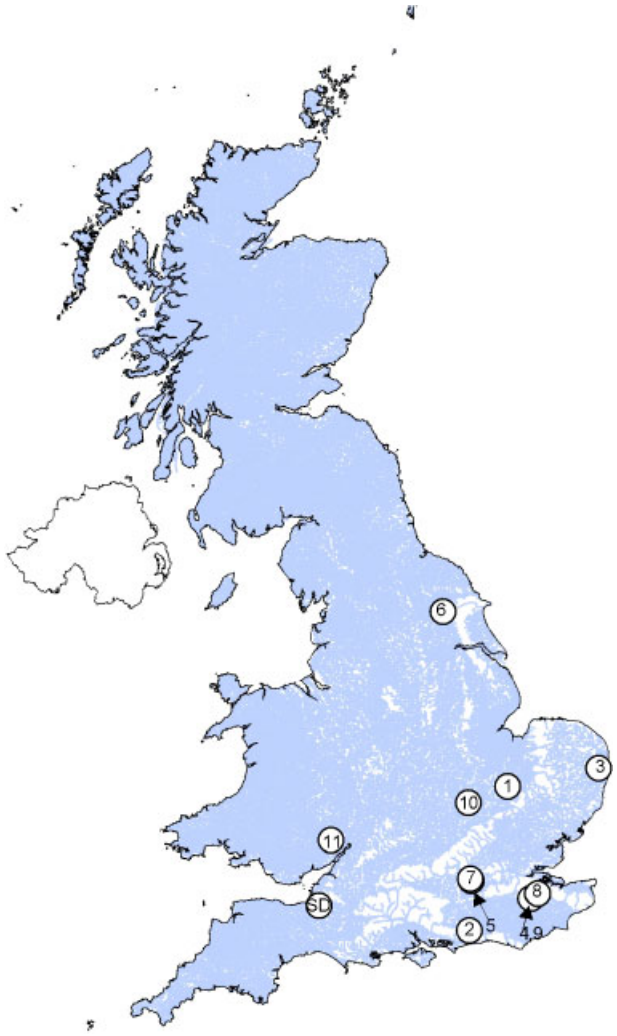

Figure 1. Map of sampling locations of *Pseudanodonta complanata*. Some features of this map are based on digital spatial data licensed from the Centre for Ecology and Hydrology, © NERC (CEH). © Crown copyright. All rights reserved, licence number 100017897. See Table 1 for population designations.

overnight, followed by a standard phenol–chloroform extraction. This was digested sequentially with *TaqI* and then *EcoRI* under conditions specified by the manufacturers, and ligated to linkers. The samples were then genotyped using direct radioactive incorporation in four different selective AFLP primer combinations (see Tables 2 and 3 for primer sequences and combinations used). Resulting AFLP products were resolved by electrophoresis through 6% acrylamide gels, visualized by autoradiography. Polymorphic bands that were discernible in >95% of individuals (i.e. where there was obvious polymorphism) were scored as present, 1, or absent, 0. All bands were scored independently by two observers, and any discrepancies resolved by consultation.

Population differentiation

A consensus tree for the *P. complanata* populations were created using Phylip version 3.66 (Felsenstein, 1991). Genetic distances were calculated using the program RESTDIST using the method of Nei and Li, which generates a distance matrix (Nei and Li, 1979). Neighbour-joining trees were created using the program

Table 2. Primers used for selective amplification

Primer	Primer sequence (5′–3′)
TaqI-CCA	GAT GAC TCC TGA CCG ACCA
TaqI-CAG	GAT GAG TCC TGA CCG ACAG
TaqI-CAC	GAT GAG TCC TGA CCG ACAC
EcoRI-AGC	GAC TGC GTA CCA ATT CAGC
EcoRI-ATG	GAC TGC GTA CCA ATT CATG
EcoRI-ACA	GAC TGC GTA CCA ATT CACA

Table 3. Combinations of primers used in the selective amplification, and resulting numbers of polymorphic bands scored

	EcoRI	TaqI	Number of polymorphic bands in Pseudanodonta complanata
Primer combination 1	AGC	CCA	58
Primer combination 2	ATG	CCA	22
Primer combination 3	ACA	CAG	40
Primer combination 4	AGC	CAC	42
		Total	162

NEIGHBOUR which implements the neighbour-joining method of Saitou and Nei (Saitou and Nei, 1987). The reliability of the trees was assessed by bootstrapping using the program SEQBOOT with 100 replicates and a consensus tree following the 50% majority rule, generated using the program CONSENSE. The resulting tree was visualized using the program TREEVIEW (Page, 1996) as an unrooted tree. Pairwise distances among populations were estimated as F_{ST} using the program Arlequin 3.11 (Excoffier *et al.*, 2005). To analyse the significance of correlations between genetic, morphological and geographic (land) distances while controlling for non-independence, Mantel tests were used, implemented in the program GENALEX (Peakall and Smouse, 2006).

Genetic diversity

AFLP bands are generally inherited as unidominant markers, band absence indicating the lack of a band on either chromosome, with band presence indicating either homozygous present or heterozygous present/absent. Where this holds, all heterozygotes are included within the band-present phenotype, and band number therefore correlates loosely with heterozygosity. Several methods exist for interpreting band counts in terms of either genome-wide heterozygosity or the individual's inbreeding coefficient, *F*. A recently developed method that is effective even in the presence of an unknown number of inbred individuals (Dasmahapatra *et al.*, 2008) was used in this study. This method is based on a simulation approach that seeks the best fit between the data and the proportion of inbred individuals sampled. The output of the program FAFLPcalc is given in terms of estimates of F_{AFLP} for each sampled individual.

RESULTS

Of the 82 *P. complanata* samples, 58 amplified successfully at all of the primer combinations, generating a total of 162 polymorphic loci (Table 3). F_{ST} values (Table 4) were mainly not significant, suggesting low levels of differentiation between most populations, a result supported by the neighbour-joining tree

Table 4. Pairwise F_{ST} values among *Pseudanodonta complanata* populations. Significant ($P<0.05$) values are highlighted in bold

	1	2	3	4	5	6	7	8	9	10	11	SD
1	*											
2	0.055	*										
3	0.091	**0.058**	*									
4	0.107	0.015	0.045	*								
5	0.171	**0.079**	**0.164**	0.074	*							
6	0.047	0.022	0.063	0.043	0.047	*						
7	0.051	**0.080**	**0.154**	0.045	0.013	0.001	*					
8	0.049	0.050	0.303	0.123	0.276	0.028	0.023	*				
9	0.017	0.059	0.219	0.021	0.089	0.055	0.074	0.137	*			
10	0.210	0.062	**0.138**	0.121	0.085	0.047	0.098	0.358	0.098	*		
11	0.351	**0.193**	**0.313**	0.292	**0.335**	0.218	**0.175**	0.531	0.186	0.338	*	
SD	**0.336**	**0.277**	**0.381**	**0.314**	**0.371**	**0.306**	**0.227**	0.405	**0.211**	**0.401**	**0.274**	*

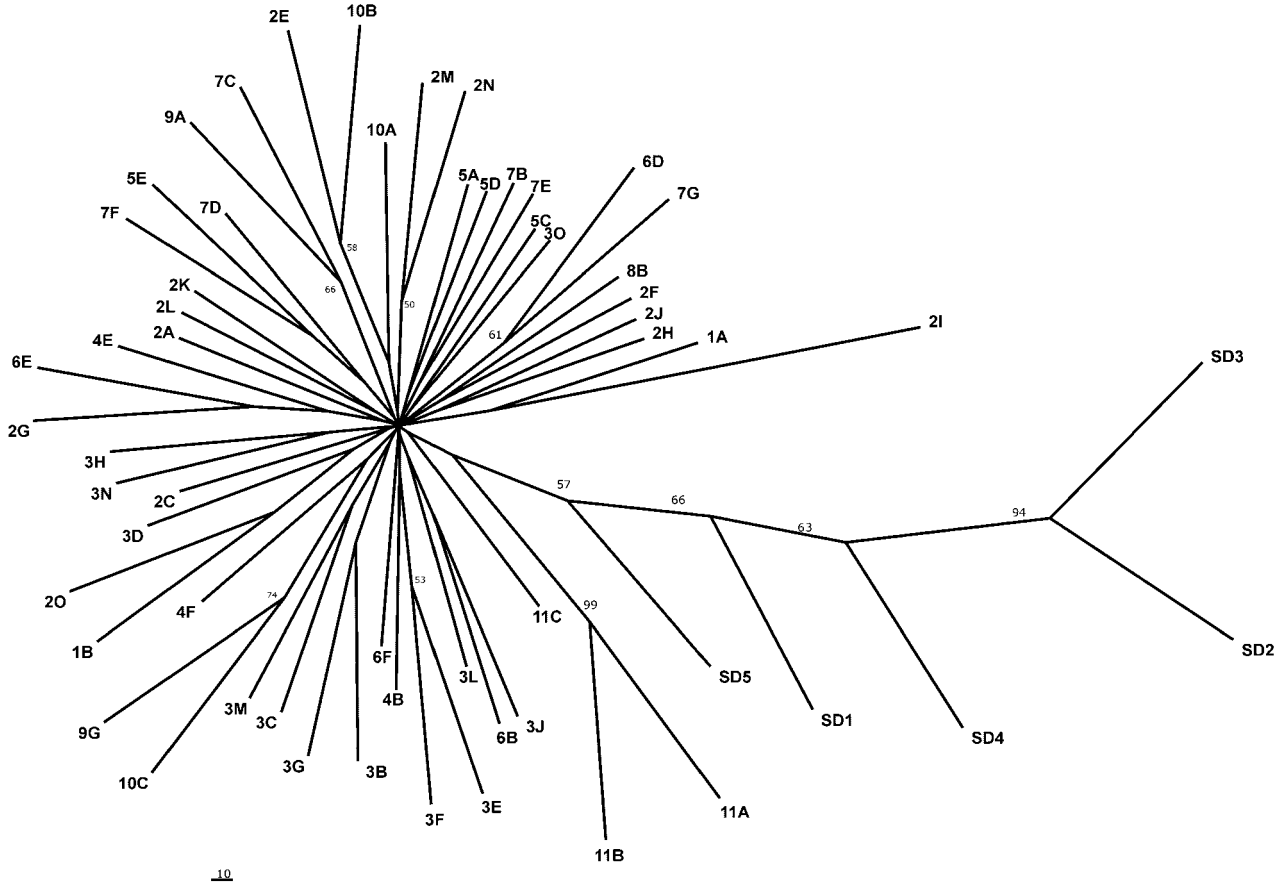

Figure 2. Unrooted consensus tree using the neighbour-joining method showing the relationships among 58 individuals from 12 populations of *Pseudanodonta complanata*. Bootstrap values >50 are shown, based on 100 replicates. See Table 1 for population designations.

(Figure 2). Of those population pairs that did show significant pairwise differences, half involved population SD and this population also appears in the tree as the only one to stand out in an otherwise starlike pattern (Figure 2). Moreover, although some other clustering of individuals from the same population can be seen (e.g. population 3), only population SD and, to a lesser extent population 11, form monophyletic lineages.

Despite the rather low levels of differentiation found, *P. complanata* might still follow a classic isolation-by-distance model of dispersal. Testing this hypothesis is important because it has implications for how dispersal occurs in this species. A strong pattern indicates that most dispersal events involve movement between geographically adjacent sites, while a very weak pattern indicates either high levels of gene flow acting to reduce differentiation, or that an appreciable proportion of dispersal events are long distance. In practice, the correlation between geographic and genetic distance is not statistically significant (Figure 3, Mantel test, $r = 0.260$, $n = 12$ populations with 1000 permutations, $P = 0.139$). However, it is noticeable that one population, River Derwent (population 6), contributes many points with low F_{ST} values regardless of geographic distance. Removing this Derwent population leaves a highly significant correlation (Figure 3, Mantel test, $r = -0.489$, $n = 11$, $P<0.001$).

Given a general pattern of isolation by distance, it is interesting to ask further whether the somewhat restricted gene flow this implies is also reflected in morphological differentiation.

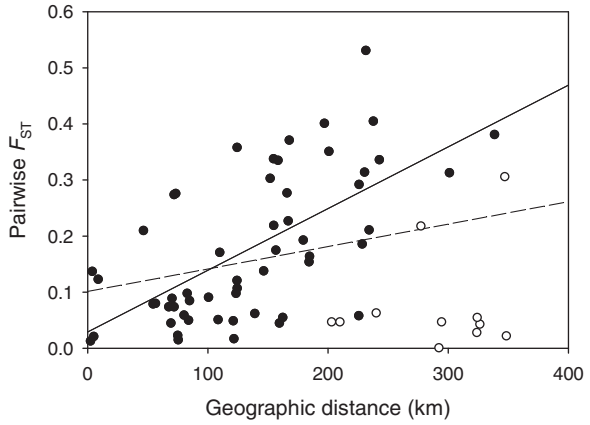

Figure 3. The relationship between pairwise F_{ST} values and geographic distance among 12 *Pseudanodonta complanata* populations. A dashed linear regression line is shown to indicate the underlying trend ($r^2 = 0.068$). Excluding comparisons involving River Derwent (unfilled circles) substantially improves the fit of the regression (solid line, $r^2 = 0.393$).

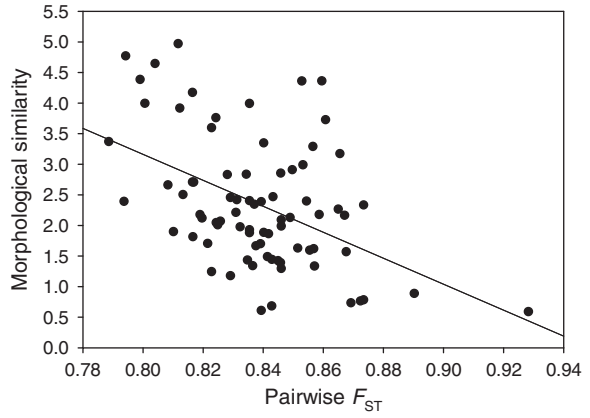

Figure 4. The relationship between pairwise F_{ST} values and morphological similarity among 12 *Pseudanodonta complanata* populations. A linear regression line is shown to indicate the underlying trend ($r^2 = 0.216$).

Consequently, the analysis was repeated after replacing the matrix of geographic distances with a corresponding matrix of morphological distances based on the Euclidean distance between PC1 and PC2 from the PCA. A significant negative relationship between genetic distance and morphological distance was found (Figure 4, Mantel test, $r = 0.465$, $n = 12$, $P < 0.001$).

Overall, F_{AFLP} values do not vary significantly between sites. However, when plotted against rank order catch per unit effort, CPUE, an interesting pattern emerges (Figure 5), with the highest F_{AFLP} values (= greatest inbreeding) occurring in populations with intermediate CPUE. An index of relative shell width for each population was generated by dividing the square root of shell height by width. A mirror pattern emerges with the narrowest mussels occurring at intermediate CPUE values.

DISCUSSION

AFLP polymorphisms have been analysed in *P. complanata* from different UK locations. Genetic differentiation among UK populations is slight, but the relatively isolated South Drain population appears different while the River Derwent population, which is frequently subject to fish stocking (D. Barber, Environment Agency, pers. commun.), is relatively similar to all other populations. Despite this, a pattern of isolation by distance is found and genetic distance also correlates negatively with morphological similarity.

As is often inevitable with threatened species, the sample sizes of this study tended to be small. This problem is further exacerbated by the fact that appreciable numbers (18%) of samples failed to produce reliable banding patterns even after repeated DNA extractions using a range of different extraction protocols. As yet the exact reason why amplification was so variable is unclear, but extraction of DNA from mollusc tissues is known to be difficult in some species. In addition, there may have been issues with preservation. For the most part material preserved in 95% ethanol was used; however, earlier samples were stored in 70% ethanol and it is also possible that penetration of the ethanol into the tissue in some cases occurred too slowly to prevent degradation. More work is needed to develop a reliable method. One recent possibility is a non-invasive approach for DNA isolation from living freshwater mussels (Henley *et al.*, 2006), that could be of use

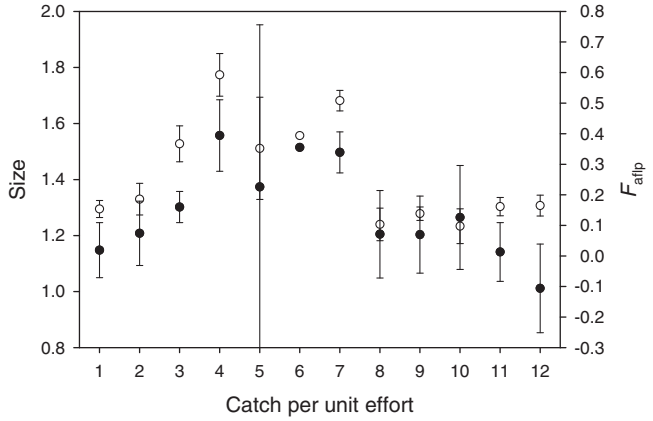

Figure 5. The relationship between catch per unit effort and mean +/− SE relative width (filled circles) and mean +/− SE F_{AFLP} (open circles).

in future studies, particularly those based on endangered species.

Within the UK, levels of genetic differentiation are modest with only the South Drain population showing significant evidence of isolation. The modest levels of genetic differentiation show that there is surprisingly good gene flow between widely dispersed populations of unionid mussels. These patterns can be explained by human activities; while fish stocking may contribute to genetic mixing of unionids between some UK rivers, it is likely that greater facilitation of dispersal comes from the interconnectivity of the different drainages. Previous studies show that the construction of the canal systems across England during the late 18th and early 19th centuries played an important role in the rapid spread of the invasive zebra mussel (*Dreisssena polymorpha*) across the country (Aldridge *et al*., 2004). The South Drain is notable for its clear isolation from other catchments. Moreover, the South Drain and broader Somerset Levels remains one of the few mussel-rich systems in England yet to be invaded by *D. polymorpha*, further illustrating its isolation.

These results suggest that waterways which remain hydrologically isolated, such as the South Drain, may require special attention in conservation programmes as they can harbour genetically distinct populations. The primary threat to mussel populations in the UK comes from river management operations. Aldridge (2000) showed that weed cutting and dredging operations can remove and displace mussels from the river bed. Mussel removal by dredging is of particular concern in the South Drain (Francis Farr Cox, Environment Agency, Pers. commun.), with large numbers of *P. complanata* removed on an annual basis. Given the relative genetic importance of the South Drain population on a national scale, the balance between flood defence and conservation priorities needs careful consideration.

Despite the low level of differentiation observed, there remains a significant indication of isolation-by-distance, suggesting the majority of gene flow does still occur between geographically close sites. In this context, the Derwent population is of particular interest because it exhibits little or no tendency to be more similar to nearer populations and indeed the isolation by distance pattern is only significant if this population is excluded. The most likely explanation for this is the effects of fish stocking. The River Derwent was most recently subject to stocking of 1000 chub (*Leuciscus cephalus*) and 1000 barbel (*Barbus barbus*) from the Midlands in December 2003 (D. Barber, Environment Agency, pers. commun.). Stocking was carried out at Howsham Bridge, the precise collection point of the *P. complanata* used for this study. Cyprinid fishes are known to be suitable hosts to *P. complanata* (Hüby, 1988; Aldridge, 1997) and *P. complanata* in Britain releases mature glochidia between September and April (McIvor and Aldridge, 2007). It is therefore possible that introduced fishes have been vectors for introducing at least some of the *P. complanata* into the Derwent. Conservation often involves management of populations in isolation to avoid irreversibly mixing genetic lineages and to maintain the historic integrity of each population. The implications of fish stocking for the loss of genetic identity within mussel populations has not been previously considered, but may have important consequences for the protection of threatened species.

P. complanata is known to be highly variable morphologically and this study reinforces this view. In addition to the correlation between geographic and genetic distance, a significant trend is found for genetically similar populations to be more morphologically similar. This suggests that, despite local gene flow, the species is evolving adaptations to local environments. However, the situation is by no means clear. The correlation might be in part due to some level of non-independence, in that individuals sampled from the same river system will tend both to experience similar habitats and to be geographically close. If *P. complanata* in similar habitats tend to assume similar morphologies, as was illustrated for other unionid mussels across a much smaller scale (Zieritz and Aldridge, 2009), a correlation comparable with the one reported here could arise without needing to invoke adaptation. To test this hypothesis would require further work, ideally the culture of geographically diverse individuals under a range of uniform laboratory conditions.

The pattern of finding the widest mussels in populations at the two extremes of the CPUE scale may reflect a number of important factors. First, bivalve shell morphology is affected strongly by hydrology, with wider specimens often being associated with relatively low velocities (Eager, 1978; Zieritz and Aldridge, 2009). Individuals occurring in faster flowing water become more slender. Anodontine mussels, including *Pseudanodonta*, are typically found at highest densities where relatively sluggish flows do not confine individuals to infrequent, localized patches of slower velocity or deeper sediments (Killeen *et al*., 2004). Wide mussels at the other end of the CPUE scale may reflect the tendency for many anodontines to become predominantly hermaphroditic at low densities (Heard, 1975). Female and hermaphroditic mussels sometimes display greater widths than males to accommodate the swollen marsupial gills during gravid periods (Saha and Layzer, 2008). While histological examination of gonads by McIvor and Aldridge (2007) found no evidence of hermaphroditism in *P. complanata*, the studies focused on recruiting populations with especially high density (Rivers Waveney and Great Ouse; populations 1, 3 and 10 in this study).

It was surprising to find the greatest homozygosity at intermediate densities. One possible explanation could be that high density populations have high diversity due to their size while the lowest density populations tend to be potentially transient groups of immigrants from some other, larger population. Across a wide range of species, including bivalves, outbreeding and the high heterozygosity this induces, are both linked to increased fitness. Conversely, inbreeding and low heterozygosity tend to be detrimental. Consequently, the finding of populations with low diversity and also low recruitment could have important implications for conservation. On the one hand, these relatively inbred populations might potentially benefit disproportionately from the introduction of individuals from other, thriving populations. On the other, a better understanding is needed of the importance of local adaptation to fitness, since any benefits of outbreeding might be outweighed by loss of fitness due to the introduction of genes associated with adaptation to a different environment. Clearly this is a fertile area for further research.

It is clear that human activity has a profound effect on the genetic structure of freshwater mussel populations, especially in highly managed river systems. It is likely that construction of canals and stocking of fish will facilitate mixing among

populations, but modern dredging and weed-cutting activities can alter population structure and compromise recruitment (Aldridge, 2000; Carr and Aldridge, unpublished data). The impact of river management on the conservation genetics of freshwater mussels has received little previous attention, but understanding the relationships between management and gene flow could be crucial in the future protection of some of the world's most imperilled fauna.

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