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Using genetic monitoring to inform best practice in a captive breeding programme: inbreeding and potential genetic rescue in the freshwater pearl mussel *Margaritifera margaritifera*

Rebecca Kyle[1,2] · Gemma E. Beatty[1,2,3] · Dai Roberts[1,2] · Jim Provan[1,2,4]

[1] School of Biological Sciences, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL

[2] *Quercus*, School of Biological Sciences, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL

[3] School of Education and Lifelong Learning, Aberystwyth University, Aberystwyth SY23 3UX

[4] Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth SY23 3DA

Corresponding author: Dr. Jim Provan

Aberystwyth University

E-mail: J.Provan@aber.ac.uk

Tel: +44 (0)1970 622324

Running title: Ex situ conservation genetics of *Margaritifera margaritifera*

Freshwater pearl mussel (*Margaritifera margaritifera*) populations are declining in Northern Ireland to the extent that a captive breeding programme was established on the Upper Ballinderry river in 1998. Previous genetic analysis of the hatchery broodstock and their first cohort of offspring showed significant levels of inbreeding (F_{IS} = 0.166). The broodstock, which currently numbers *ca.* 90 individuals, was supplemented with new individual mussels, whilst in 2013, a previously unknown population was discovered on the Lower Ballinderry river. The aim of the present study was to determine whether the rotation of the broodstock has led to a decrease in the levels of inbreeding in the second cohort of juveniles, and to determine whether the new population found in the Lower Ballinderry was genetically distinct from the captive bred population and populations from the Upper Ballinderry, which represent the source of the hatchery broodstock. Genotyping using eight microsatellite markers indicated that levels of inbreeding in the second cohort of captive-bred mussels were high, (F_{IS} = 0.629), and were comparable to those sampled from the original cohort and the hatchery broodstock (F_{IS} = 0.527 and 0.636 respectively). Bayesian analysis of population structure indicated that the newly discovered Lower Ballinderry population was genetically distinct from the broodstock and its source populations on the Upper Ballinderry. The observed differentiation was primarily due to differences in allele frequencies, and was most likely a result of genetic drift. The occurrence of ten alleles, albeit at low frequency, in the Lower Ballinderry population, including four private alleles, suggests that this new population could be incorporated into the broodstock with the aim of decreasing levels of inbreeding in the future.

Keywords Ex situ conservation · genetic monitoring · genetic rescue · inbreeding · *Margaritifera margaritifera* · microsatellites

Introduction

Species and habitat declines in the 21st century have brought conservation biology into the spotlight (Hedrick, 2001), with European legislation such as the European Habitats and Species Directive (Directive/92/43/EEC) being implemented to try to reduce declines and protect species and habitats which are already threatened. Global biodiversity is currently under serious threat from a range of factors such as overexploitation, habitat loss and fragmentation, climate change and the introduction of invasive species (Coleman & Williams, 2002; Clavero et al., 2009; Kingsford et al., 2009; Bellard et al., 2012). Freshwater ecosystems are considered amongst the most endangered ecosystems in the world (Dudgeon et al., 2006), with extinction rates being five times greater than terrestrial systems and three times greater than marine coastal systems (Saunders et al., 2002; Dextrase & Mandrak, 2006).

A number of methods have been used to try and combat biodiversity loss such as habitat restoration (Krauss et al., 2010), changes to policy (Mace & Baillie, 2007; Alkemade et al., 2009), increasing habitat connectivity (Luoto et al., 2003) and developing *ex situ* captive breeding programmes (Preston et al., 2007; Fraser, 2008). Captive breeding is widely regarded as a last resort (Snyder et al., 1996) due to the number of associated problems. Guidelines for captive breeding programmes set out by Jones et al. (2006) recommend that before beginning a captive breeding programme, all threats to the populations persistence should be identified and remedied, where possible, to provide suitable habitat and allow early release of propagated juveniles to avoid domestication (McPhee, 2004; Frankham, 2008). Augmentation of populations should use adults from the closest genetically similar population and an appropriate number of adults should be selected to form the broodstock and rotated periodically (Hedrick & Fredrickson, 2010; Kubota et al., 2010). Allendorf and

Luikart (2007) recommend a minimum of 30 founders should be used to maintain 98% of the original heterozygosity but preferably at least 50 should be used. In addition, to maintain population fitness and avoid potential outbreeding depression, evolutionarily significant units (i.e. strongly differentiated populations) should not be mixed (Edmands, 2007; Kubota et al., 2010). One of the most important recommendations is that all augmentations and reintroductions should be sufficiently monitored to ascertain the effectiveness of the captive breeding programme (Seddon et al., 2007).

The freshwater pearl mussel, *Margaritifera margaritifera*, a long-lived unionid mussel, is widely distributed throughout its holarctic range in the Northern hemisphere (Reis, 2003). Throughout the 20th century, dramatic declines have been recorded throughout its range (Beasley and Roberts, 1996; Bolland et al., 2010; Österling et al., 2010). A number of factors have contributed to declines of the freshwater pearl mussel, including overexploitation by pearl fishing (Geist, 2010), eutrophication (Beasley & Roberts, 1999), degradation of habitat (Hastie et al., 2003) and declines of suitable host fish (Geist et al., 2006). The freshwater pearl mussel has a complex, partially parasitic lifecycle during which juvenile mussels, known as glochidia, live on the gills of a suitable host fish, normally salmon (*Salmo salar*) or trout (*Salmo trutta*; Geist et al., 2006) and it is the post-parasitic stage which is widely considered the most vulnerable stage in the lifecycle due to sensitivity to siltation (Buddensiek, 1995). *M. margaritifera* is listed by the IUCN as "critically endangered" therefore it is included in Annexes II and V of the European Union Habitats and Species Directive (Directive 92/43/EEC) and Appendix III of the Berne Convention (JNCC, 2007). It is listed as a Priority Species by the United Kingdom (Habitas, 2006) and has a Species Action Plan in Northern Ireland (DOE, 2005). This species is an indicator of good river ecosystem health and can be classified as an ecosystem engineer, a keystone species, and an umbrella species (Bolland et al., 2010; Geist, 2010).

Freshwater pearl mussel populations in Northern Ireland are regarded as "non-functional" due to a lack of recruitment (Reid et al., 2013), and are now extinct in ten rivers in the province including the Blackwater (G), Bush (H), Colebrook (I), Derg (J), Drumragh (K), Finn (L), Glenelly (M), Mourne/Stroule (N), Moyola (O) and the Upper Bann (P). Currently, populations only exist in six rivers west of Lough Neagh; Ballinderry (A), Owenkillew (B), Owenreagh (C), Swanlinbar (D), Tempo (E) and Waterfoot (F; Figure 1). Surveys carried out in the 1990s (Beasley & Roberts, 1996; Beasley et al., 1998) revealed that virtually no wild mussels in Northern Ireland were under ten years old and that most individuals were in excess of 50 years old, suggesting that freshwater pearl mussels would disappear completely from Northern Ireland rivers unless "adequate protection and management are provided" (Beasley & Roberts, 1996). As a result of this recommendation, a captive breeding programme was initiated in the Ballinderry Fish Hatchery on the Upper Ballinderry in 1998 in an attempt to propagate *M. margaritifera* for restocking purposes. The captive breeding programme uses a semi-natural approach in which water drains from a tank containing 90 adult broodstock mussels into tanks containing suitable juvenile host fish. This allows fertilisation of the mussels and infection of the fish to occur in a natural manner. Fish are held in the tanks for approximately nine months until the glochidia are ready to excyst, a process which is temperature dependent (Scheder et al., 2014). The fish are then transported to a vivarium to allow the glochidia to excyst naturally and burrow into the sediment (Preston et al., 2007).

Integrating fundamental concepts of population genetics into both the establishment and implementation of conservation programmes ensures the preservation or even the enhancement of intraspecfic diversity (Kohn et al., 2006). Population genetics has been shown to have many practical uses in conservation (Schwartz et al., 2006; Jackson et al., 2012), ranging from forensic wildlife protection (Baker et al., 2010) to determining the range

of an endangered species (McKelvey et al., 2006) but one of its most fundamental applications is in determining conservation management units (Schwartz et al., 2006; Jackson et al., 2012), which is especially pertinent in the case of *ex situ* breeding programmes. When establishing a programme, individuals should be selected to represent the diversity of the population whilst limiting the risks of inbreeding and outbreeding depression (Amos and Balmford, 2001; Edmands, 2007). Consequently, understanding management units plays an important role in maintaining the diversity and selecting appropriate individuals to breed from (Schwartz et al., 2006; Jackson et al., 2012). Subsequent genetic monitoring of the broodstock and offspring will determine whether this has had a beneficial impact i.e. increasing diversity and reducing levels of inbreeding.

A study by Wilson et al. (2012) revealed that the captive breeding programme for *M. margaritifera* at the Ballinderry Fish Hatchery showed significant levels of inbreeding. The study also reported the genetic relationships between extant populations in Northern Ireland, revealing three genetic clusters: (1) Ballinderry, including both the wild river and hatchery mussels (River A in Figure 1); (2) Waterfoot (River F) and (3) Owenkillew, Owenreagh, Swanlinbar and Tempo Rivers (B,C, D and E). These clusters were proposed as separate management conservation units. A recent survey carried out in the Lower Ballinderry (Figure 1) discovered a previously unknown population of freshwater pearl mussels which have not been analysed with regards to these genetic clusters. Given the potential for genetic approaches to inform best practice conservation strategies with respect to *ex situ* breeding, the aims of the present study were to determine: (1) whether the rotation of the hatchery broodstock has reduced the level of inbreeding previously reported within the captive population; (2) the contribution of parental broodstock to the next generations; and (3) whether the newly discovered Lower Ballinderry population can be incorporated into the

captive breeding population to increase diversity, or whether it is sufficiently differentiated that it should be managed as a separate unit to minimise the risk of outbreeding depression.

Materials and methods

Surveys, sampling and DNA extraction

A survey carried out in the Summer of 2013 discovered a previously unknown population of freshwater pearl mussels in the lower stretches of the Ballinderry River (Figure 1). Surveyors moved upstream, using bathyscopes to survey the whole width of the river. All 24 mussels of the population were collected and brought to the Ballinderry Rivers Trust hatchery facility, since the habitat quality in the area was considered to be very poor. Individuals were tagged and measurements collected (length, width, depth and mass). A tank was set up to house these mussels separately from the Upper Ballinderry mussels. A non-destructive sampling method (Henley et al., 2006) was used to collect 0.1 - 0.3 ml of haemolymph from the foot of each individual mussel using a 1 ml syringe (Geist and Kuehn, 2005; Karlsson et al., 2013). Haemolymph samples were collected from the current hatchery broodstock adults (n = 74), hatchery "teenagers" bred from the first group of broodstock adults (n=48) in 1998, hatchery "juveniles" (n = 32) bred from the second group of broodstock adults between 2010 and 2014, and the mussels found in the Lower Ballinderry (n = 24). Samples were stored in 1.5 mL Eppendorf tubes in a fridge and extracted the following day to minimise DNA degradation. DNA was extracted following the High Salt Extraction Protocol described in Paxton et al. (1996). In addition, DNA previously collected and extracted from the wild Upper Ballinderry (n = 87) and hatchery broodstock adult (n = 33) for the Wilson et al. (2012) study was used for genotyping and comparing to the Lower Ballinderry population.

Microsatellite genotyping

Initial screening of the nine microsatellite described by Geist et al. (2003) exhibited eight (MarMa3050, MarMa 2671, MarMa 5167, MarMa5280, MarMa4322, MarMa4277, MarMa4315, MarMa4726) which consistently amplified scorable products. Forward primers included a 19 bp M13 tail (CACGACGTTGTAAAACGAC) and reverse primers included a 7 bp tail (GTGTCTT). For all loci, polymerase chain reaction (PCR) was carried out in a total volume of 10 µl containing 100 ng genomic DNA, 10 pmol of HEX-labelled M13 primer, 1pmol of tailed forward primer, 10 pmol reverse primer, 1x PCR reaction buffer, 200 µM each dNTP, 2.5 mM $MgCl_2$ and 0.25 U GoTaq Flexi DNA polymerase (Promega). PCR was carried out on a MWG Primus thermal cycler using the following parameters: initial denaturation at 94°C for 3 min followed by 60 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 5 min. Genotyping was carried out on an AB3730xl capillary genotyping system (Life Technologies; Carlsbad, California, USA). Allele sizes were scored using LIZ size standards and were checked by comparison with previously sized control samples.

Data analysis

Tests for linkage disequilibrium between pairs of microsatellite loci were carried out in the program FSTAT (V2.9.3.2; Goudet, 2001). For all populations, levels of Allelic Richness (A_R), and observed (H_O) and expected (H_E) heterozygosity were calculated using FSTAT (V2.9.3.2; Goudet 2001) and ARLEQUIN (V3.5.1.2; Excoffier and Lischer, 2010) software packages respectively. Inbreeding coefficients (F_{IS}) were estimated using FSTAT. In addition, levels of overall population differentiation were estimated from microsatellite allele

frequencies using Φ-statistics, which give an analogue of F-statistics (Weir and Cockerham, 1984) calculated within the analysis of molecular variance (AMOVA) framework (Excoffier et al., 1992), using the ARLEQUIN software package. To allow for potential biases based on multi-allelic markers such as microsatellites, we also calculated Hedrick's G'_{ST} and Jost's D using the SMOGD software package (Crawford 2010). Population-pairwise estimates of genetic differentiation were calculated using ARLEQUIN and SMOGD.

A likelihood-based approach for determining the parentage of the juveniles with the current broodstock was implemented in the CERVUS software package (v3.0; Kalinowski et al., 2007). The program can allow for potential genotyping errors, and the fact that not all putative parents may be sampled. Simulations were run for 10,000 iterations, with a genotyping error rate of 0.01, since all markers were scored manually to check for automated miscalls and allelic dropout, and assuming 85% sampling of putative parents. Parent-pairs or individual parents were assigned based on the critical values for the 95% strict log-likelihood (LOD) scores.

The software package BAPS (V5; Corander et al., 2003) was used to determine whether the newly discovered Lower Ballinderry population was genetically differentiated from the Upper Ballinderry populations and the hatchery broodstock based on the microsatellite data. BAPS uses a greedy stochastic optimization algorithm to determine K, the most likely number of genetic clusters based on the data. Ten replicates were run for all possible values of the maximum number of clusters (K) up to K = 5, the number of populations sampled in the study, with a burn-in period of 10 000 iterations followed by 50 000 iterations. Multiple independent runs always gave the same outcome.

Results

Levels of diversity in the current broodstock and captive-bred offspring

Mean levels of allelic richness (A_R) were 6.350 (J), 5.667 (T) and 6.978 (BALH), whilst mean expected heterozygosity values (H_E) were 0.537 (J), 0.545 (T) and 0.590 (BALH). High levels of inbreeding were detected within each group, with mean F_{IS} values of 0.629 (J), 0.527 (T) and 0.636 (BALH; Table 1). Diversity values by locus and population are given in Table S1. 74 adults, accounting for 85% of the putative parents, and 32 juveniles from the hatchery breeding programme were genotyped with only four individuals being assigned parentage.

Comparison of the newly discovered Lower Ballinderry population with existing populations and broodstock

Mean levels of allelic richness (A_R) ranged from 4.007 (LB) to 5.889 (BAL3), whilst mean expected heterozygosity values (H_E) ranged from 0.463 (BAL3) to 0.590 (BALH). High levels of inbreeding were indicated in all populations, with mean F_{IS} values ranging from 0.349 (LB) to 0.587 (BAL2; Table 1). The BAPS analysis indicated two genetic clusters, one corresponding to the newly discovered Lower Ballinderry population, and the other made up of the wild Upper Ballinderry populations and the Hatchery broodstock. The AMOVA indicated low levels of population differentiation overall (Table 3), with less than 3 % of the total variation occurring between populations (Φ_{ST} = 0.029), and mean values for Hedrick's G'_{ST} and Jost's D were 0.140 and 0.089 respectively. This was largely due to differences in allele frequencies across populations (Figure 2), but four private alleles were detected in the

Lower Ballinderry population; one at locus MarMa3050 (117 bp), two at locus MarMa4315 (228 bp and 236 bp) and one at locus MarMa4726 (180 bp). All private alleles were found at low frequencies. In total, ten alleles were found in the Lower Ballinderry population that were not detected in the broodstock (117 bp at MarMa3050; 203 bp at MarMa4277; 187 bp, 228 bp,232 bp and 236 bp at MarMa4315; 180 bp at MarMa4726; 147 bp, 153 bp and 160 bp at MarMa5167). Population-pairwise levels of differentiation based on Φ_{ST} ranged from -0.033 (BAL1 vs. BAL3) to 0.120 (J vs. T; Table S2a), from 0.032 (BAL3 vs. LB) to 0.243 (T vs. BAL2; Table S2b) based on Hedrick's G'_{ST}, and from 0.021 (BAL3 vs. LB) to 0.209 (T vs. BAL2; Table S2c) based on Jost's *D*.

Discussion

The findings of the present study highlight the importance of ongoing genetic monitoring of threatened populations to maintain efficient best-practice conservation and management strategies. Analysis showed all groups within the breeding facility have similar levels of allelic richness, with the broodstock showing the highest level of diversity. High levels of inbreeding were detected within all groups examined, which Wilson et al. (2012) previously attributed to a founder effect; a population bottleneck which is common in reintroduced and captive bred populations (Frankham et al., 1999). Numerous studies recommend regular rotation of the broodstock to ensure that genetic diversity within the population is maintained, helping to reduce the founder effect (Jones et al., 2006) and to ensure that the genetic diversity of wild population, if not extinct, is well represented within captive breeding programmes (Brummett and Ponzoni, 2009). Breeding programmes by their very nature have been developed as a last resort to save a species from the brink of extinction (Wilson et al., 2012), therefore genetic diversity is often already greatly diminished within these threatened populations. Consequently, it is important to try and maintain the remaining diversity, as limited as it may be, and to reduce the inbreeding depression and maintain fitness within the population (Reed and Frankham, 2003).

Ex situ conservation programmes are a last resort method of maintaining threatened species with effective monitoring of success and failures valuable tools for future projects (Snyder et al., 1996). A number of risks are associated with *ex situ* conservation, including the loss of genetic diversity, producing deleterious allele combinations, behavioural changes and the transfer of pathogens between captive and threatened populations (Ebenhard, 1995; Zippel et al., 2011). Ballinderry has adhered to a number of the guidelines laid out by Jones et al., (2006) for the rearing of freshwater mussels, including identifying and remedying

threats in the catchment which has been carried out by the Freshwater Pearl Mussel Project (Horton et al., 2015) and addressing the risk that have been highlighted by a number of studies that individuals could become adapted to captivity (Frankham, 2008; Robert, 2009). Wilson (2010) released 350 mussels ranging from 10-13 years old to three locations within the Ballinderry catchment and used Passive Integrated Transponders (PIT) to aid with their recovery, subsequent surveys have found individuals at each site suggesting individuals in the programme have undergone little adaption to captivity. In fact all ten of the guidelines put forward by Jones et al. (2006) have been addressed through the semi-natural propagation method used (Preston et al., 2007) and projects such as the Freshwater Pearl Mussel Breeding Re-introduction Project and the Freshwater Pearl Mussel Rescue Project (Horton et al., 2015).

Juveniles bred from the "second" broodstock (after rotation) were found to be more inbred than the teenagers from the "first" broodstock and parental assignment was only possible for four individuals. This is due to the high inbreeding exhibited by the juveniles, teenagers and broodstock making it difficult to distinguish which juveniles came from each member of the broodstock (Lacy et al., 1993). Throughout the three groups there are relatively few alleles at high frequencies for many loci which are shared by many individuals. At MarMa3050, the teenagers show a different dominant allele than both the juveniles and broodstock, which is representative of the broodstock before it was rotated.

The newly discovered Lower Ballinderry population appears to be genetically distinct from the Upper Ballinderry and Hatchery populations, suggesting this population could be maintained as a separate conservation management unit; however, it should be noted that BAPS often overestimates the number of clusters (Latch et al., 2006). Three private alleles were detected at low frequencies in the Lower Ballinderry population, but the differentiation between this and the remaining populations was primarily due to differences in allele

frequencies, which have most likely arisen through genetic drift as a result of the small size of the population. The genetic distinctiveness of the Lower Ballinderry population is most likely due its isolation until the 1960s, when this stretch of the river was separated from the Upper Ballinderry by a waterfall (Bells Rock) which was impassable to fish except in periods of exceptionally high flow. Although the populations are within the same catchment basin, the minimal interaction and mixing between populations and their host fish resulted in a lack of gene flow between the Lower Ballinderry and the rest of the wild Upper Ballinderry populations.

This study has shown that significant levels of inbreeding remain within the breeding programme even after the rotation of broodstock adults, and the level of inbreeding within the juveniles has actually increased. Although BAPS shows the Lower Ballinderry as a separate population, which could be developed and maintained as a separate management unit, the differences between the Lower and Upper Ballinderry populations attributed to differences in allele frequencies rather than allele composition. As the Lower Ballinderry is such as small population, consisting of only 24 individuals, maintaining them as a separate management unit may actually increase the level of inbreeding; therefore it would be recommended that the Lower Ballinderry population is incorporated into the Upper Ballinderry breeding population. Small, isolated populations such as the Lower Ballinderry are more vulnerable to inbreeding and loss of genetic diversity (Keller and Waller, 2002) which can lead to an increased risk of extinction (Bijlsma et al., 2000). As the breeding population is also small and exhibiting significant levels of inbreeding, combining the two populations will act as a type of "genetic rescue" by introducing "immigrants" and helping to alleviate inbreeding depression (Tallmon et al., 2004; Hedrick, 2005). This will increase the frequency of rarer alleles already found in the Upper Ballinderry population, as well as introducing the ten

alleles (including four private alleles) found in the Lower Ballinderry population that were not detected in the broodstock (Shen et al., 2009).

A number of studies have highlighted the risks associated with mixing management units including outbreeding depression which can decrease the fitness of future generations (Edmands, 2007). However, Mortiz (1999) has stated that it would be appropriate to mix management units if it is for the purposes of augmentation of remnant populations that show inbreeding depression or populations that are becoming increasingly fragmented. There have been examples of success stories of mixing management units such as the Mexican wolf, *Canis lupis bailyei* (Fredrickson et al., 2007; Hedrick and Fredrickson, 2010).

The findings of this study are applicable to other *ex situ* conservation programmes, for example, a project in Upper Austria which has similar numbers of wild adults (Scheder and Gumpinger, 2008). We recommend that the breeding population of *M. margaritifera* held at Ballinderry Rivers Trust should continue to undergo genetic monitoring and that any individuals which are introduced in the future are also examined. It would be prudent to continue rotating the broodstock every 5-10 years with wild Upper Ballinderry adults to reduce the level of inbreeding, and in particular to "pre-screen" new individuals to maximise genetic diversity. To further increase the diversity of the broodstock, the Lower Ballinderry population should be incorporated into the breeding population to further help reduce the level of inbreeding through genetic rescue.

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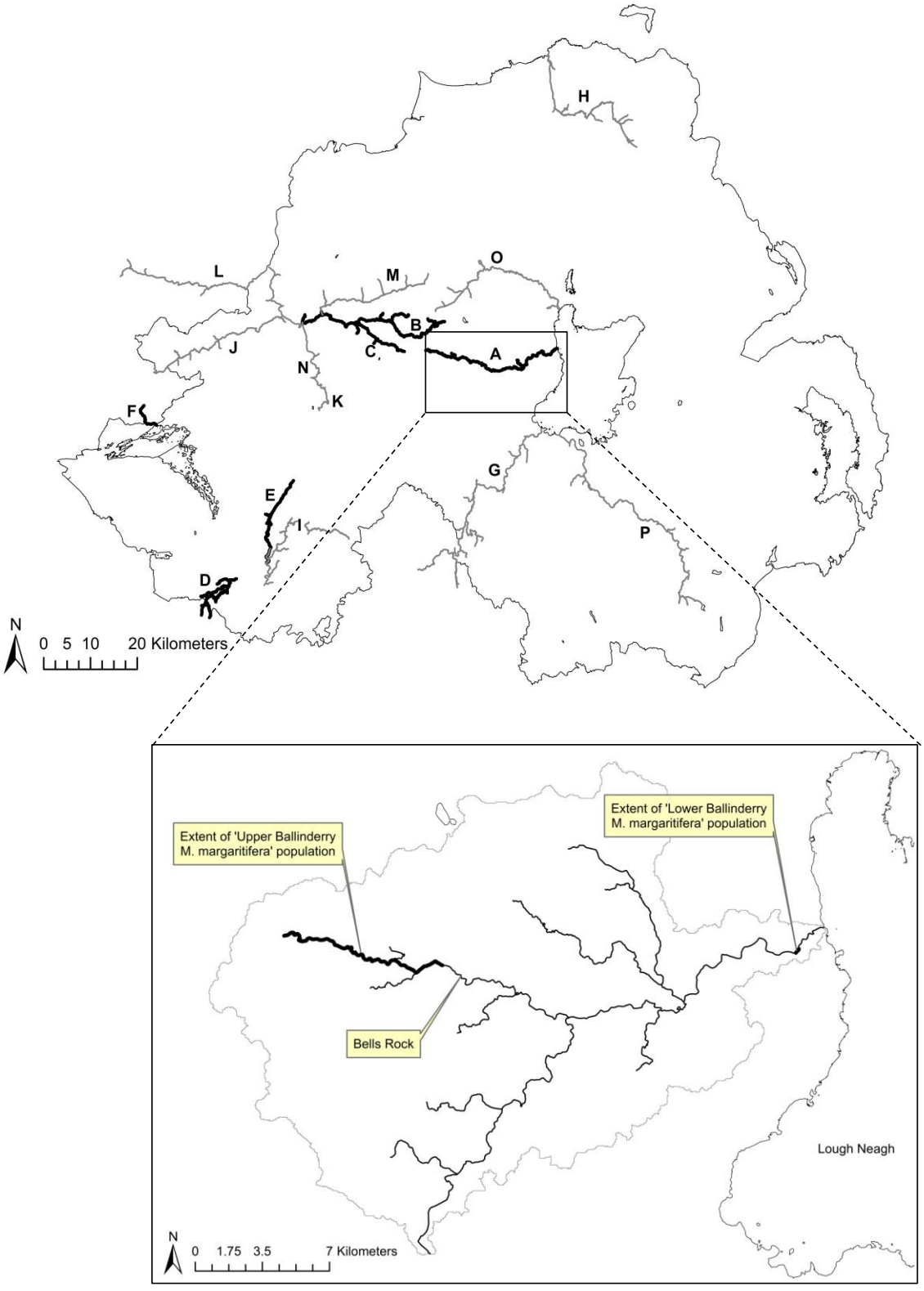
Figure captions

Fig. 1. Rivers with extant *Margaritifera margaritifera* populations labelled A-F (black) and those whose *M. margaritifera* populations are now extinct labelled G-P (grey). See text for river codes. Inset shows the location of the newly discovered Lower Ballinderry population in relation to the historic impassable fish waterfalls (Bells Rock) and the main Upper Ballinderry population.

Fig. 2. Bubble plots showing allele frequencies at the eight microsatellite loci analysed for the Lower Ballinderry (LB), the wild Upper Ballinderry populations (BAL1, BAL2 and BAL3), and the current broodstock (BALH). Y-axes indicate allele size in base pairs.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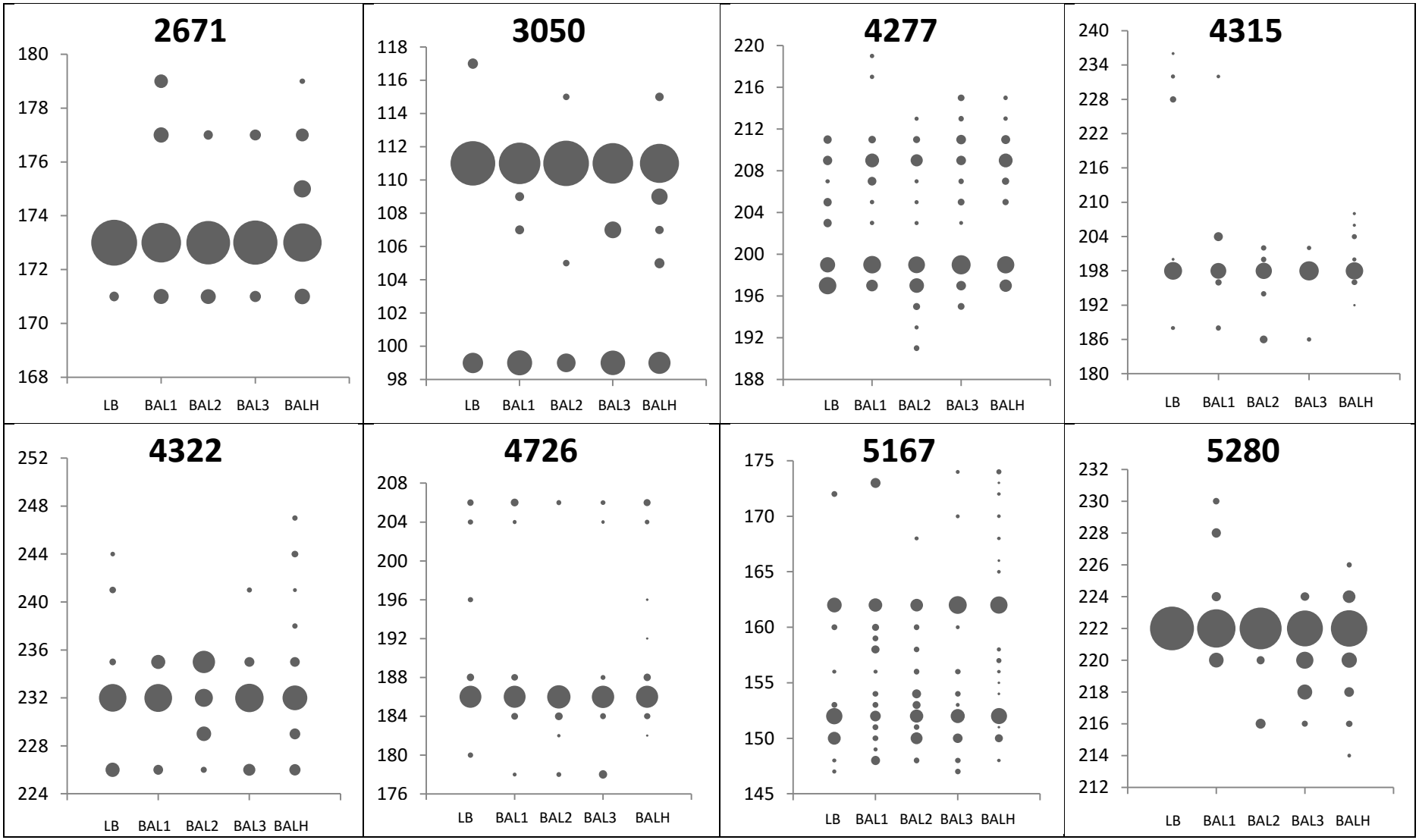
Extent of 'Upper Ballinderry M. margaritifera' population
Extent of 'Lower Ballinderry M. margaritifera' population
Bells Rock
Lough Neagh
N
0 1.75 3.5 7 Kilometers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LB BAL1 BAL2 BAL3 BALH
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LB BAL1 BAL2 BAL3 BALH

Table 1 Summary statistics. *N*- sample size; A_R- allelic richness; H_O- observed heterozygosity; H_E- expected heterozygosity; F_{IS}- inbreeding coefficient.

Population	Code	*N*	A_R	H_O	H_E	F_{IS}
Juveniles	J	32	6.350	0.201	0.537	0.629***
Teenagers	T	48	5.667	0.259	0.545	0.527***
Hatchery broodstock	BALH	74	6.987	0.216	0.590	0.636***
Upper Ballinderry 1	BAL1	28	5.560	0.265	0.571	0.542***
Upper Ballinderry 2	BAL2	29	4.627	0.211	0.503	0.587***
Upper Ballinderry 3	BAL3	27	5.889	0.265	0.463	0.433***
Lower Ballinderry	LB	24	4.007	0.315	0.481	0.349***

*** $P < 0.001$

Table 2 Analysis of molecular variance (AMOVA)

Source of variation	d.f.	Sum of squares	Variance	% variation
Among populations	4	16.477	0.047	2.94
Within populations	267	415.571	1.556	97.06