Unique sympatric quartet of limnetic, benthic, profundal and piscivorous brown trout populations resolved by 3D sampling and focused molecular marker selection

Eric Verspoor1 | Mark W. Coulson1 | Ronald B. Greer2 | David Knox3

1Rivers and Lochs Institute, Inverness College, University of Highlands and Islands, Inverness, UK
2Natural Resources Scotland, Blair Atholl, UK
3Ardblair, Blair Atholl, UK

Correspondence
Eric Verspoor, Rivers and Lochs Institute, University of Highlands and Islands Inverness, Inverness, UK.
Email: eric.verspoor.ic@uhi.ac.uk

Abstract
1. The full extent of sympatric intraspecific population diversity (SIPD) i.e. structuring into multiple genetically distinct Mendelian populations, remains uncertain for most lacustrine fish species, particularly in northern lakes. However, increasing application of molecular genetics is advancing understanding and has shown both that many known intraspecific sympatric lacustrine morphological and behavioural polymorphisms represent SIPD, and resolved the existence of phenotypically cryptic structuring.

2. Uncertainty remains as only a few northern lakes have been comprehensively surveyed and existing studies focus on known phenotypic polymorphisms or exploit ad hoc field sampling, marker selection and data analysis methods. Such unfocused approaches constrain resolving power and increase the likelihood of failing to detect SIPD when present.

3. Brown trout (Salmo trutta L.) in a previously unstudied Scottish lake were collected by 3D stratified random netting. They were screened with both an arbitrary set of molecular markers and markers pre-selected for their ability to resolve regional allopatric brown trout population structuring and data analysed by both Bayesian and non-Bayesian approaches.

4. Depth clines for mitochondrial and microsatellite markers variation were observed and found to arise from different, but overlapping, depth distributions of four genetically distinct piscivorous, limnetic, shallow benthic, and profundal benthic populations. This is the highest number of ecologically and phenotypically distinct sympatric brown populations identified in any lake to date, and includes the first reported profundal benthic form.

5. The detection of the SIPD in Loch Laidon depended critically on the random 3D sampling and using markers preselected for their power to differentiate regional allopatric populations of trout, as well as aided by using both Bayesian and non-Bayesian analytical approaches.

6. The findings support the view that the extent of ecologically and evolutionarily significant SIPD is probably underestimated in brown trout and other fish species, and probably is a significant component of the biodiversity in many northern hemisphere lakes.

Keywords
cryptic biodiversity, lacustrine fish, molecular genetics, sympatric populations
Effective biological conservation requires accurate accounting of the earth’s evolutionary population diversity i.e. its division into distinct genetic (Mendelian) populations, both at a global and local level (Cook, Page, & Hughes, 2008; Delić, Trontelj, Rendos, & Fiser, 2017; Giangrande, 2003). A broad insight into its nature and extent is essential to guide conservation policy development while specific local understanding is essential to effectively guide practical implementation; broad, generic conservation approaches alone cannot address the issue that some intraspecific diversity will be locally unique such that functional roles and biological community contexts may vary in each locale (Dickie et al., 2011; Lentini & Wintle, 2015). However, cataloguing intraspecific population diversity both globally and locally remains an urgent conservation priority, even for large organisms in relatively accessible terrestrial biomes this remains an on-going challenge (Fennessy et al., 2016; Nater et al., 2017; Poulakakis et al., 2015).

Freshwater biomes are estimated to encompass c. 0.8% of the earth’s surface area and c. 6% of its species (Dudgeon et al., 2006). However, for various reasons, developing a full understanding of their diversity is particularly challenging. These include their wide-ranging distribution and, in many areas such as the northern hemisphere, their extreme numbers, as well as the relative inaccessibility of many, and the physical and psychological remoteness of the underwater world for humans. Furthermore, aquatic taxa tend to show less overt phenotypic differentiation than other groups (Seidel, Lang, & Berg, 2009), such that it is more likely to be cryptic (or pseudocryptic) (Cardoso, Erwin, Borges, & New, 2011; Lajus, Sukhikh, & Alekseev, 2015; Poulin & Pérez-Ponce de León, 2017). This is problematic for species accounts given an historical bias towards using morphological criteria to define taxa and subjectivity in species designations (Bickford et al., 2007; Fujita, Leaché, Burbink, McGuire, & Moritz, 2012; Hey, 2006; Pante et al., 2015). As such, diversity is more likely to be underestimated than in terrestrial biomes. It also generates a bias against the assignment of populations to species, with frequent reference to species complexes (e.g. brown trout—Pustovrh, Snoj, & Bajec, 2014; Arctic char—Taylor, 2016) and often differing views on the classification of distinct populations as species (e.g. in northern hemisphere fishes (Adams & Maitland, 2007; Kottelat & Freyhof, 2007).

Within currently designated species, novel sympatric infraspecific population diversity (SIPD) in lakes, i.e. structuring into genetic populations, has been increasingly resolved by molecular genetic analyses. It is now widely reported across most taxonomic groups, including fish and other freshwater taxa (Poulin & Pérez-Ponce de León, 2017), and has led some to view SIPD as common and widespread (Bickford et al., 2007; Seehausen & Wagner, 2014; Vonlanthen et al., 2009). Arguably, this might be expected. Like terrestrial islands, lakes are quasi-isolated ecosystems with a predisposition to generate endemic population diversity (e.g. cichlid fishes in African great lakes—Salzburger, Van Bocxlaer, & Cohen, 2014; brown trout in Ireland—Ferguson & Taggart, 1991; Arctic char—Sandlund et al., 1992). Furthermore, at least in the northern hemisphere, only a small fraction of its millions of lakes (Verpoorter, Kutser, Seekell, & Tranvik, 2014) have been studied and very few comprehensively. Thus, the actual extent of SIPD in northern hemisphere fish species remains uncertain and is probably underestimated.

The brown trout (Salmo trutta L.), a widely distributed species in European lakes, provides some of the earliest confirmed cases of SIPD. One of the first, in Lough Melvin, Ireland, relates to known, overt phenotypic and ecological polymorphisms (Ferguson & Mason, 1981), while another in Lough Neagh, Ireland, has no demonstrable link with phenotypic variation (Anon, 2015; Crozier & Ferguson, 1986); a third, in a Swedish lake, describes phenotypically cryptic structuring (Allendorf, Ryman, Stennek, & Ståhl, 1976; Ryman, Allendorf, & Ståhl, 1979). However, since these early studies, relatively few further cases have been reported. In two Scottish lakes, the large piscivorous ferox form has been found to be genetically distinct from other trout (Duguid, Ferguson, & Prodohl, 2006) and phenotypically cryptic SIPD resolved in a number of further Fennoscandian lakes (Allendorf et al., 1976; Andersson et al., 2017; Mákinen, Niva, Koljonen, Swatdipong, & Primmer, 2015; Palmé, Laikre, & Ryman, 2013; Swatdipong, Vasemägi, Niva, Koljonen, & Primmer, 2010, 2013). In all these cases, structuring appears to be associated with natal homing of spawners and the spatial separation of spawning habitat in different lake tributaries or lake areas. Unfortunately, extensive and wide-ranging surveys of structuring across lakes are lacking for brown trout as well as other northern species (see Wilson et al., 2004 for an exception).

Most molecular genetic studies addressing SIPD have been based on phenotypically targeted or ad hoc spatial sampling and, at least initially, arbitrary molecular marker sets, probably limiting their potential informativeness in respect of overall levels of SIPD. Resolving power can be expected to vary depending on the representativeness of sampling of a species stock in a lake and on marker choice—given that not all markers are likely to be equally informative (Bradbury et al., 2013; Burri et al., 2015; Feulner et al., 2015). Furthermore, the power of statistical approaches to detect structuring can depend upon the actual degree and pattern of population differentiation (e.g. Kalinowski, 2011; Puechmaille, 2016; Putman & Carbone, 2014; Wang, 2017). Thus existing studies provide a poor guide to the full extent of SIPD.

The described study aims to extend understanding of SIPD in brown trout and assess the advantages of using focused, systematic methods to increase detection power, particularly where prior knowledge of structuring is lacking. The work centres on Loch Laidon, a previously unstudied lake in Scotland selected based on the incidental netting of a phenotypically unusual trout in the lake’s profundal zone when sampling for Arctic char (Salvelinus alpinus L.). Historical reports document typical brown trout caught by anglers as well as ferox trout being present (Campbell, 1979). The study exploits a preliminary 3-D random-stratified netting of the lake used as the first step in the method recommended under the Water Framework Directive for assessing interspecific fish diversity in northern European lakes (Degerman, Nyberg, & Appelberg, 1989; Olin et al., 2014), similar to the broad-scale monitoring method used in North America (Sandstrom, Rawson, & Lester, 2013); preliminary netting provides basic local information for guiding the design of
the main sampling programme (Appelberg, 2000; Appelberg et al., 1995). The study encompassed two sets of molecular markers, one assembled without prior knowledge of its population resolving power and another pre-selected for its capacity to resolve allopatric structuring in brown trout stocks in Ireland (Keenan et al., 2013). The assessment of structuring was made using both Bayesian and non-Bayesian data analysis approaches. The specific study objectives were to establish: (1) how many distinct genetic populations were present; and (2) if more than one, whether sampling method, marker selection or analysis approach influenced SIPD detectability.

2 | METHODS

2.1 | Study location

Loch Laidon, is situated 280 m above mean sea level on Rannoch Moor, in the upper River Tummel catchment of the River Tay in Scotland (Figure 1a), and c. 9.5 km long and c. 4.5 km² with mean and maximum depths of 10.5 and 39 m (Figure 1b). The main basin is >15 m deep, 5 km long and underlies 26% of the loch; the unsampled western arm is c. 2 km in length and averages 2–3 m in depth. Laidon has two main inlet streams, one at the SW end and the other entering western arm, with numerous smaller inflows as well as the outflow. The only published scientific account of the lake, on its bathymetry (Murray, Pullar, & Chumley, 1910), noted it as “...one of the best trouting lochs in the district, or perhaps in Scotland”.

2.2 | Samples

The primary sampling for the study was carried out using the preliminary netting protocol for the Nordic Gill Net Survey (NGNS) approach (Appelberg, 2000; Appelberg et al., 1995). Benthic nets—30 m long by 1.5 m deep with 12 different 2.5 m wide mesh
size panels ranging from 5 to 55 mm knot to knot, and pelagic nets—27.5 m long and 6 m deep, divided into upper (0–3 m) and lower (3–6 m) depth zones, without a 5 mm mesh panel. Nets were set between 1700 and 1900 h (Table 1, Figure 1b), lifted the next morning between 09:00 and 11:00 h. Fish were removed, and net of origin, species, weight and length recorded, c. 5 mm² of caudal fin removed and stored in 99% ethanol, and carcasses frozen.

The analysis also integrated unpublished mitochondrial (mt)DNA restriction fragment polymorphism data from prior ad hoc sampling carried out for non-genetic studies (Table 1). Additionally, data was included from preliminary NGNS netting of two lakes, Lochs Affric and Beinn A’ Mheadhoin, on the River Glass in the River Beauly catchment (Figure 1a), which are lakes of a similar size to Laidon. Their populations probably derive from historical stocking as they exist above an impassable falls and there are landscape constraints on historical headwater connections to other catchments. These latter data were included by way of contrast and to provide outlier populations for rooting the phylogenetic trees.

All samples used in the study in accordance with Scottish law derive from wild fish collected and killed with permission from the owner of the riparian rights to net Loch Laidon.

2.3 | Genetic typing

DNA was extracted using Qiagen kits following manufacturer’s instructions and following Knox, Lehmann, Reddin, and Verspoor (2002) for older samples typed for mtDNA variation. Three mtDNA regions—Cyto-b, D-loop and 16sRNA-ND1—were screened (Supporting Information Table S1 (Verspoor, Knox, Greer, & Hammar, 2010) for known restriction polymorphisms (Supporting Information Tables S2 and S3). For microsatellite variation, screening encompassed nine arbitrarily selected loci (Supporting Information Table S4) as well as an overlapping panel of 19 loci (Supporting Information Table S5) preselected based on their power to resolve allopatric structuring in brown trout (Keenan et al., 2013), with 23 different loci overall. Microsatellite fragments were separated and sized by MegaBace DNA analyser following the manufacturer’s instructions, with double-blind genotyping and inconsistencies resolved by re-running/re-extracting problematic samples.

2.4 | Statistical analysis

2.4.1 | mtDNA

A minimum spanning mtDNA haplotype network was constructed manually and haplotype heterogeneity among samples assessed using either a Fisher’s exact test (FET) or χ² test with Monte Carlo estimation of p (PAST 3.13—Hammer, Harper, & Ryan, 2008). A test for a linear trend (Everitt, 1992) across a contingency table of net depth and mtDNA haplotype frequencies used an Excel macro (Cannon, 2001).

2.4.2 | Microsatellites

Genotyping was assessed for allelic drop-out, null alleles and miss-scoring due to stutter peaks using MICROCHECKER V2.2

<table>
<thead>
<tr>
<th>Label on map</th>
<th>Method</th>
<th>Depth Zone (m)</th>
<th>Number of Trout</th>
<th>Latitude GPS</th>
<th>Longitude GPS</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Benthic net</td>
<td>0–3</td>
<td>22</td>
<td>56°38.865′N</td>
<td>4°39.166′W</td>
<td>19–20/06/2008</td>
</tr>
<tr>
<td>B</td>
<td>Benthic net</td>
<td>3–6</td>
<td>21</td>
<td>56°38.867′N</td>
<td>4°39.147′W</td>
<td>19–20/06/2008</td>
</tr>
<tr>
<td>C</td>
<td>Benthic net</td>
<td>6–12</td>
<td>23</td>
<td>56°38.911′N</td>
<td>4°39.124′W</td>
<td>19–20/06/2008</td>
</tr>
<tr>
<td>D</td>
<td>Benthic net</td>
<td>12–20</td>
<td>10</td>
<td>56°38.867′N</td>
<td>4°38.789′W</td>
<td>19–20/06/2008</td>
</tr>
<tr>
<td>E</td>
<td>Benthic net</td>
<td>20–35</td>
<td>17</td>
<td>56°38.912′N</td>
<td>4°38.782′W</td>
<td>19–20/06/2008</td>
</tr>
<tr>
<td>F</td>
<td>Benthic net</td>
<td>35+</td>
<td>13</td>
<td>56°39.208′N</td>
<td>4°38.290′W</td>
<td>19–20/06/2008</td>
</tr>
<tr>
<td>G</td>
<td>Benthic net</td>
<td>6–12</td>
<td>15</td>
<td>56°40.699′N</td>
<td>4°35.765′W</td>
<td>19–20/06/2008</td>
</tr>
<tr>
<td>H</td>
<td>Benthic net</td>
<td>3–6</td>
<td>12</td>
<td>56°40.718′N</td>
<td>4°35.704′W</td>
<td>19–20/06/2008</td>
</tr>
<tr>
<td>I</td>
<td>Benthic net</td>
<td>0–3</td>
<td>11</td>
<td>56°40.929′N</td>
<td>4°35.397′W</td>
<td>19–20/06/2008</td>
</tr>
<tr>
<td>P</td>
<td>Upper pelagic</td>
<td>0–3</td>
<td>19</td>
<td>56°39.278′N</td>
<td>4°38.197′W</td>
<td>19–20/06/2008</td>
</tr>
<tr>
<td>P</td>
<td>Lower pelagic</td>
<td>3–6</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Label on map</th>
<th>Method</th>
<th>Depth Zone (m)</th>
<th>Number of Trout</th>
<th>Latitude GPS</th>
<th>Longitude GPS</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>Benthic net</td>
<td>35+</td>
<td>33</td>
<td>56°38.912′N</td>
<td>4°38.782′W</td>
<td>24–25/08/2006</td>
</tr>
<tr>
<td>S</td>
<td>Fly fishing</td>
<td>0–3</td>
<td>10</td>
<td>56°40.951′N</td>
<td>4°35.600′W</td>
<td>02/09/2006</td>
</tr>
<tr>
<td>T</td>
<td>Benthic net</td>
<td>0–3</td>
<td>19</td>
<td>56°40.951′N</td>
<td>4°35.600′W</td>
<td>08/2005</td>
</tr>
<tr>
<td>Z</td>
<td>Electrofishing</td>
<td>Stream</td>
<td>31</td>
<td>56°38.957′N</td>
<td>4°41.305′W</td>
<td>08/2006</td>
</tr>
</tbody>
</table>
(Van Oosterhout, Hutchinson, Wills, & Shipley, 2004); heterozygote deficiencies alone were deemed insufficient for null allele identification given potential Wahlund effects (Garnier-Géré & Chikhi, 2001). Individual samples and defined genetic populations were assessed for Hardy–Weinberg (H-W) departures, inter locus linkage disequilibrium (LD), and heterogeneity of genotype proportions using Genepop 4.2 and tested using the default Markov chain parameters (Raymond & Rousset, 1995; Rousset, 2008), with a sequential Bonferroni correction (SBC) applied to multiple tests (Lessios, 1992). The overall lake sample was screened for full sibs using COLONY 2.0.6.1 (Jones & Wang, 2010) with only one full sib retained in population structure analyses. A neighbour joining (NJ) tree was constructed (MEGA4—Tamura, Dudley, Nei, & Kumar, 2007) using pairwise $F_{ST}$ among samples across loci calculated using Genepop 4.2 to visualise sample differentiation to depth. Spearman rank correlation coefficients ($r_{s}$) were tested for association of major alleles (defined as those with mean net sample frequencies of $>0.100$) with depth and among allele frequencies.

Non-Bayesian hierarchical clustering (complete linkage on individual pairwise Euclidean distances) was carried out with identified groups assessed by discriminant analysis of principal components (DAPC; adegenet 2.0.1—Jombart, 2008) using cross-validation to optimise the retained number of principal components. Bayesian clustering was carried out with STRUCTURE (Hubisz, Falush, Stephens, & Pritchard, 2009; Pritchard, Stephens, & Donnelly, 2000) using admixture, correlated frequencies, and no prior population information with 10 runs for each K (number of groups) for $K = 1$–5 to 30,000 burn in, 300,000 iterations. Mean and standard deviation of the likelihood of K (L(K)) and $\Delta K$ (Evanno, Regnaut, & Goudet, 2005) were plotted using CLUMPAK (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015). For the most probable K, 10 runs were also made for each of: (a) admixture plus sample information; (b) no admixture; and (c) no admixture plus sample information, and results compared. The most probable population membership of individuals was determined using the STRUCTURE ancestry model with the most likely being the group to which an individual was assigned (Falush, Stephens, & Pritchard, 2007), in respect of either the Bayesian or non-Bayesian method. An NJ tree was constructed for the defined populations (MEGA4—Tamura et al., 2007) using genetic distances ($D_{A}$) among Laidon groups using Affric and Beinn A’ Mheadhoin to root the tree.

Population correspondence of individuals between the two clustering methods was tested using a $\chi^2$ with Monte Carlo estimation of $p$ (PAST 3.13—Hammer et al., 2008). Genetic similarity of populations defined by the two methods was visualised using NJ trees constructed from pairwise $F_{ST}$ values, and the distribution of individual locus $F_{ST}$ values compared for the two methods.

Overall STRUCTURE results were compared to three alternate loch sampling strategies using: (a) 0–3-m shoreline nets only (those typically exploited by small boat and shore anglers); (b) 0–12-m nets (reflecting typical ad hoc shallow benthic netting samples); and (c) nets below 12 m (typical of bycatch from targeted Arctic char netting). The basic admixture model was run with correlated frequencies and no prior population information, for $K = 1$–5 to 10 runs each K using 30,000 burn in and 300,000 iterations.

3 | RESULTS

3.1 | Intersample variation

3.1.1 | mtDNA

The nine haplotypes found (Supporting Information Figure S1, Table S6) differed by single restriction site changes, with >85% of trout being either Hap2 or Hap8 (Supporting Information Table S6), and haplotype richness highest in the 6–20 m zone where the rare haplotypes 4, 5, 6, 7 and 9 were most common. Following pooling of related rare haplotypes (Hap1 & 2, Hap4 & 5, Hap6, 7 & 9), pairwise $F_{ST}$'s (Figure 2a, Supporting Information Table S7) showed the upper pelagic to be the most differentiated sample, most notably from deep benthic samples, and closest to the 2006 shore angled (S) and 2008 NGNS 0–3 m (I) net samples, c. 3 km away (Figures 1b and 2a). The deep benthic samples do not differ significantly from each other or the adjacent 20–35 m sample. However, the latter is suggested to be different from the adjacent 12–20 m sample and it in turn from the adjacent 6–12 m sample. Stream samples X and Y were not different but differed from stream Z. The latter clustered with the deep benthic samples while the former did so with samples from the shallow benthic zones in both lake sectors. Samples from the same depth and location but different years (I & T, W & F), and same depth but different locations (A & I/T, B & H, C & G), showed no heterogeneity and were pooled. After pooling, the remaining location samples displayed a highly significant heterogeneity ($\chi^2 = 156.06, p < 0.0001$), progressive increase in Hap8 ($\chi^2 = 23.6, df = 1, p < 0.0001$) and progressive decline in Hap1/2 ($\chi^2 = 32.6, df = 1, p < 0.0001$) with depth. There were with no significant departures from these latter linear trends. Significant parallel differences were also found between the upper and lower pelagic net samples (Figure 3a, FET $p = 0.01$).

3.1.2 | Microsatellites

Locus Ssa171 had an irregular repeat size, confounding genotyping, and was discarded. The 22 remaining loci showed no significant H-W excesses across loci or across samples though after SBC six loci had individually significant deficiencies—MHCI, Ssa407, SsaD48, BG9254B8, CB512797 and Ssa289. However, tests were significant in one or more samples for only two loci (MHCI: three samples—A, B, C; SsaD48: two samples—B, I plus A, approach significance), all from <6 m depth.

Four samples had significant departures across loci (A, D, E and I), including both shallow inshore benthic and both deep benthic samples. The overall deficiency across loci for pooled nets was...
highly significant ($p < 0.0001$). LD tests were significant for five of 154 sample following SBC—SsaD48/Ssa289, BG935488/MHC-I, UT, SsaD71/CBS12797, CocLAV4/SasaTAP2, and MHC-I/SasaTAP2, but none in the overall lake sample.

Significant heterogeneity of genotype frequencies was present across net samples ($\chi^2$ infinity, $df = 36, p < 0.0001$) with 34/55 significant pairwise $F_{ST}$ between nets, although just 12 after SBC (Supporting Information Table S8). $F_{ST}$ divergence also was positively associated with depth (Figure 2b) and nets from the same lake sectors tending to cluster together. Five NJ tree nodes had moderate bootstrapping values with the only notable exception being the 0–3-m net from the eastern sector, which clusters with the central upper pelagic net. Locus differentiation was greatest for One103, Ssa417 and MHC-I ($F_{ST}$ values—0.0758, 0.036, and 0.306, respectively; Supporting Information Table S9). Major alleles at six loci (CAO48828, MHC I, MHC I UTR, One102, One103, SsaD48, Ssa407, SsaD71) show clinal variation with depth, with significant allelic clines at the same loci highly negatively correlated. The most marked clines are for One103*$176 and MHC-I *131 (Figure 3b).

3.2 Population structure

Seven families composed of two individuals and one of four individuals were resolved in the trout collected in the net samples. Duplicate family representatives were removed from the data set used for the structure analysis.

Visual inspection of non-Bayesian clustering suggested four higher-level clusters of individuals (Figure 4a). One, made up of two outlier individuals, encompassed the two largest and only morphologically typical ferox trout (Campbell, 1979) (Figure 1c). The remaining three groups, were composed of 52, 36 and 76 individuals. DAPC (Figure 4b), showed good separation of the ferox individuals with 100% posterior probability of assignment back to group. DAPC of the remaining three groups also found good separation, but with lower posterior individual assignments and 17 trout reassigned compared to the four groups DAPC. Bayesian STRUCTURE analysis, based on both mean $L(K)$ and $\Delta K$, gave $K = 3$ as the best supported cluster number (Figure 5a), with clusters encompassing 66, 66 and 44 individuals (Figure 6a). However, $K = 4$ (Figure 6b) resolves a fourth group encompassing the two piscivorous trout and two smaller individuals from Net 1. In contrast, no evidence of structuring was found for Lochs Affric or Beinn A’Mheadhoin (Supporting Information Figure S2a,b).

With few exceptions, the probable group ancestries of individuals in the STRUCTURE and DAPC groups (STRUCTURE ancestry model—migration priors 0.05 and 0.10) agreed with initial group assignments. For STRUCTURE groups, one Group 2 individual had a higher probability of Group 1 ancestry and one Group 1 individual was indicated to have first- or second-generation ancestry from cluster 4 (Figure 6c). For DAPC groups, one individual, identified by STRUCTURE ($K = 4$) as belonging to a fourth population encompassing the two large trout, had a higher probability of a common ancestry with those trout (Figure 6d); for both methods it showed a distinct shared ancestry.

Excluding the two ferox trout, there was a moderate (56.4%) significant ($\chi^2 = 45.10, df = 4, p < 0.0001$; Supporting Information Table S10) agreement of assignment of individuals by the two methods. DAPC places c. 50% of individuals from each of STRUCTURE groups 1 and 2 into DAPC group 3. However, the three groups defined by each method did not markedly differ in their genotype or haplotype frequencies. This was evidenced by close clustering of groups defined by the two methods (Figure 7), which have a similar level of microsatellite differentiation ($F_{ST}$—across groups:

**FIGURE 2** Neighbour joining trees of genetic differentiation showing the relatedness of net samples based on pairwise $F_{ST}$ among samples: (a) mitochondrial DNA haplotype frequencies, (b) microsatellite allele variation. Letters and numbers denote net and depth (see Table 1) and rectangle those samples from the northeast end of lake (Figure 1b); nodal values for microsatellite variation are bootstrap % values.
STRUCTURE—0.0366, DAPC—0.0377). Some differences occurred in respect of the loci underlying group differentiation by the two methods (Figure 8). However, for both methods differentiation is most marked for One103 and MHC I. In respect of mtDNA, group differentiation was greater for STRUCTURE ($\chi^2 = 41.03$, $df = 14$, $p = 0.0001$) than DAPC ($\chi^2 = 24.26$, $df = 14$, $p = 0.0167$); two DAPC defined groups not differing in haplotype frequencies (DAPC1 and DAPC3—FET $p = 0.914$).

### 3.3 Spatial distribution of groups

Distributions of groups defined by both methods are heterogeneous (DAPC: $\chi^2 = 42.4$, $df = 20$, $p < 0.0017$, STRUCTURE: $\chi^2 = 122.8$, $df = 20$, $p < 0.0001$), and associate with net depth (Supporting Information Figure S3). However, heterogeneity for the STRUCTURE groups is more marked. For both methods, Group 1 dominated in deeper benthic nets, Group 2 in pelagic and littoral nets, and Group 3 in shallow and intermediate benthic nets; the two large ferox trout occurred in different nets at different depths.

### 3.4 Effects of genomic and spatial sampling

STRUCTURE analysis of the eight microsatellites in the initial panel screened failed to resolve different genetic groups (Supporting Information Figure S2c). In contrast, the eight Beaufort Panel loci showing the highest group differentiation in the overall analysis, were sufficient alone to resolve the genetic clusters (Supporting Information Figure S2d) but structuring was more marked using all 22 loci. Results with the 22 loci for
different and more restricted subsamples, using the STRUCTURE admixture analysis, failed to detect structuring. Neither was structuring found for four stratified random subsets of 52 trout, encompassing c. 1/4 of individuals from each net (Figure 5b–e), in respect of either log \( P/K \) or \( \Delta K \). This was also the case where the analysis encompassed only nets from the shallow benthic eastern (A, B, C), mid-lake (G, H, I) or deep benthic (D, E, F) nets (Figure 5f–h). The analysis of trout from nets A, E, uP or from A, E, I, was suggestive but equivocal (Figure 5i,j).

### 3.5 | Phylogenetic divergence of populations

The three main groups, defined by the two clustering methods, formed a clade distinct from the Affric and Beinn A’ Mheadhoin populations, for both microsatellite and mitochondrial variation (Figure 9). For microsatellites, a subclade within the Laidon groups was also supported, although this differed between clustering methods. For microsatellite variation, the Affric/Beinn A’ Mheadhoin cluster was closest to STR3/DAPD3 while for mtDNA it is closest to STR2/DAPC2.

### 4 | DISCUSSION

#### 4.1 | Sympatric population structuring

Brown trout occur in lakes in a wide range of depths and habitat zones e.g. benthic, pelagic, littoral (Jonsson, 2006; Jonsson & Jonsson, 2011; Langeland, L’Abée-Lund, Jonsson, & Jonsson, 1991). However, as evidenced here and elsewhere, it cannot be assumed that a lake’s stock will constitute a single Mendelian population. To make such an assumption can lead to inaccurate assessments of local species biodiversity and inappropriate management regimes (Schindler et al., 2010).

The current study found compelling evidence of four differentially distributed genetically distinct sympatric populations in Loch Laidon. Their inferred differential spatial distribution is able to account for the significant genetic heterogeneity among nets, both for microsatellites and mtDNA variation, as well as depth clines in variant frequencies and minor genetic differences among lake sectors. The differential distributions of the three
numerically dominant populations strongly indicate they have distinct ecologies, something also suggested for the fourth population based on its encompassing individuals with morphological traits typical of the piscivorous ferox form of brown trout. Alternative explanations for the observed genetic heterogeneity are highly improbable. Genetic structuring within a panmictic population in sessile life history stages can occur if selection pressures are spatially patchy or vary clinally (Gorospe & Karl, 2015; Schmidt & Rand, 2001) or, for mobile organisms, by assortative use of habitat by genotypes (e.g. Jaenike & Holt, 1991). However, other than egg and early yolk-sac fry stages, lacustrine trout are highly mobile (e.g. Nettles, Haynes, Olson, & Winter, 1987). Furthermore, such explanations cannot easily explain the correlated depth clines at multiple independent microsatellite loci and for mitochondrial haplotypes. Additionally, as the structure relates to DNA sequence variation, it does not involve the differential expression of genes, but this may underlie phenotypic diversity among the genetic populations. (e.g. Giger et al., 2006).

The population diversity in Laidon contrasts with its absence in Lochs Affric and Beinn A’ Mheadhoin, lakes of a similar size and depth, assessed using the same methodology. In neither of these two lakes is there genetic or spatial evidence of structuring and, in both, trout display a surprisingly uniform superficial appearance. This situation accords with their trout stocks having been established by recent historical introductions as also indicated by their isolation above impassable falls. However, despite being separated by <1 km of river, trout stocks in the two lakes are genetically different and have a level of divergence ($F_{ST} = 0.0239$) similar to that among the Laidon populations.

The rarest population in Laidon encompassed the only two large ferox trout caught and was the resolved first in the DAPC analysis. Given that the two fish are not indicated to be siblings, the probability of their clustering together by chance is small ($p < 0.0001$). In contrast, in the STRUCTURE analysis, this group resolves last but encompasses a small number of further individuals. Its less marked resolution by STRUCTURE probably reflects the lower power of this
Bayesian approach to detect groups encompassing small numbers of individuals (Evanno et al., 2005; Puechmaille, 2016). Given that ferox forms have also been found elsewhere to be genetically distinct from other co-occurring trout (Duguid et al., 2006; Ferguson & Taggart, 1991), the evidence for a distinct ferox population in Loch Laidon is compelling. However, a recent paper suggests that ferox-like forms can also arise within genetic populations in some situations (Wollebaek, Heggenes, & Roed, 2018).

The four populations were resolved by both methods even with the removal of siblings from the analysis, which may or may not be problematic (Waples & Anderson, 2017); only 10 trout from eight sib groups were excluded. Their reality is also consistently across both analysis methods with similar genetic patterns associated with structuring i.e. H-W and LD departures, as well as levels of differentiation and patterns of spatial distribution among their defined populations. In contrast, only moderate agreement was observed at the individual level (c. 56%), similar to levels found using the same loci in a study of allopatric sea trout populations—46–88% (Keenan et al., 2013; Prodöhl et al., 2017). However, if population differentiation encompasses few private alleles and largely involves relatively small differences in allele frequencies, different algorithms can give differing assignments for some individuals without substantively changing the population metrics for the groups. Interestingly, individual assignments for STRUCTURE defined groups show a more marked spatial separation and better account for the clinal variation with depth than those seen for DAPC.

4.2 Importance of focused methodology to SIPD detection

The extended population structure analysis carried out using various subsets of the net samples, as expected, evidences that the use of 3D random stratified sampling will be critical in assessments of SIPD in lacustrine species of fish and other aquatic organisms. Where sympatric fish populations have been resolved, even if cryptic, they have inevitably differed to some degree in distribution, due
to different feeding ecologies e.g. trout (Ferguson, 1989), Arctic char (Walker, Greer, & Gardner, 1988), or spatial or temporal differences in spawning (e.g. Ferguson, 1989; Mäkinen et al., 2015). Thus there is a general risk in SIPD studies of biased, under or non-sampling of some populations where assessment only involves the use of known phenotypes or opportunistically collected samples (e.g. by angling, or netting or trapping a few depth zones based on general accounts of a species’ ecology). The result, a failure to detect diversity that is present, is a recognised risk addressed in species level assessments (Gotelli & Colwell, 2001; Schwartz & McKelvey, 2009; Sites & Marshall, 2003). However, to date this issue has been largely ignored in studies of lacustrine SIPD, even though it is tangentially considered in studies of the power of different data analysis methods (e.g. Evanno et al., 2005; Wang, 2017). Yet, as evidenced, this

**FIGURE 7** Neighbour joining trees for corrected pairwise $F_{ST}$ distances among defined STRUCTURE and K means discriminant analysis of principal components in defined populations (excluding ferox) based on (a) microsatellite loci and (b) mitochondrial DNA.

**FIGURE 8** Jitter plot of microsatellite $F_{ST}$ values for three main STRUCTURE and K means discriminant analysis of principal components in defined groups with loci above the overall mean locus $F_{ST}$ named.

**FIGURE 9** Neighbour joining trees based on genetic distance ($D_{ij}$) of three main populations for microsatellites (a, b) and mitochondrial DNA (c, d), including Affric and Beinn A’ Mheadhoin populations as outliers; for link of group numbers to population types see Figure 1c.
risk can be addressed using the same 3D random stratified sampling approaches already used for interspecific assessments of lacustrine fish communities (e.g. Olin et al., 2014; Sandstrom et al., 2013).

The same risk of missing or underestimating SIPD can occur from using small arbitrary sets of loci for genetic screening as also evidenced. To a degree, this can be alleviated by using more markers so as to increase the likelihood of including informative loci (Bekkevold et al., 2015) but, given the size of most genomes, arbitrary sets of loci remain to a large degree a shot in the dark. Unfortunately, informative loci for recently diverged populations may be restricted largely to a few adaptively differentiated regions (Palmé et al., 2013) with a very restricted genomic distribution (Bradbury et al., 2013; Burri et al., 2015; Feulner et al., 2015). As done here, this risk can, to some extent, be reduced by using DNA loci with a known capacity to resolve local allopatric structuring, particularly when populations are recently evolved or incompletely reproductively isolated, and levels of divergence are low.

Resolving power can also vary among analysis methods particularly when population divergence is low (Carstens, Pelletier, Reid, & Satler, 2013; Hubisz et al., 2009; Latch, Dharmarajan, Glaubitz, & Rhodes, 2006). Thus the risk of missing SIPD can be further reduced by using multiple analytical approaches when carrying out assessments. Where results agree, as in the current study, which encompassed two fundamentally different Bayesian and non-Bayesian methods, there will be greater confidence in findings.

Interestingly, in the Laidon analysis, two microsatellites in the pre-selected panel, that showed some of the highest levels of population differentiation, are physically linked to the MHC immune genes whose variation is known to often be adaptive (Hansen, Skaala, Jensen, Bekkevold, & Mensberg, 2007). This adaptation can be related to factors such as differential parasite exposure due to different diets, or different thermal conditions, the latter possibly underlying some of the observed mtDNA differentiation of populations (e.g. Consuegra, John, Verspoor, & de Leaniz, 2015). In contrast, the One103 microsatellite, which shows the highest differentiation among the Laidon populations, has no known link to a functional gene and may be driven by genetic drift. Differentiation caused by genetic drift at neutral loci may be more widespread and marked across the genome where population divergence is more ancient e.g. where sympatric populations derive from secondary contact of older allopatric lineages that predate the LGM.

4.3 | Ecological character of Laidon populations

The two large trout netted in the study assigned to a separate population, were visually typical of ferox forms (i.e. elongated jaws, big square tail and large size) and distinctive from other trout. They have previously been reported as present in Laidon (Campbell, 1979), and both expected and widely observed in medium to large Scottish lakes, displaying a wide ranging depth distribution (Hughes, Dodd, Maitland, & Adams, 2016). In contrast, the other three populations have distinctive depth distributions, which point to different ecologies—one profundal benthic (STR1/DAPC1), one limnetic (STR1/DAPC1), and one more typical littoral benthic type (STR1/DAPC1). The first of these showed the most robust body, largest eye, fewest and largest spots, and lighter colouration; the second the smallest eye, a more streamlined body and higher spot density; with the third being intermediate in these characters (Figure 1c). Interestingly, the phenotypic differences of three of the Laidon populations appear to parallel the three described trout forms in Lough Melvin, Ireland—ferox, planktivorous sonaghan and shallow benthivorous gillaroo, respectively (Cawdrey & Ferguson, 1988; Ferguson, 1989; Ferguson & Taggart, 1991). In contrast, the profundal benthic form of brown trout seen in Laidon has not previously been described. These morphological as well as dietary differences have been analysed in detail by Piggott et al. (2018).

The novel profundal benthic form found in Laidon appears to be the ecological and morphological equivalent to profundal benthic forms of Arctic char as found in adjacent Lochs Rannoch and Erich (Adams et al., 1998, 2007; Walker et al., 1988). Although unverified second-hand accounts of its presence exist (Campbell, 1979), char appear absent from Laidon. The species was not found in targeted netting of Laidon in 1974 (R. B. Greer, unpublished data) or in the current study. They have been widely recorded for most of the other large lakes in the River Tay and adjacent catchments, particularly in deep, open waters and, in the profundal zones in other char lakes, the only fish found have been char (E. Verspoor and R. B. Greer, unpublished data). Char do occur in Lough Melvin (Maitland, Winfield, McCarthy, & Igoe, 2007) but its profundal zone has not be surveyed (A. Ferguson, personal communication, 2018) and whether it has a profoundal trout form remains an open question. However, trout tend to be excluded from the pelagic and deep benthic zones by char (Jonsson & Jonsson, 2011; Klementsen et al., 2003; Langeland et al., 1991) suggesting that the evolution of a profundal trout form may not co-occur where Arctic char are present.

The detection in Laidon of four populations with distinct morphologies and ecologies makes it the highest number of sympatric morphs so far found for brown trout in a single lake and exceeds the three reported for Lough Melvin (Ferguson & Taggart, 1991). However, it is uncertain in the latter case if this encompasses all distinct forms in this lake. Unfortunately, as for most lakes so far studied for brown trout, the profundal zone and the lake overall, have not been comprehensively sampled. Four sympatric forms of Arctic char have been resolved in Lake Thingvallavatn, Iceland (Skúlason, Snorrasson, Noakes, & Ferguson, 1996) and seven have recently been reported in Lake Kronotskoe, Russia (Markevich, Esin, & Anisimova, 2018). In Scotland, a few further lacustrine studies of brown trout have found differentiation of ferox forms from other trout (Duguid et al., 2006), or involved ad hoc samples and been uninformative (e.g. Stephen & McAndrew, 1990). Thus whether the diversity seen in Loch Laidon is typical or exceptional is unclear, either in respect of Scotland or the species’ range overall. Less overt, phenotypically cryptic structuring has been widely reported including for lacustrine trout in Ireland (Anon., 2015; Crozier & Ferguson, 1986), Sweden (Allendorf et al., 1976; Andersson et al., 2017; Palmé et al., 2013) and Finland (Mäkinen et al., 2015; Swatdipong et al., 2010, 2013).
The preliminary and somewhat cursory nature of the sampling on which the current study is based means that the full extent of SIPD in Laidon may not have been resolved, particularly in respect of cryptic structuring within the identified ecological forms. Thus the analysis needs to be extended to encompass a full 3D field sampling of the lake and all its sectors as set out in the NGNSs (Appelberg, 2000). It also needs to include targeted sampling of ferox forms to increase the numbers of this less abundant form and of trout in all potential spawning streams. Such a further assessment should also be based on a larger, higher resolution suite of markers identified by undertaking a general genomic screening of population types (Moura et al., 2014), using methods such as RAD sequencing (Andrews, Good, Miller, Luikart, & Hohenlohe, 2016; Matala, Ackerman, Campbell, & Narum, 2014). This is to give a more accurate assignment of individuals so that a more robust determination of the ecological differences among populations (e.g. Benestan et al., 2015). It would also allow a more exact assessment of their historical and contemporary level of reproductive isolation.

4.4 | Evolution of Laidon trout populations

The DAPC analysis indicated that the ferox population is highly divergent from the other three population types, consistent with the population having a distinct and separate allopatric origin that predates the formation of the lake at the end of the LGM. This has been suggested for Lough Melvin where they are postulated to have colonised first (Cawdrey & Ferguson, 1988) although the sympatric Melvin gillaroo and sonaghen populations also appear to derive from different allopatric lineages. Published evidence does point to ferox trout being a distinct lineage in Britain and Ireland, and ferox in three lakes adjacent to Loch Laidon show mtDNA profiles typical of the proposed ferox lineage (McKeown, Hynes, Duguid, Ferguson, & Prodöhl, 2010). By contrast, the absence of Arctic char in Laidon, a species present in Lough Melvin, suggests the possible situation in Laidon of populations adapted to the limnetic and profundal benthic niches. However, to properly address these questions requires a more informative set of population markers that can provide a more detailed molecular genetic characterisation of the Laidon trout populations, as well as of populations elsewhere in the River Tay and adjacent catchments.

4.5 | Conservation implications

The brown trout population diversity found in Loch Laidon is unique in published accounts of SIPD, in both the number and type of populations present. However, such diversity has to varying degrees also been observed in most northern fish species and in many of the relatively few lakes so far studied (see Piggott et al., 2018). It has been reported in large (e.g. Great Bear Lake—Harris et al., 2015) and small lakes (Chavarie et al., 2017), and associated with depth, fragmentation of spawning habitat, and diversity of foraging opportunities (Bergek & Björklund, 2007; Recknagel, Hooker, Adams, & Elmer, 2017). Furthermore, it occurs in both older and recently deglaciated lakes >10,000 years old, a timeframe sufficient for interspecific diversity to evolve (e.g. Lake Victoria—Salzburger et al., 2014). Even shorter time frames are sufficient for adaptive divergence to evolve, as illustrated by studies of transplanted populations (Hendry, Wen burg, Bentzen, Volk, & Quinn, 2000; Kautt, Machado-Schiaffino, Torres-Dow dall, & Meyer, 2016; Seehausen & Wagner, 2014).

These considerations strongly point to SIPD being widespread in northern lacustrine fish species and, potentially, in other freshwater taxa. Unfortunately, such structuring is seldom specifically addressed, and generally ignored, in conservation legislation and practice such that it is implicitly assumed to be absent, even though available evidence points to it being widespread. This suggests that the disproportionate contribution of freshwater ecosystems to global diversity is likely to be even greater than indicated accounts based on current species assessments (Dudgeon et al., 2006). As such it is also likely to be of general importance to the functional integrity and productivity of many if not most northern lacustrine ecosystems (e.g. Martin, Gido, Bello, Dodds, & Veach, 2016). To take SIPD into account in general conservation policy and local conservation initiatives (Poulin & Pérez-Ponce de León, 2017), it is necessary to understand its full extent and nature in lakes, as well as its evolutionary origins and significance. This is only possible by undertaking wide ranging surveys of fish communities, in representative but randomly selected sets of lakes. However, these must encompass genetic methodologies sufficiently powerful to resolve not only phenotypically overt but also cryptic SIPD. Doing so is particularly important given that a high proportion diversity is likely to be cryptic due to the generally lower morphological divergence found among aquatic taxa (Seidel et al., 2009).

ACKNOWLEDGMENTS

We are indebted to Malcolm Pearson for permission to net Loch Laidon and Paulo Prohdöl for providing pre-publication access to information on a beta version of the Celtic Sea Trout microsatellite panel. Field work and genetic screening was funded by the Scottish Government. We are also grateful to the referees for their comments, which have led to a much improved manuscript. The authors are not aware of any conflicts of interest they have in relation to the described work.

ORCID

Eric Verspoor http://orcid.org/0000-0001-8460-4327

REFERENCES

