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Research paper

Strong neutral genetic differentiation in a host, but not in its parasite

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ABSTRACT

The genetic diversity and population structure of a parasite with a complex life cycle generally depends on the dispersal by its most motile host. Given that high gene flow is assumed to hinder local adaptation, this can impose significant constraints on a parasite's potential to adapt to local environmental conditions, intermediate host populations, and ultimately to host-parasite coevolution. Here, we aimed to examine the population genetic basis for local host-parasite interactions between the eye fluke *Diplostomum* lineage 6, a digenean trematode with a multi-host life cycle (including a snail, a fish, and a bird) and its second intermediate host, the three-spined stickleback *Gasterosteus aculeatus* L. We developed the first microsatellite primers for *D.* lineage 6 and used them together with published stickleback markers to analyse host and parasite population structures in 19 freshwater lakes, which differ in their local environmental characteristics regarding water chemistry and *Diplostomum* abundance. Our analyses suggest that one parasite population successfully infects a range of genetically differentiated stickleback populations. The lack of neutral genetic differentiation in *D.* lineage 6, which could be attributed to the motility of the parasite's definitive host as well as its life cycle characteristics, makes local host-parasite co-adaptations seem more likely on a larger geographical scale than among the lakes of our study site. Our study provides a suitable background for future studies in this system and the first microsatellite primers for a widespread fish parasite.

1. Introduction

In host-parasite interactions both parasites and hosts are expected to adapt not only to changes in their respective environments, but also to changes in each other's defence mechanisms. Since the balance between selection and gene flow is considered the strongest determinant of local adaptation (e.g. Tigano and Friesen, 2016), investigating the rate of genetic exchange among host and parasite populations can help to understand the local adaptive potential in a host-parasite system. Generally, it is assumed that high migration rates and gene flow can hinder adaptation to (temporally stable) habitats where selection by environmental factors is weak (Slatkin, 1987; Lenormand, 2002; Kawecki and Ebert, 2004). While limited gene flow reduces the introduction of maladapted alleles and thus favours local adaptation, genetic drift, which can cause the loss of potentially beneficial alleles, is expected to decrease local adaptation (Blanquart et al., 2012). Host-parasite systems add a further dimension of (reciprocal) adaptations because host populations that adapt their defence mechanisms to the parasites present in their habitat constitute an environment that changes not only in space, but also in time. In temporally variable environments, on the other hand, intermediate levels of gene flow can even maximise adaptation by contributing to genetic variation (Blanquart et al., 2013). Interestingly, a recent meta-analysis found a general trend towards stronger genetic differentiation in hosts than in parasites across a wide range of taxa (Mazé-Guilmo et al., 2016). In light of this, identifying the mechanisms which determine dispersal and genetic differentiation in parasites remains a key question in the study of host-parasite interactions.

The distribution and population structure of a parasite (here we refer to macroparasites) depends on a range of different factors. Host dispersal is commonly considered the most obvious determinant of parasite dispersal (Blouin et al., 1995). Although gene flow requires physical movement between populations and dispersal is usually expected to correlate positively with gene flow (Räsänen and Hendry, 2008; but see Edelaar and Bolnick, 2012), dispersal per se is not the only factor determining parasite genetic structure (Mazé-Guilmo et al., 2016). Host-specificity and life-history traits like the mode of reproduction, the existence of free-living stages, or life-cycle complexity also affect parasite population structures and genetic diversity (see e.g. Barrett et al., 2008; Blasco-Costa and Poulin, 2013; Mazé-Guilmo et al., 2016 for a review and meta-analyses). Since different factors (partly with opposed effects) act on different stages in the life cycle, parasites with complex (multi-host) life cycles are particularly interesting, in this regard. By providing additional dispersal opportunities (intermediate/alternate host(s), water current), life-cycle complexity, host specificity, and the presence and number of free-living stages are expected to contribute

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to weaker parasite genetic differentiation compared to each single host (Mazé-Guilmo et al., 2016). Theoretical models indicate that in parasite species with alternating sexual and asexual reproduction self-fertilisation in the sexual phase results in higher inbreeding coefficients whereas variance in reproductive success among different clones decreases inbreeding coefficients (Prugnolle et al., 2005a). In a recent meta-analysis hermaphroditic parasites were less genetically differentiated than their hosts, which was attributed to a homogenising effect of higher dispersal rates in the (mostly bird-infecting) parasites (Mazé-Guilmo et al., 2016). Quite a few theoretical and empirical studies have focussed on genetic diversity in digenean trematodes, a subclass of parasitic flatworms (Platyhelminthes), which exhibit complex life cycles and comprise many human and livestock infecting species. In general, in digenean trematodes the host with the largest geographic range, i.e. usually the definitive host, is assumed to determine dispersal and genetic structure. This has been shown e.g. in salmon and eel infecting trematodes (Criscione and Blouin, 2004; Blasco-Costa et al., 2012), *Schistosoma mansoni* (Prugnolle et al., 2005b; van den Broeck et al., 2015), *Diplostomum pseudospathaceum* (Louhi et al., 2010), and in marine trematodes (Feis et al., 2015). Further, parasites completing their entire life cycle in aquatic habitats tend show more pronounced population structuring than parasites which use birds or (terrestrial) mammals as definitive host since these facilitate dispersal across aquatic habitat boundaries (Criscione and Blouin, 2004; Blasco-Costa and Poulin, 2013; Feis et al., 2015).

Here, we investigate the population structure of the digenean trematode *Diplostomum* lineage 6. Adult *Diplostomum* sexually reproduce in the intestines of piscivorous birds either through self-fertilisation or outcrossing (facultative hermaphrodites). With the bird's faeces, their eggs are released into the water where larvae (miracidia) hatch and infect lymnaeid snails. Inside the snail host, miracidia develop to sporocysts which clonally multiply and develop further into cercariae. These leave the snail, penetrate the skin of fish within eight minutes or less (Williams, 1966) and move within hours to the lens or to the retina. Thus, the parasite is exposed to the immune system of its host only for a short period of time before it reaches the immune-privileged eye. Despite this short time frame, innate resistance of the three-spined stickleback *Gasterosteus aculeatus* L. against *D. pseudospathaceum* is based on genotype-genotype interactions and (indirectly) involves the adaptive immune system of the host (Rauch et al., 2006; Haase et al., 2014; Haase et al., 2015). Research on host-parasite interactions of *Diplostomum* mainly focuses on lens-infecting species, which form cataracts and can have severe consequences for the competitive ability, growth and mortality of their host, particularly in fish farms (Chappell et al., 1994). *Diplostomum* species infecting the non-lens region have rarely been investigated, although recent molecular studies suggest that *Diplostomum* species diversity within the non-lens region might be higher than previously thought (Locke et al., 2010b; Blasco-Costa et al., 2014; Locke et al., 2015). In the only population genetic study on a *Diplostomum* species of which we are aware, Louhi et al. (2010) analysed the population genetic structure of *D. pseudospathaceum* over a geographic range of >300 km between sampling sites and failed to detect evidence for population structure despite the presence of population genetic structuring in the snail host *Lymnaea stagnalis* (Puurtinen et al., 2004).

In this study, we aimed to compare the population genetic structure of *Diplostomum* lineage 6—an eye fluke from the non-lens region in fishes—with that of its second intermediate host, the three-spined stickleback *Gasterosteus aculeatus* L., on the Scottish island of North Uist. The three-spined stickleback has frequently colonised freshwater habitats from the sea and is known to diverge into genetically differentiated populations within relatively short periods of time (e.g. Lescak et al., 2015). Thus, we expected strong population genetic structuring in the fish host, while we expected the parasite's highly motile definitive host to impede the formation of distinct populations in *D.* lineage 6. The three-spined sticklebacks on North Uist have proven interesting models for various research questions in the recent past regarding e.g. morphology (MacColl et al., 2013; Smith et al., 2014), UV-signalling (Hiermes et al., 2015), patterns of macroparasite distribution (de Roij and MacColl, 2012; Rahn et al., 2016), and spatial differences in susceptibility to a monogenean parasite (de Roij et al., 2010). Therefore, we additionally aimed to establish a useful basis for further studies in this system.

2. Methods

2.1. Study site and sampling

North Uist (Outer Hebrides, Scotland) measures about 300 km^2 and is covered with >180 lakes (Giles, 1983). Due to the influence of shell sediment and peat, these lakes comprise habitats ranging from alkaline clear water lakes in the west to lakes with acidic tea-stained water in the central and eastern part of the island (Giles, 1983). The lakes were likely recolonised by sticklebacks from the North Atlantic (Ravinet et al., 2014) during the last deglaciation approximately 15,000 years ago (Giles, 1983; Ballantyne, 2010) and have been isolated from each other ever since. The North Uist sticklebacks are mostly annual with about 10% experiencing a second winter (Abdul Rahman & Andrew MacColl unpublished data). De Roij and MacColl (2012) and Rahn et al. (2016) have examined the distribution of stickleback macroparasites on North Uist and found substantial differences in *Diplostomum* spp. abundances among lakes, which were largely consistent over several years. As these differences could not be explained by general abiotic habitat characteristics such as geographic distance, pH or the amount of dissolved calcium, they were attributed to local host-parasite dynamics. Prevalences (% fish infected) of *Diplostomum* spp. of the non-lens region (present in all lakes sampled in this study, not identified to species level) ranged from 14 to 100% (55, 31.5, 90; median, 1st, 3rd quartiles) (Table 1; see also Rahn et al., 2016).

We caught approximately 21 (median; 20, 25 1st, 3rd quartiles) adult male and female three-spined sticklebacks per sampling location from 19 freshwater lakes and from three coastal lagoons with open access to the sea (see Fig. 1 and Table 1 for sampling locations and sample sizes). Lakes were chosen with the aim of covering a geographically large part of the island as well as a broad spectrum of sampling locations representing the habitat diversity found on North Uist with regard to *Diplostomum* spp. abundance and presumably resistance to parasites (de Roij et al., 2010; de Roij and MacColl, 2012; Rahn et al., 2016), water chemistry, and stickleback morphology. Fish were caught using minnow traps (Jenzi: green nylon mesh (3–4 mm), Gee: galvanized steel mesh, G40 M, G48 M), which were set overnight in shallow water near the shoreline in spring 2010 (April and May) and 2011 (April). This time of the year marks the beginning of the breeding season when marine sticklebacks enter the coastal bays. At the three brackish water sites resident as well as morphologically distinct (significantly larger, fully plated) anadromous sticklebacks were caught. Therefore, we will speak of a total of 25 sampling locations. We additionally collected fish from the freshwater lakes in summer 2012 (August) to obtain sufficient *Diplostomum* spp. sample sizes.

For dissection, fish were killed by decapitation followed immediately by a cut through the brain and placed under a microscope (Novex RZ-Range, 6.5–45× magnification, illuminated by a cold-light source (Schott KL 1500)). The eyes of the sticklebacks were carefully checked for metacercariae within the intact lenses as well as outside the lens. Fins and metacercariae were conserved in 98% EtOH and stored at room temperature.

2.2. Microsatellite genotyping of the sticklebacks

2.2.1. Amplification

Microsatellite analysis was based on 600 fish caught in spring 2010 and 2011 as well as 25 anadromous sticklebacks from one of the three coastal lagoons ('Aileodair') in 2007 some of which had

Table 1
Sampling locations (19 freshwater lakes, three coastal lagoons with anadromous and resident fish) with three letter codes (LocID), lake surface area in km^2 (Area), pH, prevalence of infections with *Diplostomum* outside the lens (in %, D_{prev}), and sample sizes of genotyped individuals given as N_s MS number of sticklebacks genotyped at nine microsatellite (MS) loci, N_s mt number of sticklebacks sequenced at cytochrome *b* and control region of the mitochondrial DNA, and N_D MS number of *Diplostomum* spp. genotyped at six microsatellite loci. pH and *Diplostomum* prevalence (based on an average of 20.8 ± 2.3 dissected fish (mean ± standard deviation)) were taken from Rahn et al. (2016).

Location name	Geographic coordinates	LocID	Area	pH	D_{prev}	N_s MS	N_s mt	N_D MS
Aileodair *anadromous*	57°38′7″N, 7°12′54″W	1ana	–	–	0[d]	58	7	–
Aileodair *resident*		1res	–	–	0[c]	28	5	–
Aird Heisgeir *anadromous*	57°34′48″N, 7°24′48″W	2ana	–	–	0[d]	19	6	–
Aird Heisgeir *resident*		2res	–	–	0[d]	20	5	–
nan Clachan *anadromous*	57°38′14″N, 7°24′45″W	3ana	–	–	0[d]	21	5	–
nan Clachan *resident*		3res	–	–	0[d]	19	5	–
Croghearraidh	57°36′54″N, 7°30′40″W	4GRO	0.108	7.94	14[d]	22	5	19
Eubhal	57°37′6″N, 7°29′42″W	5EUB	0.379	7.89	35[d]	20	5	15
nam Magarlan	57°36′10″N, 7°28′54″W	6MAG	0.066	7.19	100[c]	22	5	20
Hosta	57°37′40″N, 7°29′18″W	7HOS	0.247	8.34	14[d]	20	5	22
Sanndaraigh	57°35′12″N, 7°27′48″W	8SAN	0.157	8.10	51[b]	41	5	18
Olabhat	57°39′8″N, 7°26′48″W	9OLA	0.141	7.47	29[d]	20	5	6
na Gearrachun	57°38′34″N, 7°25′18″W	10GEA	0.070	6.89	100[d]	33	5	20
Mhic Gille-bhride	57°36′6″N, 7°24′36″W	11MGB	0.142	6.77	90[c]	21	5	19
a' Charra	57°35′45″N, 7°23′42″W	12ACH	0.093	6.62	95[c]	21	5	17
Mhic a' Roin	57°35′42″N, 7°25′48″W	13MOI	0.064	6.30	15[d]	20	5	6
Dubhasairidh	57°34′54″N, 7°24′12″W	14DUB	0.234	6.67	55[d]	25	5	7
Tormasad	57°33′45″N, 7°19′W	15TOR	0.213	6.87	72[c]	40	5	11
a' Bharpa	57°34′24″N, 7°17′42″W	16BHA	0.482	6.10	30[d]	20	5	5
na Moracha	57°34′30″N, 7°16′18″W	17MOR	0.367	6.53	95[d]	30	5	22
Sgadabhagh[a]	57°35′6″N, 7°14′10″W	18SCD	5.516	6.16	45[d]	20	4	9
nan Ceithir Eilean	57°34′24″N, 7°15′30″W	19EIL	0.033	7.37	90[d]	21	5	20
an Daimh	57°35′35″N, 7°12′35″W	20DAI	0.034	6.87	55[d]	20	4	6
na Maighdein	57°35′42″N, 7°12′6″W	21MAI	0.095	6.30	33[d]	24	5	6
na Buaile	57°38′48″N, 7°11′48″W	22BUA	0.020	6.29	60[c]	20	5	5

[a] Referred to as "South Sgadabhagh" by Spence et al. (2013).
[b] Average of two sampling years (2010, 2011).
[c] Sampled in 2010.
[d] Sampled in 2011.

been freshly killed, some had been conserved after they had died in captivity, some had been frozen (−20 °C), and some were stored in 70% denatured EtOH. Genomic DNA was extracted using blood and tissue kits (Macherey and Nagel, Qiagen) following the companies' protocols. DNA concentration was determined using a spectrophotometer (NanoDrop™ 1000, Peqlab) and adjusted to a concentration of 20 ng/μl. DNA samples were stored at −20 °C. Sticklebacks were genotyped at nine microsatellite loci developed at the University of Bern, Switzerland (Gac7010PBBE (Heckel et al., 2002), Gac1097PBBE, Gac1116PBBE, Gac1125PBBE, Gac3133PBBE, Gac4170PBBE, Gac4174PBBE, Gac5196PBBE, Gac7033PBBE (Largiadèr et al., 1999)). DNA was amplified using the tailed primer method (Schuelke, 2000, see Supplemetary Table S1 for detailed PCR conditions). PCR products were analysed on a CEQ™ 8800 capillary sequencer (Beckman Coulter) with GenomeLab™ GeXP (version 10.2) software. To estimate the reliability of our genotyping method, 10% of all analysed samples (62 randomly chosen fish) were genotyped again. Ambiguities were found for five individuals at one locus each, resulting in an error rate of 0.9%.

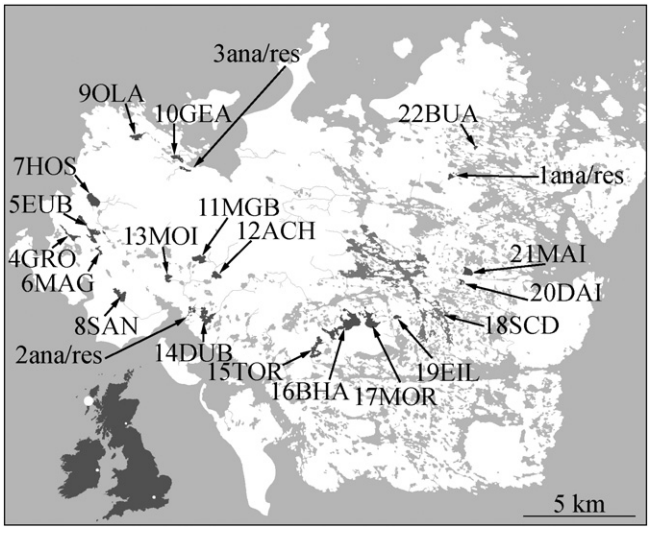

Fig. 1. Distribution of the sampling locations across North Uist. See Table 1 for full lake names.

2.2.2. Analysis

Allele frequencies were checked for possible scoring errors using the program Micro-Checker (van Oosterhout et al., 2004; 1000 randomisations, Bonferroni correction). The web-based version of Genepop (Genepop on the web 4.2, Raymond and Rousset, 1995; Rousset, 2008) was used to test for linkage disequilibrium as well as for deviation from Hardy-Weinberg equilibrium (10,000 steps dememorization, 1000 batches, 10,000 iterations) and to calculate the inbreeding coefficient F_{IS} according to Weir and Cockerham (1984). Observed and expected heterozygosity (Nei's unbiased gene diversity, Nei, 1987), and pairwise F_{ST} values as a measure for genetic differentiation between sampling locations were calculated in Arlequin 3.5.1.3 (Excoffier and Lischer, 2010; 1000 permutations). Expected heterozygosities of the freshwater populations were regressed against lake surface areas (determined from a 1:25,000 Ordnance Survey map using ImageJ 1.45s; Rasband, 1997-2009) in *R*3.0.1 (R-Core-Team, 2013). Spearman rank correlations were used as surface area data significantly deviated from normal distribution ($P < 0.05$, Shapiro-Wilk test).

Due to the colonisation history of the island, we followed a Bayesian cluster assignment approach to infer population structure using the programs STRUCTURE 2.3.3 (Pritchard et al., 2000; Falush et al., 2003) and BAPS (Corander and Marttinen, 2006; Corander et al., 2008). Cluster analyses were based solely on allele frequencies. Spatial information was not considered. STRUCTURE analysis was run using an admixture model with correlated allele frequencies with 10^6 Markov Chain

Monte Carlo (MCMC) repetitions preceded by a burn-in of 100,000 repetitions. One to 20 clusters were assumed and each number of clusters (K) was tested five times. The most likely K was estimated using the Delta K method (Evanno et al., 2005) as implemented in STRUCTURE Harvester (Earl and von Holdt, 2012). As Delta K indicated a first level of population structure for K = 4 clusters, we additionally performed a hierarchical structure analysis following Coulon et al. (2008, see Supplementary Fig. S1). For finding mean cluster membership coefficients of the five runs for each individual, we used the LargeKGreedy method in CLUMPP (Jakobsson and Rosenberg, 2007; random input order, 1000 repeats). Admixture analysis in BAPS was based on 100,000 simulations. The number of reference individuals per cluster was set to ten. Maximum numbers of clusters from one to 20 were tested ten times.

The microsatellite primers used in this study have proven informative in several other studies, but according to Colosimo et al. (2004) and DeFaveri et al. (2011) loci 4174 and 1125 may be linked to variation in number and pattern of lateral plates (but also see Mäkinen et al., 2008). As North Uist fish differ strongly in these traits (Giles, 1983; Campbell, 1985; Spence et al., 2013; MacColl and Aucott, 2014; Smith et al., 2014), using these loci might have biased our analysis and potentially resulted in overestimating population structure. We therefore additionally ran our STRUCTURE analysis without these loci.

To visualise genetic relationships among fish from the different lakes, a Neighbor-Joining tree was constructed using the software package PHYLIP and the programs therein (Felsenstein, 2013). First, allele frequencies were boostrapped 1000 times using SEQBOOT. The newly generated data sets were then used to calculate pairwise genetic distances (Cavalli-Sforza's and Edwards' chord distance D_C, Cavalli-Sforza and Edwards, 1967) in GENDIST. NEIGHBOR and CONSENSE (all PHYLIP) were used to assemble a consensus tree based on majority criteria. The final tree was visualised in FigTree 1.3.1 (Rambaut, 2006).

2.3. Mitochondrial DNA sequencing of the sticklebacks

2.3.1. Amplification

Mitochondrial DNA analysis was based on five randomly chosen individuals per sampling location and three morphologically deviating fish found in two of the coastal areas (one partially plated, 'Aird Heisgeir', two of intermediate body size compared to anadromous fish and residents, 'Aileodair', 128 fish in total, Table 1). We considered these sample sizes sufficient as theory suggests that even small samples can describe distribution patterns of allele frequencies and limit standard deviations of haplotype and nucleotide diversity (Tajima, 1983).

Partial sequences of the cytochrome *b* and control region of the mitochondrial DNA were amplified using the primers published in Mäkinen and Merilä (2008). We did not make use of the nested primer method suggested by the authors. Separate PCRs were carried out for cytochrome *b* and control region sequences respectively. PCR conditions can be found in Table S1 of the supporting information. Amplification success was confirmed on 1.5% agarose gel before purified (MN NucleoSpin® PCR clean-up kit) PCR products were sent to a commercial sequencing service (LGC Genomics GmbH, Berlin).

2.3.2. Analysis

Electropherograms of the raw sequences were visually checked for ambiguities and manually edited and aligned in BioEdit 7.2.5 (Hall, 1999). Final cytochrome *b* sequences (1014 bp) and sequences of the control region (453 bp) were concatenated to a sequence with a total length of 1467 bp. Diversity indices (haplotype diversity (Hd), nucleotide diversity (π) (Nei, 1987) and average number of nucleotide differences (k) (Tajima, 1983)) were calculated in DnaSP 5.10.01 (Librado and Rozas, 2009). Arlequin was used to calculate average pairwise nucleotide differences between sampling locations and to compare these with pairwise F_{ST}-values calculated from microsatellite data using a Mantel test with 1000 permutations. A median-joining network of all haplotypes that occurred at least twice in the data set was constructed using the program Network 4.6.1.3 (http://www.fluxus-engineering.com; Bandelt et al., 1999; Polzin and Vahdati Daneshmand, 2003). Epsilon was set to 10 as suggested by the program's manual (page 17) and all variable sites were weighted equally.

2.4. Establishing microsatellite primers for Diplostomum spp.

To our knowledge, no microsatellite primers have so far been published for any *Diplostomum* species from the non-lens region of the eyes of freshwater fish. The only available primers for *Diplostomum* spp. are those Reusch et al. (2004) published for the lens infecting *D. pseudospathaceum*. We therefore tested their applicability for our *Diplostomum* species and additionally developed own primers. For this, a pooled DNA sample of metacercariae from stickleback eyes was enriched for simple sequence repeats and sequenced. Sequences suitable for primer design were checked against published fish sequences and tested for amplification on stickleback DNA. Please refer to the supplementary material for a more detailed description of the procedure. Five markers proved to be *Diplostomum* spp. specific, i.e. they yielded a product within the size range expected from sequencing for *Diplostomum* spp., while not amplifying stickleback DNA. Final PCR conditions can be found in Table S2 of the supplementary material.

For primer tests and subsequent genotyping, DNA was extracted by incubating individual metacercariae for two hours at 56 °C in a lysis solution consisting of 0.25 μl 1 M Tris (pH 8), 0.05 μl 0.5 M EDTA, 0.625 μl 20% SDS, 24.075 μl H_2O (LiChrosolv®, Merck), and 2.27 μl Proteinase K (20 mg/ml). After incubation samples were vortexed for 20 s, incubated for 15 min at 100 °C, vortexed for 20 s, and shortly centrifuged before 25 μl of 20% Tween 20 were added. Samples were stored at −20 °C.

2.5. Microsatellite genotyping of Diplostomum spp.

We analysed only one metacercaria per infected stickleback to keep the *Diplostomum* spp. individuals analysed in this study as genetically independent as possible. As *Diplostomum* spp. reproduces clonally inside its snail host and snails are able to release hundreds of cercariae at a time—*Lymnaea stagnalis*, for example, has been shown to shed several thousand *D. spathaceum* cercariae per day (Karvonen et al., 2004)—it is theoretically possible that one individual stickleback contracts several genetically identical parasites. We tested metacercariae from all infected fish caught for this study until either a target sample size of 20 worms per lake had been successfully genotyped at at least five of the six loci or until all available worms had been tested. In total 253 metacercariae from North Uist were successfully genotyped. In addition, to examine geographically extended population structure, we genotyped 26 metacercariae from 26 sticklebacks caught on Iceland (65°37′42″N, 16°55′17″W), which were kindly provided by Frederik Franke.

2.5.1. Analysis

Considering all 253 metacercariae as belonging to one population, we estimated expected and observed heterozygosity, linkage disequilibrium and indications of possible scoring errors for each locus using the same programs and settings as for the stickleback analysis. As this study is the first application of the new markers, we also calculated PIC values (polymorphism information content, Botstein et al., 1980) using the Microsatellite Toolkit (Park, 2001) for Microsoft® Excel. Genetic diversity at the different sampling locations as well as the degree of population genetic structuring was estimated as described for the sticklebacks.

2.6. Molecular Diplostomum species identification and marker specificity

Morphological *Diplostomum* species identification based on metacercariae is nearly impossible. We therefore confirmed species

identity of our samples and three additional metacercariae from the non-lens region of three nine-spined sticklebacks, *Pungitius pungitius*, from lake 8SAN by sequencing the barcode region of the cytochrome *c* oxidase subunit 1 (*cox*1) of the mitochondrial DNA using the PlatdiploCOX1 primers published by Moszczynska et al. (2009, see supplementary material for details).

3. Results

3.1. Population structure of the sticklebacks

3.1.1. Microsatellite analysis

Genotyping success was 99.4% (4 of the 625 fish could not be genotyped at one locus each). For one locus (Gac7010PBBE) scoring errors due to stuttering were suspected. Furthermore, for all loci the presence of null alleles was suspected, due to a general excess of homozygotes. These results did not occur (except for the null alleles at locus Gac1097PBBE) when only anadromous fish were considered in the analysis. No significant evidence for large allele dropout or linkage disequilibrium between the loci was found. Significant deviations from Hardy-Weinberg equilibrium were found at four sampling locations (13MOI, 17MOR, 18SCD, 21MAI; Table 2). Observed heterozygosity was significantly lower than expected heterozygosity at these locations and inbreeding coefficients were positive but small, ranging from 0.059 to 0.175 (Table 2). Expected heterozygosity was significantly positively correlated with lake surface area (Spearman rank correlation: $r_S = 0.84$, $N = 19$, $P < 0.0001$, Fig. 2) indicating limited genetic diversity in small lakes. This correlation stayed significant if 18SCD was excluded (Spearman rank correlation: $r_S = 0.81$, $N = 18$, $P < 0.0001$) and also if the regression was based on the 15 freshwater population clusters suggested by the Bayesian analyses (see below, Spearman rank correlation: $r_S = 0.82$, $N = 15$, $P < 0.001$). In this case, mean expected heterozygosities were regressed against the sum of the surface areas of the contributing lakes.

In general, pairwise F_{ST}-values (Supplementary Table S4) as well as Bayesian cluster analyses (Fig. 3) clearly show the presence of structuring into distinct freshwater populations. No significant genetic differentiation was found between western lakes 4GRO, 5EUB and 6MAG (same cluster, all $F_{ST} < 0.01$), and between 11MGB and 12ACH (same cluster, after Bonferroni correction, $F_{ST} = 0.017$). Between 17MOR and 18SCD there was only little ($F_{ST} = 0.022$) but significant genetic differentiation. Fish in 18SCD showed signs of admixture as only eleven of the 20 genotyped individuals could be assigned to a certain cluster (proportion >0.5, STRUCTURE). Of these, seven were assigned to the same cluster as 17MOR fish. Pairwise F_{ST} values and Bayesian clustering analysis did not suggest population structuring among the anadromous fish, but significant reproductive isolation from resident fish caught at the same sampling locations was found with the highest value ($F_{ST} = 0.051$) found between anadromous and resident sticklebacks at the north-western site (3ana/res in Fig. 1).

Population assignments by BAPS (16 clusters) and STRUCTURE (17 clusters) generally showed similar patterns. However, BAPS assigned fish from 13MOI, 19EIL and 20DAI to distinct clusters, while STRUCTURE assigned 19EIL and 20DAI fish to the same cluster, although genetic differentiation between fish of these lakes was high ($F_{ST} = 0.328$). Also, 17 of the 20 13MOI fish were assigned to the same cluster as 11MGB and 12ACH (two lakes in the same catchment as 13MOI; F_{ST} 13MOI-12ACH = 0.236, F_{ST} 13MOI-11MGB = 0.207) with an average proportion of 0.6. Fish from 16BHA formed their own cluster in STRUCTURE, but not in BAPS. Both programs clearly separated resident fish caught at the north-western site (3ana/res) from all other fish, but resident fish from the southwest (2ana/res) were only assigned to their own cluster by STRUCTURE. Resident fish from the north-eastern site showed high degrees of admixture as 14 (BAPS) and 19 (STRUCTURE)

Table 2
Summary of basic diversity indices calculated from microsatellite data and mtDNA sequences given as N_s MS (number of sticklebacks genotyped at nine microsatellite (MS) loci), A (average number of alleles per locus rounded to the nearest integer), H_e (expected heterozygosity), H_o (observed heterozygosity), deviation from HWE (Hardy-Weinberg equilibrium (χ^2, df degrees of freedom, *P*, *P* values significant after Bonferroni correction printed in bold)), mean F_{IS} (inbreeding coefficient), N_s mt (number of fish for which composite mtDNA sequences were obtained, see text for details), h (number of mtDNA haplotypes), Hd (Haplotype diversity), SD (standard deviation), π (nucleotide diversity), k (average number of nucleotide differences). Statistics are given for all sample origins separately as well as for all anadromous, resident, and freshwater fish treated as one population, respectively.

					HWE								
LocID	N_s MS	A	H_e	H_o	χ^2	df	*P*	F_{IS}	N_s mt	h	Hd ± SD	π ± SD	k
1ana	58	18	0.86	0.84	16.17	18	0.581	0.027	7	6	0.95 ± 0.10	0.0049 ± 0.0007	7.2
1res	28	14	0.88	0.85	29.53	18	0.042	0.026	5	5	1.00 ± 0.13	0.0022 ± 0.0004	3.2
2ana	19	14	0.90	0.86	28.47	18	0.055	0.041	6	4	0.80 ± 0.17	0.0031 ± 0.0008	4.5
2res	20	13	0.89	0.87	13.87	18	0.737	0.021	5	2	0.40 ± 0.24	0.0014 ± 0.0008	2.0
3ana	21	14	0.88	0.86	21.19	18	0.270	0.024	5	5	1.00 ± 0.13	0.0060 ± 0.0011	8.8
3res	19	10	0.84	0.82	19.16	18	0.382	0.024	5	2	0.40 ± 0.24	0.0011 ± 0.0007	1.6
4GRO	22	11	0.80	0.75	28.95	18	0.049	0.065	5	4	0.90 ± 0.16	0.0027 ± 0.0013	4.0
5EUB	20	11	0.81	0.84	12.69	18	0.810	−0.039	5	3	0.80 ± 0.16	0.0015 ± 0.0006	2.2
6MAG	22	10	0.80	0.80	17.94	18	0.460	−0.007	5	4	0.90 ± 0.16	0.0008 ± 0.0002	1.2
7HOS	20	10	0.83	0.79	16.18	18	0.580	0.038	5	4	0.90 ± 0.16	0.0012 ± 0.0003	1.8
8SAN	41	13	0.82	0.82	15.46	18	0.630	0.006	5	3	0.70 ± 0.22	0.0008 ± 0.0003	1.2
9OLA	20	7	0.63	0.57	24.00	18	0.155	0.086	5	2	0.60 ± 0.18	0.0004 ± 0.0001	0.6
10GEA	33	11	0.74	0.73	18.04	18	0.453	0.024	5	4	0.90 ± 0.16	0.0010 ± 0.0002	1.4
11MGB	21	8	0.70	0.70	9.33	18	0.952	−0.004	5	2	0.40 ± 0.24	0.0014 ± 0.0008	2.0
12ACH	21	7	0.64	0.61	21.07	18	0.276	0.062	5	3	0.80 ± 0.16	0.0008 ± 0.0002	1.2
13MOI	20	8	0.69	0.59	70.83	18	<**0.0001**	0.175	5	2	0.40 ± 0.24	0.0014 ± 0.0008	2.0
14DUB	25	10	0.79	0.79	16.84	18	0.534	0.004	5	4	0.90 ± 0.16	0.0019 ± 0.0008	2.8
15TOR	40	11	0.80	0.81	17.86	18	0.465	−0.019	5	4	0.90 ± 0.16	0.0008 ± 0.0002	1.2
16BHA	20	9	0.81	0.76	22.82	18	0.198	0.064	5	2	0.40 ± 0.24	0.0003 ± 0.0002	0.4
17MOR	30	12	0.82	0.75	∞	18	<**0.0001**	0.096	5	2	0.40 ± 0.24	0.0003 ± 0.0002	0.4
18SCD	20	13	0.87	0.78	∞	18	<**0.0001**	0.101	4	3	0.83 ± 0.22	0.0017 ± 0.0007	2.5
19EIL	21	5	0.56	0.57	21.37	18	0.261	−0.036	5	2	0.60 ± 0.18	0.0012 ± 0.0004	1.8
20DAI	20	4	0.56	0.54	17.41	16	0.359	0.020	4	2	0.50 ± 0.27	0.0007 ± 0.0004	1.0
21MAI	24	9	0.77	0.72	48.57	18	**0.0001**	0.059	5	1	0 ± 0	0 ± 0	0
22BUA	20	3	0.45	0.44	13.47	14	0.490	0.045	5	1	0 ± 0	0 ± 0	0
anadromous	98	22	0.88	0.85	24.17	18	0.150	0.032	18	12	0.92 ± 0.05	0.0046 ± 0.0005	7
resident	67	19	0.90	0.85	44.70	18	**0.001**	0.056	15	8	0.73 ± 0.12	0.0026 ± 0.0004	4
freshwater	460	28	0.89	0.71	∞	18	<**0.0001**	0.204	93	38	0.96 ± 0.01	0.0037 ± 0.0002	5
all	625	32	0.90	0.75	∞	18	<**0.0001**	0.169	126	53	0.97 ± 0.01	0.0039 ± 0.0002	5.7

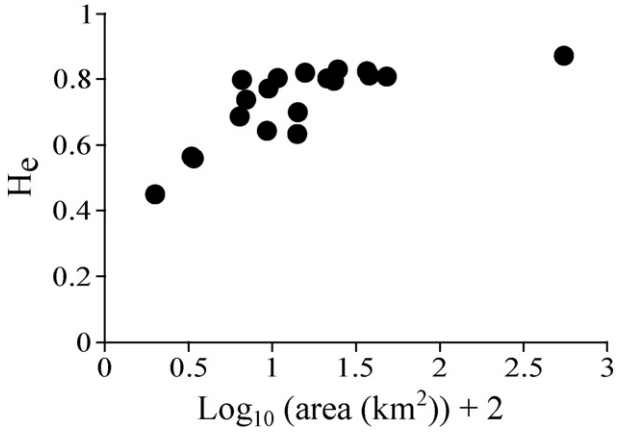

Fig. 2. Relationship between lake surface area in km^2, given as log(area) + 2, and expected heterozygosity calculated from stickleback microsatellite data.

of the 28 analysed fish could not be assigned to a cluster at all (proportions <0.5).

Excluding the two loci that might be linked to plate morphology resulted in an estimated number of two clusters according to Delta K (Supplementary Fig. S2), assigning fish of the freshwater lakes 4GRO, 5EUB, 6MAG, 10GEA, 11MGB, 12ACH, 13MOI, 15TOR, and 22BUA to one cluster and all brackish water fish together with fish from the remaining freshwater lakes to another cluster. For K = 17, STRUCTURE results showed a similar pattern to that based on all nine loci (Supplementary Fig. S2) with the exception that the 17 13MOI fish mentioned earlier were now assigned to their own cluster with an average proportion of 0.5.

In over 90% of all generated Neighbor-Joining trees anadromous fish as well as resident fish from the coastal lagoon in the Northeast of the island were assigned to the same branch. Also, fish from lakes 4GEO, 5EUB and 6MAG, and fish from 11MGB and 12 ACH originated from a common branch (Fig. 4). Bootstrap support for close relatedness of fish from lakes 13MOI and 14DUB, and from lakes 17MOR, 18SCD and 21MAI was 74% and 75%, respectively (Fig. 4).

3.1.2. Mitochondrial DNA analysis

Analysis of mitochondrial DNA was based on 126 individuals, because cytochrome *b* sequences were incomplete for two fish (one 18SCD, one 20DAI). Overall, 53 different haplotypes with 54 polymorphic sites were found, resulting in a sequence divergence of only 0.39%. Comparison with composite haplotypes previously published by Mäkinen and Merilä (2008) and Ravinet et al. (2014) revealed that ten haplotypes of the North Uist fish correspond to sequences from the European, Irish and Trans-Atlantic lineage (see Supplementary Table S5 for all haplotypes from this study and their GenBank accession numbers). Although mean haplotype diversity was relatively high

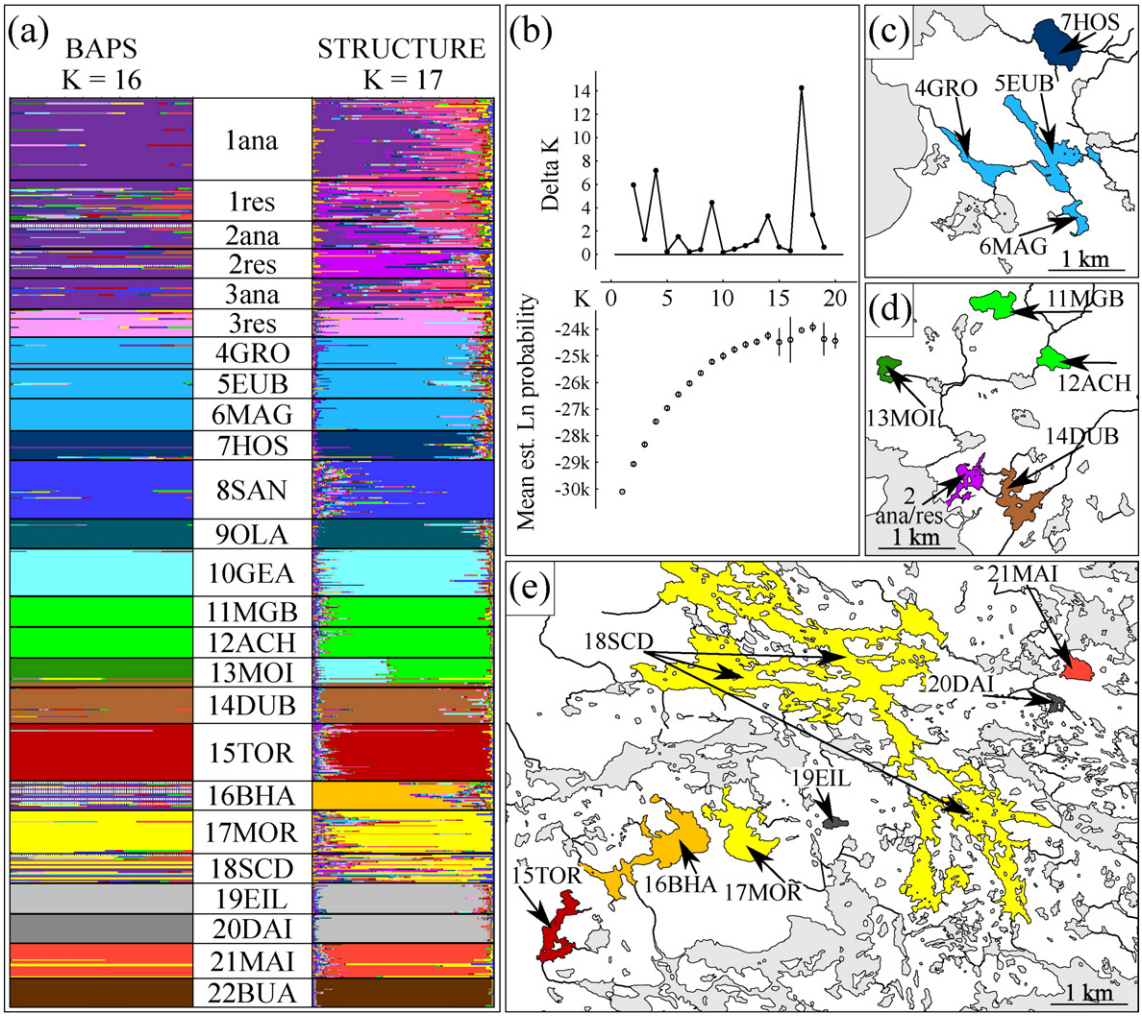

Fig. 3. Results of the Bayesian cluster analysis based on nine microsatellite loci. (a) Cluster membership proportions of the sticklebacks according to BAPS and STRUCTURE, (b) Delta K values and Ln probabilities (mean of five runs with standard deviation), (c)–(e) regional maps depicting sampling locations contributing to population clusters and connecting streams; sampled lakes have been coloured for better visibility.

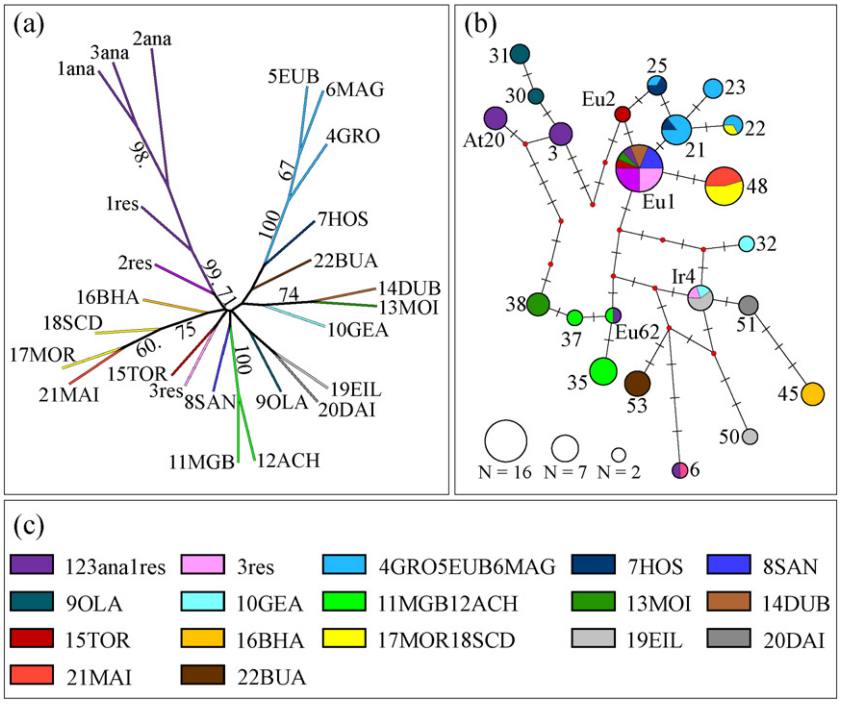

Fig. 4. Visualisation of the relationships among sticklebacks of 25 sampling locations on North Uist. (a) Neighbor-Joining (NJ) tree based on Cavalli-Sforza's and Edwards' chord distance calculated from microsatellite data. Bootstrap (1000×) values ≥50% are given next to branching points. (b) Median-Joining (MJ) network based on composite mitochondrial (cytochrome *b* and control region) haplotypes. Red dots depict median vectors, dashes depict mutation steps. Numbers correspond to haplotype numbers in Table S5, i.e. 3 = NU3 etc. Haplotypes identical to published sequences retained their original names (See text for details.). Circle widths relate to haplotype frequency (three examples are shown). Note that only haplotypes occurring at least twice in the data set were considered. (c) Colour codes used for NJ tree and MJ network. Coding is based on Bayesian clustering results and was applied to all fish caught at the respective sampling sites, regardless of an individual's cluster membership.

(0.7 ± 0.3, mean ± standard deviation over all samples), this was mostly due to differences in only a few nucleotides (0–9, average diversity per sampling location) resulting in a very low mean nucleotide diversity (π) of 0.0015 ± 0.0014 (Table 2). The correlation between pairwise F_{ST}-values calculated from microsatellite data and average differences in mitochondrial DNA nucleotide diversity was positive, but failed to reach statistical significance ($r = 0.24$, $P = 0.081$). Although genetic differentiation was not very pronounced at the mitochondrial DNA level, the median-joining network shown in Fig. 4 generally supported the population clusters of the microsatellite analysis.

3.2. Population structure of Diplostomum spp.

3.2.1. Polymorphism of the new microsatellite loci

All six markers were polymorphic with five to fifteen alleles per locus (see Table S3 of the supporting information for general marker characteristics). Diga4 was difficult to interpret due to heavy stuttering. To avoid overestimating polymorphism, we reduced its genotype profile to six different patterns thereby artificially increasing homozygosity at this locus. There was no significant indication of large allele dropout or linkage disequilibrium between the six loci. Generally, fewer heterozygotes were detected than would have been expected by chance. Therefore, scoring errors due to stuttering or the presence of null alleles were suspected at all loci (stuttering: all markers except for Diga3).

3.2.2. Molecular Diplostomum species identification and marker specificity

*Cox*1 sequences could be obtained for 260 of the 279 individuals that were included in the analyses. All worms, including the three worms from nine-spined sticklebacks, were identified as *Diplostomum* lineage 6 (following naming from Blasco-Costa et al. (2014). This name is most likely a synonym for *D. gasterostei* (Williams, 1966)), which was first described in three-spined sticklebacks from Scotland. Closest similarity was found to samples from Norway collected by Kuhn et al. (2015). Five of the metacercariae that could not be genotyped at any of the six loci were also sequenced at the barcode region and were identified as *D. baeri* 2 sensu Georgieva et al. (2013).

3.2.3. Population structure Diplostomum spp.

Observed heterozygosity was significantly lower than expected at nearly all sampling locations (see Table 3) resulting in relatively high inbreeding coefficients. The only pairwise coefficients of genetic differentiation (F_{ST}) that remained significant after Bonferroni correction were found between individuals from Iceland and lakes 8SAN, 15TOR, and 19EIL, and indicated moderate genetic differentiation (F_{ST} (ICE–8SAN) = 0.062, F_{ST} (ICE–15TOR) = 0.078, and F_{ST} (ICE–19EIL) = 0.073, respectively; Supplementary Table S4). *Diplostomum* spp. samples were best clustered into four groups according to the Evanno method (note that K(optimal) = 1 is not possible with this method). Generally, the results of the Bayesian cluster analysis did not indicate structuring into distinct populations and differentiation between worms from Iceland and from North Uist was only marginal (Fig. 5).

4. Discussion

4.1. Population structure of the sticklebacks

As expected, our results show strong neutral genetic differentiation in the North Uist sticklebacks. Cluster analyses suggest the presence of different levels of population structure: some lakes seem to occasionally receive gene flow from the sea, while others are completely isolated. This was indicated by the cluster membership proportions for K = 4 clusters (Fig. S1), but also by the high degree of admixture in lake 18SCD, which is indirectly connected to the sea through streams and neighbouring lakes. Small streams connecting 18SCD and 17MOR seem to facilitate genetic exchange between the fish in these lakes, which are genetically isolated from fish in other freshwater lakes. The same applies for lakes 11MGB and 12ACH, and for lakes 4GRO, 5EUB, and 6MAG (Fig. 1). Although spatial information was not considered

Table 3
Summary of basic diversity indices calculated from microsatellite data, N_D MS number of *Diplostomum* spp. genotyped, A average number of alleles per locus rounded to the nearest integer, H_e expected heterozygosity, H_o observed heterozygosity, deviation from HWE (Hardy-Weinberg equilibrium (χ^2, df degrees of freedom, *P*, *P* values significant after Bonferroni correction printed in bold)), mean F_{IS} (inbreeding coefficient).

					HWE			
LocID	N_D MS	A	H_e	H_o	χ^2	df	*P*	F_{IS}
4GRO	19	6	0.64	0.49	36.43	12	<**0.001**	0.219
5EUB	15	5	0.68	0.49	30.16	12	0.003	0.233
6MAG	20	5	0.59	0.38	61.04	12	<**0.001**	0.296
7HOS	22	6	0.66	0.40	∞	12	<**0.001**	0.367
8SAN	18	6	0.65	0.49	34.55	12	<**0.001**	0.223
9OLA	6	5	0.67	0.56	10.54	10	0.394	0.174
10GEA	20	6	0.67	0.44	∞	12	<**0.001**	0.251
11MGB	19	5	0.65	0.43	47.75	12	<**0.001**	0.323
12ACH	17	6	0.70	0.57	41.42	12	<**0.001**	0.152
13MOI	6	4	0.71	0.38	26.20	12	0.010	0.481
14DUB	7	4	0.63	0.33	40.48	12	<**0.001**	0.506
15TOR	11	4	0.59	0.38	35.64	12	<**0.001**	0.259
16BHA	5	4	0.57	0.43	12.90	10	0.229	0.262
17MOR	22	6	0.66	0.46	52.89	12	<**0.001**	0.314
18SCD	9	5	0.72	0.55	22.56	12	**0.032**	0.254
19EIL	20	6	0.63	0.40	61.68	12	<**0.001**	0.332
20DAI	6	3	0.60	0.64	15.10	10	0.129	−0.145
21MAI	6	5	0.72	0.47	25.81	12	0.011	0.359
22BUA	5	3	0.74	0.56	19.83	10	0.031	0.271
ICE	26	5	0.61	0.48	45.27	12	<**0.001**	0.178
all	279	10	0.67	0.46	∞	12	<**0.001**	0.285

in the analyses, lakes 7HOS, 8SAN, 9OLA, 10GEA, 14DUB, 15TOR, 21MAI, and 22BUA clearly form distinct populations. If strong population structures are present in a data set, this can affect the clustering algorithms in a way that subtle population structures might not be detected. This seems to be the reason why STRUCTURE assigned fish from lakes 19EIL and 20DAI to the same cluster despite significant evidence for differentiation between the two lakes provided by pairwise F_{ST} values and the BAPS analysis. That fish of 19EIL and 20DAI belong to separate populations is also supported by mitochondrial data (Fig. 4) as well as by differences in morphology (19EIL: ventral spines not present, 20DAI: ventral spines present; Giles, 1983; Spence et al., 2013). The positive correlation between lake surface area and expected heterozygosity, which mainly seemed to be driven by lakes 22BUA, 19EIL, and 20DAI (Fig. 2), point to an influence of genetic drift, brought about by small population sizes, on genetic differentiation. The present results suggest that the anadromous sticklebacks around North Uist belong to a single population. Differentiation from resident sticklebacks was significant but relatively low (highest $F_{ST} = 0.051$), which is comparable to a study on Irish anadromous and resident sticklebacks ($F_{ST} = 0.07$; Ravinet et al., 2015). That BAPS and STRUCTURE detected substantial proportions of admixture among the saltwater fish and (at least BAPS) did not assign resident fish to separate clusters as clearly as freshwater fish, might indicate occasional gene flow. The network analyses revealed striking similarity of the relationships between mitochondrial composite haplotypes and population clusters derived from microsatellite genotypes. Given the lower mutation rates of mitochondrial DNA compared to nuclear loci, this underlines the results of the microsatellite analysis and confirms the presence of strong population genetic structuring.

4.2. Population structure of Diplostomum and conditions for local host-parasite (co-)-adaptations

Bayesian cluster analysis as well as small (mean $F_{ST} = 0.04$) and mostly not significant pairwise F_{ST} values indicated the absence of population genetic structuring of *D.* lineage 6 on the island of North Uist despite evidence for strong neutral genetic differentiation in its fish host in the same area. Significant F_{ST} values between Iceland and 8SAN, 15TOR, and 19EIL indicate that the newly established markers were able to detect (weak) genetic differentiation between Iceland and North Uist. Our observation is congruent with the study by Louhi et al. (2010) on the lens-infecting *D. pseudospathaceum*. Despite a geographic range of 300 km, the authors did not find evidence for population genetic structuring. The lack of structuring into distinct populations on a relatively small island is not surprising for a bird-infecting parasite (Blasco-Costa and Poulin, 2013)—especially, since some of the fish-eating birds on North Uist (e.g. gulls, terns, divers; Giles, 1981) are migratory and presumably disperse the parasite over large geographic areas. Also, this result supports theoretical predictions that parasites with complex life cycles are generally less structured than their (intermediate) hosts (Mazé-Guilmo et al., 2016). Further, our results would be in line with the hypothesis that less host-specific parasites show weaker genetic differentiation than their single hosts. Although recent surveys have suggested a narrow fish host range of *D.* lineage 6 (Locke et al., 2010a; Blasco-Costa et al., 2014)—to this date, it has only been found in *G. aculeatus*—we can confirm that this *Diplostomum* species infects at least two different stickleback species.

The lack of population genetic structuring in *D.* lineage 6 does not completely rule out parasite local adaptation. An increasing number of

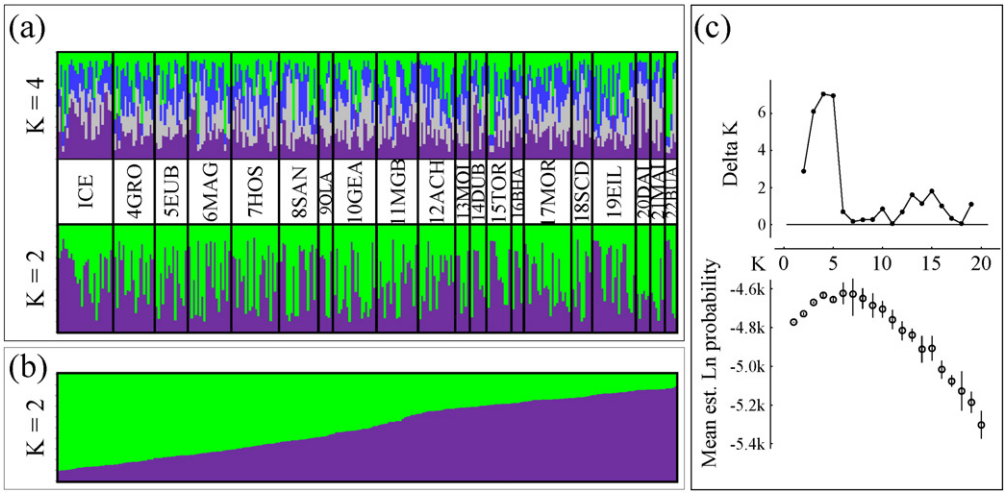

Fig. 5. Results of the Bayesian cluster analysis based on six *Diplostomum* spp. microsatellite loci. (a) Cluster membership proportions for K = 4 clusters as suggested by Delta K values and for K = 2 clusters sorted by sampling location, (b) results for K = 2 sorted after cluster membership proportion, (c) Delta K values and Ln probabilities (mean of five runs with standard deviation).

studies have shown that gene flow does not necessarily disrupt local adaptation and that it can even promote adaptation (see e.g. Tigano and Friesen, 2016 and citations therein). But in that case, natural selection favouring local genotypes must have been strong as gene flow is generally assumed to hinder local adaptation (Lenormand, 2002; Kawecki and Ebert, 2004; Räsänen and Hendry, 2008). It appears more likely that gene flow across (freshwater) habitat boundaries provides the parasite with the genetic diversity necessary to successfully infect a range of genetically differentiated host populations.

The absence of population genetic structuring does not suggest local adaptation of the parasite to local fish populations as a cause of the different *Diplostomum* spp. abundances found in de Roij and MacColl (2012) and Rahn et al. (2016). Instead, it is possible that the stickleback populations differ in their *Diplostomum* susceptibility. However, our results indicate that such differences in susceptibility, should they exist, would be the result of adaptation to a diversity of *D.* lineage 6 genotypes rather than to specific genotypes. Spatial heterogeneity in host resistance to a certain parasite genotype would have led to a non-random distribution of parasite genotypes and therefore parasite genetic differentiation within the fish host despite continuous mixing in the bird host (Edelaar and Bolnick, 2012). Additional analyses of genotypes of immune relevant genes, e.g. those of the major histocompatibility complex (MHC; but see Scharsack and Kalbe, 2014), in relation to parasite abundances could shed light on the mechanisms responsible for *Diplostomum* spp. distribution patterns. Alternative explanations include the distribution of the snail host, site preferences of the fish-eating birds (e.g. gulls and terns; Giles, 1981), which serve as definitive host, and/or the direct or indirect influence of abiotic conditions (de Roij and MacColl, 2012; Rahn et al., 2016).

Louhi et al. (2010) found inbreeding coefficients to be low in *D. pseudospathaceum* (between −0,029 and 0,050). This was attributed to high numbers of parasites and high genetic diversity among parasites inside the intestines of the definitive hosts, *Larus argentatus* and *L. canus* (common gull and herring gull, respectively; Karvonen et al., 2006; Louhi et al., 2010). Given the high dispersal rates and frequent encounters of worms from distant lakes owing to the mobility of the definitive host, the significant and positive inbreeding coefficients found in this study (0.289 across all samples) appear counterintuitive. Self-fertilisation within the bird host, probably due to low prevalence and/or diversity in the definitive host, which again might partially be due to clonal reproduction in the snail host, seems the most likely reason (Prugnolle et al., 2005a). Such an influence of prevalence on parasite mating patterns and, as a consequence, parasite genetic differentiation (Barrett et al., 2008) has been found e.g. in the malaria parasite *Plasmodium falciparum* (Anderson et al., 2000). All but one (Diga4) of the newly developed markers were polymorphic and fairly good to analyse. Still, our approach does not allow to decide whether homozygosity was high because of the presence of null alleles (David et al., 2007) or whether the presence of null alleles was suspected because of the high number of homozygotes. The fact that five of the metacercariae which had not yielded a product with any of the markers were identified as *D. baeri* 2 suggests that the markers could be used as a tool for discriminating *D.* lineage 6 and *D. baeri* 2.

5. Conclusion

Our results are congruent with the hypotheses that predict high gene flow and low genetic differentiation in hermaphroditic parasites with complex life cycles including free-living stages, several host species, and birds as final hosts. The lack of neutral genetic differentiation in the parasite makes local host-parasite co-adaptations between *D.* lineage 6 and its fish host seem more likely on a larger geographical scale than among the lakes of a relatively small island.

The microsatellite primers established for this study are the first for *Diplostomum* lineage 6 and can provide a useful tool for studying host-parasite interactions with this geographically widespread parasite found in three-spined and nine-spined sticklebacks. Additionally, our description of the stickleback population structures could be used for choosing genetically independent lakes for studies investigating the ecological causes underlying the evolution of sticklebacks on this island and elsewhere.

Data accessibility

Stickleback mitochondrial haplotypes are available as separate cytochrome *b* and control region sequences under GenBank accession numbers KT971020-KT971072 and KT971073-KT971125, *Diplostomum* microsatellite sequences under GenBank accession numbers KT971126-KT971130. Stickleback and *Diplostomum* microsatellite genotypes are available from the Mendeley Digital Repository DOI: 10.17632/rr434xd2dm.1 and DOI: 10.17632/5tftys6ww5.1. *Diplostomum* Cox 1 sequences can be found on GenBank (accession numbers KX037874-KX037915 and KX140051-KX140055).

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Appendix A. Supplementary data

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