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Two multiplexed sets of 21 and 18 microsatellites for *Phoxinus phoxinus* (L.) and *Gobio gobio* (L.) developed by cross-species amplification

Rémi Grenier · Caroline Costedoat · Rémi Chappaz · Vincent Dubut

Abstract A total of 68 cyprinid microsatellites were screened for cross-species amplification in order to develop three PCR multiplex kits for the Eurasian minnow (*Phoxinus phoxinus* L.) and the gudgeon (*Gobio gobio* L.). The multiplex kits cluster 21 and 18 microsatellites for *P. phoxinus* and *G. gobio*, respectively. All loci were evaluated using ~80 individuals sampled from three distinct populations. By multiplexing the markers, we aim to decrease genotyping costs and increase time efficiency. These two microsatellite sets represent a valuable tool for ecological, evolutionary, conservation and management issues.

Keywords Microsatellite · Cyprinid · Population genetics · Conservation · Management

Introduction

The Eurasian minnow (*Phoxinus phoxinus* L. 1758) and the gudgeon (*Gobio gobio* L. 1758) are both widespread and common freshwater cyprinid species in Europe. They are commonly stocked and are used by anglers as fry, bait or forage species (Kottelat and Freyhof 2007). *Phoxinus*

phoxinus and *G. gobio* are therefore prone to recurrent translocation and are considered as invasive species in some European areas where they are not native (Crivelli 1995; Elvira and Alomodóvar 2001; Hesthagen and Sanlund 2007; Museth et al. 2007). In their native range however, they proved to be valuable models either for studying the impact of water pollution on freshwater fishes (Knapen et al. 2009) or for investigating the processes involved in local adaptive variation (Collin and Fumagalli 2011). Furthermore, it was shown that anthropogenic habitat fragmentation impacts the genetic diversity and structure of these two species differently (Blanchet et al. 2010). Together, *P. phoxinus* and *G. gobio* thus represent a valuable system for an array of management, conservation and evolutionary purposes. Additionally, molecular and morphological studies recently suggested revisions of the taxonomy of both *P. phoxinus* and *G. gobio* (Kottelat and Persat 2005; Kottelat 2007; Mendel et al. 2008), implying critical conservation and management issues.

To date, no specific microsatellites have been developed for *P. phoxinus* and only a limited set of markers is available for *G. gobio* (six reliable markers; Knapen et al. 2006, 2009). The development of additional markers would permit the evolutionary history of these species to be deciphered and would allow a straightforward evaluation of the genetic diversity and structure in both evolutionary ecology and conservation frameworks. From this perspective, cross-species amplification represents a cost- and time-effective approach (e.g. Wang et al. 2009; Rosenbom et al. 2012) and has proven especially valuable for cyprinid species (Hamilton and Tyler 2008; Dubut et al. 2010). Cross-species amplification was previously used for *P. phoxinus* and *G. gobio* in order to identify amplifiable and polymorphic microsatellites (Holmen et al. 2005, 2009). Nevertheless, the screening was conducted on a limited

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number of individuals for most of the markers, which prevented full validation and characterisation. Using cross-species amplification, our study aimed to (1) identify a sufficient number of loci that would allow for discriminating populations at a microgeographic scale (see Nikolic et al. 2009), (2) standardise PCR conditions (one single PCR protocol) and (3) multiplex loci in order to minimise the genotyping time and cost.

Materials and methods

Fish were caught by electrofishing, and caudal fin samples were collected before the fish were returned to their original sampling site. Two distinct locations from the Rhone River system (Isere River and Saone River, Eastern France) and one location from the Meuse River (Northern France) were sampled. At each location, 25 to 33 individuals of *P. phoxinus* and *G. gobio* were collected (see Table 1). Additional samples were collected in the Garonne and Loire River systems (France) for preliminary amplifiability and polymorphism testing. DNA was extracted from ~0.25 cm² of fin using the Gentra® Puregene™ Tissue Kit (QIAGEN).

To identify amplifiable loci, 56 published primer sets developed for Cyprinidae species were tested: BL1-2b, BL1-30, BL1-36, BL1-44, BL1-61, BL1-84, BL1-98, BL1-153, BL1-158, BL1-T2 and BL2-114 (Dubut et al. 2009a, 2012); Ca1 and Ca3 (Dimsoski et al. 2000); CnaB-030, CnaD-112, CnaF-177, CtoA-254, CtoE-249, CtoF-172, CtoG-075 and CtoG-216 (Dubut et al. 2010); CypG9, CypG24 and CypG30 (Baerwald and May 2004); IV04 (Salgueiro et al. 2003); LC27 (Vyskočilová et al. 2007); LceC1 and LceA149 (Lamo et al. 2005); LleA-029, LleA-071, LleA-131, LleA-150, LleA-191, LleB-048, LleB-071, LleB-072, LleC-049, LleC-090 and LleC-184 (Dubut et al. 2009b, 2010); Lco1, Lco3 and Lco5 (Turner et al. 2004); Lid8 and Rru4 (Barinova et al. 2004); Lsou5, Lsou8, Lsou10, Lsou19, Lsou29 and Lsou34 (Muenzel et al. 2007); MFW1 (Crooijmans et al. 1997); N7K4 (Mesquita et al. 2003); Ppro132 (Bessert and Ortí 2003); Rhca20 (Girard and Angers 2006); Rser10 (Dawson et al. 2003); and Z21908 (Shimoda et al. 1999). These primer pairs were initially tested for amplification using four individuals, stemming from Garonne, Loire, Meuse and Rhone drainages (France). Thermocycling was performed using one single protocol: 95 °C for 15 min, followed by 30 cycles (94 °C for 30 s, 56 °C for 90 s, 72 °C for 60 s) and 60 °C for 45 min. PCR were conducted in a total volume of 10 µL containing ~20 ng of DNA and using the QIAGEN Multiplex PCR Kit (QIAGEN). Visualisation of the amplicons was performed by electrophoresis on 1.5 % agarose gels. We retained loci that amplified in all individuals and that gave clean PCR products, i.e. discrete single bands or at most two bands when a large size difference between alleles existed.

The selected loci were then amplified using forward primers labelled with fluorescent dye 6-FAM (Eurogentec), PET, NED or VIC (Applied Biosystems) with PCR conditions as described above and using eight individuals (two individuals from each of the Garonne, Loire, Meuse and Rhone River systems). Visualisation of the amplicons was conducted on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Allele sizes were scored against an internal GeneScan-500 LIZ® Size Standard (Applied Biosystems) and genotypes were obtained using GeneMapper® 3.7 (Applied Biosystems). We retained loci that (1) amplified in all individuals, (2) had unambiguous genotype profiles and (3) were polymorphic.

At this stage, 22 loci were retained for *P. phoxinus*. However, only 10 loci were selected for *G. gobio*. Therefore, 12 additional primer pairs for loci previously detected as polymorphic for *G. gobio* (Holmen et al. 2005; Vyskočilová et al. 2007; Hamilton and Tyler 2008) were tested: Ca13 and Ca14 (Dimsoski et al. 2000); LC293 (Vyskočilová et al. 2007); Lco4 (Turner et al. 2004); Lid1 (Barinova et al. 2004); and Z1206, Z6804, Z8249, Z10362, Z11841, Z14008 and Z15401 (Shimoda et al. 1999). These 12 markers were tested on *G. gobio*, permitting the selection of three additional loci using the protocols and criteria described above. Furthermore, five specific microsatellites (Gob12, Gob15, Gob16, Gob22, Gob28; Knapen et al. 2006) were included, resulting in a total of 18 polymorphic loci for *G. gobio*. Finally, the primer pairs selected for *P. phoxinus* and *G. gobio* were combined into three PCR multiplex kits for each species (Table 1).

The microsatellites were evaluated using three distinct populations of *P. phoxinus* and *G. gobio* (Table 1). GENEPOP 4.0 (Rousset 2008) was used to (1) test Hardy–Weinberg (HW) equilibrium, (2) estimate the observed (H_o) and expected (H_e) heterozygosities for all loci and populations and (3) test for linkage disequilibrium (LD) among loci within populations. MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) was used to analyse the causes of departures from HW equilibrium. In order to graphically prospect the discrimination power of the two sets of markers at population scale, factorial correspondence analyses (FCA) were conducted on genotypes using GENETIX 4.05 (Belkhir et al. 2004) (Fig. 1). For FCA, the locus Ca3 was discarded from the *P. phoxinus* dataset since null alleles were detected in all three populations (see below).

Results and discussion

Based on three population samples, the total number of alleles ranged from 3 to 42 for *P. phoxinus* (excluding locus Ca3) and from 2 to 33 for *G. gobio*, and the expected heterozygosity of polymorphic loci ranged from 0.04 to 0.95 for *P. phoxinus* and from 0.09 to 0.90 for *G. gobio*

Table 1 Microsatellites developed by cross-species amplification for *P. phoxinus* and *G. gobio*: characteristics and polymorphism

Locus (GenBank ID)		Primers (5'-3')	Core motif	Allele size range (N _A)	Multiplex PCR		H _o /H _e		Reference
					Kit	Primer conc. (nM)	Isera River (n=25)	Saone River (n=27)	
CtoA-247 (GU254031)	F-VIC: TGCAACATATAAACTGAAACAAGG R: GCAGGTATATCCAGCC	(ATC) ₇	163–182 (12)	Pp1	30	0.40/0.35	0.41/0.41	0.56/0.70	Dubut et al. (2010)
CypG9 (AY439127)	F-VIC: GCAGTCAGTATTAAGGCGAGCAG R: GAGCGGACTCTCAGGCACCTACC	(CAGA) ₅	106–119 (7)	Pp1	30	0.68/0.58	0.56/0.64	0.40/0.41	Baerwald and May (2004)
LcoC1 (AY962241)	F-6FAM: AGGTGTTGGTTCCTCCCG R: TGTTATCTCGGTTTCACGAGC	(CA) ₁₅	87–127 (21)	Pp1	200	0.72/0.92	0.93/0.94	0.92/0.80	Lamo et al. (2005)
Lco3 (AY318779)	F-NED: GCAGGAGCGAAACCAATAAT R: AAACAGCAGGACACAAAAG	(TG) ₉	238–255 (7)	Pp1	400	0.16/0.15	0.30/0.27	0.52/0.62	Turner et al. (2004)
LieB-072 (FJ601720)	F-PET: TCATTAGGAGGCTGCTTATTC R: CCTTTTCAACAATTTGTCACGG	(TG) ₁₃	161–194 (13)	Pp1	75	0.76/0.77	1.00/0.85	0.76/0.71	Dubut et al. (2009b)
LieC-090 (FJ601722)	F-VIC: TCAGACAACTAACCGACC R: GGGCTGTCCAGAACTGA	(TC) ₁₅ GG(TC) ₃	228–313 (24)	Pp1	100	0.72/0.76	0.93/0.89	0.60/0.87	Dubut et al. (2009b)
Ppro132 (AY254354)	F-PET: GCATTCCTTTTGTGTGAAAGTCTCAA R: GGTTTAACCCGATCAATGGCTGTGC	(CT) ₁₈	119–123 (3)	Pp1	100	0.20/0.19	0.00/0.00	0.40/0.44	Bessert and Orti (2003)
Rru4 (AB112740)	F-6FAM: TAAGCAGTGACCAGAAATCCA R: CAAAAGCCTCAAAAAGCACAA	(CA) ₁₅	169–197 (13)	Pp1	150	0.72/0.83*	0.85/0.88	0.60/0.60	Barinova et al. (2004)
BLI-44 (FJ468355)	F-6FAM: AAGACCAGCATGTGCTT R: ACATAGACTAACGAGTTTCACTT	(CA) ₁₃	115–161 (21)	Pp2	50	0.80/0.81	0.81/0.91	0.56/0.88*	Dubut et al. (2009a)
BLI-84 (FJ468346)	F-NED: CATTACTCGGCAACCACAT R: GCGAAAAGGAAAAGAGACTGA	(AC) ₄ N ₂₄ (CA) ₉	178–195 (9)	Pp2	100	0.44/0.45	0.41/0.42	0.84/0.73	Dubut et al. (2009a)
BLI-98 (FJ468349)	F-PET: ATTGTTTTCATTTTGTGAG R: CCGAGTGTGAGAGTTATT	(CA) ₉ N ₄ (CA) ₃ N ₅₀ (CA) ₄	274–395 (42)	Pp2	400	0.68/0.83	0.89/0.94	0.96/0.95	Dubut et al. (2009a)
CtoG-075 (GU254035)	F-VIC: TCATTTGGATAACAATCCATCATCAC R: ACTATGTTAGATCCACACC	(GC) ₄	217–227 (7)	Pp2	50	0.48/0.61	0.48/0.59	0.28/0.54*	Dubut et al. (2010)
Lsou5 (EF209002)	F-6FAM: CTGAAGAAGACCTGGTTCCG R: CCCCACATCTGCTACTGAC	(CA) ₁₇	178–261 (30)	Pp2	200	0.84/0.90	0.96/0.95	0.92/0.95	Muenzel et al. (2007)
Lsou19 (EF209008)	F-PET: TCCCGTGGAGAACTACAGG R: TTCCTCGGTGAGTGTGATG	(GT) ₃₂	164–199 (18)	Pp2	50	0.48/0.75*	0.67/0.74	0.84/0.89	Muenzel et al. (2007)
Rhea20 (DQ106915)	F-NED: CTACATCTGCAAGAAAGGC R: CAGTGAGGTATAAAGCAAGG	(GA) ₁₇	101–128 (11)	Pp2	50	0.84/0.73	0.89/0.82	0.72/0.65	Girard and Angers (2006)
BLI-153 (FJ468350)	F-NED: GCACAGCTTAATCCGTCACT R: TATGGTCAAAACACGGGTCAA	(AC) ₂₀	216–275 (16)	Pp3	50	0.72/0.81	0.52/0.76*	0.84/0.88	Dubut et al. (2009a)
Ca3 (AF277575)	F-6FAM: GGACAGTGAGGACGCAGAC R: TCTAGCCCCCAAATTTTACGG	(TAGA) ₁₄	207–387 (74)	Pp3	100	0.44/0.96*	0.59/0.97*	0.80/0.98*	Dimoski et al. (2000)
	F-PET: GAAAAACCTGAGAAATTCAAAAGA	(TAGA) ₇	126–227 (19)	Pp3	100	0.76/0.82	0.89/0.94	0.60/0.70	Baerwald and May (2004)

Table 1 (continued)

Locus (GenBank ID)	Primers (5'-3')	Core motif	Allele size range (N_A)	Multiplex PCR Kit	H_o/H_e			Reference
					Primer conc. (nM)	Isere River ($n=30$)	Saone River ($n=32$)	
CypG30 (AY439148)	R: GGACAGGTAAATGGATGAGGAGATA	(CT) ₂₂ (CACT) ₃	141–153 (3)	Pp3 50	0.00/0.00	0.04/0.04	0.32/0.33	Vyskočilová et al. (2007)
LC27 (EF362792)	F-6FAM: TCCAGTCTTCCTTCCCTAAAT R: GCGGAGGGAGAGTATGTCAA	(CA) ₆ T(AC) ₁₀	340–432 (33)	Pp3 100	0.88/0.86	0.48/0.89*	0.88/0.95	Dubut et al. (2009b)
LleA-071 (FJ601715)	F-PET: GTCCTAGATTGTGACGGG R: ACTTCAGTTACTAAGAGATTAGTGA	(GT) ₁₇	178–202 (13)	Pp3 50	0.80/0.78	0.67/0.76	0.88/0.85	Muenzel et al. (2007)
Lsou8 (EF209003)	F-NED: GCGGTGAACAGGCTTAACTC R: TAGGAACGAAGAGCCTGTGG	(GT) ₁₄ N ₃ (GA) ₄	164–215 (19)	Pp3 400	0.76/0.89	0.85/0.91	0.28/0.29	Crooijmans et al. (1997); Tong et al. (2005)
MFW1 (AY703054)	F-VIC: GTCCAGACTGTCAATCAGGAG R: GAGGTGTACACTGAGTCACGC							
<i>Gobio gobio</i>								
Locus (GenBank ID)	Primers (5'-3')	Core motif	Allele size range (N_A)	Multiplex PCR Kit	H_o/H_e			Reference
Ca1 (AF277573)	F-NED: AAGACGATGCTGGATGTTTAC R: CTATAGCTTATCCCGGCAGTA	(CA) ₂₄	89–163 (18)	Gg1 100	0.57/0.62	0.63/0.62	0.48/0.53	Dimoski et al. (2000)
Gob16 (DQ207803)	F-NED: CCAAGATCTCCAAGCCACAT R: TGGAGAACTGCTCTCACCAA	(TG) ₆	185–187 (2)	Gg1 30	0.50/0.48	0.09/0.09	0.30/0.37	Knapen et al. (2006)
Lid8 (AB112735)	F-VIC: AAATGCTAATGTTTCAATCCATA R: AAGCCTTCCCTCTTGTTCC	(GA) ₁₁	120–207 (33)	Gg1 500	0.87/0.77	0.69/0.72	0.91/0.93	Barinova et al. (2004)
Lsou5 (EF209002)	F-6FAM: CTGAAGAAGACCCTGGTTCG R: CCCACATCTGCTGACTTGAC	(CA) ₁₇	179–202 (8)	Gg1 200	0.47/0.38	0.66/0.67	0.64/0.67	Muenzel et al. (2007)
Z10362 (G39785)	F-6FAM: GGTGACCTCATGGAAAGCAAT R: AGCTACTGAAAACCCCTTGGC	(CA) ₂₁	111–151 (14)	Gg1 250	0.57/0.69	0.66/0.71	0.85/0.77	Shimoda et al. (1999)
Z21908 (G40277)	F-PET: ATTGATTAGGTCATGGCCG R: AGGAGTCATCGCTGGTGAGT	(CA) ₆	157–214 (10)	Gg1 100	0.73/0.67	0.34/0.40	0.55/0.63	Shimoda et al. (1999)
CtoA-247 (GU254031)	F-VIC: TGC AAA CATATAA AACTGAAACAAGG R: GCAGGTATATCCAGCC	(ATC) ₇	158–170 (5)	Gg2 50	0.17/0.21	0.09/0.09	0.55/0.53	Dubut et al. (2010)
CypG24 (AY439142)	F-PET: CTGCCGCATCAGAGATAAACACTT R: TGGCGGTAAAGGTAGACCAC	(CAGA) ₁₉	176–189 (6)	Gg2 50	0.67/0.64	0.44/0.42	0.61/0.58	Baerwald and May (2004)
Gob12 (DQ207801)	F-6FAM: AAGGAAATGCAGAATCACAAAATTAC R: GAACTTGCAAAATAGCAGGGTG	(CA) ₇ AA(CA) ₁₉	180–243 (17)	Gg2 200	0.63/0.49	0.59/0.68	0.85/0.86	Knapen et al. (2006)
Gob28 (DQ207805)	F-NED: CGCACAAAACAGCTCAGACTC R: CCGTATGTACAAGCCAGATGAA	(GT) ₉ (GA) ₄ (GT) ₃	191–197 (4)	Gg2 50	0.07/0.13	0.16/0.20	0.24/0.22	Knapen et al. (2006)
Leo4 (AY318780)	F-PET: ATCAGGTCAGGGGTGTCACG R: TGTATTGGGGTCTGTGT	(GT) ₅ N ₂ (GT) ₂ (GA) ₁₁	244–281 (15)	Gg2 100	0.73/0.72	0.78/0.85	0.82/0.90	Turner et al. (2004)
	F-NED: CTACATCTGCAAGAAAGGC	(GA) ₁₇	115–158 (15)	Gg2 100	0.70/0.76	0.75/0.79	0.88/0.89	Girard and Angers (2006)

Table 1 (continued)

Rhea20 (DQ106915)	R: CAGTGAGGTATAAAGCAAGG	(AC) ₂₀	50	0.37/0.35	0.41/0.52	0.39/0.39	Dubut et al. (2009a)
BL1-153 (FJ468350)	F-6FAM: GCACAGCTCTAATCGGTCACT R: TATGGTCAAAACACGGGTCAA	(CA) ₁₀	100	0.53/0.59	0.63/0.61	0.61/0.57	Knapen et al. (2006)
Gob15 (DQ207802)	F-6FAM: TGCCACGTTCTGGTCACAG R: CTTTAAATGTTATTTGGTCTTTGCAG	(CA) ₉ N ₁₈ (CA) ₁₀	50	0.73/0.66	0.63/0.75	0.79/0.82	Knapen et al. (2006)
Gob22 (DQ207804)	F-NED: GATTGCCATGGTTACCGAAT R: TCTCCACCTCGAAATCATCC	(CT) ₅ N ₈ (CT) ₁₆	50	0.00/0.00	0.00/0.00	0.00/0.00	Vyskočilová et al. (2007)
LC293 (EF362795)	F-NED: TTGCCCTCACCCACTAAACA R: CACAGATGCAGATCGAGGAG	(TG) ₁₃	100	0.00/0.00	0.22/0.20	0.36/0.31	Dubut et al. (2009b)
LieB-072 (FJ601720)	F-PET: TCATTAGGGAGGCTGCTTATTC R: CCTTTTCAACAATTTGTCACGG	(GT) ₁₄ N ₃ (GA) ₄	400	0.40/0.44	0.69/0.71	0.94/0.86	Crooijmans et al. (1997); Tong et al. (2005)
MFW1 (AY703054)	F-VIC: GTCCAGACTGTCAATCAGGAG R: GAGGTGTACACTGAGTCACGC						

N_A number of alleles; H_o , observed heterozygosity; H_e , expected heterozygosity

* HW disequilibrium significant at 0.05 level after FDR controlling procedure

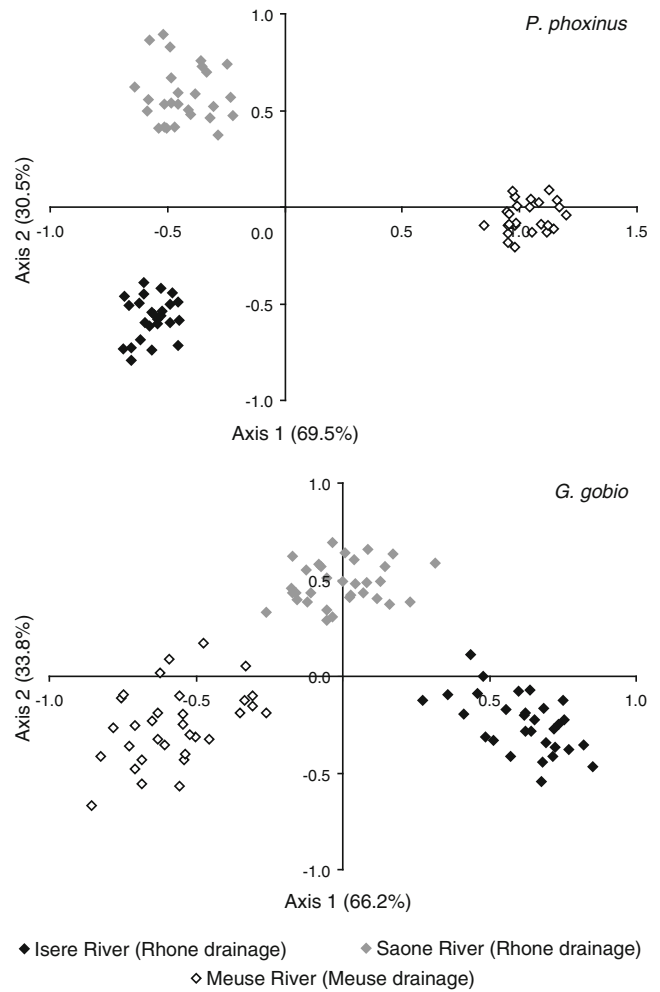


Fig. 1 Factorial correspondence analyses (FCA) of microsatellite genotypes of three populations of *P. phoxinus* (21 loci) and *G. gobio* (18 loci)

(Table 1). For *G. gobio*, LC293 was monomorphic in the three population samples, although polymorphic (including heterozygotes) in the eight individual samples used for the preliminary tests. After applying the false discovery rate (FDR) controlling procedure (Benjamini and Hochberg 1995), no significant HW disequilibrium was detected for *G. gobio*. On the contrary, significant HW disequilibrium was detected for *P. phoxinus* in one out of three populations: loci BL1-44 (Meuse population), BL1-153 (Saone), CtoG-075 (Meuse), LieA-071 (Saone), Lsou19 (Isere) and Rru4 (Isere). Additionally, Ca3 exhibits HW disequilibrium in all three *P. phoxinus* populations (Table 1). For loci at HW disequilibrium, MICRO-CHECKER detected neither evidence for scoring error due to stuttering nor evidence for large allele dropout. However, null alleles may be present at all loci (which present heterozygotes deficiency), except BL1-153 and Rru4. After FDR correction, no LD was detected in *P. phoxinus*. In *G. gobio*, Lco4 and CypG24 showed significant LD in the Isere River population only, likely indicating a local demographic event or local selection.

Furthermore, using 18 microsatellites and 21 microsatellites, FCA permitted the unambiguous discrimination of the populations of *G. gobio* and *P. phoxinus*, respectively. Although some population samples were collected from the same drainage, any overlap was observed between the distinct populations (Fig. 1).

Using cross-species amplification and five specific markers, we were able to develop 18 microsatellites for *G. gobio* and 21 microsatellites (excluding Ca3) for *P. phoxinus*. To catch the widest allelic range and to minimise the retention of loci with null alleles, individuals from four different hydrographical systems (Garonne, Loire, Rhone and Meuse Rivers) were included in the microsatellite development protocol (see Sinama et al. 2011). For *P. phoxinus* however, punctual occurrences of null alleles were detected at loci BL1-44, CtoG-075, LleA-071 and Lsou19. This suggests that these four loci must be used with caution and should be further evaluated depending on the geographic location of samples and the objectives of the study.

Finally, standardising the PCR protocol and multiplexing the microsatellite loci make their use more time- and cost-effective and minimise errors during the compilation of genotypes (see Guichoux et al. 2011). Both sets of loci also proved to correctly discriminate populations. These two sets of microsatellites therefore represent a useful tool for an array of ecological, evolutionary, conservation and management applications. For instance, these markers could be useful in developing molecular tools for relatedness and parentage analyses (as initiated by Bernhardt et al. 2012).

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