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SHORT COMMUNICATION

A rapid and reliable detection procedure of Atlantic trout introgression at the diagnostic lactate dehydrogenase chain-1 gene

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Abstract

The Italian-native Mediterranean brown trout (*Salmo ghigii*) is a seriously threatened freshwater fish, especially by anthropogenic hybridisation with the domestic strains of Atlantic origin that have been repeatedly released into the wild for angling. A PCR-restriction fragment length polymorphism (RFLP) assay of the diagnostic lactate dehydrogenase chain-1 (LDH-C1) gene sequences has been routinely applied to distinguish exotic from native brown trout lineages and detect Atlantic introgression signals in the Mediterranean wild populations. Here, we used dermal swab DNA obtained from 28 wild trout to improve laboratory procedures to genetically characterise trout samples at the LDH-C1gene through (1) a capillary electrophoresis analysis of the RFLP fragments and (2) the optimisation of a diagnostic single nucleotide polymorphism analysable through mini-sequencing approaches. The developed methods were fully consistent with those obtained through the traditional approach, but their analytical process is almost entirely automated and digitalised, thus improving result readability and accuracy in the detection of alien introgressed traces in wild Mediterranean brown trout populations.

KEYWORDS
dermal swab, fish, LDH-C1 genotyping, non-invasive genetic sampling, *Salmo trutta* complex, SNP

1 | BACKGROUND

The brown trout (*Salmo trutta* complex) is considered a complex of incipient species, including vulnerable and/or endemic taxa whose taxonomy is still debated (Lobón-Cerviá & Sanz, 2017). Wild local populations of the Italian-native Mediterranean brown trout, currently referred to as *S. ghigii*, Pomini, 1941 (previously referred to as *S. cettii* Rafinesque, 1810), are seriously declining mainly due to multiple anthropogenic factors such as water pollution and abstraction, habitat modification, overfishing and poaching, global increase in water temperatures and hybridisation (Bassem, 2020; Carosi et al., 2022; Habitat Directive Reporting, 2022) with Atlantic *S. trutta*,

Linnaeus, 1758 (Lobón-cerviá et al., 2019). Such massive introductions were repeatedly performed to support fisheries and contrast Indigenous population decline since the 19th century, resulting in deep introgressive hybridisation, competition and spread of pathologies (Barbat-Leterrier et al., 1989). Similarly, even across the Italian Peninsula, some wild *S. ghigii* populations have become extinct and have been replaced by domestic trout of Atlantic origin or hybrids between the two taxa (Splendiani et al., 2016). The remaining native populations are currently rare, fragmented and mostly isolated (Rossi et al., 2022). To restore the habitat, distribution and genetic diversity of native Mediterranean brown trout, several conservation projects have been started, whose actions often rely on the evaluation of the genetic status of wild populations, through a number of informative molecular markers. Among them, the protein-coding lactate dehydrogenase chain-1 (LDH-C1) nuclear gene has been routinely typed, through a restriction fragment length polymorphism (RFLP)-based procedure, to distinguish between 'modern' (LDH-C1*90 allele) and 'ancestral' (LDH-C1*100 allele) lineages, as well as the occurrence of their hybrids (García-Marín et al., 1999; Hamilton et al., 1989; McMeel et al., 2001). The former occurs in wild brown trout populations from northern Europe (and is almost fixed within European hatchery stocks), while the latter is found in native populations of the Mediterranean area (McMeel et al., 2001). The two alleles can be identified through the RFLP technique, by cutting the amplified LDH-C1 gene at a specific diagnostic site with a restriction enzyme (BslI; McMeel et al., 2001) that produces: two fragments in homozygote Atlantic individuals (*90/*90); a not-cut single fragment in homozygote Mediterranean individuals (*100/*100); all the three fragments in heterozygotes (*90/*100). In this study, we proposed two alternative procedures to improve the genotyping of the LDH-C1 gene: (1) RFLP followed by capillary electrophoresis in automated sequencers and (2) a Sanger mini-sequencing for the direct screening of the above-mentioned diagnostic single nucleotide polymorphism (SNP).

2 | SAMPLING AND DNA EXTRACTION

Sampling was carried out in the framework of the LIFE-STREAMS project (LifeSTREAMS, 2022), within the Foreste Casentinesi National Park (Parco Nazionale Foreste Casentinesi, Monte Falterona e Campigna, 2022) and the Maiella National Park (Parco Nazionale della Maiella, 2022).

Dermal swabs (Breacker et al., 2017; Le Vin et al., 2011; Tilley et al., 2020) from 28 *Salmo* individuals, as authorised by the Osservatorio Faunistico Regionale—Dipartimento Agricoltura (Authorisation No. 93_21_prot.3327–28.05.2021 and no. DPD023-319–01.12.2020), were collected and individually stored in Longmire buffer at −20°C until DNA isolation.

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. After digestion in 180 μL ATL buffer and 20 μL proteinase K, samples were incubated at 56°C, and then the lysates were loaded in a QIAcube HT robotic station (Qiagen) for further purification steps. DNA quality was checked by electrophoresis through a 2% E-Gel Agarose Gels with SYBR Safe (Invitrogen, Thermo Fisher Scientific).

3 | SETTING UP AN AUTOMATED METHOD FOR LDH-C1 FRAGMENT ANALYSIS

The LDH-C1 gene was amplified in a total volume of 8 μL with 20 ng of DNA as the template, 0.025 U of HotStarTaq (Qiagen), 0.8 μL of 10X PCR (Polymerase Chain Reaction) Buffer, 0.8 μL of 0.2% BSA (Bovine Serum Albumin), 0.48 μL of 25 mM MgCl2, 0.4 μL of 2.5 mM dNTP (deoxynucleotide triphosphate) mix, 0.1 μL of 10 μM of each primer (Table 1). DNA was amplified in a Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific) applying the following cycling protocol: 94°C for 15 min, 35 cycles at 94°C for 40 s, 60°C for 40 s and 72°C for 40 s, with a final extension at 72°C for 10 min.

For RFLP-based procedures, LDH-C1 amplicons were digested in 1X Buffer Tango with 0.33 U/μL of BslI restriction enzyme (Thermo Fisher Scientific) for 1 h at 55°C. LDH-C1 pattern was assessed by the traditional method based on visual inspection of fragment size on an agarose gel. As a novel, potentially more accurate and reliable genotyping protocol, we developed a fragment analysis procedure, using fluorescent ABI dyes to label forward, reverse or both primers. Labelled amplicons were separated through capillary electrophoresis in an ABI 3130xl genetic Analyzer (Thermo Fisher Scientific), and alleles were scored in GeneMapper 5.0 using GeneScan 500 ROX size standard (Thermo Fisher Scientific).

The Mediterranean LDH-C1*100 allele results in a single peak at 426 bp, while the Atlantic LDH-C1*90 allele results in one or two peaks according to primer labelling: a 78 bp peak when labelling the reverse primer; a 348 bp peak when labelling the forward primer; two peaks when labelling both the forward and reverse primers. However, better results were obtained by labelling only the LDHXON4R primer with a 6Fam fluorescent dye, which allowed unambiguously identifying the *90 allele (peak of 78 bp) and/or the *100 allele (peak of 426 bp). Fragment analysis through capillary electrophoresis was performed in two independent laboratories for method validation. Results returned by automated capillary analysis were fully consistent with those obtained from visual inspection of gel electrophoresis, supporting its employment as a routine diagnostic practice (Table 2). However, this implemented protocol offers practical and technical advantages, compared to traditional agarose gel electrophoresis: (1) a potentially improved sensitivity and discriminatory power; (2) higher throughput (96 samples run in a few hours); (3) results not affected by the subjective interpretation of the operator; (4) digitalised data, relevant for quality standards and data traceability.

4 | DISCOVERY AND ANALYSIS OF LDH-C1 SNP

As a second alternative approach, the LDH-C1 was directly sequenced to identify the Atlantic–Mediterranean diagnostic SNP through a

TABLE 1 Primers and conditions used for amplification of lactate dehydrogenase chain-1 (LDH-C1) gene.

Primer	Primer sequence 5′–3′	Primer [nM]	Ta (°C)	Reference
LDHXON3_F	GGCAGCCTCTTCCTCAAAACGCCCAA	125	60	(McMeel et al., 2001)
LDHXON4_R	CAACCTGCTCTCTCCCTCCTGCTGACGAA	125		
41LDH-I	TGATTGATTGTTCTCCCACGGTCAG	250	50	This study

TABLE 2 Results of restriction fragment length polymorphism-based fragment analysis and single nucleotide polymorphism (SNP) sequence inspection of LDH-C1 gene.

Sample	Fragment analysis BsII restriction		SNP analysis		Strain
TRO_1	78	–	C	C	ATL
TRO_2	78	–	C	C	ATL
TRO_3	78	426	C	T	Hyb
TRO_4	78	–	C	C	ATL
TRO_5	78	–	C	C	ATL
TRO_6	78	–	C	C	ATL
TRO_7	78	–	C	C	ATL
TRO_8	78	–	C	C	ATL
TRO_9	78	–	C	C	ATL
TRO_10	78	426	C	T	Hyb
TRO_11	78	–	C	C	ATL
TRO_12	78	426	C	T	Hyb
TRO_13	78	426	C	T	Hyb
TRO_14	/	/	T	T	MED
TRO_15	–	426	T	T	MED
TRO_16	78	426	C	T	Hyb
TRO_17	78	426	C	T	Hyb
TRO_18	–	426	T	T	MED
TRO_19	–	426	T	T	MED
TRO_20	78	426	C	T	Hyb
TRO_21	78	426	C	T	Hyb
TRO_22	–	426	T	T	MED
TRO_23	78	426	C	T	Hyb
TRO_24	78	426	C	T	Hyb
TRO_25	–	426	T	T	MED
TRO_26	78	426	C	T	Hyb
TRO_27	78	–	C	C	ATL
TRO_28	78	-	C	C	ATL

Note: Strain definition according to genotypes obtained by fragment analysis and SNP-41: Atlantic (ATL), Mediterranean (MED), hybrid (Hyb).

primer extension-based method. DNA amplification was performed using reagents and conditions reported above. PCR products were purified with A'SAP (Shrimp Alkaline Phosphatase) PCR clean-up kit (ArcticZymes Technologies ASA) and then sequenced in both directions using the BigDye Terminator Cycle Sequencing chemistry (Applied-Biosystems). Sequences were displayed using the software Chromas 2.6.6 (Technolysium Pty Ltd.). Obtained sequences were phased via DnaSP6 (PHASE model with recombination, iterations = 1000; burn-in = 100; Rozas et al., 2017) and then aligned in MEGA-11 (Tamura et al., 2021) with two reference alleles—AF488541 for the Mediterranean native strain and AF488539 for the Atlantic strain—to score the restriction site mutation discriminating Atlantic and Mediterranean strains (41T in Ad strain AF488541, 41C in At strain AF488539). Once obtained a full alignment, an internal primer was manually designed

to screen the diagnostic SNP (viz., the one distinguishing between LDH-C1*90 and *100 alleles; Table 1) by a mini-sequencing analysis (SNaPshot Multiplex Kit, Applied Biosystems) with the following protocol: 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 30 s. Products were treated with 1 U of SAP at 37°C for 30 min followed by a denaturation step at 80°C for 15 min. SNaPshot products were detected through capillary electrophoresis in an ABI 3130xl genetic Analyzer (Thermo Fisher Scientific) and scored in GeneMapper 5.0 using GeneScan 120 LIZ size standard (Applied Biosystems). The SNP at position 41C/T allowed distinguishing Atlantic (i.e., nucleotide C), Mediterranean (i.e., nucleotide T) and hybrid strains (Table 2).

The consistency of the results obtained from the SNP analysis with those retrieved from the RFLP-based protocol confirms the robustness and sensitivity of the former in routine diagnostic practice. Anyhow, even if the analysis of the SNP at position 41 returns the same information obtainable from RFLP analysis, the former is a simpler, faster and automatable working protocol, compared to the latter that remains a labour-intensive and time-consuming genotyping procedure (either based on fragment analysis or visual inspection of electrophoresis gel).

5 | CONCLUSION

Direct sequencing of the SNP at position 41 remains a more efficient working protocol as compared to RFLP-based procedures. On the other hand, genotyping by capillary analysis of RFLP-derived fragments could be conveniently coupled with typing of other commonly employed markers, such as microsatellite loci, hence reducing costs and laboratory effort.

In summary, we proposed two implemented working protocols to rapidly detect and monitor the LDH-C1-based introgression of Atlantic strains in Mediterranean brown trout, a vulnerable taxon of high conservation and economic value.

AUTHOR CONTRIBUTIONS

Anna Padula: Data curation; formal analysis; investigation; methodology; supervision; validation; visualisation; writing—original draft. **Claudia Greco**: Conceptualisation; investigation; writing—review and editing. **Lorenzo Talarico**: Methodology; writing—review and editing. **Romolo Caniglia**: Conceptualisation; investigation. **Caterina Maria Antognazza**: Investigation; validation; writing—review and editing. **Susanna D'Antoni**: Funding acquisition; project administration; resources; writing—review and editing. **Massimo Lorenzoni**: Funding acquisition; project administration; resources; writing—review and editing. **Isabella Vanetti**: Investigation; validation; writing—review and editing. **Serena Zaccara**: Investigation; validation; writing—review and editing. **Nadia Mucci**: Conceptualisation; methodology; supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

Sampling was conducted in the framework of LIFE18 442 NAT/IT/000931 STREAMS ('Salmo ceTtii REcovery Actions in Mediterranean Streams') project, according to official guidelines 'LIFE-STREAMS—LIFE18 NAT/IT/000931 (Salmo ceTtii REcovery Actions in Mediterranean Streams)—ACTION A.2 – FIELD MANUAL AND TECHNICAL PERSONNEL TRAINING'. Authorisation no. 93_21_prot.3327 – 28.05.2021 and no. DPD023-319 – 01.12.2020.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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PEER REVIEW

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