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Fast and cost-efficient species identification of Atlantic salmon (*Salmo salar* L.), brown trout (*Salmo trutta*), and their hybrids using a single SNP marker

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Abstract

A workflow for developing a cost- and time-efficient, single nucleotide polymorphism (SNP)-based assay for species and hybrid identification is described. In a reference set ($n = 46$), the developed assay identified individuals of two closely related species, the Atlantic salmon (*Salmo salar* L., $n = 23$) and brown trout (*Salmo trutta*, $n = 23$), with 100% accuracy. Furthermore, species and hybrid identification using field-collected embryos had 98.1% concordance (155/158) to more expensive and time-consuming methods that utilized multiple SNP markers. The method can be integrated into management and conservation plans to quantify species' spawning distribution and hybridization rates.

KEYWORDS
conservation genetics, diagnostic locus, hybridization, KASP SNP-assays, species identification

Accurate species identification is a cornerstone of conservation biology, but distinguishing even well-known species can be challenging. As an example, two closely related salmonid species with overlapping distributions and habitats, Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*), or cutthroat trout (*Oncorhynchus clarki*) and rainbow trout (*Oncorhynchus mykiss*), are difficult to differentiate visually in early life stages (Allendorf et al., 2001; Makhrov, 2008). Further, the occurrence of hybrids between these species pairs makes accurate identification even more difficult. Hybridization may reduce parents' fitness via reduced reproductive success (with conspecifics), and decrease population viability and productivity due to introgression (Allendorf et al., 2001; Garcia-Vazquez et al., 2004; Makhrov, 2008;

TABLE 1 Species identification and concordance.

Sample	Assigned using multi-SNP panel (AS/BT/hybrid/NA)	Assigned using KASP assay (AS/BT/hybrid/NA)	Concordance between methods
Atlantic salmon	23/0/0/0	23/0/0/0	100%
Brown trout	0/0/23/0	0/0/23/0	100%
Embryos (*dataset1*)	18/33/12/0	19/34/10/0	96.8%
Embryos (*dataset2*)	60/24/10/0	59/24/10/1	98.9%

Abbreviations: AS, Atlantic salmon; BT, brown trout; NA, unidentified individuals.

McKelvey et al., 2016). Thus, hybridization may have important eco-evolutionary consequences for species with low pre- or post-zygotic barriers, and represents a major conservation challenge, particularly in small populations (Allendorf et al., 2001).

The hybrids of Atlantic salmon and brown trout are functionally sterile either in the first or second generation (Galbreath & Thorgaard, 1995; Makhrov, 2008). The anthropogenic drivers of river regulation (e.g., changes in downstream water regime as a result of dam constructions, and subsequent water flow regulations) and spawning habitat limitations, combined with a typically limited number of spawners (i.e., shortage of potential mates), may increase the hybridization rates between salmon and trout populations, thus emphasizing the need for continued monitoring (Allendorf et al., 2001). Existing visual identification methods for salmon-trout hybrids require expertise and may not always be accurate (Makhrov, 2008; Koljonen & Koskiniemi, 2020). For example, despite brown trout embryos being typically larger due to earlier spawning periods than Atlantic salmon, size distributions at early life stages overlap considerably (Heggberget et al., 1988). While genetically based species and hybrid detection methods provide a powerful alternative, the current single-marker method to identify Atlantic salmon and brown trout is based on detecting a species-specific length polymorphism in a microsatellite marker (12 bp difference between two species), which requires a cost- and time-consuming fragment analysis step (Perrier et al., 2010).

Assays targeting differences in single nucleotide polymorphism such as the PCR-based Kompetitive Allele-Specific PCR (KASP, Semagn et al., 2013) are fast and cost-efficient methods that have been widely used in ecological and evolutionary research (Wenne, 2023). In salmonids, such a method has been applied to detect single gene polymorphisms within controlled crosses (Prokkola et al., 2022). These methods are also suitable for identifying two closely related species, that is, by exploiting fixed single-nucleotide differences between species (e.g., Devran & Göknur, 2020; Çatalkaya et al., 2023).

In this study, we describe a workflow to develop and validate a SNP-based KASP assay for species and hybrid identification using a dataset from endemic landlocked Atlantic salmon and brown trout populations in the River Gullspång, Sweden as the case system (*dataset1*). The assay was further verified using another dataset (*dataset2*), from the River Pielisjoki (Laurinvirta stream), Finland, which is the original spawning area of the critically endangered Lake Saimaa salmon and of the highly endangered migratory population of brown trout. *Dataset1* was composed of a reference set that consists of 23 Atlantic salmon spawners and 23 brown trout juveniles reared in a conservation fish hatchery, and a test set that consists of 65 fertilized eggs (eyed embryos) sampled in the field from salmon/trout redds (Supporting Information Data S1). Gullspång in February 2023. *Dataset2* was composed of 94 fertilized eggs (eyed embryos) sampled in the field from salmon/trout redds in 2022 and 2023.

Species and hybrid identification using multi-SNP assays: We first conducted species and hybrid identification analysis using multi-SNP genetic markers. Samples from *dataset1* were genotyped with 146 SNP markers initially designed for Atlantic salmon (Aykanat et al., 2016) using the genotyping-by-sequencing approach (described in Aykanat et al., 2020), and *dataset2* was initially genotyped for 4457 SNP markers using DNA TRACEBACK® Fisheries SNP genotyping array FSHSTK1D (IdentiGEN Limited, Dublin, Ireland), designed for genotyping seven fish species (MultiFishSNPChip_1.0 array; Andersson et al., 2024).

For *dataset1*, we employed coverage-based species identification methods, in which mean SNP coverage for Atlantic salmon-specific SNP markers (SNP markers that did not amplify in brown trout, $n = 15$) standardized to the mean coverage of interspecific SNP markers (SNP markers that amplified equally well in both species, $N = 74$) were used as a criteria to identify species and their hybrids (Figure S1a and Supporting Information Data S1). Out of 63 embryo samples, 18, 33, and 12 were assigned as Atlantic salmon, brown trout, and hybrids, respectively (Table 1 and Figure 1a). For *dataset2*, principal component analysis (PCA) was performed using *dudi.pca* function in the ade4 package (version 1.7–22, Dray & Dufour, 2007) in R version 4.4.1 (R Core Team 2024), which separated species and hybrid groups into three clusters. Out of 94 embryo samples, 60, 24, 10 were assigned as Atlantic salmon, brown trout, and hybrids, respectively (Table 1 and Figure 1b).

Designig the KASP assay and genotyping: SNP markers amplified in Atlantic salmon but not in brown trout were candidates for designing a species-specific KASP assay (Figure S1a and Supporting Information Data S1). Approximately 100 bp DNA sequence around one of these Atlantic salmon specific markers was aligned to the brown trout (Genome assembly fSalTru1.1) and Atlantic salmon genomes (Genome assembly Ssal_v3.1) using the NCBI BLAST tool, and nucleotide

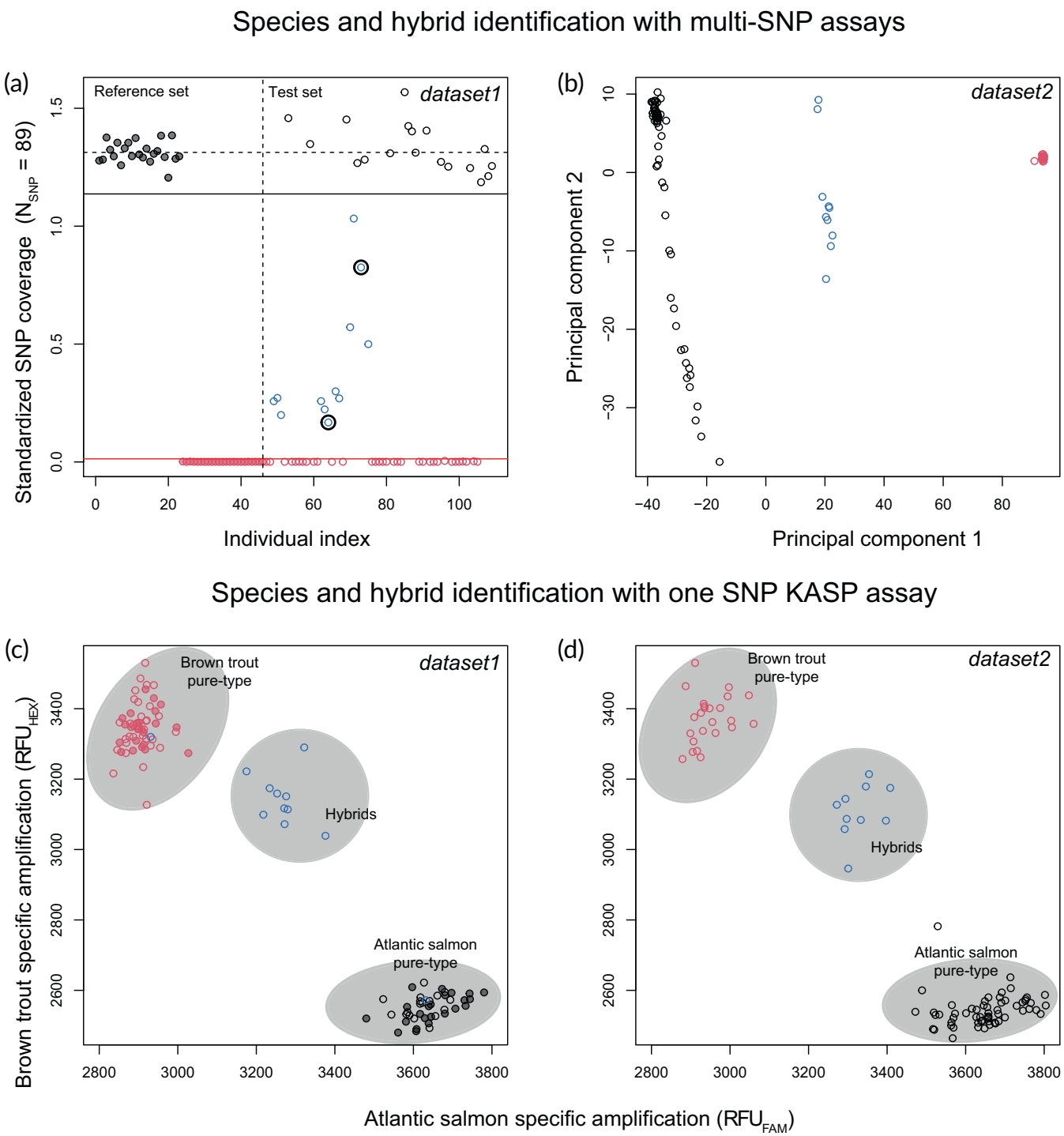

FIGURE 1 Accuracy and concordance of species and hybrid identification across multi-SNP and one-SNP KASP assays. (a) Species identification using standardized coverage of individuals using the genotyping-by-sequencing method in *dataset1*. The reference set is indicated with filled black and red points for Atlantic salmon and brown trout, respectively. Black, red, and blue circles are embryos in the test set identified as Atlantic salmon, brown trout, and hybrids, respectively. The solid black line is four standard deviations below the average standardized coverage (dashed black line) obtained from the Atlantic salmon reference set and is used as the cut-off value for pure-type Atlantic salmon identification. The solid red line is the cut-off value for pure-type brown trout identification. Samples located in between the two solid lines are identified as hybrids. Circled samples indicate mismatches to KASP identification. (b) Species identification using principal component analysis using 4457 SNP markers in *dataset1*. Black, red, and blue circles are individuals in the test set identified as Atlantic salmon, brown trout, and hybrids, respectively. (c, d) Species identification using one SNP marker KASP assay (TN_1579_SNP_M). The *x* and *y* axes indicate Atlantic salmon- and brown trout-specific fluorescence intensities, respectively. Filled black and red circles in (c) are Atlantic salmon and brown trout individuals in the reference set, respectively. Black, red, and blue empty circles indicate embryos in each test set identified as Atlantic salmon, brown trout, and hybrids, respectively, using the multi-SNP dataset. The grey areas are three visual clusters representing the pure-type species and the hybrids.

differences between the two species were identified. Primers were then designed by LGC genomics (UK) for a KASP assay (named as TN_1579_SNP_M), whereby the species-specific nucleotide polymorphism is located in chromosome 30 at position 41097526 in brown trout, and in chromosome 13 at position 8608040 in Atlantic salmon. A common reverse primer was designed to perfectly align with both species' DNA (5′ GTTAGAAACACATCCACACCCRAAACAT 3′), while two forward primers were designed with a species-specific nucleotide at the 3′ end of the primers (Atlantic salmon specific forward primer: 5′ CAGGGTGGATTTGGTGGAAGTA 3′ and brown trout specific forward: 5′ CAGGGTGGATTTGGTGGAAGTC 3′; Figure S1b). These forward primers were fluorescently labelled with FAM and HEX dyes, respectively, to generate species-specific optical signals.

Using *dataset1* and *dataset2*, the PCR reaction for the assay was performed in a 384-well plate in 4 μL (2 μL of KASP 2× Mastermix and 0.05 μL of primer mix, supplied by LGC Genomics, UK), and 2 μL of 1/20 diluted genomic DNA. The reactions were conducted using a quantitative PCR (qPCR) machine (C1000 Thermal cycler with CFX384 Real-Time System; Bio-Rad) under the following thermal cycling conditions: an initial denaturation step at 94°C for 15 min, 10 cycles each consisting of a denaturation at 94°C for 20 s and annealing/extension at 61°C for 1 min (decreasing temperature by 0.6°C/cycle), 35 cycles of denaturation at 94°C for 20 s and annealing/extension at 55°C for 1 min. Fluorescence signal analysis for allelic discrimination was performed using the CFX Maestro software (Bio-Rad).

The KASP assay clearly identified brown trout and Atlantic salmon reference sets as two distinct clusters (Figure 1c,d). The genetic assignment of the test subjects was also in concordance with the results obtained with the SNP set, with 98.5% (62/64) and 99.0% (93/94) accuracy between the two methods for *dataset1* and *dataset2*, respectively (Figure 1c,d and Table 1). In *dataset1*, two individuals identified as Atlantic salmon and brown trout using the multi-SNP analysis were identified as hybrids in the KASP assays (Figure 1b,d). In *dataset2*, one individual identified as Atlantic salmon was unidentified in the KASP assays (Figure 1d). In general, species and hybrid specific clusters were remarkably distinct in the KASP assay.

The assay is expected to be highly reliable for species identification and to quantify hybridization across the overlapping range of these species, since the SNP sequences that we designed primers for were retrieved from the reference genomes of Atlantic salmon and brown trout obtained from individuals from large geographical areas, and verified in two independent systems.

The KASP assay is a very cost-effective method for species identification. Given the institution has appropriate equipment, such as a 384-well plate optical thermocycler, the cost of sequencing, including primers and master mix, and a 384-well optical plate, are 49.17€ per plate (i.e., 384 samples), which is 0.13€ per sample, not including the costs of DNA extraction and personnel. (Costs will be likely higher for the 96-well plate since higher reaction volumes are generally required.) While we used a high-quality DNA extraction method, KASP assays are robust to more economical alternatives such as salt extraction and commercial quick extraction methods such as Lucigen QuickExtract kits (e.g., Prokkola et al., 2022). Likewise, preparing the assays requires only setting up the PCR reactions, which can be completed in a few hours, reducing personal costs and turnaround times. The use of a pipetting robot for dispensing reagents on plates can further reduce the working time needed, but it is not required for running the assay.

In conclusion, we demonstrated a workflow for designing and validating a single SNP marker-based species and hybrid identification assay. The designed assay confidently identified two closely related salmonid species, the Atlantic salmon and brown trout, and their reciprocal hybrids quickly and cost-efficiently compared to the methods that rely on detecting length polymorphisms (e.g., Perrier et al., 2010). The assay is especially suitable for identifying samples collected in the field during early development (embryos, newly-hatched alevins and parr), but also at later life stages, and for monitoring the relative rates of hybridization between species. Integrating the developed species identification methods to redd counting (Syrjänen et al., 2014) and embryo sampling provides a robust methodology to estimate the sizes of spawning stocks of sympatric stream-spawning salmonids, which, for example, can be beneficial for more efficient conservation of endangered salmon and trout stocks. Lowering the cost and decreasing the turnaround times of analysis are important factors in expanding this set of methods in the management and monitoring of sympatric, especially endangered, salmonid stocks, and in improving and verifying channel restoration methods (e.g. Piccolo et al., 2012). The workflow can also be used to discover multiple species-specific SNPs, which can be used to study the viability and fertility of second-generation hybrids in the wild. Furthermore, when used in combination with mitochondrial assays (e.g., Karlsson et al., 2012), one can also infer the parental origin of hybrid individuals.

Monitoring the coexistence and demogenetics of sympatric salmonid species pairs can have far-reaching consequences for conservation. In particular, for our case study in River Gullspång (*dataset1*), we found a remarkably high number of hybrids from the embryo samples from the field, indicating a significant level of hybridization for the populations with potential fitness consequences. On a broader scale, anthropogenic drivers, such as river regulation and climate change, may break down historic temporal or spatial ecological barriers between co-evolved sympatric salmonid species, allowing for an increased rate of hybridization (Garcia-Vazquez et al., 2003) and in some cases the loss of species integrity (McKelvey et al., 2016). Our workflow can be easily implemented with other studies that aim to identify species of interest and their potential hybrids.

AUTHOR CONTRIBUTIONS

Concept: T.A., J.M.P., J.N., and J.J.P. Sample collection: J.T.S., M.J., J.N., and J.J.P. Laboratory work and analyses: T.A., A.B., K.K., and T.L. Writing: T.A., J.T.S., M.J., J.M.P., and J.J.P.

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DATA AVAILABILITY STATEMENT

The compiled datasets and R-codes to reproduce the results are available from the figshare repository at https://doi.org/10.6084/m9.figshare.27679287.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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