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Short communication

# Buccal swabs for long-term DNA storage in conservation genetics of fish: One-and-a-half-year analysis timeframe

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ABSTRACT

Conservation genetic research is essential for the management and recovery of endangered taxa. However, the invasive collection of biological material for DNA analysis is controversial. From an ethical perspective, non-destructive sampling methods leave the aquatic specimen alive and less invasive procedures minimize stress on the animals. DNA can be obtained from fish using minimally invasive techniques such as buccal swabs. Here we evaluated the performance of buccal swabs for long-term storage of DNA obtained from brown trout (*Salmo trutta*). The buccal swabs were stored at room temperature and cut into pieces, one part of which was used for extraction of an aliquot and the others were stored as a "biobank" of biological material. The elapsed time from sampling to molecular analysis was one and half year. The amplification of three different DNA targets was tested to assess the effectiveness of the extraction: mitochondrial DNA (the D-LOOP region), nuclear DNA (the *LDH* gene) and microsatellite DNA at multiple loci. The results showed high quantification (mean value: 281.84±72.4 ng/μL), indicating that DNA could be effectively extracted from the buccal swabs. Our study results suggest that buccal swabs for long-term storage of DNA at room temperature are promising for use in field conservation studies.

## 1. Introduction

Anthropogenic and environmental disturbances (e.g., climate change, overfishing, habitat destruction, introduction of invasive species) pose a threat to the conservation of the biodiversity of many aquatic species (Gangloff et al., 2016; Costa et al., 2021; Ahmed et al., 2022). Recreational fishing and the release of non-native species have altered native fish populations, resulting in genetic changes, due to interspecific hybridization favouring genetic introgression (Gozlan et al., 2010; Britton, 2022). Conservation genetic research is essential for the management and recovery of endangered taxa, while competitive replacement and genetic swamping of native species alter aquatic ecosystems (Howes et al., 2009; Frankham, 2018; Russello et al., 2020; Bernos et al., 2022).

Genetic variation in fish populations first attracted scientific interest in the late nineteenth century. Genetics could be used to protect and conserve endangered taxa and to reveal diversity within and between geographic populations (Rossi et al., 2022; Seth

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et al., 2022). Knowledge gained from genetic studies of endangered species can now be used to inform management plans and prevent the extinction of endangered taxa by avoiding inbreeding and maintaining genetic variability (Hurt and Hedrick, 2004). With the advent of new molecular techniques, genetic diversity within and between populations can be assessed for many more threatened species (Willi et al., 2022). Such data contribute to biodiversity conservation and have been extensively applied in population genetics studies of aquatic animals over the past decade (Yi et al., 2019). A wide range of molecular markers can be used to determine population history or species status and to predict change (Loeschcke et al., 2013). Mitochondrial DNA (mtDNA) can be present in multiple copies within each mitochondrion, evolves much faster than nuclear DNA markers due to its rapid mutation rate, and it is remarkably resistant to environmental degradation. For this reason, mtDNA analysis is widely used in many scientific fields, including forensic genetics anthropology and molecular ecology, where it has become the gold standard (Kowalczyk et al., 2021). In contrast, nuclear DNA (nDNA) exists in a limited number of copies (typically two in diploid individuals), is organized into chromosomes, is surrounded by nuclear membranes, and is inherited from both the maternal and the paternal lines. Unlike mtDNA, it is not resistant to degradation (Allentoft et al., 2012). These differences between mtDNA and nDNA in their structural and functional properties are exploited in different ways: mtDNA is an important source of data, but it provides only part of the information needed to characterize species and to make informed conservation decisions (Rubinoff, 2006).

DNA is the starting material for biodiversity and genetic conservation studies (Theissinger et al., 2023). However, the collection of biological material for DNA analysis is controversial (Levy, 2012). From an ethical perspective, non-destructive sampling methods leave aquatic specimens alive (Robinson et al., 2019), and less invasive procedures minimize stress on individuals, avoiding adverse physiological and behavioural effects, while providing effective conservation without compromising survival or affecting animal welfare (Reid et al., 2012; Fernandes and Pedroso, 2017).

Sources of DNA in non-destructive sampling in fish include fin clips, scales, barbels, muscle, blood, and sperm (Wasko et al., 2003). Although non-destructive, they are still invasive, and they should be replaced with minimally invasive methods. The terms "alternative methods to animal testing" encompasses methods that replace animals in experiments, reduce their use and are refined to limit or eliminate their suffering. To be effective, alternative methods follow the principle of the 3Rs (replacement, reduction, refinement) (Russell and Burch, 1959).

More than 25 species of the genus *Salmo* (Linnaeus, 1758) have been described in Europe, all of which are of socio-economic importance (Tougard et al., 2018; Carosi et al., 2020). Salmonids include game species that are popular for sport fishing and play an important role in the outdoor recreation economy (Brown et al., 2019). Because of their high recreational and commercial value, fish of the genus *Salmo* are a focus of conservation and management of aquatic ecosystems (Filipe et al., 2013). Some populations have experienced habitat degradation due to water pollution or hydraulic works not designed with naturalistic criteria (Carosi et al., 2020). Where overfishing has reduced natural salmonid populations, artificial restocking is used to support sport fisheries, without considering the genetic variability of trout or the introgression of allochthonous genomes into autochthonous lines (Carosi et al., 2020).

The most widely used method for genetic identification of salmonids today is the removal of a portion of tissue and the removal of adipose tissue and the anal fin, which has implications for the health and welfare of the fish (Koll et al., 2019). DNA can be obtained from fish using minimally invasive techniques such as buccal swabs (Reid et al., 2012). Studies have demonstrated the effective use of this sampling method with various buffers and carrier fluids; however, its technical limitation is that the swab is destroyed after use (Colussi et al., 2017). Once processed, the swab is effectively discarded, precluding subsequent extraction, unlike other biological matrices (tissue and blood) from which multiple extractions can be made.

Swabs used to collect biological materials serve different purposes in different species. For example, Pidancier et al. (2003) described the use of buccal swabs as a non-destructive DNA sampling method for genetic studies in amphibians in conservation genetics. Smalley and Campanella (2005) reported the use of buccal swabs in pumpkinseed (*Lepomis gibbosus*) for DNA extraction and PCR analysis. Broquet et al. (2007) reported the successful use of buccal swabs for microsatellite amplification in two amphibian species, *Triturus alpestris* and *Hyla arborea*. Ford et al. (2022) described the use of buccal swabs to detect Ranavirus infection in frogs. They used GenoTube livestock swabs (ThermoFisher Scientific, Waltham, MA, USA) to produce aliquots from the original swabs for biobanking and multiple analysis, without the need for further sampling.

The aim of this study was to evaluate the efficacy of long-term DNA storage swabs for conservation purposes and as a biobanking resource in fish species. GenoTube livestock swabs (ThermoFisher Scientific, Waltham, MA, USA) were used to produce aliquots from the original swabs for biobanking or multiplex analysis without the need for further sampling. We evaluated buccal swabs from a population of brown trout (*Salmo trutta*) reared on a fish farm in north-western Italy. Different molecular markers were used to test the efficacy of the swabs: the mitochondrial region of the D-LOOP (Suárez et al., 2001) and the nuclear *LDH* gene (McMeel et al., 2001) were analyzed for lineage and hybridization information, while microsatellite analysis was used to gain insight into gene introgression (Lerceteau-Köhler and Weiss, 2006).

## 2. Materials and Methods

### 2.1. Sampling

A total of 100 specimens were sampled from a population of brown trout (*Salmo trutta*) from a mountain fish farm located in northwest Italy. GenoTube livestock swabs (ThermoFisher Scientific, Waltham, MA, USA) were used; this patented drying system blocks enzyme activity, thus preventing degradation of nucleic acids and proteins. Swabs were taken from the oral mucosa of the cheeks and palate for approximately 10 s. To minimize manipulation stress, the subjects were lightly sedated (10 mg/L) with a bath of tricaine methane sulphonate (MS-222; Sigma-Aldrich, Milan, Italy). Fish were kept in a separate tank until the recovery before the

release in the farm. From each individual one swab was taken.

The elapsed time from sampling to DNA extraction molecular analysis was one and half year. After this period, the swab was divided in three aliquots: the first was used for molecular analysis, while the remaining two aliquots were further stored. Swabs were stored dry at room temperature (20 °C) to ensure adequate preservation. Once dried, samples can be stored without refrigeration for easy and cost-effective transport (ThermoFisher Scientific Livestock Geno Tube Flyer; www.thermofisher.com).

### 2.2. DNA extraction and quantification

A MagMAX™ DNA Multi-Sample Ultra Kit was used to extract DNA from the swab, with rapid extraction of high-quality DNA. Extraction was performed according to the kit protocol guidelines. The swab aliquot (around 0.5 cm) was placed in a 96-well plate, then 100 μL of PBS buffer 1X was added to each well, followed by 200 μL of PK Mix, that is composed by 8 μL Proteinase K and 192 μL of PK buffer to each sample. Samples were incubated for 2.5 h at 65 °C, then 200 μL of lysis buffer was added; after spunt at 950 rpm for 5 min, the lysates were transferred to a new 96-well plate. Then 240 μL of isopropanol was added to each sample and the plate was spunt at 950 rpm for 5 min. Finally, 40 μL of binding bead mix prepared from 16 μL of DNA binding beads and 24 μL of MilliQ water was added to each sample. A dedicated programme on the King-Fisher extractor extracted 96 samples in approximately 40 min. Samples were eluted in 100 μL of Elution Buffer and quantified with a NanoDrop™ spectrophotometer (ThermoFisher Scientific). Since nucleotides, RNA, ssDNA, and dsDNA all absorb at 260 nm, they constituted the total absorbance of the sample. The ratio of absorbance at 260 nm and 280 nm was used to assess DNA purity. A ratio of ~1.8 is generally accepted as "pure" for DNA; lower values may indicate the presence of proteins, phenols or other contaminants that absorb strongly at or near 280 nm; higher values indicate the presence of RNA (ThermoFisher Scientific T042-TECHNICAL BULLETIN NanoDrop Spectrophotometers).

### 2.3. D-LOOP mt-DNA control region

The variability of the D-LOOP control region was analyzed by PCR followed by sequencing of the amplified product according to the protocol described in Suárez et al. (2001) and in Sabatini et al. (2011). Amplification was performed in a volume of 50 μL using the following reaction mix: buffer 5X, $MgCl_2$ 2 mM, dNTP's 0.25 mM, primer (L15998-PRO: 5' TACCCCAAACTCCCAAAGCTA 3'; HDL-C-1: 5' CCTGAAGTAGGAACCAGATGCCAG 3') 20 pmol, Taq 1,25 U; $H_2O$ 9.15 μL, and DNA 50 ng. Amplicons were purified using an ExtractMe DNA Clean-up and Gel-out kit (Blirt, Poland) according to the manufacturer's instructions. The purified PCR products were sequenced bidirectionally using BigDye™ Terminator v1.1 Cycle Sequencing Kit (ThermoFisher Scientific) and the same primers as for PCR amplification. Cycle sequencing products were purified using a BigDye™ Terminator v1.1 Cycle Sequencing Kit and sequenced on an ABI 3130xl Genetic Analyzer (ThermoFisher Scientific). Contig assembly of forward and reverse DNA sequences for each isolate was performed using the Lasergene software package (DNASTAR, Madison, WI, USA). Sequences from the GenBank database were compared using the basic local alignment search tool (BLAST) search algorithm.

### 2.4. LDH nuclear gene

PCR-RFLP analysis of the nuclear gene *LDH* was performed according to the protocol described in Mc Meel et al. (2001). An initial amplification by PCR of a 440 bp region within the *LDH* gene containing the polymorphism was performed in a volume of 25 μL using the following reaction mix: buffer 5X, $MgCl_2$ 3 mM, dNTP's 0.2 mM, primer (LDHXON3F: 5' GGCAGCCTCTTCCTCAAAACGCCCAA 3'; LDHXON4R: 5' CAACCTGCTCTCTCCCTCCTGACGAA 3') 100 ng, Taq 1 U; $H_2O$ 19.6 μL, and DNA 50 ng. The amplicons were sheared with restriction enzyme BsII (NEB) to distinguish between the Atlantic and the Mediterranean allele. The restriction reaction was performed in a volume of 50 μL with 1X BsII buffer, BsII enzyme 1.5 μL, DNA 7 μL, and $H_2O$ 36.5 μL. The thermal profile of the reaction was 55 °C for 30 minutes. The digested PCR product was analysed by agarose gel electrophoresis.

### 2.5. Microsatellite loci

Microsatellite locus analysis by fluorophore labelling was performed according to the protocol described in Lerceteau-Kohler and Weiss (2006), which includes 9 microsatellite loci. The microsatellites were: -Ssa85 (101−113) -Str60INRA (93−103) -Str73INRA (138−144) -SsoSL417 (169−195) -SsaD190 (115−157) -SsaD71 (183−239) -SSsp2213 (159−229) -OMM1064 (163−286) -Ssa408Uos (205−305). For each microsatellite, the forward primer was labelled with different fluorophore with emission at a wavelength corresponding to the colors in the visible spectrum (190 blue; 71 blue; 2213 green; 1064 yellow; 408 green). Amplification was performed in a volume of 11.5 μL using the following reaction mix: buffer 5X, $MgCl_2$ 1.5 mM, dNTP's 0.2 mM, primer 0.25 μM, Taq 0.5 U; $H_2O$ 3.85 μL, and DNA 50 ng.

### 2.6. Ethics approval statement

The experimental protocol was designed according to the guidelines of the European Union Council 2010/63/EU for the use and care of experimental animals. The animal study protocol was approved by the Institutional Review Board of the Italian Ministry of Health (authorization n. 196/2020-PR).

## 3. Results and discussion

Extraction from the sectioned swabs was effective and with sufficient yield with high quantification values, demonstrating the efficacy of DNA extraction from swabs (Table 1) as already reported by other Authors (i.e., Foley et al., 2011). The mean SD of DNA quantification was 281.84±72.4 ng/mL, indicating that subsequent molecular analysis is likely to be effective. In addition, the ratio of absorbance at 260 nm and 280 nm showed a mean SD of 1.84 ± 0.03, indicating DNA purity.

The three genetic targets we tested to evaluate the effectiveness of the extraction gave good results. Fig. 1 shows the image of the *LDH* nuclear gene amplification. Cutting with restriction enzymes highlights three bands (at 440 bp, 360 bp and 80 bp) which identify the Mediterranean (single band at 440 bp) and the Atlantic lineages (two bands, one at 360 bp and another at 80 bp), and the hybrid individuals (three bands, at 440 bp, 360 bp, and 80 bp). The figure shows two types of restriction patterns: the one characteristic of homozygous Atlantic strain individuals and that characteristic of hybrid individuals. Fig. 2 shows the results of mitochondrial gene amplification by D-LOOP analysis. Fig. 3 shows an example of a microsatellite: for each microsatellite in diploid chromosomal order, the trace consists of two peaks in a range of size characteristic for each locus.

Based on mtDNA, we have done additional analysis to assign a lineage to each specimen; analysis of the nuclear gene *LDH* allowed the identification of hybrid individuals, which cannot be obtained from the D-LOOP due to the matrilineal origin of mtDNA; finally, microsatellites analysis allowed us to assess gene introgression between the populations of interest for conservation purposes.

Rapid non-destructive techniques to study DNA without animal sacrifice are fundamental to conservation genetic studies (Reid et al., 2012; Fernandes and Pedroso, 2017). The swabs are easy to use and can be handled by non-experts. They have a proprietary drying system that rapidly reduces enzyme activity and prevents degradation of nucleic acids and proteins. The DNA contained in the samples can remain stable for years, which is essential for many diagnostic laboratory procedures (Thermo Fisher Scientific Livestock Geno Tube Flyer; www.thermofisher.com).

As mentioned above, the effectiveness of swabbing as a sampling method has been evaluated (Colussi et al., 2017). However, a critical issue, the swab is discarded after processing, unlike biological tissue samples from which multiple extractions can be made. In this study, the method was refined, after a storage of one-and-a-half year; after this period, the swab was divided in three parts. with one aliquot extracted and the other two sections further stored at room temperature. In this way, a biobank can be established for further study or to replicate the analysis in the event of an error, while avoiding the need for further sampling. It also eliminates the need for reagents such as ethanol for preservation. An additional benefit is the cost saving. Long-term storage at room temperature is

**Table 1**
DNA quantification (ng/μL) from extracted swabs and 260/280 ratio.

| Sample | Quantification | 260/280 ratio | Sample | Quantification | 260/280 ratio | Sample | Quantification | 260/280 ratio |
|---|---|---|---|---|---|---|---|---|
| **1** | 79 | 1.83 | **35** | 344 | 1.89 | **69** | 380 | 1.84 |
| **2** | 705.5 | 1.87 | **36** | 271 | 1.89 | **70** | 584 | 1.83 |
| **3** | 283 | 1.86 | **37** | 273.5 | 1.89 | **71** | 108 | 1.89 |
| **4** | 689 | 1.86 | **38** | 252.5 | 1.85 | **72** | 200 | 1.89 |
| **5** | 218.5 | 1.89 | **39** | 224 | 1.89 | **73** | 562.5 | 1.83 |
| **6** | 223.5 | 1.76 | **40** | 161 | 1.83 | **74** | 309.5 | 1.81 |
| **7** | 460 | 1.83 | **41** | 265 | 1.85 | **75** | 378 | 1.86 |
| **8** | 291.5 | 1.84 | **42** | 169.5 | 1.85 | **76** | 403 | 1.86 |
| **9** | 197.5 | 1.84 | **43** | 469 | 1.89 | **77** | 422 | 1.87 |
| **10** | 259 | 1.89 | **44** | 194 | 1.89 | **78** | 311.5 | 1.88 |
| **11** | 263.5 | 1.81 | **45** | 350 | 1.87 | **79** | 177 | 1.88 |
| **12** | 517.5 | 1.89 | **46** | 308.5 | 1.84 | **80** | 312.5 | 1.82 |
| **13** | 132.5 | 1.82 | **47** | 413 | 1.82 | **81** | 182.5 | 1.76 |
| **14** | 298.5 | 1.86 | **48** | 370 | 1.84 | **82** | 542 | 1.84 |
| **15** | 166.5 | 1.75 | **49** | 149.5 | 1.89 | **83** | 244.5 | 1.83 |
| **16** | 543.5 | 1.83 | **50** | 143 | 1.74 | **84** | 334.5 | 1.89 |
| **17** | 258.5 | 1.89 | **51** | 175 | 1.78 | **85** | 355 | 1.89 |
| **18** | 390.5 | 1.85 | **52** | 206.5 | 1.78 | **86** | 140 | 1.81 |
| **19** | 156.5 | 1.87 | **53** | 362 | 1.87 | **87** | 102.3 | 1.85 |
| **20** | 342.5 | 1.891 | **54** | 643 | 1.82 | **88** | 145 | 1.78 |
| **21** | 85.5 | 1.80 | **55** | 116.8 | 1.82 | **89** | 236 | 1.82 |
| **22** | 168.5 | 1.80 | **56** | 357.5 | 1.88 | **90** | 369.5 | 1.83 |
| **23** | 115 | 1.85 | **57** | 205 | 1.89 | **91** | 305.5 | 1.85 |
| **24** | 211 | 1.75 | **58** | 229 | 1.76 | **92** | 199.5 | 1.78 |
| **25** | 312.5 | 1.89 | **59** | 146 | 1.81 | **93** | 253.5 | 1.89 |
| **26** | 201 | 1.76 | **60** | 289 | 1.84 | **94** | 173 | 1.84 |
| **27** | 144 | 1.89 | **61** | 166 | 1.79 | **95** | 175 | 1.88 |
| **28** | 291.5 | 1.81 | **62** | 296.5 | 1.89 | **96** | 277 | 1.82 |
| **29** | 439 | 1.82 | **63** | 149.5 | 1.88 | **97** | 118 | 1.88 |
| **30** | 316.5 | 1.88 | **64** | 172 | 1.85 | **98** | 450.5 | 1.88 |
| **31** | 129,5 | 1.87 | **65** | 229.5 | 1.82 | **99** | 298 | 1.85 |
| **32** | 186 | 1.82 | **66** | 593.5 | 1.83 | **100** | 381 | 1.84 |
| **33** | 102,5 | 1.83 | **67** | 185 | 1.89 | | | |
| **34** | 191 | 1.85 | **68** | 504 | 1.87 | | | |

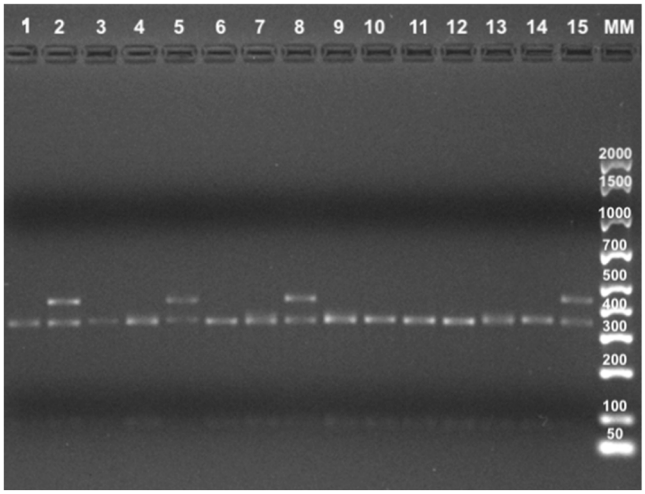


**Fig. 1..** *LDH* nuclear gene amplification. Two types of restriction pattern are present: the pattern characteristic of homozygous Atlantic strain individuals and the pattern characteristic of hybrid subjects. 1–15= samples; MM= DNA Molecular Weight Marker (50–2000 pb).

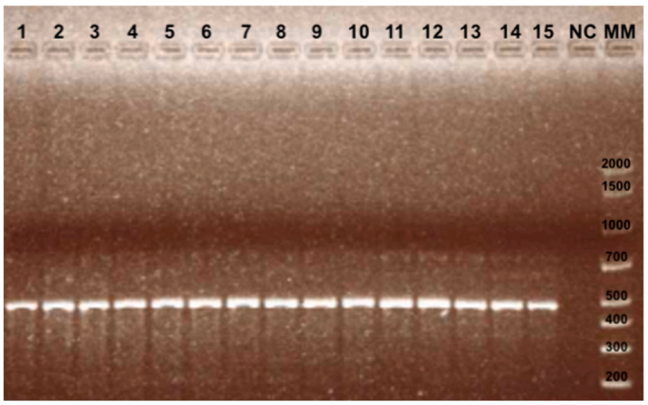


**Fig. 2.** Mitochondrial gene amplification: D-LOOP. All samples showed the presence of the D-LOOP gene. 1–15= samples; NC=negative control; MM= DNA Molecular Weight Marker.

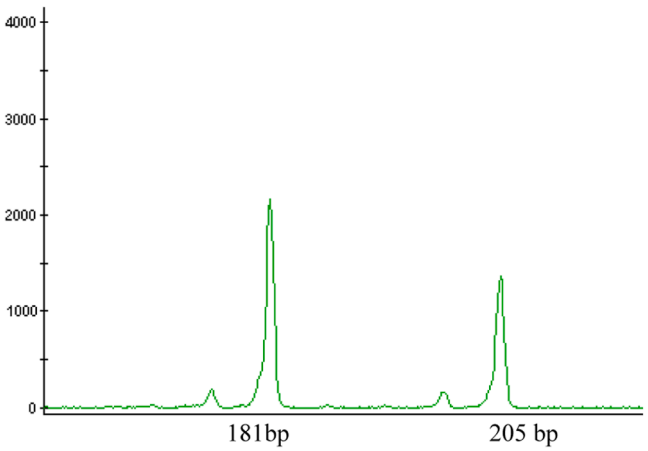


**Fig. 3.** Microsatellite image: SSsp2213 (size range 159–229).

convenient because the swabs can be transported without temperature-related problems and swabbing can be done directly in the field, even in areas distant far from the analytical laboratory.

## 4. Conclusions

Buccal swabs designed for the extended storage of DNA at room temperature can prove to be a valuable tool for laboratory procedures and conservation studies. This paper specifically examines the performance of the Livestock Geno Tube, but various other swabs for long-term DNA storage, manufactured by different companies, are readily available in the market. The establishment of a biobank containing biological material through a minimally invasive method is crucial for preserving scientific knowledge. However, additional research is required to assess the performance and applicability of different swabs for conservation purposes. Specifically, standardizing swabbing techniques, validating accuracy across species, and optimizing for diverse environments are essential steps to establish swabbing as a promising alternative for studies on fish population genetics. Considerations such as ethical implications, cost-effectiveness, and collaboration with fisheries agencies play a significant role in enhancing the credibility of this method. By addressing these aspects, swabbing emerges as a humane, efficient, and cost-effective approach to advance research in fish population genetics, with the potential for seamless integration into routine assessments and monitoring programs.

## CRediT authorship contribution statement

**Simona Sciuto:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Sivia Colussi:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Giuseppe Esposito:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Arianna Meletiadis:** Writing – review & editing, Methodology, Investigation. **Marino Prearo:** Writing – review & editing, Methodology, Investigation. **Elisabetta Pizzul:** Writing – review & editing, Methodology, Investigation. **Pier Luigi Acutis:** Writing – review & editing, Methodology, Investigation. **Rodolphe Elie Gozlan:** Writing – review & editing, Investigation, Conceptualization. **Paolo Pastorino:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

## Acknowledgement

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