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Coral-ID: A forensically validated genetic test to identify precious coral material and its application to objects seized from illegal traffic

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ABSTRACT

The production and trade of objects manufactured from the skeletal axis of coralid precious corals is a historically, culturally and economically important global industry. Coralids are members of the diverse Coralliidae family, which contains several species complexes and morphospecies. For most precious coral found in the jewelry trade, the color remains the sole clue and link to the taxonomic identity of the individual. Different coralid species have however similar or overlapping colors resulting in difficulty to taxonomically identify jewelry objects, including four species listed by the Convention on the International Trade of Endangered Species (CITES) whose international transport and trade requires species-specific and country of origin documentation. We aimed at developing a reliable method to taxonomically identify coralid material with the objective of distinguishing CITES protected species from their non-protected counterparts. We present *Coral*-ID, a genetic assay to taxonomically classify coralid objects using quasi non-destructive sampling. The assay classifies the analyzed sample in one of six taxonomic categories and performs at least presumptive separation of CITES-listed and non-listed species in all cases. Developmental validation experiments prove that *Coral*-ID is a specific, accurate and very sensitive method. As the first attempt to randomly sample corals in the trade to identify them, we applied *Coral*-ID on 20 precious coral objects seized by custom authorities upon import to in Switzerland. Thirteen (65%) of these samples could be analyzed; three of these were found to be presumptively CITES-listed, and 10 of them have proven to originate from non-CITES-listed species.

1. Introduction

The shiny and colorful polished calcite skeleton of precious corals is among the most valuable products derived from living organisms. Coralid corals (members of the Coralliidae, Octocorallia) have been used to produce luxury products since antiquity [1–3]. Although coralid colonies are distributed in seabeds worldwide, their commercial harvest is centered in three specific regions: the Mediterranean (Mediterranean Sea and East-Atlantic), the West Pacific Ocean (mainly the waters surrounding Japan and Taiwan) and the North Pacific (Hawaiian Islands and the Emperor Seamount chain) [4]. The yearly quantity of the precious coral landings in the past decade was estimated around 70 tons with the value of the raw harvested precious corals reaching 500 million € [5,6].

Until recent decades, the beds of the slow-growing precious corals were fished unselectively and uncontrolledly, which has led to depletion of some of their populations [7,8]. With the recognition of the unsustainable nature of the coral harvesting starting from the 1970s, local fishing regulations were put in place to ensure more controlled fishing practices for precious corals. These include a variety of measures made by local authorities including specifying allowed harvesting areas and dates, setting fishing quotas and restricting allowed fishing methods and minimal coral sizes [9–12]. In parallel, a debate began whether these actions are sufficient to achieve a sustainable precious coral jewelry industry, or if restrictions of their trade should also be envisaged [13–15]. The monitoring and control of the trade of vulnerable and

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Table 1
The six taxonomic groups distinguishable by the *Coral*-ID assay. Note that species in the species complexes cannot be differentiated based on mitochondrial markers. Species used in the trade according to [4] are marked with asterisk.

Taxonomic group	Species within group	CITES-listed	Primary color of skeletal axis	Distribution area
Corallium rubrum	*C. rubrum* *	No	uniform red to deep orange	Mediterranean Sea, North-East Atlantic [5]
***Corallium japonicum* species complex**	*C. japonicum* *	Yes	dark red with white center [29]	Japan, Taiwan [30]
	C. nix	No	dark red or pink, white center, white tip [31,32]	New Caledonia [31]
	C. tortuosum	No	pale pink [33], white-transparent [26]	Hawaiian Islands [33], New Caledonia [26], Taiwan [26]
Hemicorallium	*H. abyssale*	No	pale pink, darker center [33]	Hawaiian Islands [33]
	H. aurantiacum	No	pale pinkish – orange [26]	New Caledonia [26]
	H. bathyrubrum	No	deep pink to red [34]	North-West Atlantic [34]
	H. bayeri	No	white [34]	North-West Atlantic [34]
	H. ducale	No	dark pink [35]	East-Pacific [35]
	H. guttatum	No	milk white [26]	Hawaiian Islands [26]
	H. imperiale	No	rich pink [35]	East-Pacific [35]
	H. laauense *	No	white [33]	Hawaiian Islands [33], Emperor Seamount [4]
	H. niobe	No	white [36]	Western Atlantic [36]
	H. regale *	No	pale pink [33]	Hawaiian Islands [33]
	H. sulcatum *	No	pink [26]	Taiwan, Japan [5], Philippines [4]
***Pleurocorallium elatius* species complex**	*P. elatius* *	Yes	red to pink with white center [29], orange [37]	Taiwan, Japan, Vietnam [5]
	P. konojoi *	Yes	milky white, pinkish center [37]	Japan, Taiwan, Vietnam [5]
	P. carusrubrum *	No	crimson, orange [26,37]	Taiwan [37]
Pleurocorallium secundum	*P. secundum* *	Yes	pale pink, often almost white [33]	Hawaiian Islands [33], Taiwan [38]
other *Pleurocorallium*	*P. bonsaiarborum*	No	pure white - transparent [26]	New Caledonia [26]
	P. borneense	No	white with pink center [39]	Malaysia [39]
	P. clavatum	No	white [26]	New Caledonia [26]
	P. inutile	No	white [29]	Japan [29]
	P. porcellanum	No	white [29]	Hawaiian Islands [29]
	P. niveum *	No	white [33]	Hawaiian Islands [33]
	P. norfolkicum	No	white [26]	New Caledonia [26]
	P. thrinax	No	white [31]	New Caledonia [31]

endangered species is implemented by The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), an agreement signed and regularly updated by the parties of the United Nations. The trade regulations are codified by national legislation and implemented by local law enforcement authorities. The first attempt to list the historically most abundantly fished precious coral species, *Corallium rubrum*, in CITES was made by Spain in 1987 but was voted against by the CITES parties [16]. Similarly, the later proposals to include the *Corallium* genus (by the USA in 2007) and the entire Coralliidae family (by the USA and the EU in 2010) in CITES Appendix II, were both rejected [17,18]. China, however added four species to CITES Appendix III in 2008, which is in effect since then [19]. These four species are *C. japonicum*, *C. elatius*, *C. konojoi* and *C. secundum*, of which the latter three have subsequently been suggested to belong to the *Pleurocorallium* genus [20,21]. This means that the international trade of any specimen or part of these four species requires a certificate of origin issued by the management authority of the exporting country for customs clearance if it is to be legally traded at an international level.

To implement CITES regulations, the proper identification of the species plays a key role. Traditionally, the identification and separation of the coralid species has relied on microscopic examination of specific morphologic traits. These traits get lost in processed (i.e. cut, carved and polished) objects, and although still easily distinguishable from imitations and other coral groups, the morphological species identification of precious corals often becomes difficult or even impossible [3,22]. A reliable method of species identification would therefore be essential for effective implementation of CITES regulations [5,23].

The technical study of Lendvay et al. [24] demonstrated the presence of DNA preserved in the internal skeletal axis of precious corals. These authors concluded that genetic testing of precious coral jewelry objects may be a powerful tool for taxonomic identification of coral jewelry when using optimized laboratory techniques combined with a "quasi non-destructive" sampling method that keeps the market value of the objects.

The Coralliidae corals form a phylogenetically diverse family containing species complexes, morphospecies and cryptic species and the mitochondrial genomes of precious coral species are exceptionally similar to each other in comparison to species of other groups of animals [20,21]. Within the *Corallium* genus, *Corallium rubrum* is genetically distinguishable from the other species of the genus, however *Corallium japonicum* (CITES-listed) forms a genetically unresolved species complex with *Corallium nix* and *Corallium tortuosum* [21]. The species of the *Hemicorallium* genus are genetically similar and the species cannot be confidently delimited by mitochondrial markers [5]. Within the *Pleurocorallium* genus, *Pleurocorallium secundum* (CITES-listed) can be well distinguished from the other species, as well as the *Pleurocorallium elatius* species complex, which however contains three species; *Pleurocorallium elatius* (CITES-listed), *Pleurocorallium konojoi* (CITES-listed) and *Pleurocorallium carusrubrum*, whose separation based on mitochondrial markers is not feasible [21]. Given the phylogenetic limitations outlined above, it is possible to distinguish six categories of coralids: *Corallium rubrum*, *Corallium japonicum* species complex, *Hemicorallium*, *Pleurocorallium secundum*, *Pleurocorallium elatius* species complex and the group of all other *Pleurocorallium* species apart from the latter two groups, hereinafter referred to as "other *Pleurocorallium*" (Table 1).

In the current study, we aimed at further developing the methodology for genetic testing of precious coral objects. Our intention is to develop a single genetic marker with maximal resolution power to distinguish precious coral species with an emphasis on differentiating CITES-listed from non-CITES-listed species. The newly designed marker is a short fragment, located on the mitochondrial genome to ensure the high sensitivity of the marker and its applicability to degraded DNA.

We determine a framework for execution of the wet lab procedures and data analysis protocols and call the defined system *Coral*-ID. We perform a thorough developmental validation of *Coral*-ID by following the validation criteria recommended by the Society of Wildlife Forensic Science [25]. Specifically, we test *Coral*-ID's phylogenetic resolution, sensitivity, specificity, accuracy, precision and resilience to contamination. Finally, we implement *Coral*-ID on a set of 20 precious coral objects confiscated by the Swiss customs authorities due to the lack of valid CITES documentation and discuss the performance of *Coral*-ID on real-world samples.

2. Materials and methods

2.1. Coralid species

The Coralliidae family contains 45 described species in the three genera, *Corallium*, *Hemicorallium* and *Pleurocorallium* [26,27]. Eleven species are estimated to be present in the commercial trade, however there is no consensus on the exact number of species used in jewelry [4,5,24]. The color of the skeletal axis of some species is known to be highly variable. As two examples of commercially important species, *C. japonicum* can be dark blackish red to pale whitish red, whiles *P. elatius* can vary from bright red and salmon to orange and flesh color [5,28].

2.2. Development of the taxonomic identification marker

The single-locus *Coral*-ID marker was designed based on the most complete phylogenetic and taxonomic data available of coralids [21,26]. The DNA sequence data generated by Tu et al. [21] was downloaded from NCBI GenBank and was used to design the marker. This data set contained the DNA sequences of six mitochondrial regions (16S mt-rRNA, mtND1, 16S mt-rRNA – mtND2, mtND3 – mtND6, mtND6 – mtCOI and mtMutS) pertaining 109 Coralliidae individuals. We performed a prior phylogenetic screening of the downloaded DNA sequences to check the expected monophyletic clustering of the individuals and to discover any potentially mislabeled samples. This screening identified that each DNA sequence of one individual ("*Hemicorallium* sp1_EP_3″) clustered together with the *Pleurocorallium* sequences, therefore the data of this likely mislabeled individual were omitted from further processes. The sequences of all other individuals were assigned to one of the six taxonomic groups of Table 1 and a consensus DNA sequence was generated for each mitochondrial gene of each group. These consensus sequences, hereon group consensus sequences, were generated by considering all existing nucleotide variations within the six groups. The number of nucleotide differences between the taxonomic groups was evaluated on a 150 base pairs (bp) sliding window on the six mitochondrial regions (altogether 3650 bp). The final region was then selected by choosing a region where at least two mismatches were present between the members of any to species. Primers were designed flanking a fragment of the mitochondrial DNA mismatch repair protein (mtMutS) gene with the length of a 149 bp in all coralids. Degenerate bases were inserted into the primers to assure complete match to the priming sites of all sequences. The designed primers *Coral*-ID-F 5′-TACGYTCATAAATTATHCCT-3′ and *Coral*-ID-R 5′-AGATTTGCCATGGYACAGAA-3′ flank the nucleotide positions 14,802 and 14,950 of the *Corallium rubrum* complete mitochondrial reference genome (GenBank ID NC_022864).

2.3. Laboratory and data analysis protocols of the Coral-ID assay

The primer pair produced a single band on agarose gels when using annealing temperatures ranging from 50 °C to 62 °C. PCR reactions were performed in 25 μl final volume containing 1 × Qiagen Multiplex PCR Master Mix (Qiagen) and 0.4 μM of the forward and reverse primers, respectively. Input volume of the template DNA was set to 5 μl in the validation experiments. This relatively large volume was set as standard to minimize pipetting errors. The same volume was used for DNA extracts of the confiscated precious coral objects, which was subsequently elevated to 8 μl in the lack of initial amplification. Thermal cycling

Table 2
The number of reference samples used in this study and the diversity within the six coralid taxonomic groups.

	Number of reference individuals			Number of unique genotypes	Mean number of pairwise differences within group	Gene diversity
	From literature	From this study	Total number			
Corallium rubrum	15	47	62	1	0	0
Corallium japonicum species complex	10	91	101	1	0	0
Hemicorallium	46	17	63	3	0.313	0.063
Pleurocorallium elatius species complex	38	11	49	1	0	0
Pleurocorallium secundum	3	4	7	1	0	0
other *Pleurocorallium*	35	3	38	12	3.788	0.876
Total number	147	173	320			

commenced with enzyme activation at 95 °C for 10 min followed by 50 cycles of denaturation 94 °C for 45 s, 53 °C annealing for 1 min 30 s and elongation at 72 °C for 45 s, and a final elongation of 72 °C at 10 min was applied. PCR amplicons were purified with the AmPure XP bead system (Beckman Coulter) and quantified with Qubit fluorimeter device (Invitrogen). 0.25–1 ng of the resulting PCR product was sequenced using the BigDye terminator 1.1 cycle sequencing kit (Applied Biosystems) on an ABI3130xl instrument (Applied Biosystems) on both DNA strands. Raw sequences were processed using Geneious 11.1.5 (https://www.geneious.com) in an automated procedure for speedy and unbiased analysis of the raw sequencing data. Forward and reverse sequences were assembled with trimming primer sequences and the sequence ends containing bases with more than 5% probability of erroneous base calling and a consensus sequence was generated.

The taxonomic classification of the consensus sequence was performed with the Classify Sequences plugin in Geneious using a reference data set consisting of the six mtMutS gene group consensus sequences. The classification algorithm was set to allow classification only if the query sequence is complete and a single mismatch compared to the reference sequence was allowed. This permits classification of variants with single mismatches that may occur due to postmortem DNA damages (typically cytosine to thymine changes) or if the query sequence belongs to a previously undiscovered sequence variant. Theoretically, DNA sequences could exist that are equally one mismatch distant from reference sequences of two separate groups, and whose classification would thus result in being assigned into two groups. Such double assignments were not allowed and considered unsuccessful classification. The DNA extract of each analyzed coral sample was analyzed with the *Coral*-ID assay in two replicates. The results were approved and accepted only if the same classification was obtained by both analyses.

2.4. Characterization of the marker polymorphism

In order to accurately characterize the polymorphism of the *Coral*-ID marker within and between the six predefined coralid taxonomic groups, the sequence data of Tu et al. [21] was complemented with additional available sequence data of peer-reviewed scientific literature and a large set of newly generated sequence data from voucher specimens (Table 2, for details see Supporting Table 1). DNA of voucher specimens was isolated from alcohol-stored polyps using the QIAamp DNA Mini kit (Qiagen) and a 624 bp long fragment of the mtMutS gene was amplified and sequenced using the primers MSH-Co-F and MSH-Co-R as described by Tu et al. [21]. The obtained DNA sequences contained the locus of the *Coral*-ID marker together with its primer sites. The complete reference data set contained 320 coralid sequences of which 147 were obtained from former studies and 173 were generated for this study. All DNA sequence data of the voucher samples with their pertaining metadata were deposited in the NCBI GenBank (see Supporting Table 1).

To characterize the inter-group diversity of the six defined taxonomic groups, we calculated the minimal difference between any two species of two different groups. The intra-group diversity was described as number of unique genotypes, mean number of pairwise differences within group and gene diversity. All these calculations were performed in Arlequin 3.5 [40].

2.5. Assay specificity tests

The specificity of *Coral*-ID was tested using DNA of 38 non-coralid octocorals covering a wide range of taxonomic groups (see Supporting Table 2). DNA of each sample was amplified and sequenced with the primers MSH-Co-F and MSH-Co-R. In case this pair of primer did not produce amplicons, then alternatively the MSH-Co2-F 5′-AYAAGGGRATGYTAATAYTYTC-3′ and MSH-Co2-R 5′-KAGNRTTATATTTAGARGGRCG-3′ or MSH-Co3-F 5′-GGTHGAAATGAGAGATCTYTC-3′ and MSH-Co3-R 5′-CCCATWACTTCRATGCCATA-3′ primer pairs were used with identical PCR conditions. The resulting DNA sequences were mapped to the mitochondrial reference genome of *C. rubrum* (GenBank ID NC_022864) and the polymorphisms of the *Coral*-ID-F and *Coral*-ID-R priming sites were documented. The DNA sequence data of the non-coralid octocoral samples with their pertaining metadata were deposited in the NCBI GenBank (see the Supporting Table 2).

At the same time, the *Coral*-ID assay was performed on each of the 38 non-coralid samples in triplicates using 0.5–1 ng DNA input. The minimal difference of the non-coralid DNA sequences from any reference sequence of each of the six coralid groups was calculated in Arlequin.

2.6. Assay sensitivity test

The recent study by Lendvay et al. [24] has shown that the number of short mitochondrial DNA copies recovered following a minimally destructive sampling of precious coral objects spans over the range of 1–10^4. We tested the sensitivity of the *Coral*-ID assay using DNA of *C. rubrum*, *C. japonicum* and *P. elatius*. These three species were selected because they represent three different variants of the *Coral*-ID-R priming site and are often used in jewelry. To adjust potential individual variations of DNA purity, a DNA cocktail was prepared for each of the three species by pooling DNA of 3–5 samples with known identical mtMutS sequences. Then, absolute quantification of a 172–198 bp long fragment of the mitochondrial ribosomal large subunit RNA gene was carried out using quantitative real-time PCR technology as in agreement with the MIQE guidelines [41] as described by Lendvay et al. [24]. Following the quantification, each of the three DNA cocktails were serially diluted to 10^4, 10^3, 10^2 and 10 copies per 5 µl volume. We performed PCR amplification with the *Coral*-ID primers for each of the three species with the four dilutions 10 times. The amplification success was tested by

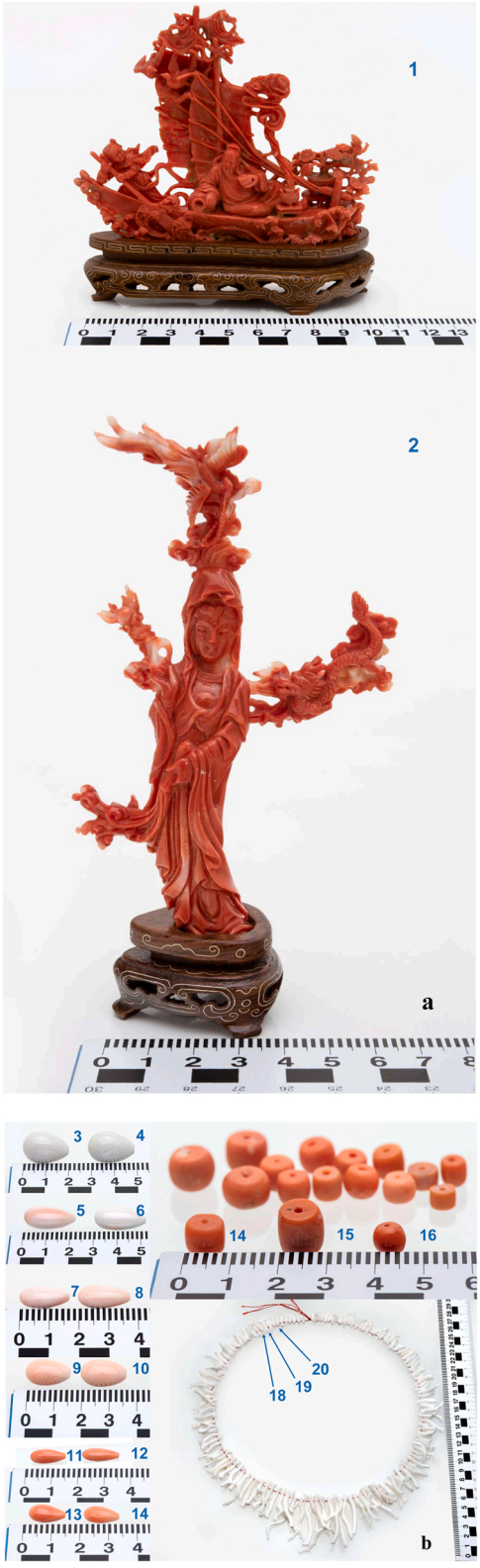

Fig. 1. The precious coral objects analyzed with the *Coral*-ID assay. All objects have been seized by Swiss customs authorities. Sampling was performed either from the bottom of the objects (a) or from the inner surface of existing drill-holes (b). Blue numbers indicate each object sampled for this study. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

GelRed (Biotium) stained agarose gel electrophoresis. In advance, a test of the detection limit of the electrophoresis system proved that 0.025 ng DNA, the lower limit of DNA input in the sequencing reaction that can produce clear sequence results, still produces visible bands. The number of successful amplifications were counted and to confirm that the PCRs indeed produced the required *Coral*-ID amplicons, the PCR products generated with 10 template copies were sequenced.

2.7. Assay accuracy tests using low copy number samples

We tested the accuracy of the *Coral*-ID assay by repeating it 10 times using 10 copies of DNA molecules and comparing the results for consistency. The test was carried out with 10 independently prepared template DNA dilutions of each of *C. rubrum*, *C. japonicum* and *P. elatius*, respectively as described in the Assay sensitivity test section. For each of the three species, the resulting 10 DNA sequences were aligned and checked for variation.

2.8. Assay precision tests using case-type samples

Precision of the *Coral*-ID assay was tested as the degree of agreement among results of a series of individual measurements. We assessed precision by analyzing each of 10 case-type precious coral jewelry samples 10 times with the *Coral*-ID assay. All 10 case-types test samples originated from the coral collection of the Swiss Gemmological Institute SSEF and were analyzed previously by Lendvay et al. [24]. In their study, moderate levels of DNA – ranging from c. 250–750 mtDNA copies per µl of the 172–198 bp long fragment of the mitochondrial ribosomal large subunit RNA gene – was measured in each of these samples. Each DNA sample was diluted 5 times before using 5 µl as PCR template to retain the formerly measured moderate DNA amount as PCR template. For each of the 10 samples, the results of the 10 assay replicates were checked for consistency.

2.9. Assay resilience to external contamination

Cross-contamination between coral samples e.g. from carry-over during sample preparation or contamination by human DNA can compromise the accurate interpretation of the results. To assess the resilience of *Coral*-ID to contamination, we performed *Coral*-ID assay using mixtures of known quantity DNA samples.

DNA mixtures containing two species with known DNA concentrations were prepared using pairs of DNA samples of *C. rubrum, C. japonicum* and *P. elatius* following their absolute quantification as described in the Assay sensitivity test section. *Coral*-ID was performed with mixed samples using combinations of 10:1, 100:1 and 1000:1 ratio DNA inputs in each six potential pairwise combination of the three DNA samples in triplicates. To test for the influence of human DNA on the performance of *Coral*-ID, 100 copies of each of the three DNA cocktails were amplified in the presence of 1 ng, 0.1 and 0.01 ng human DNA (AmpFℓSTR DNA Control 007, Applied Biosystems), respectively.

2.10. Application of the Coral-ID assay to seized precious coral objects

We applied the *Coral*-ID assay on a set of 20 items of precious coral jewelry and decoration objects seized by Swiss customs authorities (Fig. 1) to evaluate the performance of the *Coral*-ID assay on real-world samples. The objects were seized in five cases, due to the lack of valid CITES documentation during their import to Switzerland in five different years between 2009 and 2017. The samples were obtained from the Federal Food Safety and Veterinary Office of Switzerland (FSVO, Berne). Each object has a claimed taxon name, which were those used by the importers. Alternatively, if the objects lacked import documentation, then they were registered as *Corallium sp.* in the FSVO documentation.

Sampling of the objects and DNA extraction was carried out as

Table 3
Minimum number of nucleotide differences between any two members of the six coralid taxonomic groups.

	Corallium rubrum	*Corallium japonicum* species complex	*Hemicorallium*	*Pleurocorallium elatius* species complex	*Pleurocorallium secundum*	other *Pleurocorallium*
Corallium rubrum						
Corallium japonicum species complex	2					
Hemicorallium	5	3				
Pleurocorallium elatius species complex	8	6	5			
Pleurocorallium secundum	7	5	6	7		
other *Pleurocorallium*	6	4	5	2	4	
Related non-coralid octocorals	5	5	7	9	9	7

Table 4
Variable positions of the *Coral*-ID priming sites of coralids and related octocorals. Each sample was analyzed three times with the *Coral*-ID assay. Filled boxes indicate the number of successful tests. Asterisk indicates amplification of unspecific PCR products.

		Coral-ID	Forward primer nucleotide bases 5` - 3`																			Reverse primer nucleotide bases 3` - 5`																			
			20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	Primer sequence		A	G	A	T	T	T	G	C	C	A	T	G	G	Y	A	C	A	G	A	A	A	G	G	D	A	T	A	A	T	T	T	A	T	G	A	R	C	G	T	A
Coralids	*Corallium rubrum*	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Corallium japonicum species complex	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	G	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Hemicorallium	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Pleurocorallium elatius species complex	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	G	C	G	T	A
	Pleurocorallium secundum	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	G	C	G	T	A
	other *Pleurocorallium*	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	Y	A	C	A	G	A	A	A	G	G	D	A	T	A	A	T	T	T	A	T	G	A	G	C	G	T	A
Non-coralid octocorals	*Sibobagorgia* sp.	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Paragorgia sp. 1	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	D	A	T	A	A	T	T	T	A	T	G	A	R	C	G	T	A
	Paragorgia sp. 2	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	G	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Paragorgia sp. 3	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Paragorgia sp. 4	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Paragorgia sp. 5	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Paragorgia arborea	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Paragorgia sp. 6	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Paragorgia sp. 7	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Paragorgia sp. 8	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	T	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Paragorgia sp. 9	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	T	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Paragorgia sp. 10	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	T	A	C	A	G	A	A	A	G	G	T	T	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Calcigorgia spiculifera	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	G	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Isididae sp. 1	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	G	C	G	T	A
	Isididae sp. 2	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	G	C	G	T	A
	Isididae sp. 3	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	G	C	G	T	A
	Isidella sp.	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	G	C	G	T	A
	Keratoisis sp.	■■□	A	G	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	G	C	G	T	A
	Primnoa pacifica	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	G	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Narella sp.	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	G	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Plumarella longispina	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	C	G	G	G	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Chrysogorgia sp.	■■■	A	G	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	T	T	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Fanellia compressa	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	T	T	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Distichoptilum gracile	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	G	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Pennatula phosphorea	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	A	A	C	A	G	A	A	A	G	G	G	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Halipteris sp.	■■□	A	A	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	G	C	G	T	A
	Anthoptilum grandiflorum	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Funiculina quadrangularis	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	T	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Thouarella cristata	■■■	A	A	A	T	T	T	G	C	C	A	T	G	G	G	A	C	A	G	A	A	A	G	G	G	A	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Gersemia sp. 1	□□□	A	A	A	T	T	T	G	C	C	A	T	G	G	A	A	C	A	G	A	A	A	G	G	G	C	C	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Gersemia sp. 2	□□□	A	A	A	T	T	T	G	T	C	A	T	G	G	A	A	C	A	G	A	A	A	G	G	G	C	C	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Swiftia kofoidi	***	A	A	A	T	T	T	G	T	C	A	T	G	G	A	A	C	A	G	A	A	A	G	G	G	C	C	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Swiftia simplex	***	A	A	A	T	T	T	G	T	C	A	T	G	G	A	A	C	A	G	A	A	A	G	G	G	C	C	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Swiftia pacifica	***	A	A	A	T	T	T	G	T	C	A	T	G	G	A	A	C	A	G	A	A	A	G	G	G	C	C	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Swiftia spauldingi	□□□	A	A	A	T	T	T	G	T	C	A	T	G	G	A	A	C	A	G	A	A	A	G	G	G	C	C	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Adelogorgia cf. *phyllosclera*	□□□	A	A	A	T	C	T	G	T	C	A	T	G	G	A	A	C	A	G	A	A	A	G	G	G	C	C	T	A	A	T	T	T	A	T	G	A	A	C	G	T	A
	Acanthogorgia sp.	**	A	A	A	T	T	T	G	T	C	A	T	G	G	A	A	C	A	G	A	A	A	T	G	G	C	C	T	A	A	T	C	T	A	T	G	A	A	C	G	T	A
	Eugorgia rubens	***	A	A	A	T	T	T	G	T	C	A	T	G	G	A	A	C	A	G	A	A	A	G	G	G	C	C	T	T	A	T	T	T	A	T	G	A	A	C	G	T	A

described by Lendvay et al. [24]. Briefly, minimally destructive sampling was applied using an electric drill following thorough decontamination of the utensils and the objects' surfaces. Powder sample was taken from the bottom of the object of two larger carvings (Fig. 1a), whereas powder was taken from the existing drill-holes of the other 18 samples (Fig. 1b). Maximum five PCR attempts were carried out and only those samples were further analyzed that produced PCR amplicons in two occasions as checked on GelRed stained agarose gels. Strict cleaning of the laboratory utensils and inclusion of blank controls at all stages of the laboratory work were applied to avoid contamination and ensure the authenticity of the results [cf. 24].

3. Results

3.1. Marker polymorphism

Each of the four taxonomic groups *Corallium rubrum*, *Corallium japonicum* species complex, *Pleurocorallium elatius* species complex and

Table 5
Precision of the Coral-ID assay was evaluated by performing the *Coral*-ID assay ten times on ten case-type test samples with moderate DNA content.

Case-type test sample		DNA copies	Identified group	Similarity to group in 10 replicates									
				1	2	3	4	5	6	7	8	9	10
I		259	other *Pleurocorallium*	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
II		350	other *Pleurocorallium*	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
III		536	*Pleurocorallium elatius* species complex	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
IV		753	*Hemicorallium*	99.3%	99.3%	99.3%	99.3%	100%	99.3%	100%	100%	100%	100%
V		349	*Hemicorallium*	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
VI		257	*Corallium rubrum*	99.3%	99.3%	99.3%	99.3%	99.3%	99.3%	99.3%	99.3%	99.3%	99.3%
VII		464	*Corallium japonicum* species complex	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
VIII		625	*Corallium rubrum*	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
IX		406	*Corallium japonicum* species complex	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
X		680	*Hemicorallium*	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

Table 6
Characteristics of the 20 seized precious coral items analyzed using the *Coral*-ID assay. The objects originate from five shipments seized by the Swiss customs authorities in five different years.

Object	Year seized	Claimed taxon	Weight of item (g)	Sample weight (mg)	Group identified with *Coral*-ID
1	2017	*Corallium sp.*	78.14	24.4	*Pleurocorallium elatius* species complex
2	2017	*Corallium sp.*	43.00	18.2	*Pleurocorallium elatius* species complex
3	2013	*Corallium konojoi*	5.60	10.4	no result
4	2013	*Corallium konojoi*	5.33	14.0	no result
5	2013	*Corallium konojoi*	3.34	12.1	no result
6	2013	*Corallium konojoi*	3.27	15.2	no result
7	2013	*Corallium konojoi*	1.30	6.8	*Hemicorallium*
8	2013	*Corallium konojoi*	1.41	6.9	no result
9	2013	*Corallium konojoi*	1.95	5.7	other *Pleurocorallium*
10	2013	*Corallium konojoi*	2.06	5.1	other *Pleurocorallium*
11	2009	*Corallium elatius*	0.78	9.8	*Hemicorallium*
12	2009	*Corallium elatius*	0.80	8.8	*Hemicorallium*
13	2009	*Corallium elatius*	0.99	5.6	*Hemicorallium*
14	2009	*Corallium elatius*	0.79	15.9	no result
15	2010	*Corallium sp.*	1.70	8.5	*Corallium rubrum*
16	2010	*Corallium sp.*	1.02	12.0	*Corallium rubrum*
17	2010	*Corallium sp.*	0.48	8.2	*Corallium rubrum*
18	2015	*Corallium sp.*	0.61	6.2	no result
19	2015	*Corallium sp.*	0.88	5.3	other *Pleurocorallium*
20	2015	*Corallium sp.*	0.49	4.4	*Pleurocorallium elatius* species complex

Pleurocorallium secundum had a single *Coral*-ID marker DNA sequence, respectively. Multiple genotypes exist in the *Hemicorallium* and "other *Pleurocorallium*" group (Table 2). Any two specimens from two separate groups have at least two mismatches (Table 3). The difference compared to *Paragorgia* and *Sibobagorgia* species, the members most closely related to the coralids, is at least five bases.

3.2. Assay specificity

The mtMutS gene used in the *Coral*-ID assay is specific to the octocoral class of the Anthozoa phylum. We therefore tested whether the *Coral*-ID assay performs with a range of off-target, non-coralid octocorals. We sequenced a longer stretch of the mtMutS gene of 38 non-coralid octocorals to characterize the fit of their priming sites used for the *Coral*-ID assay. Then, we performed the *Coral*-ID assay for these species in triplicates. 29 DNA extracts amplified well in two or three replicates and gave clear sequence results with the *Coral*-ID assay (Table 4). These samples had priming sites with maximal three mismatches to the primers that were at least five bases distant from the 3′ end of the priming site with one exception (*Plumarella longispina*, see Table 4). Nine phylogenetically more distant samples did not amplify at all with the *Coral*-ID assay or their sequence did not align to the homologous mtMutS gene sequence. All of these samples had mismatches at three out of the five nucleotides closest to the 3′ priming site of the reverse primer.

3.3. Assay sensitivity

We performed PCR amplification with the *Coral*-ID primers using 10^4, 10^3, 10^2 and 10 template DNA molecules in 10 replicates for three samples from three different species. Amplification was successful with 10^4, 10^3 and 10^2 template molecules in each of the 10 replicates for each three species. Furthermore, samples with 10 template DNA molecules produced strong and clear amplification bands for all 10 replicates of *Pleurocorallium elatius*, and nine replicates of *Corallium rubrum* and *C. japonicum*, respectively.

3.4. Assay accuracy

We tested the accuracy of the *Coral*-ID assay by running the assay 10 times and comparing the results for consistency using a DNA sample of *Corallium rubrum*, *C. japonicum* and *Pleurocorallium elatius*, respectively. In order to carry out the accuracy test with very low DNA template inputs, the PCR amplifications were performed with 10 template molecules. The 10 DNA sequences obtained from each of the three DNA samples showed complete identity with each other and with the previously gained DNA mtMutS gene sequence of these DNA samples with high template input.

3.5. Assay precision

Precision test of the *Coral*-ID assay was performed using DNA samples of 10 case-type test samples that have been previously measured to contain moderate levels (between 10^2 and 10^3 copies per µl volume) of a c. 200 bp long mitochondrial gene fragment. *Coral*-ID was run with each case-type test sample 10 times. Results of the precision test show that each of the 10 replicates were successfully assigned to the same group for all 10 ten samples (Table 5). In eight out of the ten samples, all ten respective resulting DNA sequences were identical and completely matched with a reference sequence. The two exceptions are test samples IV and VI. Two mismatches were present in test sample IV, one in a single replicate (adenine instead of guanine at position 5 of the *Coral*-ID fragment), and one in four other replicates (guanine instead of adenine at position 114). In test sample VI, an adenine to guanine mismatch at position 46 was present in all 10 replicates.

3.6. Assay resilience to external contamination

Mixture tests were conducted to estimate the tolerance of the *Coral*-ID assay to cross-contamination by other coral samples and human DNA. Of the nine reciprocal mixture tests among three coral samples with ratios of 10:1, 100:1 and 1000:1, each but one test resulted in the DNA sequence of the major contributor. The exceptional case was the 10:1 template ratio of a *Corallium rubrum* – *Corallium japonicum*, which resulted in a chimeric sequence equally one base distant from the reference sequence of contributors leading to unsuccessful analysis according to our evaluation criteria.

In the tests where the *Coral*-ID assay was performed by mixing known coral and human DNA, the correct coral DNA sequence was recovered even at adding as much as 1 ng human DNA to the PCR reaction.

3.7. Application of the Coral-ID assay to confiscated precious coral objects

The sample amounts removed for analyses varied between 4.4 mg and 24.4 mg, which corresponds to 0.03–2% (0.69% on average) weight loss of the objects (Table 6). Altogether 13 samples (65%) gave repeated results and were assigned to one of four groups: *Corallium rubrum* (3), *Hemicorallium* (4), *Pleurocorallium elatius* species complex (3), "other *Pleurocorallium*" (3). Seven of the successfully analyzed objects had no previously specified documented species names. In each of the remaining six samples, the identified taxon differed from the documented species name. In two out of the five coral shipments that were seized, more than one single taxonomic group was discovered: *Hemicorallium* plus "other *Pleurocorallium*" and *Pleurocorallium elatius* species complex plus "other *Pleurocorallium*", moreover the latter two samples were taken from a single necklace. For 10 samples, the analyses revealed that the specimens originated from a non-CITES listed taxa (*Corallium rubrum*, *Hemicorallium* and "other *Pleurocorallium*"). Three samples were identified as *Pleurocorallium elatius* species complex, which is a group that consists of the red-pink colored *Pleurocorallium elatius* (CITES-listed), the white colored *Pleurocorallium konojoi* (CITES-listed) and the crimson to orange colored *Pleurocorallium carusrubrum* (non-CITES-listed).

4. Discussion

The high demand for coral jewelry has made high quality raw material to reach values of 10,000 USD per kilogram [13]; manufacturing and trade of precious corals is a multi-million dollar global industry. The consequent strong fishing pressure and the parallel slow renewal rate of precious coral populations cause them to be a vulnerable resource prone to overexploitation [9,42]. The imposed fishery-level and trade regulations have contributed to a more responsible harvesting of coral beds [5]. However, poaching has not completely disappeared, which has led to the appearance of illegally sourced material on the market [5,7,12,30,43–48]. In the pursuit to curb illegal trafficking, corals are globally among the taxa most frequently confiscated without the ability to properly identify the species [49]. Adherence to CITES regulations is particularly difficult in the coralid family, as only a part of the internationally traded species are CITES-listed. A species identification technique for coralids is therefore essential. There is no established list yet of which precious coral species have been used in jewelry industry; *Hemicorallium laauense* was first designated as such species in 2017 and a *Pleurocorallium niveum*-like taxon was only identified based on genetic testing of jewelry objects in 2020 [24,28]. Hence, an identification method must consider all potential species.

4.1. Validation of the Coral-ID assay

The *Coral*-ID assay assigns any coralid sample into one of six taxonomic categories. The assay was designed to have the maximal potential of distinguishing CITES-listed and non-CITES-listed species within the existing phylogenetic limitations. The DNA fragment targeted by the assay is a part of the mtMutS gene, which is known to be the most variable gene of the otherwise exceptionally slowly evolving mitochondrial genome of the octocorals [50–52]. The mtMutS gene has also been the choice for several previous evolutionary and taxonomic studies of octocorals [21,53–60]. A large number of reference data support that at least two bp differences will separate any two samples belonging to different taxonomic categories. Therefore, one mismatch from the reference genotype of any group (owing to novel variants or sequence artefacts due to the postmortem damages) can be tolerated given that double hits are not allowed. The latter requirement is necessary to exclude the possibility of a single mismatch at a discriminating position to cause the query sequence to be equally one bp difference from the reference sequence of two distinct groups.

The assay is designed to be specific to octocorals by targeting the idiosyncratic mtMutS gene. In the performed specificity test, any off-target (non-coralid) species where the assay produces DNA sequence, a minimally five mismatch gaps from any of the six coralid group's reference sequence assures that false identifications are not probable. The assay proved to be very sensitive by generating results in 90%, 90% and 100% of the cases when using 10 copies of three respective DNA samples as template input. At the same low template input, the *Coral*-ID assay was completely accurate by producing the correct DNA sequence in each case.

The precision test with case-type test samples resulted in the uniform group assignment for each respective 10 replicates of each 10 samples, implying high precision. However, in two test samples mismatches were present compared to the reference sequence. In test sample IV, both mismatches can be interpreted as a partial cytosine deamination of the DNA molecules (leading to C>T and G>A mismatches), which is typical for post-mortem DNA degradation in aging biological material. Cytosine deamination is a known phenomenon in forensic genetics, especially in decade-old low copy-number samples (initial template number <1000) [61]. Nevertheless, one of the two mismatches can only be explained by this phenomenon if the original DNA has a mismatch at this position

compared to the reference DNA sequence of the identified *Hemicorallium* group, meaning that this is as a novel, previously undetected variant. The DNA of test sample VI, for which all replicates are identical but with a mismatch compared to the *Corallium rubrum* reference sequence, presumably represents a novel variant, as well. Our reference data set contains 62 *C. rubrum* and 63 *Hemicorallium* specimens, which originate from a geographically stratified sampling (see Supporting Table 1). However, some areas relevant to the coral fishery industry are not included, such as waters off North Africa in the case of *C. rubrum* and the Emperor Seamounts in the case of *Hemicorallium*. The two putative novel variants highlight the benefit of assignment criteria allowing a mismatch compared to the reference sequences.

Analysis of mixture samples is common in genetic validation studies where the detectability of the minor contributor DNA sample is of question. In the case of coral objects, sample mixtures are not expected *per se*, however during the manufacturing process objects may be contaminated with material of previously processed items. Particularly, drilling holes for hanging and threading using uncleaned drill bits could lead to cross-contamination between samples. Complete removal of such contaminating material may be difficult before sampling for genetic testing. In only one of the mixture tests with the highest proportion of the minor contributor in the mixtures (10:1), the assay returned a mixture result. Nevertheless, proper cleaning of items to avoid cross-contamination has to remain a priority to avoid contamination of low-DNA content samples with DNA of other coral material.

Human DNA is among the most common laboratory DNA contaminants and precious coral objects are often touched. The performed tests indicate that the *Coral*-ID assay is very robust in tolerating the presence of human DNA and the correct results are not compromised.

4.2. Application of the Coral-ID assay to confiscated precious coral objects

We sampled precious coral objects from five different seized shipments by Swiss customs authorities. The minimally invasive sampling of 20 items covered objects of different shapes and colors. The sampling caused no visible damage of the objects. The 65% success rate (13 items out of 20) is very similar to the 64% success rate found in the previous study by Lendvay et al. [24]. Given the high sensitivity of the assay and the expected lack of enzyme inhibition of the DNA extracts (as demonstrated previously [24]), we assume that the DNA of the samples that did not give results was too degraded to allow amplification of the *Coral*-ID fragment, potentially due to the age of the samples. The presence of decayed DNA in some samples would not be surprising given that a skeletal axis is built over decades and a large portion of precious corals is fished already dead or even fossilized [9,22,38,62–64].

The successfully analyzed samples belonged to four different taxonomic groups. Of the 12 samples claimed as CITES-listed *Corallium elatius* and *Corallium konojoi*, each successfully analyzed sample was found to be from non-CITES-listed species. Of the seven successfully analyzed samples documented as *Corallium* sp., four could be excluded as originating from CITES-listed species, and three others are from the *Corallium elatius* species complex, which contains both CITES-listed and non-CITES-listed species. Multiple taxonomic groups were found among similar-looking samples analyzed in two seized shipments (from the years 2013 and 2015), highlighting the importance of sampling multiple items even if they look very much alike.

Three of the 13 successfully analyzed samples belong to the “other *Pleurocorallium*” group, which contains eight non-CITES-listed *Pleurocorallium* species, none of which are listed by CIBJO as used in jewelry trade. The former study by Lendvay et al. [24] already found *Pleurocorallium* samples of taxa previously thought to be irrelevant for jewelry and shows the importance of considering all coralid species in the *Coral*-ID assay.

4.3. Presumptive species identification

According to our knowledge of coral phylogenetics, reliable separation of certain coral species based on DNA analysis is not possible. This is particularly problematic in the case of the two species complexes, *Corallium japonicum* species complex and *Pleurocorallium elatius* species complex, which contain both CITES-listed and non-CITES-listed species. The *Coral*-ID assay was designed to conservatively handle the taxonomic uncertainties and deliver modest conclusions about the taxonomic identity of an object. Considering supplemental non-genetic information, a presumptive species identification may be possible in certain cases. Such information may be the color of the object, the distribution area of the potential species and the location of known commercial precious coral fishing grounds.

For a sample identified as originating from the *C. japonicum* species complex the species can be *C. japonicum* (CITES-listed) and two morphologically very much alike species, *C. nix* and *C. tortuosum* (both non-CITES-listed) [32]. The red colored *Corallium japonicum* has been fished in large quantities from waters off Japan and Taiwan (Table 1). *Corallium tortuosum* has a white or pale pink skeletal axis and is found around the Hawaiian Islands, New Caledonia and Taiwan [26]. It is also the most abundant precious coral in Hawaiian waters but, due to its small size (maximum 3 in. high) and usually deformed axis, it is less likely to be fished for commercial purposes [33]. *Corallium nix* can have a dark red skeletal axis, but has only been described during scientific surveys from the Norfolk ridge (New Caledonia), where commercial coral fishing has never occurred. Therefore, if an object is red and identified as originating from the *Corallium japonicum* species complex, then it presumptively originates from the CITES-listed *Corallium japonicum* species.

In the case of samples that were identified as originating from the *Pleurocorallium elatius* species complex, three morphologically similar species are possible: the red-pink *P. elatius* and the mainly white *P. konojoi* (both CITES-listed) and the red *Pleurocorallium carusrubrum* (non-CITES-listed). The first two species have been fished in large quantities in Japan and Taiwan [30]. The latter has been found exclusively in the waters of northern Taiwan and its material is expected to be circulating on the market mixed in stocks of *P. elatius* [5]. Therefore, if a sample is respectively white or pink, then it presumably originates from the CITES-listed *P. konojoi* or *P. elatius*, while if red then it can be both *P. elatius* or *P. carusrubrum*. Three of the confiscated objects we analyzed fell in the *Pleurocorallium elatius* species complex: Object 1 (pure dark red), Object 2 (red with white parts) and Object 20 (pure white). Combining the genetic results with morphological cues, we may draw the presumptive conclusion that Object 20 is from the CITES-listed *P. konojoi*, while Objects 1 and 2 can both be either *P. elatius* or *P. carusrubrum*.

4.4. Conclusions

The here presented *Coral*-ID assay is a forensically validated genetic tool. By applying *Coral*-ID and considering additional non-genetic information, it is possible to taxonomically assign precious coral artifacts with unprecedented confidence. The use of *Coral*-ID allows assignment of any precious coral object to six taxonomic groups, specifically to decide whether an object originates from a CITES-listed or non-CITES-listed species. The here presented system is supported by developmental forensic validation based on a broad set of reference samples and a large body of experimental data. The assay is designed to consider the phylogenetic constrains of the shallowly diverged coralid family and deliver conservative results. By taking into account the known color and distribution of the particular species, more detailed, presumptive species-level identification may be possible in certain cases. The main potential limitation for the use of the *Coral*-ID system are samples with decayed DNA preventing the assay from performing successfully. However, the amount of the sampled material should not exceed what is

minimally necessary. According to our experience, owners accept minimally destructive sampling in most cases and it would be possible to test the majority of precious coral items in the trade including any unprocessed raw material. Overall, the *Coral*-ID assay will be a powerful tool for law enforcement authorities, traders and jewelry owners to conform to the legal requirements and for the better understanding of the occurrence and predominance of taxa in the precious coral trade. If the most expensive reaction of the assay, the sequencing of the single gene fragment, is carried out in an optimized down-scaled volume, the assay can be used as a cost-effective tool. With the data collected from the future analyses of a larger set of samples, *Coral*-ID will contribute to a more accurate insight in the trade statistics of precious corals.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2022.102663.

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