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DNA fingerprinting: an effective tool for taxonomic identification of precious corals in jewelry

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Precious coral species have been used to produce jewelry and ornaments since antiquity. Due to the high value and demand for corals, some coral beds have been heavily fished over past centuries. Fishing and international trade regulations were put in place to regulate fishing practices in recent decades. To this date, the control of precious coral exploitation and enforcement of trade rules have been somewhat impaired by the fact that different species of worked coral samples can be extremely difficult to distinguish, even for trained experts. Here, we developed methods to use DNA recovered from precious coral samples worked for jewelry to identify their species. We evaluated purity and quantity of DNA extracted using five different techniques. Then, a minimally invasive sampling protocol was tested, which allowed genetic analysis without compromising the value of the worked coral objects. The best performing DNA extraction technique applies decalcification of the skeletal material with EDTA in the presence of laurylsarcosyl and proteinase, and purification of the DNA with a commercial silica membrane. This method yielded pure DNA in all cases using 100 mg coral material and in over half of the cases when using “quasi non-destructive” sampling with sampled material amounts as low as 2.3 mg. Sequence data of the recovered DNA gave an indication that the range of precious coral species present in the trade is broader than previously anticipated.

Precious corals are among the most appreciated and oldest known gems. They are valued for their color, texture and workability (polishing, carving), and have thus been collected and used for adornment for millennia^{1–3}. Growing demand, particularly in Asia in recent years, has led to an increase in prices of precious corals used in jewelry^{4–6}.

The most valuable precious coral species belong to the Coralliidae family within the Octocorallia subclass of the Anthozoa. The precious coral material used for jewelry is the worked (i.e. cut, carved and polished) hard coral skeletal axis, which is a biogenic material created by a biomineralization process⁷. In this process, closely packed magnesium-rich calcite crystals are secreted by coral polyps (1–2 mm in size) to build up a skeleton over decades. The polyps can thrive on the surface of the skeleton as colonies connected and surrounded by a 0.5–1 mm thick surface tissue (coenenchyme)⁸. The Coral Commission of The World Jewellery Confederation (CIBJO) lists eight Coralliidae species as significant in the precious coral jewelry industry^{9,10}. Precious coral products are sold worldwide, with production centers located in Italy, Japan and Taiwan and large-scale trade of raw material between these areas^{5,6,11}.

Until recent decades, the populations of these highly coveted marine animals experienced exploitation in boom and bust cycles where the discovery of precious coral beds led to rushes by coral fishers and these beds were exploited as long as it remained economically feasible^{12,13}. Local and international regulations were put in place to control both fishing and international trade of precious corals, among which four Pacific species were listed in Appendix III of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) at the request of China^{4,13–15} (Table 1). It has been reported that traders may often not be aware of the origin and species of their coral jewelry products^{4,6}. At the same time, consumers and jewelers increasingly request specific information about precious corals, particularly their geographic origin and species, mainly due to the perceptions of value that different types of coral have in the market and possible sustainability considerations¹⁶.

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Species	Distribution	CITES listed	Traditional trade names for sub-varieties of the species	Scientific name used in trade
<i>Corallium japonicum</i>	West-Pacific	Yes	Japanese red coral, Oxblood, Red blood, Aka, Moro.	<i>Corallium japonicum</i>
<i>Corallium rubrum</i>	Mediterranean sea, East Atlantic	No	Mediterranean red coral, Sardinian, Sardegna, Sciacca	<i>Corallium rubrum</i>
<i>Hemicorallium laauense</i>	North Pacific	No	Deep-sea Midway coral, Deep sea, New, Sensei	<i>Pleurocorallium secundum</i>
<i>Hemicorallium regale</i>	North Pacific	No	Garnet coral	<i>Pleurocorallium secundum</i>
<i>Hemicorallium sulcatum</i>	West-Pacific	No	Miss coral, Missu, Misu	<i>Pleurocorallium secundum</i>
<i>Pleurocorallium elatius</i>	West-Pacific	Yes	Pink coral, Angel skin, Satsuma, Momo, Magai, Boké, Pelle d'angelo, Cerasuolo	<i>Pleurocorallium elatius</i>
<i>Pleurocorallium konojoi</i>	West-Pacific	Yes	White coral, Pure white, Shiro, Bianco	<i>Pleurocorallium konojoi</i>
<i>Pleurocorallium secundum</i>	North Pacific	Yes	Midway coral, White/Pink, Rosato	<i>Pleurocorallium secundum</i>

Table 1. Distribution, CITES listing and trade names of the eight precious coral species considered relevant in the jewelry industry by The World Jewellery Confederation. Note that the different *Hemicorallium* species are sometimes suggested to be traded under the name *Pleurocorallium secundum*^{9,10,16,68}. Data compiled from^{6,9,10,16,59}.

Therefore, accurate taxonomic identification of precious coral products is of paramount importance for both efficient enforcement of precious coral trade regulations and for the jewelry industry. However, species of polished corals can be extremely difficult to distinguish even for trained experts based on morphological characteristics, and proper analytical tools to conclusively identify the species of worked precious corals are still lacking^{6,12,16,17}.

The various analytical methods tested to distinguish precious coral species based on skeletal material were either unable to provide clear-cut distinction among the different coral species (e.g. trace element analysis, such as X-ray fluorescence spectroscopy, LA-ICP-MS and EMPA¹⁸; and Raman spectroscopy¹⁹), or were not improved to become a standardized and easy-to-use tool (such as immunolabeling²⁰). As a novel approach, Cartier, *et al.*²¹ recently proposed DNA analysis to distinguish species, assuming that coral DNA molecules can be trapped in the organic material or adhered to the CaCO₃ crystals during the formation of the skeleton.

Genetic analyses have become a powerful analytical tool to elucidate the species identity and trace the geographic origin of various valuable artefacts of biogenic origin. These include processed products of tortoise shell²², snake skin²³, fur^{24,25}, ivory^{26,27} or tiger bones²⁸. Of greatest relevance to this present study, Meyer, *et al.*²⁹ reported quasi-nondestructive species identification of pearls based on DNA analysis, where so little amount of pearl material was used for the analyses that the market value of the pearl was not compromised. Particular biogenic materials require specific DNA extraction methods, moreover, we anticipate that DNA preserved in precious coral skeletons to be present in very small amounts and highly fragmented due to the lengthy skeleton-formation process and the degradation of the DNA after the death or the coral^{30–33}. A further challenge of using DNA to distinguish Coralliidae species may arise from the exceptionally slow evolution of the Octocorallia mitochondrial genomes, which causes different species to be genetically highly similar^{34–38}.

In the present proof of concept study, we aim to explore whether precious coral skeleton fragments cut, carved and polished for jewelry can be taxonomically identified through genetic analysis. We compare five different DNA extraction methods to find the method producing the highest purity and quantity of DNA. We then apply the most successful DNA extraction technique using a minimally destructive sampling method and amplify and sequence the recovered DNA to taxonomically identify the coral samples. We demonstrate that genetic analysis of gem-quality precious corals is a promising method to assess the identity of their species.

Results

Comparison of DNA retrieved from worked precious corals with five extraction methods. Using a set of 25 worked coral samples, we evaluated which one of five candidate DNA extraction protocols is most suited to retrieve DNA from worked precious coral samples. Each of the five tested methods (abbreviated as “W”, “F”, “B”, “E”, “Y”) have earlier proven to be useful in extracting DNA from biomineralized material^{29,39–48}. DNA was extracted from each of the 25 worked coral skeletal samples with all five techniques, and DNA purity and quantity were assessed using real-time quantitative PCR (qPCR) technology.

To test DNA extract purity, we assessed PCR inhibition with qPCR using an internal amplification control molecule. Three extraction methods, “F”, “E” and “Y”, resulted in DNA with no detectable PCR inhibition effect from any of the tested 25 samples (Fig. 1, Supplementary Results S1). In contrast, a PCR inhibition effect was observed in 15 out of 25 samples extracted with the “B” method. Of these, complete inhibition of the PCR was observed in one case. Inhibition was also detected in three DNA extracts produced with the “W” method. Of these, no PCR product was observed at all in one sample.

Absolute quantity of the DNA obtained with the five extraction techniques was tested using qPCR with a standard curve from a dilution series of a standard template DNA molecule with known concentrations. Throughout these analyses, the average qPCR efficiency was 88.5% ($\pm$ 3.6% standard deviation) and the coefficient of determination for the calibration curve was $R^2 = 0.9947$ ($\pm$ 0.0035 standard deviation).

The five extraction methods yielded highly varying amounts of DNA (Fig. 1, Supplementary Results S1.). Methods “E” and “Y” both yielded PCR amplifications for all 25 samples. Method “W” yielded PCR product for

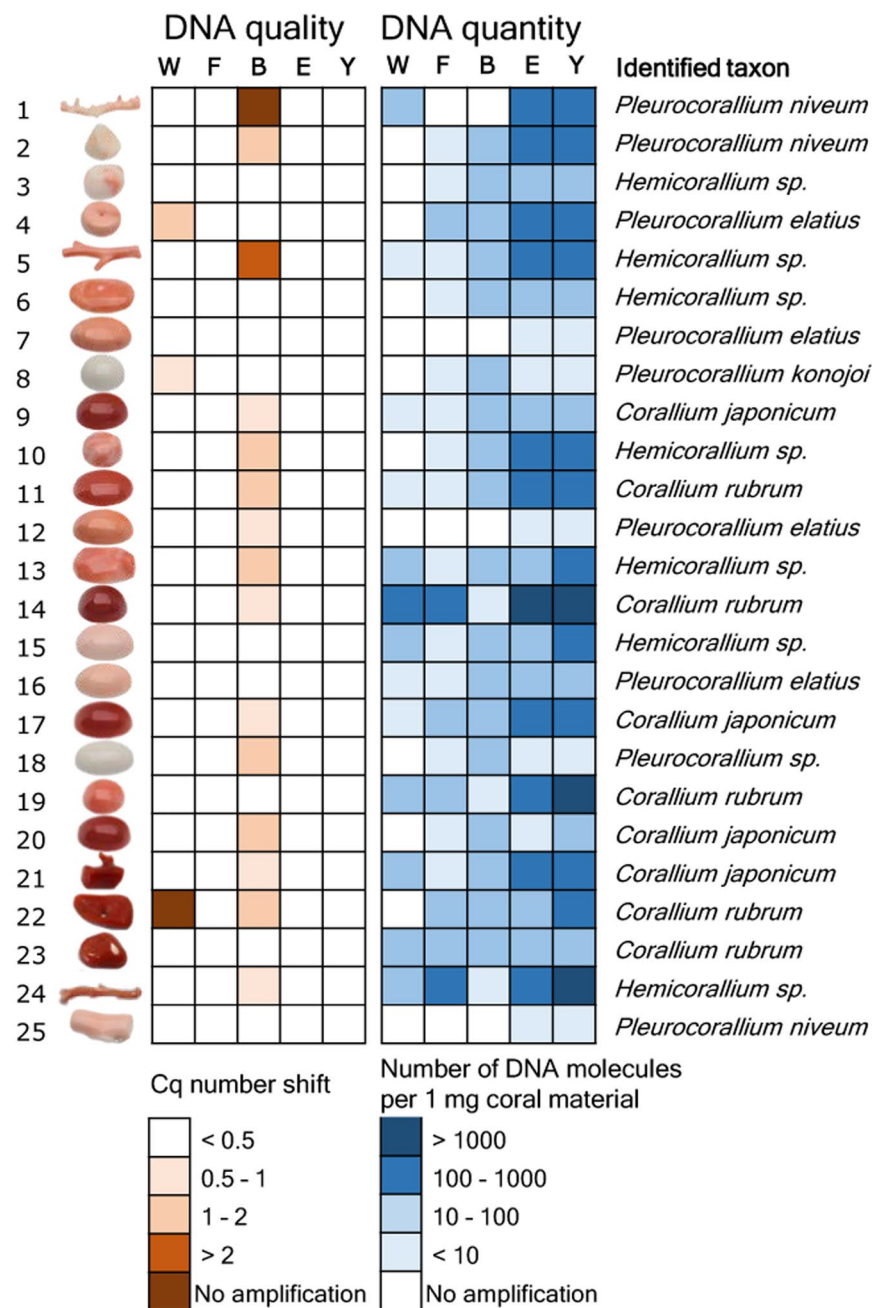


Figure 1. Results of the DNA extract purity and quantity measurement experiment and taxonomic identification of 25 worked precious coral samples. Five methods were used to extract DNA from equal amounts of material from each sample. PCR inhibition measurement and absolute template quantification was performed with quantitative real-time PCR. Two short mitochondrial DNA fragments were sequenced and each specimen was taxonomically assigned. Note that identifications as *Corallium japonicum*, *Pleurocorallium elatius* or *P. konojoi* were possible based on the combination of genetic and morphological assessments.

13 samples, while methods “F” and “B” both yielded PCR product for 21 samples. Overall, there was concordance among the amplification results; the 13 samples that amplified with method “W” also amplified with methods “F” and “B”, and the latter two methods amplified DNA of the very same 21 samples. Strong significant correlation was found between the copy numbers obtained from the same coral items with the “E” and “Y” methods ($r = 0.97$, $t = 19.223$, $df = 23$, $p < 0.001$). DNA yield was higher with method “Y” than with method “E” (595 versus 944 molecules per mg coral sample with “E” and “Y”, respectively; paired t-test: $t = -2.8832$, $df = 24$, $p = 0.008$). Focusing on the best performing “Y” method, DNA concentrations ranged between three orders of magnitude: three samples had over 10^3 DNA copies in each mg of coral skeleton material. In five other samples this value was below 10 (Fig. 1).

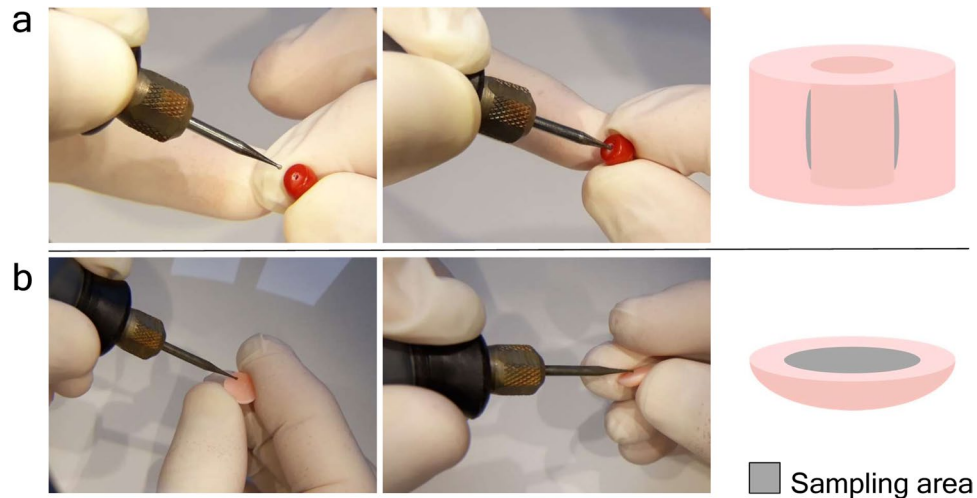


Figure 2. “Quasi non-destructive” sampling of worked coral skeletons. (a) Widening the inner surface of the existing drill-hole in a bead. (b) Sampling the back side of a cabochon item.

DNA extraction with “quasi non-destructive” sampling of worked precious coral samples. In the previous experiment, 25 samples were completely pulverized and five DNA extractions were carried out with different methods from each. The aim was to select the most suitable technique for extracting DNA from worked coral samples. In the current experiment, the best performing DNA extraction technique was used with “quasi non-destructive” sampling of worked corals. We developed a “quasi non-destructive” technique to take material for analysis from the worked corals with minimal weight loss and virtually invisible effects of the sampling (Fig. 2). A new set of 25 worked coral samples were sampled in this manner; removed material amounts ranged from 2.3 mg to 13.1 mg and were 7.9 mg on average. Modifications were applied to the lysis step of the “Y” extraction method compared to the original protocol, which resulted in an essentially complete dissolution of the coral powder. This allowed the amount of DNA that remained trapped in the undissolved powder to be kept to a minimum. Out of the 25 “quasi non-destructively” sampled worked coral objects, 16 gave qPCR amplicons at least twice (Fig. 3, Supplementary Results S1). Another two samples produced amplification only once and were omitted from further analyses. DNA copy numbers calculated per mg of coral sample were in the same range as in the case of the extractions carried out from ca. 100 mg material using the “Y” method. However, the presence of unsuccessful amplifications and lower average copy number (160 DNA copies) recovered per mg of coral skeletal material indicates that DNA recovery from low amount samples is less effective than from standard material amount, despite the amendments made in the DNA extraction protocol.

Taxonomic assignment of worked precious corals. We sequenced amplicons of the large ribosomal RNA gene subunit (LR) and the putative mismatch repair protein (MSH) fragments originating from a total of 41 worked coral skeletal samples using massively parallel sequencing. In our entire DNA sequence dataset, the sequence of altogether three OTUs were highly divergent from any of the Coralliidae LR or MSH reference sequences. NCBI BLAST search did not find any sequence entries in the NCBI database with higher than 95% sequence similarity for any of these sequences.

The lengths of the concatenated LR and MSH sequences were between 264 base-pairs (bp) and 290 bp long per coral sample (Supplementary Results S2). Bayesian phylogenetic analysis identified 10 samples (11, 14, 19, 22, 23, 31, 34, 38, 41, 45) as *Corallium rubrum*, of which nine had sequences identical to either of two the reference *C. rubrum* sequences, and one (11) had a single variable site (Fig. 4). Six samples (9, 17, 20, 21, 28, 35) were identical with reference samples of *Corallium japonicum*, but also with the reference samples of *C. nix* and *C. tortuosum*.

Three samples (6, 15, 24) formed a polytomic clade with *Hemicorallium* reference sequences. Two of these (6, 15) had sequences identical to *Hemicorallium laauense*, but also to samples of *H. abyssale*, *H. bathyrrubrum*, *H. ducale* and *H. imperiale*. The third sample (24) was one bp different from these sequences. Seven samples (3, 5, 10, 13, 37, 40, 47) with identical sequences appeared as an unresolved clade basal to the formerly mentioned samples. These had identical sequences with *H. abyssale*, *H. ducale* and *H. imperiale*.

Six samples (4, 7, 8, 12, 16, 39) had identical sequences with *Pleurocorallium carusrubrum*, *P. elatius* and *P. konojoi* reference samples. Two samples (18, 46) formed a sister clade to the former group with the posterior probability value 1. Finally, seven identical samples (1, 2, 25, 33, 42, 44, 50) were same as sequences of *Pleurocorallium niveum*. These were grouped together as an unresolved tree branch.

Discussion

Technical advancements and the growing body of reference DNA data have made genetic analyses a powerful tool to combat poaching, illegal trading and mislabeling of animal products⁴⁹. Application of genetic barcoding was suggested by Ledoux, *et al.*⁵⁰ as a forensic tool to identify species of corals. Acknowledging that the discriminatory power of standard species barcoding markers (e.g. the cytochrome c oxidase subunit I gene) is poor to distinguish the closely related precious coral species, these authors suggested development of custom designed

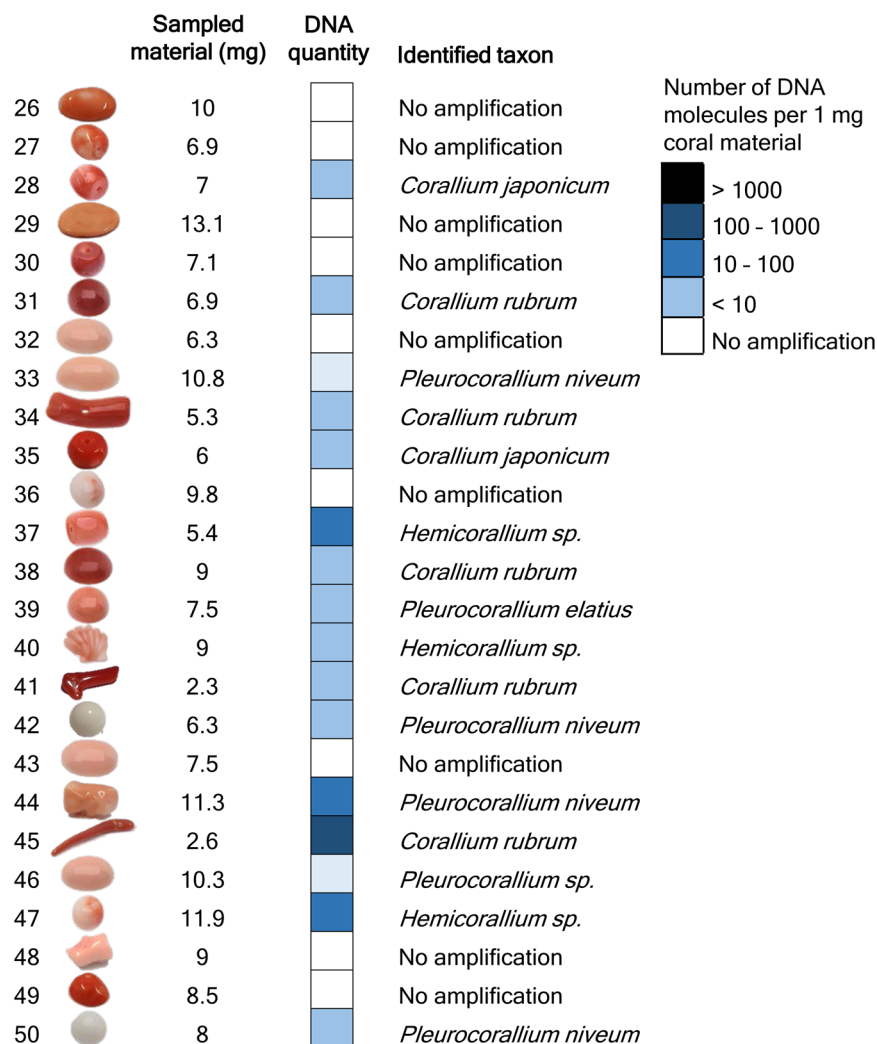


Figure 3. Results of DNA quantity measurement and taxonomic identification of 25 worked precious corals sampled by the minimally invasive technique. Absolute template quantification was performed with quantitative real-time PCR. Two short mitochondrial DNA fragments were sequenced and each specimen was taxonomically assigned. Note that identifications as *Corallium japonicum*, *Pleurocorallium elatius* or *P. konojoi* were possible based on the combination of genetic and morphological assessments.

species identification markers. Moreover, if the aim is to distinguish coral skeletal samples, then the high portion of fragmented DNA will call these markers to be as short as possible. A further challenge is if sampling of the coral sample is to be done with minimal material loss. As a consequence, the chosen DNA extraction method has to be capable of recovering DNA from a small sample amount.

In our quest to find an optimal method to recover DNA from worked coral samples, we tested the performance of five DNA extraction methods, each on equal amounts of coral material from the same set of 25 worked coral samples. We found two methods, protocol “E” and “Y” that yielded DNA that was successfully amplified and sequenced from all of the 25 tested corals. Methods “E” and “Y” are two similar techniques developed for the extraction of DNA from ancient eggshells and ancient bones. They only slightly differ in their lysis buffer ingredients and the type of DNA-binding silica column used for the purification of the recovered DNA molecules^{45,46}. These methods produced similar amounts of DNA, however method “Y” produced slightly higher DNA yield, particularly in the samples that had <50 DNA copies per mg of coral powder. The three other tested DNA extraction methods did not result in amplifiable DNA from all samples, which may be due to their inability to recover DNA coupled with PCR-inhibitory effect of co-extracted substances, which was detected in some extracts, PCR inhibition was not detected in any extracts produced with methods “W”, “E” and “Y”. By using these methods, PCR inhibition seems to be overcome in precious corals, unlike in other types of corals, where it led to technical challenges⁵¹.

DNA concentration of the extracts differed largely; while in certain samples <10 copies per mg of material was recovered, in some others this reached up to the order of magnitude of 10^3 copies per mg of material. The large variation in DNA preservation of the samples may be determined by several factors; the age of the coral when fished, whether the coral was fished dead or alive^{30–32} and the time since the coral was fished⁶. However, without specific knowledge about the age of the samples this remains hypothetical.

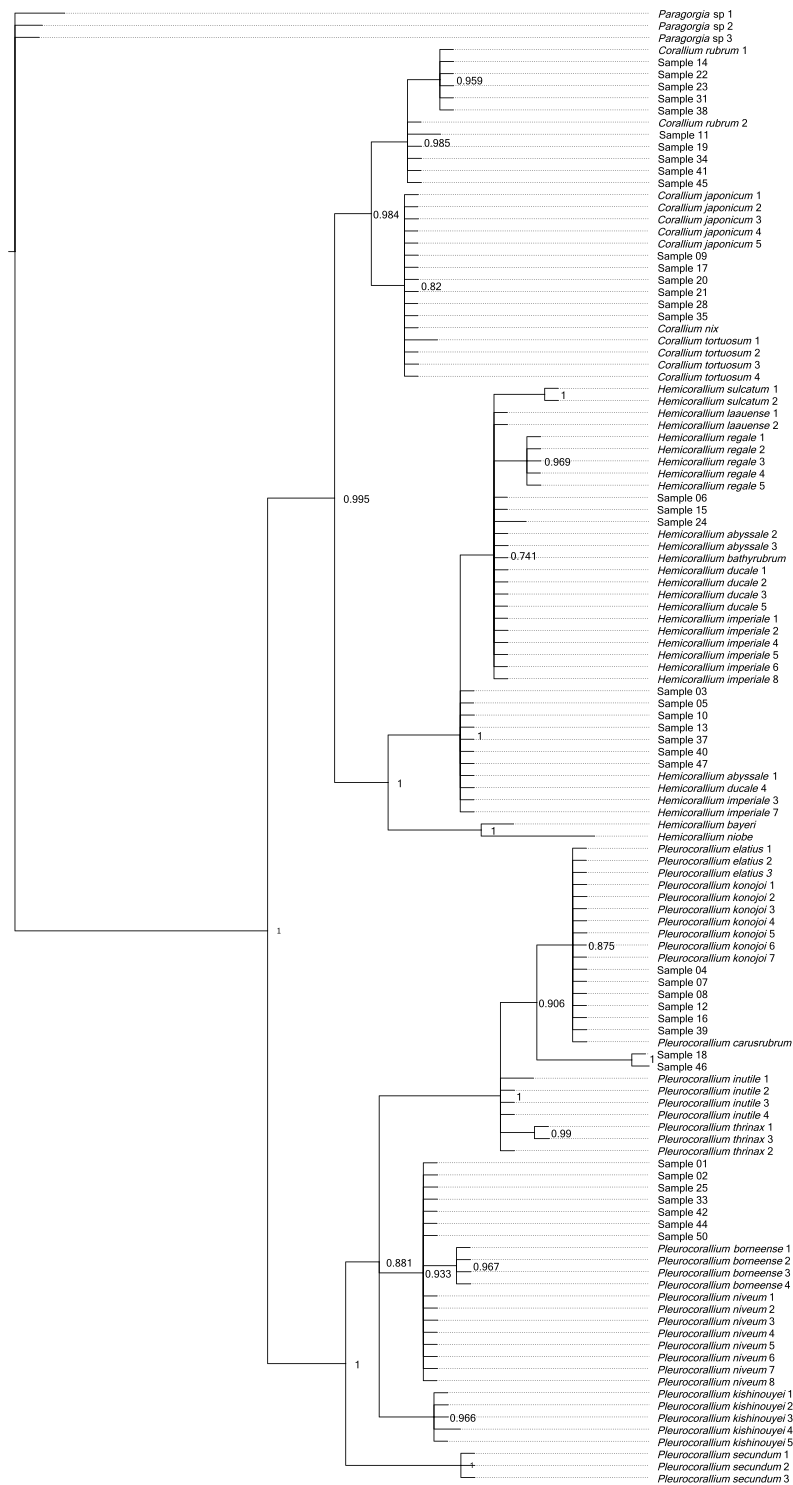


Figure 4. Majority-rule Bayesian phylogenetic tree constructed from combined mitochondrial LR and MSH region DNA sequence data of worked precious corals and reference samples. Posterior probability value is displayed after each tree node.

Our test to choose the best DNA extraction protocol from potential methods was based on 100 mg of coral skeleton material, which is a standard amount used for extracting DNA from pulverized material with the applied protocols. The essence of precious material testing would be to use as little material as possible, ideally using a “quasi non-destructive” sampling method. This means that the sampling area is not visible and the sampling does not cause significant weight loss of the coral object. Worked coral samples can be separated into two main types; the ones that have a hole drilled through the item (generally those that are strung as beads for bracelets or necklaces) and the ones that do not have a hole, instead generally have flat reverse or bottom sides (those that are mounted to a frame and

used as pendants, i.e. cabochons, or the carved figures used as ornaments). We performed “quasi non-destructive” sampling using a drill with a 0.8 mm diameter diamond engraver head taking care not to heat up the sampled object (no hard pressing of the drill and regular pauses to let the drill head cool down). With careful handling, it was possible to take sample material by slightly widening the internal surface of the ca. 1 mm wide drill-holes, which ensures that this material extraction is invisible by eye upon subsequent inspection of the sample. From the cabochons, a thin layer was removed from the reverse side; therefore the visible front side remains unaffected by the sampling. Assuming approximately 3.8 kg/dm³ density of the precious corals⁹, the removed 2.3–13.1 (on average 7.9) mg powder per sample corresponds to a 0.7–3.5 (2.1) mm³ volume loss of the items.

We were able to repeatedly produce PCR products for 16 out of the 25 “quasi non-destructively” sampled worked coral samples. We could not determine a threshold for the minimum amount of material necessary for successful genetic testing; the two samples processed with the lowest weight of coral powder, 2.3 mg and 2.6 mg, respectively, both produced results. Although it was not possible to genetically analyze all samples with the minimally destructive method, there might be a good chance that when analyzing several samples from a batch of samples, at least some will produce results.

We expected that all of the DNA sequences we generated will be identical with at least one reference sequence of the eight species listed by CIBJO as relevant in the jewelry industry⁹. However, against our expectations, we found a much higher diversity within our samples, with several of our sequences not grouping together with any of the reference sequences of the eight species. Hence, we performed an other phylogenetic analysis with a more extended reference sample set. The results of this analysis show that samples could clearly be identified as *Corallium rubrum*. The samples grouping together with *Corallium japonicum* also grouped together with two other species, *C. nix* and *C. tortuosum*, which, however, have white and pink color, respectively, unlike the dark red color of *C. japonicum*^{44,52}. Hence, we can confidently identify these red corals as *C. japonicum* based on the combination of genetic and morphological characteristics.

Samples that grouped together with the *Hemicorallium* references all had identical sequences with multiple *Hemicorallium* species. As a consequence, these samples could be identified only to the genus level as *Hemicorallium*. A part of these samples (i.e. 3, 5, 10, 13, 37, 40, 47) did not cluster with the three reportedly fished *Hemicorallium* species (*H. laauense*, *H. regale*, *H. sulcatum*), but instead had identical sequences to other species (*H. abyssale*, *H. ducale* and *H. imperiale*) that all occur around the Hawaii islands, a historically important fishing area^{44,45,47}. This result strongly suggests that *H. laauense*, *H. regale* and *H. sulcatum* are not the only *Hemicorallium* species present in the jewelry trade.

Some samples had identical sequences as the three genetically and morphologically, very similar species, *Pleurocorallium*, *P. carusrubrum*, *P. elatius* and *P. konojoi*^{47,53}. Of these species, the latter two are well known in the jewelry industry, while the former is a recently described species known from a single area of the West Pacific⁵³. To distinguish these species, the coloration of the skeletal axis may provide a partial solution. In particular, the color of *P. carusrubrum* is red, *P. elatius* varies from pale to dark pink, while *P. konojoi* is always pure white^{4,6,9,10,17}. Consequently, our specimens identified as one of these species with pink shading may be identified as *P. elatius*, while our samples with white color are determined as *P. konojoi*.

Of our multiple samples within the *Pleurocorallium* clade that did not group together with the species traditionally accepted as being present in the coral trade (*P. elatius*, *P. konojoi* and *P. secundum*), two samples (18, 46) formed an individual clade and were identified to the genus level as *Pleurocorallium*. DNA sequences of the other samples were all identical with the sequences of the *Pleurocorallium niveum* samples. This species was described from waters surrounding the Hawaii islands, which was a historically important coral fishing area^{54,55}. The 41 samples that we managed to genetically analyze from 50 samples of a single collection is not representative enough to be able to draw conclusions about the entire jewelry industry, but it indicates that there may be more species present in the trade than the eight precious coral species commonly listed as part of the jewelry industry (cf^{9,10,16}). This is conceivable, if we consider that in the Pacific Ocean different precious coral species may co-occur and coral fishing does not seek to individually separate them based on species. The presence of more than the previously anticipated eight species also implies that accurate species identification in all cases will only be possible using markers that can differentiate among all species within the Coralliidae family.

Conclusions

This study is a proof of concept demonstrating that genetic analysis can be an effective tool to taxonomically identify precious corals worked for jewelry. We demonstrated that while 100 mg coral skeletal material is sufficient for successful DNA extraction in all cases, DNA sequencing and taxonomic assignment were possible with minute amounts of “quasi non-destructive” samples in more than half of the cases. Among the worked precious corals examined in this study, DNA sequence analyses revealed several samples very likely belonging to precious coral species previously not considered to be present in the jewelry industry. Future research should focus on broadening the reference data by sequencing multiple specimens for each species identified by experts in order to substantiate their intra- and interspecific genetic diversity. Additionally, the development of more specific markers will allow for the identification of coral samples with higher accuracy. These will be essential steps in developing genetic tests that can become a reliable and standardized method to promote transparency, traceability and sustainable use of precious corals in the jewelry industry.

Materials and Methods

Studied species. The precious corals relevant to the high-end jewelry industry are Octocorallid Anthozoans that belong to the Alcyonacea order and Coralliidae family. Recent phylogenetic studies confirmed the existence of three genera in the family; *Corallium*, *Hemicorallium* and *Pleurocorallium*^{56,57}. Of the eight species listed by CIBJO as significant in the precious coral industry, a single, *Corallium rubrum*, is distributed in the Mediterranean Sea and has been fished since antiquity⁶. Four other species, *Corallium japonicum*, *Hemicorallium*

sulcatum, *Pleurocorallium elatius* and *Pleurocorallium konojoi* have been fished in the Western Pacific ocean since the early 19th century¹². The remaining three species, *Hemicorallium laauense*, *Hemicorallium regale* and *Pleurocorallium secundum* were discovered on seamounts surrounding the Hawaii archipelago and were fished in large quantities during the second half of the 20th century⁵⁸. Distribution, CITES listing and trade names of the eight precious coral species relevant to the jewelry industry are summarized in Table 1, while further details on their distribution, taxonomy, harvesting and conservation are available in Cannas, *et al.*⁵⁹.

Genetic markers used in the study. We expected that the DNA extracted from the coral skeletal samples would be highly degraded. Therefore, we used markers developed on the mitochondrial genome, which is present in each cell in multiple copies and offers the best chances of achieving positive results for fragmented DNA. Octocoral mitochondrial genomes have an exceptionally low rate of evolution and standard taxonomic markers are unable to distinguish closely related species^{34,38,60}. Hence, we designed primers for two genetic markers with the criteria that the resulting amplicon sequences are short enough to be suitable for degraded DNA and highly variable in order to maximize our ability to identify the precious coral species to the lowest possible taxonomic level. We expected each analyzed sample to originate from one of the eight precious coral species listed by CIBJO, thus chose our markers with the aim that they should be capable of distinguishing these eight species. The two mitochondrial markers were developed based on DNA sequence data of Tu, *et al.*⁵⁷, which is the most detailed study on precious coral phylogeny to date. Marker selection and procedures for designing PCR primers are detailed in Supplementary Methods S3.

Following examination of the phylogenetic resolution of multiple short mitochondrial genome fragments, we developed two sets of primers for the large ribosomal RNA gene subunit (LR gene, LR-F 5'TTCATCACAGTGAGGGTTTGT3' and LR-R 5'TGCAAAGAAGGAGAACAAAAGG3') and the putative mismatch repair protein (MSH gene, MSH-F 5'CGAAAGCGGATAAAAAGCTACC3' and MSH-R 5'CCTCACTGTCTAGGCTAATGAG3'), respectively. The LR marker was used for the assessment of DNA purity and DNA quantification. Phylogenetic analysis using the combined LR and MSH markers showed that these two short markers were able to reconstruct the phylogenetic relationships obtained by much longer sequences, and they allowed the distinction of each of the eight precious coral species from each other, except for *Pleurocorallium elatius* and *P. konojoi*. It is not possible to conclusively distinguish these two species based on the data of Tu, *et al.*⁵⁷ (Supplementary Methods S3).

Comparison of DNA purity and quantity extracted with different methods. *DNA extraction.* All laboratory work was carried out at the Forensic Genetics department of the Zurich Institute of Forensic Medicine, University of Zurich, in the laboratory facility dedicated to human and animal forensic casework. We strictly adhered to the ISO 17025 guidelines throughout the laboratory workflow with stringent rules to avoid contamination and authenticate our results (Supplementary Methods S4). Precious coral samples used in this study originated from the collection of the Swiss Gemmological Institute SSEF, Basel, Switzerland.

Twenty-five worked coral samples were selected for the experiment (named samples 1–25, Supplementary Table S5). The samples were cleaned as described in Supplementary Methods S4 and crushed in a metal mortar with a metal pestle to produce crude coral powder, which was then transferred to a porcelain mortar and ground to fine powder. The coral skeleton powder was divided into five aliquots of equal weight, 100 mg ± 1 mg in general, except for four samples that had less available powder (Supplementary Table S5). The powder aliquots were used to extract DNA using five different extraction methods, which have proven to be effective in successfully recovering DNA from biomineralized material (Table 2). For each method, we followed the protocols cited in Table 2. All DNA extracts were eluted in 100 µl and stored at –20 °C.

Assessment of the purity of the DNA extracts. We used qPCR to compare the purity of the DNA extracts produced from worked precious coral samples with five different extraction protocols. DNA purity was measured by testing the PCR inhibiting effect of the coral extracts during amplification of an internal positive control DNA fragment. We used 10³ copies of a synthetic oligonucleotide (gBlocks Gene Fragments; International DNA Technologies, Coralville, IA, USA⁶¹) as internal amplification control (IAC, Supplementary Methods S6). The 197 bp sequence of the IAC matched 151 bp of the *C. rubrum* LR gene fragment (with manual introduction of five unique mismatches for contamination detection purpose) flanked by potato-specific sequences as primer sites following Nolan, *et al.*⁶².

Following optimization (see Supplementary Methods S6), reactions were conducted in 20 µl volumes containing 1 × PowerUp SYBR Green Master Mix (Thermo Fisher), 1 µl of both 15 µM concentration primers, 10³ copies of the IAC in 3 µl and 3 µl coral DNA extract. Alongside the samples containing coral DNA extracts, we run three positive standard reactions that did not contain coral DNA. Following the manufacturer's recommendation, reactions commenced with 50 °C for 2 minutes, which was followed by initial denaturation at 95 °C for 2 minutes and 50 cycles of denaturation at 95 °C for 15 seconds, primer annealing at 60 °C for 15 seconds and elongation at 72 °C for 1 minute. A melting-curve analysis was performed at the end of the reaction by heating the PCR products from 60 °C to 95 °C with 1% ramping speed. Each coral extract was run in triplicates on an ABI 7500 qPCR instrument (Thermo Fisher).

The quantification cycle (Cq) value of each reaction containing coral DNA extract was compared to the average Cq value of the three positive standard reactions and then the three Cq shift values of each sample were averaged. The intensity of PCR inhibition in each reaction was determined as follows: we considered inhibition to be present if there was a 0.5 < cycle Cq shift compared to the positive standard Cq. Four categories of PCR inhibition were considered: 0.5–1, 1–2, 2 < cycle shifts and complete inhibition in case at least one out of the three reactions produced no PCR product.

Method	Previously used for	Demineralization	DNA binding	DNA purification	Protocol followed
“W” method	ancient bones ³⁹ , snail shells from museum collection ⁴⁰	EDTA solution with Proteinase-K	On celite particles in the presence of GuSCN containing buffer	Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA)	“WSU fast” method by Villanea, <i>et al.</i> ⁴⁰
“F” method	marine pearls ²⁹	EDTA solution	On spin column of the FastDNA SPIN Kit for Soil (MP Biomedicals, Illkirch-Graffenstaden, France)		Meyer, <i>et al.</i> ²⁹
“B” method	bones and teeth ^{41,42}	PrepFiler BTA Lysis Buffer (Thermo Fisher, Waltham, MA, USA) with DTT and Proteinase-K	On magnetic particles of the PrepFiler BTA Forensic DNA Extraction Kit (Thermo Fisher)		vendor’s protocol
“E” method	subfossil eggshells ^{43–45}	Tris-EDTA solution with Triton X-100, DTT and Proteinase-K	On the spin column of the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany)		Oskam and Bunce ⁴⁵
“Y” method	subfossil bones ⁴⁶ , subfossil and modern clam shells ^{47,48}	EDTA solution with N-laurylsarcosyl and Proteinase-K	On the spin column of the MinElute PCR Purification Kit (Qiagen)		“Y” method as described by Gamba, <i>et al.</i> ⁴⁶

Table 2. The five different methods tested to extract DNA from precious coral samples worked for jewelry. Abbreviation of chemical names: EDTA: Ethylenediaminetetraacetic acid, GuSCN: Guanidinium thiocyanate, DTT: Dithiothreitol. The abbreviated method name is followed by references of relevant studies where the method was used to extract DNA from calcified material, the chemical composition of the demineralization buffer, the method used for DNA binding and DNA purification and, finally, the exact protocol followed.

Absolute DNA quantification of the coral DNA. Absolute quantification of the coral LR gene fragment was conducted by qPCR of the coral DNA using a calibration curve prepared as a series of standard reactions with a known template DNA amount. The standards contained seven different 10-fold diluted template inputs (10^7 – 10^1 copies) of a GBLOCKS synthetic oligonucleotides of the 154 bp long sequence of the LR gene fragment characteristic to *C. rubrum* (with manual introduction of three unique mismatches for the purpose of contamination detection) flanked by the LR primer sequences (Supplementary Methods S6). Following optimization of the reaction setup (Supporting Methods S6), reactions were carried out in 20 µl volumes containing 1 × PowerUp SYBR Green Master Mix (Thermo Fisher), 1 µl of both 15 µM concentration primers and 3 µl coral DNA extract. The cycling conditions were identical to those of the DNA extract purity test.

For each sample, PCR was considered successful if at least two reactions of the triplicates amplified. The Ct values were averaged for each sample and the mean Ct values were transformed to number of DNA molecules per mg of coral sample based on the volume of the DNA template in the PCR reaction, the DNA extract elution volume and the amount of coral powder used for the DNA extraction. We compared the DNA quantities gained with the extraction methods for which DNA was successfully amplified for all 25 samples with a correlation test and paired t-test in R⁶³.

“Quasi non-destructive” sampling, DNA extraction and quantification. We define “quasi non-destructive” sampling as taking material for analysis from the worked objects without compromising its market value. A new set of 25 worked coral samples were selected from the SSEF coral collection for this experiment (named samples 26–50, Supplementary Table S7), and each was thoroughly cleaned as described in Supporting Methods S4. Two main types of samples were sampled differently: (i) beads with drill-holes: the inner surface of the drill-hole was carefully widened (Fig. 2a); (ii) worked items with no existing drill-hole: a small layer of the surface of the back side of a cabochon was removed (Fig. 2b). We used 0.8 mm diameter diamond engraver bit heads attached to a Dremel 4000–4 rotary tool (Dremel, Racine, WI, USA). The rotation speed was set to 10,000 rpm and the extracted coral powder was left to drop in 1.5 ml collection tubes.

DNA was extracted from the quasi non-destructively sampled drill-powder of the 25 samples with the “Y” method. The material amount obtained by the “quasi non-destructive” sampling was far lower than the 100 mg used in the experiment comparing extraction methods, therefore we slightly modified the “Y” protocol to accommodate it to the low material amount. In particular, 200 µl lysis buffer was added to the coral powder and incubated at 56 °C for one hour with mixing, then another 100 µl lysis buffer was added. The lysis-mixture was incubated again with mixing at 56 °C for one hour and then at 37 °C for an additional 65 hours. In the next step, the lysate was mixed with 450 µl 1 × TE buffer and 3750 µl PB buffer (Qiagen) and the entire volume of the mixture was centrifuged through a MinElute (Qiagen) column. The column was washed with PE buffer and the DNA was eluted in 35 µl EB buffer (Qiagen).

Taxonomic identification. *DNA amplification and sequencing.* We sequenced PCR products of DNA samples extracted with the “Y” method. For the LR fragment, qPCR products generated for the DNA quantity assessment were sequenced: from each sample one of the triplicate qPCR was selected for sequencing. The MSH region was amplified and sequenced for altogether 41 DNA samples: all 25 DNA samples from the DNA extraction test and those 16 DNA extracts from the “quasi non-destructive” sampling that gave amplification products for the LR region. The MSH was amplified in singlicate for each sample with identical reaction setup and cycling conditions as described above for the LR region.

The 16S and MSH PCR products were purified with the AMPure bead system (Beckman Coulter, Brea, CA, USA) and quantified with a Qubit 4 Fluorimeter (Thermo Fisher). The two amplicons of each DNA sample were

pooled with equimolar concentrations, and sequencing libraries were constructed with the Ion Plus Fragment Library Kit (Thermo Fisher) according to the vendor's protocol. The libraries were quantified with the Ion Library TaqMan Quantitation Kit (Thermo Fisher) and all samples were pooled with equimolar concentrations. Sequencing was carried out on an Ion S5 (Thermo Fisher) instrument at the Zurich Institute of Forensic Medicine, University of Zurich.

Analysis of the amplicon sequence data. Raw DNA sequence read data was exported to fastq files according to sequencing barcodes with the FileExporter plugin of the Torrent Suite software version 5.10. Primer sequences were removed from the end of the sequences of each fastq file using the cutadapt algorithm⁶⁴ implemented on the Galaxy server⁶⁵. Trimmed sequences were quality-filtered using Usearch⁶⁶ with a maximum expected error threshold of 100 and clustered into operational taxonomic units (OTUs) with Uparse⁶⁷ at 97% minimal identity threshold and minimal OTU size of 10 sequence reads, as default settings. In some cases, these settings were slightly modified for more relaxed quality filtering and clustering to allow OTU creation for samples with lower quality sequence reads. Sequences of the resulting LR and MSH OTUs were aligned and the alignments were concatenated in Geneious version 11.1.5 (<https://www.geneious.com>). Our concatenated LR-MSH sequence alignment was added to the LR-MSH alignment of reference samples of the eight precious coral species listed in Table 1. The taxonomic identity of our sequences was determined by constructing a Bayesian phylogenetic tree as described in Supporting Methods S2. We noticed that several of the DNA sequences obtained from the coral samples were not identical with any of the reference sequences of the eight precious coral species described to be found in the international trade. We therefore performed an additional phylogenetic analysis with identical settings, which included the orthologous LR-MSH DNA sequences of all Coralliidae specimens from Tu, *et al.*⁴⁷ that were identified to the species level (Supplementary Table S8).

Data availability

Raw DNA sequence data generated for this study are deposited in the NCBI Sequence Read Archive under submission number SUB6412194. Data used for the analyses is available as Supplementary Information.

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Author contributions

B.L., A.K., M.S.K., L.E.C. and N.V.M. conceived the study. M.S.K. and L.E.C. provided the coral samples. J.B.M. conducted preliminary DNA extraction and sequence analysis. B.L., N.V.M. and M.G. performed the laboratory work and analyzed the data. B.L. and L.E.C. wrote the manuscript with support from the other co-authors.

Competing interests

The authors declare no competing interests.

Additional information

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