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Applied Conservation Genomics of Military macaws (*Ara militaris*)

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ABSTRACT

The military macaw (*Ara militaris*) is bird species native to the Americas and has been historically subdivided into different subspecies, based on its geographic origin. Even though the species is endangered, its evolutionary history and its relationship to the closely related great green macaw (*Ara ambiguus*) remains uncertain, hampering potential long-term conservation initiatives. In order to reconstruct the phylogenetic history of both species, a capture enrichment strategy was used in combination with next-generation sequencing to sequence 40 mitochondrial genomes from museum samples, covering almost their complete distribution range. Bayesian analysis of these mitochondrial sequences provided robust support for a monophyletic *A. ambiguus* and *A. militaris* clade. For the *A. militaris* clade, additional diversification was found between the populations from Mexico and South-America. However, little phylogeographic structure was found within populations from either Mexico or South-America. In contrast to an earlier study, diversification was found between populations from Central-America and Ecuador for the *A. ambiguus* clade. In conclusion, a phylogenetic tree based on genetic data with an almost complete sampling was generated for a first time for military and great green macaws. Moreover, with these findings captive animals can finally be genetically screened to ascertain their geographic origin and as such can be involved in conservation programs Furthermore, we have proved that museum samples offer a unique and reliable source of DNA for phylogenetic studies.

ABSTRACT IN LAYMEN TERMS

The military macaw is an endangered parrot species native to the Americas and has been historically subdivided into different subspecies, based on its geographic origin. However, these presumed subspecies are morphologically nearly indistinguishable and their taxonomy remains a matter of debate. Moreover, their relation to the great green macaw, a similar looking parrot species, has also not been resolved. As such, the taxonomy of these species was unraveled by sequencing DNA from macaw museum samples. The amount of DNA available in these samples is often limited and damaged compared to fresh samples, and as such a specialized protocol was used in the lab. By comparing the sequences of the different museum samples, a clear difference between the military macaws and great green macaws was found, indicating that they indeed are two distinct species. Moreover, military macaws from Mexico also differentiated from those found in Central-America and as such these populations can be seen as two different subspecies. For the great green macaws, a difference was found between samples from Central-America and from Ecuador. In conclusion, in this study the complete taxonomy of these species was unraveled. Moreover, with these findings captive animals can finally be genetically screened to ascertain their geographic origin allowing them to participate in breeding programs.

SAMENVATTING

De soldatenara (*Ara militaris*) is een vogelsoort afkomstig uit Amerika die historisch gezien onderverdeeld wordt in verschillende ondersoorten, naargelang zijn geografische oorsprong. Hoewel de soort bedreigd is, blijven zowel zijn evolutionaire geschiedenis als zijn relatie tot de nauwverwante buffons ara (*Ara ambiguus*) onzeker, waardoor mogelijke conservatie-initiatieven belemmerd worden. Om de fylogenetische geschiedenis van beide soorten te reconstrueren, werd een DNA 'vangst-verrijking' strategie in combinatie met nieuwe generatie sequencing-technologieën toegepast om maar liefst 40 mitochondriale genomen van museumstalen, afkomstig uit nagenoeg de hele verspreiding van de soorten, te sequeneren. Een Bayesiaanse analyse van deze mitochondriale sequenties gaf met grote zekerheid ondersteuning voor een aparte *ambiguus* en *militaris* clade. Voor de *militaris* in het specifiek, werd nog een verdere diversificatie gevonden tussen populaties vanuit Mexico enerzijds en Zuid-Amerika anderzijds. Een verdere fylogeografische structuur werd echter noch bij populaties uit Mexico, noch uit Zuid-Amerika gevonden. In tegenstelling met een eerdere studie, werd voor de *ambiguus* clade wel nog een verdere diversificatie gevonden tussen populaties uit Centraal-Amerika en Ecuador. Tot slot, in deze studie hebben we voor een eerste maal een fylogenetische boom gegenereerd gebaseerd op genetische data die nagenoeg alle populaties omvatte. Bovendien kunnen we aan de hand van deze resultaten ara's in gevangenschap eindelijk screenen om hun geografische achtergrond met zekerheid vast te stellen. Hierdoor kunnen deze dieren betrokken worden bij internationale conservatieprogramma's. Verder hebben we ook aangetoond dat museumstalen een unieke en betrouwbare bron van DNA vormen die gebruikt kan worden bij fylogenetische studies.

1. INTRODUCTION

1.1 Genetic characterization

1.1.1 Evolutionary history

The evolutionary history of natural populations has been of interest throughout the last couple of centuries in that it tells us something about *e.g.* speciation processes. These processes arise in response to environmental changes (or by pure coincidence) and are retained in the genetic composition of a species. Recently, human activities have accelerated a dynamic resulting in a sharp decline in biodiversity (Young et al., 2005; Kim and Byrne, 2006). Moreover, biodiversity will keep declining unless drastic changes are made (Butchart et al., 2010). As such, apart from a pure fundamental interest, there's an ever increasing need for detailed information on the history of species for conservation purposes.

Even though Central America is one of the most diverse terrestrial regions in the world, its evolutionary history remains under debate. Several geologic and climatic events have been proposed to explain the vast number of species, including the Andean uplift (Hoorn et al., 2010), the linking of North and South America by the Isthmus of Panama (Bacon et al., 2015) and subsequent glacial and interglacial periods (Haffer, 1969), but both the timing and effect on biodiversity by these events remain largely uncertain (Baker et al., 2014). An increasing amount of genetic information however, in combination with geologic data, allows the opportunity of deciphering general patterns in the biogeographical and speciation processes in this region (Gutierrez-Garcia and Vazquez-Dominguez, 2013).

While the evolutionary history of parrots in America is well understood on a large scale *i.e.* genus-level (Schweizer et al., 2014), detailed information on the intragenus-level is still missing. Green macaws *i.e.* the military and great green macaw, display a remarkable distribution pattern and as such are excellent study species to help unravelling the evolutionary history of parrots. The military macaw (*A. militaris* Linnaeus, 1766) is a large green macaw native to the Americas and has a broad yet patchy distribution reaching from Mexico to Argentina (Figure 1). Interestingly, military macaws do not appear in Central America, where the great green macaw (*Ara ambiguus* Bechstein, 1811) lives (Figure 1). The great green macaw looks very similar to the military macaw, but is slightly taller, has a lighter plumage and its tail is colored in a paler red.

Based on their geologic distribution, three subspecies of the military macaw have been proposed: *Ara militaris mexicanus* (Ridgway 1915), living in Mexico, *Ara militaris bolivianus* (Reinchenow, 1908), distributed from Bolivia to northwestern Argentina and lastly *Ara militaris militaris* (Linnaeus, 1766), occurring in between the two others. Conversely, although the different subspecies (seemingly) live allopatric, the great morphological similarities and strong migratory tendencies (Parr and Juniper, 2010) suggest that the subspecies boundaries might not be as strict as previously described. To answer this question, Eberhard et al. (2015) analyzed three mitochondrial regions of recent military macaw samples of two different presumed subspecies (*A.m. militaris, A.m. mexicanus*) and found a trend towards differentiation between isolated *Ara militaris* populations, suggesting for a first time based on genetic data that different evolutionary lineages do exist. The sample size and geologic range of this study however were limited and additional research is necessary to confirm these findings.

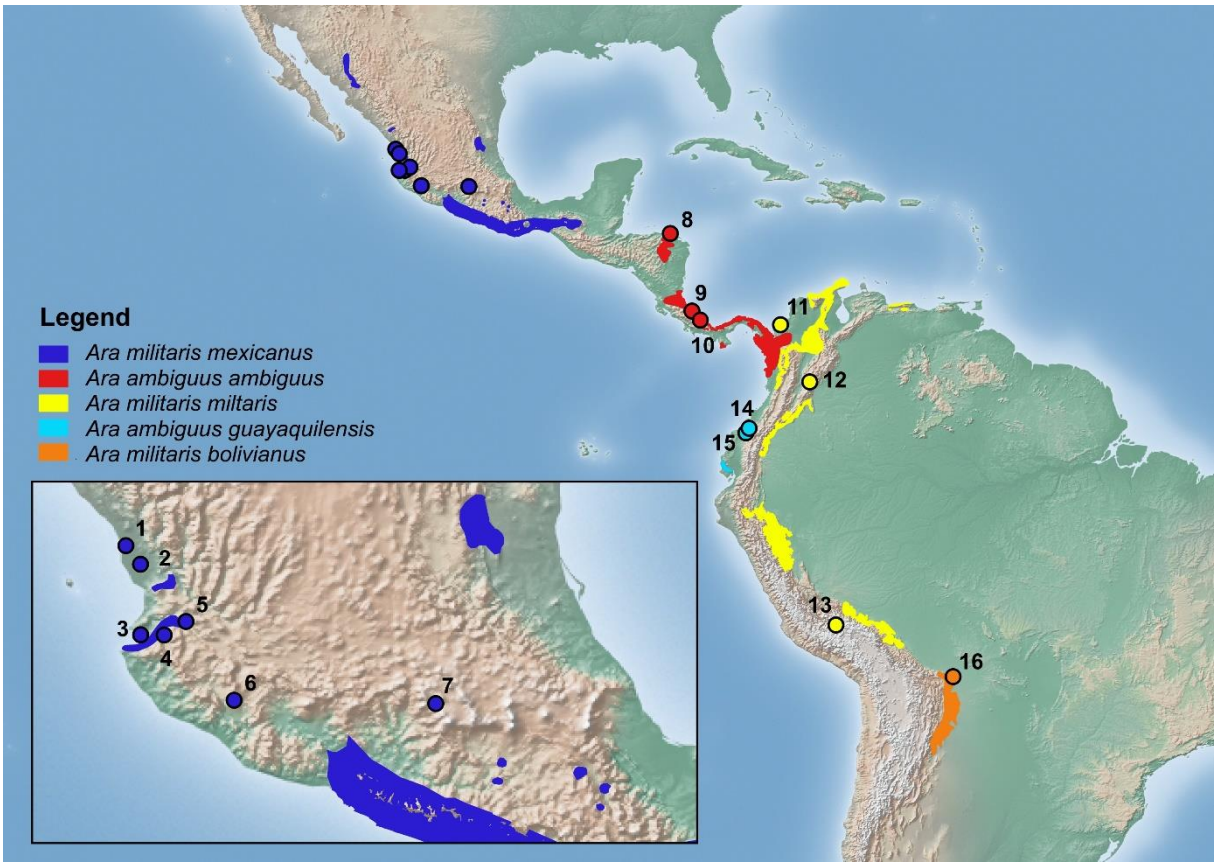

Figure 1: Current distribution of *Ara militaris* and *Ara ambiguus*. Data from Birdlife International and NatureServe (2014). The numbered points represent the localities of the museum samples examined in this study and correspond to those listed in Table 3.

The great green macaw has also been subdivided into different subspecies based on their geologic distribution: *Ara ambiguus ambiguus* (Bechstein, 1811), distributed from Honduras to

north-west Colombia and *Ara ambiguus guayaquilensis* (Chapman, 1925), occurring in western Ecuador. Although their relation to the military macaw hasn't been fully resolved yet, a phylogenetic analysis based on mitochondrial data showed that both species are closely related, but do form two distinct monophyletic clades (Eberhard et al., 2015). However, since *guayaquilensis* appears to be morphological intermediate between the two species (Fjeldsa et al., 1987), some have suggested that military and great green macaws might be conspecific (Fjeldsa et al., 1987; Parr and Juniper, 2010). A second hypothesis, namely that *guayaquilensis* is a hybrid population, has also been proposed (Fjeldsa et al., 1987).

1.1.2 Conservation genetics

While resolving the evolutionary history of these bird may provide unique insights in speciation processes in Central America, it also provides a unique chance to support current conservation programs. Both species are listed as vulnerable by the IUCN Red List of Threatened Species (IUCN, 2015) and populations keep decreasing due to capture for illegal pet trade and habitat destruction. Although reintroduction programs have been started in the past (Clubb and Clubb, 1991), additional conservation efforts are necessary to save these species from future extinction (Collar and Butchart, 2014) while keeping their taxonomic integrity intact.

Ex situ conservation, the conservation of species outside their natural habitat, is a widely applied method and has already proved to be valuable to save a range of species from extinction, such as Arabian oryx's (*Oryx leucoryx*), black-footed ferrets (*Mustela nigripes*), Przewalski's horses (*Equus ferus przewalskii*) and some plant species including Franklin trees (*Franklinia alatamaha*) and Cooke's kok'ias (*Kokia cookie*) (Frankham et al., 2010). Furthermore, habitat restoration initiatives have increased in recent years (*e.g.* Initiative 20x20, AFR100), allowing the opportunity of reintroducing *ex situ* populations into the wild.

In order to serve as a "back-up" population, captive populations part of reintroduction programs should genetically reflect their wild counterparts. Unfortunately, this issue has been largely overlooked for a long time and many breeding programs have been exclusively based on morphological rather than genetic similarities. However, even to date with the advances in molecular techniques, breeding individuals according to their (sub)species is not always possible since the necessary genetic information of the wild population is often missing and the origin of the founders is often not known. As a result many current *ex situ* populations are in

fact hybrid populations *i.e.* populations were the founders originate from genetically distinct (sub)species. Tigers (*Panthera tigris*) (Luo et al., 2008), chimpanzees (*Pan troglodytes*) (Hvilsom et al., 2013) and dwarf crocodiles (*Osteolaemus tetraspis*) (Ziegler et al., 2015) are just a few examples of species that commonly occur as hybrids in a captive setting. Although hybridization can occur in the wild and has shaped the evolution of many animal and plant species (Grant and Grant, 1992; Dowling and Secor, 1997; Soltis and Soltis, 2009), increased levels of hybridization of geographically separate taxa due to anthropogenic interventions have potential negative effects and in a worst-case scenario may even lead to the extinction of the natural population upon reintroduction (Rhymer and Simberloff, 1996; Allendorf et al., 2001). However, it is important to note that genetic mixing can be advantageous under some circumstances as well. Many inbred populations, for example, have been rescued by crossing them with a closely related outbred population (*e.g.* Madsen et al., 2004; Pimm et al., 2006). In the view of possible reintroduction, most conservation programs try to avoid hybridization as much as possible though, which can't be effectively done unless the evolutionary history of the species is completely understood.

1.1.3 Study material

One way to solve the evolutionary history of the green macaws, is by studying their genetic structure. But while sampling genetic data from wild populations can be very time- and labor-intensive *per se*, endangered species are rare by definition, making data sampling even more challenging. Museum samples on the other hand are a unique source of DNA and provide a valuable alternative. The use of ancient DNA (aDNA) in conservation studies is a relative new strategy, mainly due to the large drawbacks that are characteristic for museum samples. The amount of endogenous DNA available is often limited and consists of short degraded fragments (Higuchi et al., 1984; Paabo, 1989). Over time these fragments have been chemically modified, resulting in base misincorporations during sequencing. Despite these problems, new insights in the chemical degradation processes (Pääbo et al., 2004; Stiller et al., 2006; Briggs et al., 2007; Briggs et al., 2010) and recent technical innovations in the field of genetic research, in particular the high-throughput next generation sequencing techniques, (reviewed by and Paijmans et al., 2013; Burrell et al., 2015) have turned the use of aDNA into a viable tool.

1.1.4 Aim

The aim of this study is to resolve the evolutionary relationships of the military and great green macaw using a comprehensive genetic dataset representing all suggested subspecies. Collecting wild samples from these birds is challenging though due to their rarity, extremely wide distribution and possible local government issues. Moreover, reintroductions of confiscated animals in the past (Clubb and Clubb, 1991) may have biased the authencity of current populations. Museum samples therefore provide an elegant solution for these problems. Indeed, multiple studies have already succesfully derived evolutionary relationships from ancient samples (*e.g.* Guschanski et al., 2013; Haus et al., 2013; Springer et al., 2015). As such, the full mitochondiral genome obtained from 31 military macaws and seven great green macaws, collected from 16 different locations (see points Figure 1), was analyzed. Mitochondrial DNA is present in 100 to 1000 copies in each cell (as opposed to nuclear DNA of which only 2 copies are present), is less susceptible to degradation processes due to the double mitochondrial membrane protecting it and is suitable to study evolutionary patterns of recently diverged species because of its high mutation rate (Avise, 2009). Since the amount of endogenous mitochondrial DNA is museum samples is limited, the mitochondrial DNA was enriched via in solution hybridisation (See 2.2) using probes designed from recent military macaw samples and sequenced on an Illumina MiSeq platform. A phylogenetic tree was constructed based on the obtained sequences and in the future a timetree will be built and compared to geologic and climatic data to assess the importance of past events in the speciation of these bird species. Furthermore, by resolving the evolutionary relationships, current *ex-situ* populations can be screened for founders and different management units can be defined in a conservation framework.

1.2 Sexing

Macaws are sexually monomorphic, making sex identification on the basis of their external morphology difficult. Identifying the sex can add important information though, as it may give a broader understanding of the population structure which may be influenced by sex-specific dispersal patterns (Rollins et al., 2009; Li and Merila, 2010). The use of molecular methods to determine the sex of these birds thus provides a great advantage. Nowadays, the most commonly applied methods are based on PCR amplification of sex-linked genes that differ in length. These PCR products can be discriminated after electrophoresis, with male birds showing one band and female birds two bands due to their different chromosomal arrangement. As

opposed to mammals, male birds are homogametic and have two identical sex chromosomes (ZZ genotype) whereas female birds are heterogametic and have two different sex chromosomes (ZW genotype). Regions that are regularly used as sex markers using this method are *EE0.6* (*i.e.* 0.6 kb *Eco*RI fragment) (Itoh et al., 2001), *Wpkci* (Hori et al., 2000) and especially *CHD1*, which can be used in all bird species, with the exception of ratites (Griffiths et al., 1996; Griffiths et al., 1998). Accordingly, a number of researches have focused on the optimization of sex identification primers to routinely determine the sex in as many bird species as possible (e.g. Bantock et al., 2008; Wang et al., 2010; Vucicevic et al., 2013).

Museum samples however pose a challenge because the size of the DNA fragments decreases drastically with sample age due to degradation (Lee and Griffiths, 2003; Sefc et al., 2003). While most molecular sexing methods target regions of 300 to 400 base pairs (bp) (Griffith, 1998), previous results of the Fragment Analyzer™ (Advanced analytical, see methods) revealed that the museum extracts had an average length of approximately 100 bp. Therefore a new set of primers based on the *ATP5A1* gene was designed specifically for the macaw museum samples, potentially opening opportunities for sex determination in other sources of degraded DNA, such as fecal samples, feathers etc. in macaws and potentially other bird species. *ATP5A1* is thought to be the avian homolog of the human ATP synthase α-subunit (Fridolfsson et al., 1998) and is found as a copy on both the Z and W sex chromosomes. However, to date, this gene has received limited attention as a possible sex marker (but see Bantock et al., 2008).

Nevertheless, the strength of using this gene is largely based on the idea that it relies on (1) the amplification of a short stretch of DNA that still differs in composition between the Z and W chromosomes and as such (2) that subsequent restriction only affects one of the two amplicons. Moreover, Bantock et al. (2008) already evaluated the utility of this gene as a new sex marker in passerine birds and obtained clear and reliable results. The aim of this study is to design a new primer pair specifically for the macaw museum samples that would generate even shorter fragment lengths than the one obtained by Bantock et al. (2008). The primer pair was initially tested and optimized using recent DNA samples and subsequently used to determine the sex of a number of museum samples.

2. METHODS

2.1 Reconstructing mitochondrial genomes

2.1.1 General overview

For the sequencing of the museum samples to be economical, it is necessary to reduce the total DNA pool to the regions of interest (*i.e.* mitochondrial DNA in this study) before sequencing (Mamanova et al., 2010). Classic approaches rely on Polymerase Chain Reaction (PCR) to amplify specific regions. However, since we're working with fragmented DNA samples from a non-reference species, designing and optimizing suitable primers would be cumbersome. More specifically, considering that the museum extracts are fragmented, this would result in over 100 PCR to amplify the complete 16 988 bp (Urantowka, 2015) mitochondrial macaw genome.

An increasingly popular approach to target specific genomic regions that overcomes these problems, is capture by hybridization (Mamanova et al., 2010). Basically, illustrated on the basis of this study, single stranded DNA is used as 'bait' to capture mitochondrial DNA from the museum samples. While the mitochondrial DNA preferentially binds to the bait DNA and is retained for sequencing, the unbound (mostly nuclear) DNA molecules are washed away. As such the final library is enriched for mtDNA. An alternative term for this library preparation method is therefore 'target enrichment'. Apart from mitochondrial DNA, this method can also be used to enrich other genomic regions from other sources of DNA. In this study, the protocol by Maricic et al. (2010) was followed to enrich the museum samples. While several alternative hybridization protocols involve the initial investment in expensive laboratory materials and/or artificially synthesized specific probes, the protocol of Maricic et al. (2010) can be carried out with standard laboratory equipment, making it usable for smaller laboratories such as the one in the Zoo of Antwerp where the research was conducted. The library preparation *i.e.* the preparation of the museum samples, was carried out as described in Meyer and Kircher (2010).

The protocol is summarized in Figure 2. The left-handed side of the figure illustrates the bait preparation, whereas the right-handed side shows the preparation of the museum samples *i.e.* library preparation. Bait preparation starts with high quality recent DNA samples to produce long-range PCR products (Figure 2-I). To avoid the presence of one dominant product above the others, the overlapping long-range PCR products are first pooled in equimolar

concentrations. Subsequently these pooled amplicons are sheared into smaller fragments (Figure 2-II), blunt-end repaired and finally ligated to biotinylated adapters (Figure 2-III) (*i.e.* short DNA fragments that are covalently bound to biotin). Since the streptavidin-biotin connection is one of the strongest non-covalent bindings in nature (Weber et al., 1989), these adapters can be used to efficiently immobilize the bait DNA on streptavidin-coated magnetic beads (Figure 2-V). Although bait is created from only one of the presumed subspecies of macaws, based on previous studies we are certain that the mitochondrial DNA of the different subspecies is sufficiently similar to hybridize to the bait. More specifically, hybrid capture has been reported to work even when bait and library DNA differ up to 13% (Mason et al., 2011).

To enable sequencing of the museum sample DNA on an Illumina platform, ligation of special Illumina adapters to the samples is necessary (Figure 2-VI). These adapters allow binding to the Illumina flow cell, partly consist of a sequence complementary to the sequencing primer and include a unique index for each museum sample (*i.e.* a DNA sequence of six oligonucleotides that allows for discrimination after pooling). While pooling the indexed libraries (Figure 2-VII) has the benefit of increasing the number of samples per run without increasing the cost or time, the museum samples are subdivided into seven different capture pools according to their DNA concentration after library preparation to reduce the risk of overrepresentation of one or a few samples due to differences in initial DNA quality.

The enrichment is carried out in solution during which the single stranded pooled museum samples hybridize to the immobilized bait (Figure 2-IX). The magnetic properties of the beads on which the bait is immobilized allow stabilization of the beads while the undesired (nuclear) DNA that did not hybridize can be removed. In a next step the museum DNA is denatured from the beads (Figure 2-X) (remember that the bait is connected to the beads with a biotin-streptavidin connection which can only be broken under extreme conditions), pooled and sequenced on the Illumina platform (Figure 2-XI).

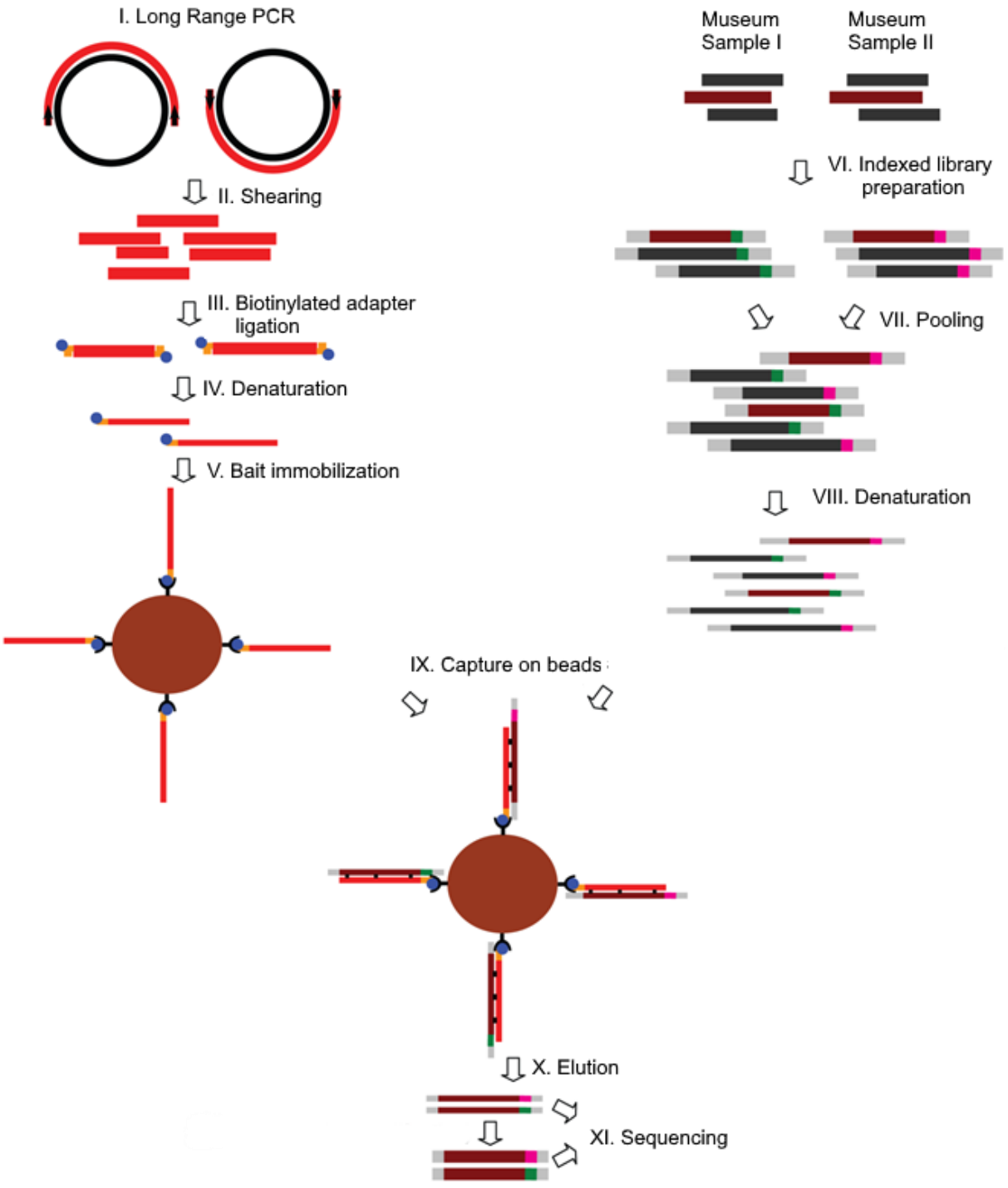

Figure 2: Overview of the capture-on-beads methods as described in Maricic et al. (2010). (I-V) Bait preparation: The bait generated from the long-range PCR products is colored in red, the adapters in orange and the biotin in blue. During bait immobilization the biotinylated PCR products bind to magnetic streptavidin (the black fork) coated beads. (VI-VIII) Library preparation: The extracted DNA contains both nuclear (black) and mitochondrial (dark red) DNA. The Illumina adapters are shown in grey and the unique indexes that allow discrimination after pooling are shown in pink and green. (IX-XI) Capture: While the mitochondrial DNA of the museum samples preferentially binds to the bait, the nuclear DNA is more likely to be washed away. After elution the desired fragments are sequenced on an Illumina platform. Figure adapted from Maricic et al. (2010).

2.1.2 Bait preparation

2.1.2.1 Start material

In order to enrich the libraries for mtDNA, three overlapping long-range PCR products, encompassing the whole mitochondrial genome, were generated from present-day *A. militaris* samples. Genomic DNA was extracted from liver and muscle tissue with the Nucleospin Tissue DNA Extraction Kit (Machery-Nagel) following the manufacturer's protocol, with the exception that the final elution was performed in two steps: one elution in 40 µL elution buffer (EB) resulting in a concentrated DNA extract and a second one in 60 µL generating a lower but still sufficiently concentrated back-up extract.

Since data of the full mitochondrial genome of *A. militaris* wasn't available at the time, conserved regions for primer design were initially based on the set of long-range primers (resulting in two long-range fragments) proposed by Meyer et al. (2007) for human mitochondrial DNA. Primers were optimized based on published sequence data of *Ara macao* (Seabury et al., 2013; Genbank: CM002021.1), a close relative of the military and great green macaw. However, due to problems with amplifying one off the long-range fragments, a third region for primer design was localized to reduce the amplicon length. Available parrot data was aligned to the *A. macao* genome using Geneious version 6 (http://www.geneious.com, Kearse et al., 2012) and the most conserved regions served as an ideal location for primer design. Uniqueness of these regions within the Ara genome was assessed by using the Basic Local Alignment Search Tool (BLAST). Finally, three long-range overlapping PCR-fragments, covering the complete mitochondrial genome, were proposed and tested (Table 1, Figure 3): long-range product 1a (5800 bp), long-range product 1b (5700 bp) and long-range product 2 (7175 bp) (Derveaux, 2014 *unpublished work*).

Table 1: Primers used for the long-range PCR's.

Primer	Direction	LR product*	Sequence (5'-3')
LR1_fwd3_no_amb	Forward	1a	CTGATTCTTCGGACACCC
LR1a_R5630	Reverse	1a	TCTTGTTGGTTGAACCTCCC
LR1b_F5173	Forward	1b	TCCTCCGTAAGCCACATAGG
REV2_Longrange1	Reverse	1b	GGATTGTATGTCCATGTCAA
ara_ChrM_LongrangeF2	Forward	2	GGTCCATTCTTTCCCCCTAC
ara_ChrM_LongrangeR2	Reverse	2	GAACCCTAGGAATCCGATTGA

* Long-range product

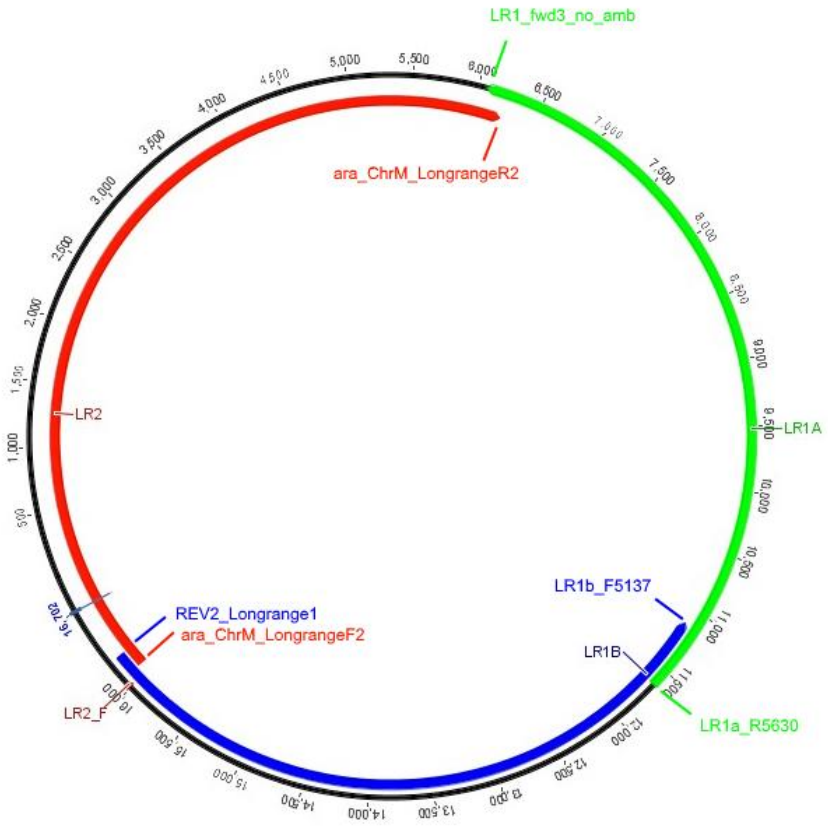

Figure 3: Overview of the three long-range PCR products covering the complete *Ara macao* mitochondrial genome. Long-range product 1a and its primer pair are colored in red, long-range product 1b and its primer pair in blue and long-range product 2 and its primer pair in green. Figure created in Geneious version 6 (http://www.geneious.com**, Kearse et al., 2012).**

High quality DNA samples were used as template for these three long-range PCR's, which were performed on a Mastercycler Epgradient S (Eppendorf). This instrument was used for all PCR's, unless mentioned differently. Each PCR was carried out in a total volume of 50 µL, containing 500 mM of each dNTP, 0.2 U of Longrange PCR enzyme mix, 0,4 µM of each primer, 5 µL of 10x Longrange PCR buffer, 10 µL of 5x Qsolution (all Qiagen LongRange PCR Kit) and 5µL of template DNA (approximately 150ng). Reaction conditions were an initial 93°C for 3 minutes, followed by 35 cycles of 93°C for 15 seconds, 53°C for 30 seconds and 68°C for 5'20'' minutes for long-range product 1a and 1b. Similar reaction conditions were used for long-range fragment 2, except for the elongation time, which was increased to 7'30'' minutes according to its longer amplicon size. Amplification success was verified by loading 3 µL of the PCR-product on a MidoriGreen© prestained 1.2% agarose gel for 60 minutes at 100 Volts. All three long-range products of the initial test PCR's were sequenced in both directions (Macrogen, the Netherlands) and aligned to the *A. macao* reference genome to ensure that the right fragments were amplified before they were processed into baits.

Next, the long-range fragments were purified with the Nucleospin Gel and PCR Clean-up Kit (Machery-Nagel) and sheared by sonication with a M220 Focused-ultrasonicator (Covaris). The shearing protocol consisted of three shearing cycles of 20 seconds with the output selector set to high in order to obtain a fragment size distribution between 100 and 1000 bp (Maricic et al., 2010). Samples were centrifuged in between cycles. To verify whether the shearing resulted in the desired fragment lengths, 3 µL of each sheared sample was loaded on a 1.2% agarose gel. Finally, in order to pool the three long-range PCR fragments (1a, 1b and 2) in equimolar amounts to a total of 1.3 µg DNA, the concentrations of the fragments were measured using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific). This apparatus was used for all DNA quantifications, unless mentioned differently.

2.1.2.2 Bait preparation

Blunt ending, adapter ligation and adding the bait to the beads procedures were all carried out as described in Maricic et al. (2010). All purification steps were performed with the MinElute PCR Purification Kit (Qiagen), unless mentioned differently. A positive control was incorporated into each step and success was assessed by running the control on an agarose gel. Before using the actual long-range products, the bait protocol was tested with a 300 bp long PCR product originating from the sex determination of a kings penguin (see 2.3 for more information on sex determinations).

Bait DNA was prepared for a total of seven different captures, with each capture targeting mtDNA of five to six individual museum sample. First, overhangs of the pooled long-range products created during shearing were removed either by filling in the 5' overhangs or removing the 3' overhangs by T4 DNA polymerase, resulting in polished (*i.e.* blunt ended) fragment ends. The 5' ends were phosphorylated by T4 DNA kinase for subsequent adapter ligation (Quick Blunting Kit, New England Biolabs). After yet another purification step, Bio-T/B adapters were ligated to the fragment ends with a Quick Ligation Kit (New England Biolabs). These adapters consist of two short complementary DNA sequences, oligo B and oligo T, of which the latter has a biotin-tag connected to its 5'end (Table 2). The biotinylated DNA was purified once more and the concentration of the resulting products were measured. Finally, the bait DNA was denatured by heating and immobilized on magnetic M-270 streptavidin coated Dynabeads (Thermo Scientific).

Table 2: Oligonucleotides used for the bait preparation and capture (Maricic et al, 2010).

Oligonucleotide	Sequence (5'-3')
Bio-T	Biotin-TCAAGGACATCC*G
B	CGGATGTCCTT*G
BO1.P5.F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCATCT- phosphate
BO2.P5.R	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATACATT-phosphate
BO3.P7.part1.F	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-phosphate
BO4.P7.part1.R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT- phosphate
BO5.P7.part2.F	ATCTCGTATGCCGTCTTCTGCTTG-phosphate
BO6.P7.part2.R	CAAGCAGAAGACGGCATACGAGAT-phosphate

* Optional phosphorothioate bond to render the oligonucleotide resistant to nuclease degradation

2.1.3 Library preparation

2.1.3.1 Start material

Toe pads from 40 different museum samples of military and great green macaws were collected. A complete overview of all samples and their localities is given in Table 3. Since modern DNA contamination remains one of the biggest problems when working with ancient DNA, all museum sample extractions were carried out in a specialized lab at the Royal Belgian Institute of Natural Sciences in Brussels by Vanja Prévot and Elien Derveaux. Because museum samples have often been chemically treated for long term preservation, additional washing steps before the actual extraction were necessary. The samples were first washed in elution buffer for 3 to 4 hours, then in 70% ethanol for 3 to 4 hours and finally in pure water overnight. DNA was extracted from the collected toe pads using Nucleospin Tissue DNA Extraction Kit (Machery-Nagel) according to the manufacturer's protocol with the following two exceptions: An additional centrifugation step for 5 minutes at 11000 x g was added after the last washing step and the final elution was performed in two steps: the first one in 40 µL and the second one in 60 µL (see 2.1.2.1). Concentrations and purity of all extracts were measured with a Nanodrop spectrophotometer and additionally eight samples were measured with a Fragment Analyzer™ (Advanced analytical) to precisely determine the average fragment length.

Table 3: List of museum samples analyzed in this study (continued on the next page).

CRC Lab Code	Country	Catalogue number	Date collected	Locality	Locality on map (Figure 1)	Scientific Name	Concentration after extraction (ng/µL)
NH_A11	Mexico	Skin-704427	18/04/1895	Quimeche River	1	*Ara militaris mexicanus*	7.78
NH_A20	Mexico	Skin-474245	12/05/1891	Tuxpan, Tepic	2	*Ara militaris mexicanus*	9.29
NH_A19	Mexico	Skin-474242	20/02/1892	Bahia de Banderas	3	*Ara militaris mexicanus*	Not measured
NH_A22	Mexico	Skin-474241	20/02/1892	Bahia de Banderas	3	*Ara militaris mexicanus*	14.72
NH_A8	Mexico	Skin-105359	23/01/1906	Los Mazos	4	*Ara militaris mexicanus*	3.09
NH_A6	Mexico	Skin-105360	7/01/1906	Los Masos	4	*Ara militaris mexicanus*	3.62
NH_A23	Mexico	Skin-474244	16/12/1891	Barranca del Oro, Tepic	5	*Ara militaris mexicanus*	7.04
NH_A21	Mexico	Skin-474243	17/12/1891	Barranca del Oro, Tepic	5	*Ara militaris mexicanus*	8.72
NH_A7	Mexico	Skin-105361	26/07/1905	Volcano Colima	6	*Ara militaris mexicanus*	Not measured
NH_A10	Mexico	Skin-105362	24/07/1905	Volcano Colima	6	*Ara militaris mexicanus*	75.31
NH_A34	Mexico	Skin-105365	24/07/1905	Volcano Colima	6	*Ara militaris mexicanus*	7.05
NH_A33	Mexico	Skin-105364	24/07/1905	Volcano Colima	6	*Ara militaris mexicanus*	8.55
NH_A9*	Mexico	Skin-105363	25/07/1905	Volcano Colima	6	*Ara militaris mexicanus*	21.54
NH_A12	Mexico	Skin-804990	5/02/1904	Volcan de Colima	6	*Ara militaris mexicanus*	32.94
NH_A31*	Mexico	Skin-393341	23/04/1909	Las Penas	7	*Ara militaris mexicanus*	7.84
NH_A14	Mexico	Skin-393340	17/04/1909	Las Penas	7	*Ara militaris mexicanus*	8.33
NH_A30	Mexico	Skin-393338	18/04/1909	Las Penas	7	*Ara militaris mexicanus*	21.78
NH_A13	Mexico	Skin-393339	26/04/1909	Las Penas	7	*Ara militaris mexicanus*	9.34
NH_A36	Honduras	Skin-812649	29/11/1977	Pisma, up Rio Platano	8	*Ara ambiguus ambiguus*	4.47
NH_A37	Costa Rica	Skin-389257	28/12/1924	Limon	9	*Ara ambiguus ambiguus*	3.97
NH_A38	Panama	Skin-247346	3/07/1927	Almirante	10	*Ara ambiguus ambiguus*	12.08
NH_A40	Panama	Skin-247345	4/07/1927	Almirante	10	*Ara ambiguus ambiguus*	12.30

* Sample analyzed in the trial run

Table 3 (continued): List of museum samples analyzed in this study

CRC Lab Code	Country	Catalogue number	Date collected	Locality	Locality on map (Figure 1)	Scientific Name	Concentration after extraction (ng/µL)
NH_A15	Nicaragua	Skin-102433	9/10/1907	Unknown		*Ara ambiguus ambiguus*	20.79
NH_A29	Colombia	Skin-474240	Unknown	Bogota	11	*Ara militaris militaris*	13.34
NH_A5	Colombia	Skin-474233	29/11/1896	Sta Marta	12	*Ara militaris militaris*	8.42
NH_A4	Colombia	Skin-474234	29/11/1896	Sta Marta	12	*Ara militaris militaris*	10.71
NH_A32	Colombia	Skin-833892	31/08/1899	Unknown		*Ara militaris militaris*	5.53
NH_A18	Colombia	Skin-474235	Unknown	Unknown		*Ara militaris militaris*	92.38
NH_A16	Peru	Skin-474238	Unknown	Loreta	13	*Ara militaris militaris*	5.35
NH_A17	Peru	Skin-474237	Unknown	Loreta	13	*Ara militaris militaris*	30.87
NH_A28	Peru	Skin-474239	Unknown	Loreta	13	*Ara militaris militaris*	31.54
NH_A27	Peru	Skin-474236	Unknown	Loreta	13	*Ara militaris militaris*	76.58
NH_A39	Ecuador	Skin-474249	14/05/1901	Rio Duranga	14	*Ara ambiguus guayaquilensis*	5.36
NH_A35	Ecuador	Skin-474250	22/11/1901	Rio Tapayo	15	*Ara ambiguus guayaquilensis*	5.36
NH_A1	Bolivia	Skin-474231	14/09/1889	Santa Cruz	16	*Ara militaris bolivianus*	17.07
NH_A26	Bolivia	Skin-474232	14/12/1889	Unknown		*Ara militaris bolivianus*	Not measured
NH_A2	Bolivia	Skin-474230	9/02/1906	High forest		*Ara militaris bolivianus*	13.54
NH_A25	Bolivia	Skin-474229	9/02/1906	High forest		*Ara militaris bolivianus*	14.96
NH_A24	Bolivia	Skin-474227	27/03/1906	High forest		*Ara militaris bolivianus*	15.5
NH_A3	Bolivia	Skin-474228	20/02/1907	High forest		*Ara militaris bolivianus*	32.82

2.1.3.2 Library preparation

The library preparation was performed according to the protocol of Meyer and Kircher (2010), with a few exceptions: all SPRI Bead reaction clean-up steps were replaced by spin column purifications with the MinElute PCR Purification Kit (Qiagen) since these are more easy to use when handling large sample sizes, all reaction volumes were halved to reduce product costs and, although Meyer and Kircher (2010) assure that amounts as small as 100 pg per sample are sufficient to successfully prepare the libraries, at least 20 ng DNA of each museum sample was used to ensure sufficient coverage across the complete genome would be obtained. A positive and negative control were incorporated for each step and the whole protocol was tested with a positive control before using actual museum samples. Initially, a PCR fragment originating from the sex determination of a male kings penguin of 300 bp was used to test the robustness of the method. However, because later analysis indicated that the museum samples have an average size of approximately 100 bp, a second control was performed with a 106 bp long PCR fragment of the gene Amelogenin (AMEL). Using a positive control of roughly the same length as the museum samples avoids any possible length biases during the experiment or additional recalculations due to length differences.

The steps involved in the library preparation are broadly the same as the ones in the bait preparation: first the samples are blunt-end repaired and subsequently the adapters are ligated. However, since the museum samples will be sequenced on an Illumina platform, some significant differences occur during adapter ligation. Another noteworthy difference is the fact that the museum samples weren't sheared before the library preparation, since their fragments are already sufficiently short due to degradation processes over time.

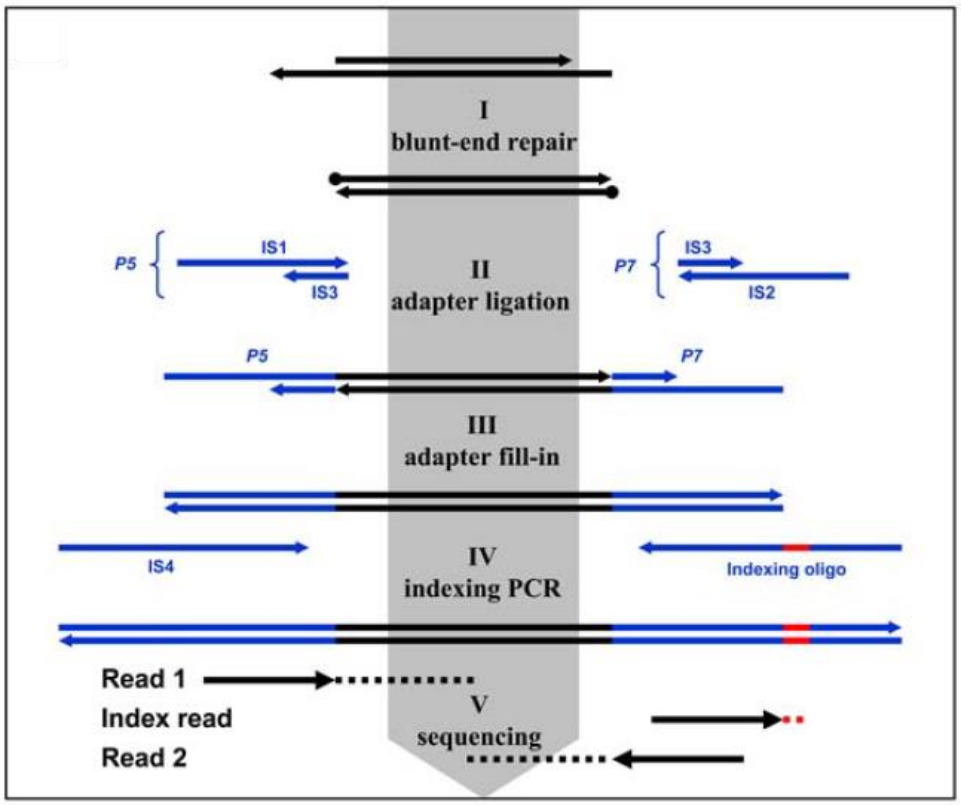

Figure 4: Overview of the library preparation (modified form Meyer & Kircher, 2010).

The full overview of the library preparation is shown in Figure 4. Analogue to the bait preparation, samples were blunt-end repaired by removing the overhangs with T4 DNA polymerase and the 5' ends were phosphorylated with T4 polynucleotide kinase (both Thermo Scientific) (Figure 4-I). Subsequently two different adapters, P5 and P7, were ligated to both ends of the blunt-ended fragments with T4 DNA ligase (Thermo Scientific) (Figure 4-II). These adapters were created in a hybridization reaction with either IS1 and IS3 or IS2 and IS3 (Table 4) and are necessary to attach the library fragments to the Illumina flow cell. The 5' overhangs of the adapters were filled in with *Bst* polymerase (New England Biolabs) (Figure 4-III). The full adapter sequences and indexes were added with a tailed-PCR (Figure 4-IV) in a total volume of 50 µL, containing 1 Unit of Phusion Hot Start DNA Polymerase (New England Biolabs), 10 µL of 5x Phusion HF Buffer (New England Biolabs), 0.2 µM of both Primer IS4 and one of the indexing primers (Table 4), 200 µM of each dNTP and 10 µL (approximately 1-10 ng) of the sample DNA. Each library was amplified with a unique indexing primer to allow discrimination of the samples after pooling. The indexing PCR started with an initial 98°C for 30 seconds, followed by 18 cycles (*i.e.* the number of cycles to reach PCR plateau, calculated from the amount of sample DNA) of 98°C for 10 seconds, 60°C for 20 seconds and 72°C for

20 seconds with a final extension step of 72°C for 10 minutes. Amplification success was verified by loading 3 µL of the PCR products on a 2.4% agarose gel.

Table 4: Oligonucleotides used for the library preparation (Meyer & Kircher, 2010).

Oligonucleotide	Sequence (5'-3')
IS1_adapter.P5	A*C*A*C*TCTTTCCCTACACGACGCTCTTCCG*A*T*C*T
IS2_adapter.P7	G*T*G*A*CTGGAGTTCAGACGTGTGCTCTTCCG*A*T*C*T
IS3_adapter.P5+P7	A*G*A*T*CGGAA*G*A*G*C
IS4_indPCR.P5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT
IS5_reamp.P5	AATGATACGGCGACCACCGA
IS6_reamp.P7	CAAGCAGAAGACGGCATACGA

* PTO bond

2.1.3.3 Quantitative Real-Time PCR (qPCR)

Successful amplified products from the indexing PCR were quantified with a quantitative real-time PCR (qPCR) using a StepOne^{TM} Real-Time PCR System (Applied Biosystems) in a total volume of 10µL. Each reaction contained 1µl of a 10^{-4} or 10^{-5} dilution of the library sample, 1x of the the Fast SYBR Green PCR mix (Thermo Scientific) and 0.5µM of primers IS5 and IS6 (Table 4). The reaction started with an initial 95°C for 10 minutes, followed by 45 cycles of 94° for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds. (Meyer and Kircher, 2010). Additionally, a dilution series of the indexed positive control, AMEL, whose concentrations was first measured on the Nanodrop was included to serve as a calibrator. To determine the efficiency of the qPCR, the C_t values of AMEL were plotted against the logarithm of the number of molecules and the resulting graph could be used to calculate the concentration of the library samples.

Before starting the targeted capture, the library were combined into seven different capture pools according to their concentration *i.e.* the first capture pool contained the six libraries with the lowest concentrations, the second capture pool the six libraries with the second lowest concentrations, etc., as such trying to avoid overexpression of high quality samples in one specific pool.

2.1.4 Targeted capture

The hybridization capture was performed as described in Maricic et al (2010). In each capture pool the DNA was denatured and hybridized to six blocking oligonucleotides (Table 2) with the Agilent aCGH Hybridization Kit (Agilent) that cover the whole adapter sequence, except of the indexes, to prevent them from cross-linking to other adapters and possible leading to the capture of unwanted (nuclear) DNA. Subsequently, each capture pool was mixed with immobilized bait and rotated in an oven at 65°C for over 30 hours. Finally the non-hybridized fragments were washed away and the enriched libraries were melted of the beads with 125mM NaOH. The eluted library pools were purified and quantified with a quantitative-PCR (see 2.1.3.3) before sequencing.

2.1.5 Sequencing

The enriched library pools were sequenced on two lanes of the Illumina MiSeq platform. Sequencing was performed paired-end *i.e.* both ends of the fragments were sequenced, with 150 cycles per forward and reverse read.

2.1.6 Trial run

Prior to the actual preparation of the 40 museum samples, two museum samples were initially prepared as described above by Veerle Lemmens (Lemmens, 2015 *unpublished work*), including some changes that have been previously described to minimize the effect degradation on quality and quantity of the samples. The full workflow of the trail run is depicted in Figure 5. Both the efficiency of enrichment as the effect of removal of deaminated cytosines on the quality of the samples were examined. The latter treatment is based on the fact that most conversions due to degradation processes in old DNA samples are causes by the deamination of cytosine, resulting in C→T and G→A conversions (Pääbo et al., 2004; Stiller et al., 2006). These sites however can be removed by treatment with USER enzyme, potentially increasing the quality of the samples.

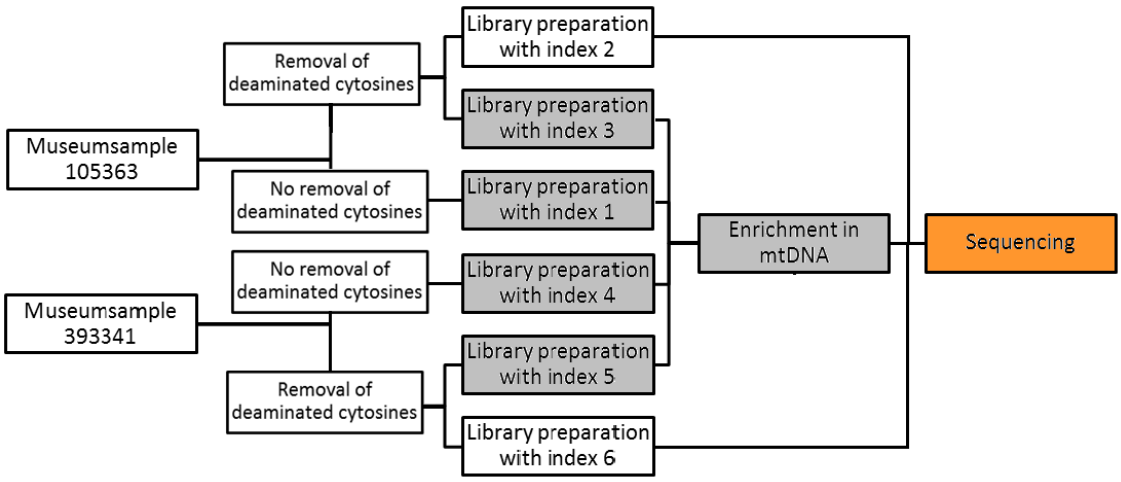

Figure 5: Overview of the steps involved in the trial run.

2.1.7 Data analysis

2.1.7.1 Mapping analysis

The reads of the pooled libraries were provided as demultiplexed files (containing a file with forward reads and a file with reverse reads), according to their barcode. A script was written in nano to process all reads of the sequenced museum samples. To properly map, align and process the reads in downstream analyses, additional quality filtering steps are necessary. The quality of the reads for example tends to dip near the read end due to desynchronization during sequencing (Fuller et al., 2009). Moreover, when fragment lengths are short, sequencing can even continue after passing the second adaptor, leading to extra noise and as a consequence these parts should be trimmed to retain high quality data. As such, ends of reads were trimmed when the quality of a position fell below the threshold of 20 on the PHRED scale. A PHRED quality score is a measure of reliability of a base-call on NGS platforms (Ewing and Green, 1998) and a score of 20 corresponds to a 99% base call accuracy. Also 'adapter read-through', the presence of parts of the adapter sequence at the read end, is a well-known cause of contamination in the sequence reads (Kircher, 2012) and should be taken into account. Therefore adapter sequences were subsequently clipped using Trimmomatic (Bolger et al., 2014). Additionally, in ancient DNA chemical modifications due to degradation processes over time may result in incorrect base-calling during sequencing. These modifications mostly occur at single-stranded overhangs though (Briggs et al., 2007) and therefore, as an alternative to the treatment with USER enzyme, additionally six base pair were trimmed from both ends of the read to minimize the number of incorrect base-callings,. PCR duplicates were removed with

RmDup (Li et al., 2009) and unique reads were mapped against the mitochondrial genome of *Ara militaris* (Eberhard and Wright, 2016; GenBank: NC_027839.1) using BWA (Li and Durbin, 2009). Reads with insertions or deletions were discarded from the alignment, as they probably do not represent real mitochondrial sequences. Finally, in the consensus sequences, sites with no or only a 1-fold coverage were considered as 'N', indication that no information existed for that particular site.

2.1.7.2 Testing for contamination

Contamination during sample preparation remains a possibility and can lead to erroneous conclusions when not corrected for, especially when processing museum samples where the initial amount of endogenous DNA is very low. As such, the prevalence of human mitochondrial DNA, the most likely source of contamination in our dataset, was checked by aligning the reads of the museum samples to both the military macaw mitochondrial genome (GenBank: NC_027839.1) and the Cambridge reference sequence of the human mitochondrial genome (GenBank: NC_012920) given as possible references. Reads that would map to the contaminant human mitochondrial reference sequence rather than the target macaw mitochondrial reference sequence were considered contaminants.

2.2 Phylogenetic analysis

2.2.1 Data Partitioning

The 40 sequences obtained from the museum samples were assembled, aligned and annotated in Geneious version 6 (Kearse et al., 2012) using the military macaw mitochondrial genome sequence (GenBank: NC_027839.1) as a reference. In addition, three sequences for outgroup species were exported from GenBank and added to the alignment: *Ara ararauna* (GenBank: KF010315), *Ara Macao* (GenBank: CM002021.1) and *Ara glaucogularis* (NC_027839).

The aligned sequences were divided into tRNA, 12s RNA, 16s RNA and the 13 protein-coding genes. With the exception of the former, all partitions were further subdivided into first, second and third codon positions, as these positions may all evolve under different conditions (Bofkin and Goldman, 2007), resulting in a total of 46 different partitions. In order to simultaneously find the best-fitting partitioning schemes and nucleotide substitution models, Partitionfinder version 1.1.0 was used (Lanfear et al., 2012). For the analysis, the model selection was based

on the Akaike information criterion corrected for finite sample sizes (AICc), with branch lengths linked, the models restricted to those implemented in MrBayes and the search algorithm set to greedy. *Ara ararauna* was set as outgroup.

2.2.2 Phylogenetic reconstruction

For the phylogenetic reconstruction, a Bayesian analysis was run with MrBayes version 3.2.6 (Ronquist et al., 2012) using the appropriate partitions and evolutionary models. The analysis was run for 10 000 000 generations and run diagnostics were sampled for each 1000th generation. Convergence was assessed by evaluating whether the average standard deviation of split frequencies was <0.001 and 25% of the first samples were discarded as burn in. Finally, the tree was visualized using FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

In addition to reconstructing a phylogenetic tree exclusively based on our own data, a second tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean Algorithm (UPGMA) with Tamura-Nei set as evolutionary model in Geneious version 6 (Kearse et al., 2012) combining both our own data and data generated by Eberhard et al. (2015). Because Eberhard et al. (2015) only focused on a subset of genes of the mitochondrial genome, *i.e.* COI, ND2 and ATPase 6 and 8, the assembling and aligning of both datasets and the resulting reconstruction were restricted to these regions.

2.3 Sexing

2.3.1 General overview

To determine the sex of the museum samples a polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) method was developed. This two-step procedure starts with an initial amplification of the target sequence of the *ATP5A1* gene, resulting in PCR-products of 127 bp and 120 bp for the W and Z sex chromosomes respectively (Figure 6 A). These products can be visualized on an agarose gel which in theory should display 1 band for male birds (ZZ genotype) and 2 bands for female birds (ZW genotype). However, due to the small difference in length between the two fragments, this could lead to ambiguous results (Figure 7 A).

As such the second part of this method consists of digestion of the Z fragment with *Smo*I, a restriction enzyme. Since the necessary restriction site is only present on the Z fragment, the digestion cuts the Z fragment in two new fragments (73 and 51 bp) while leaving the W fragment intact. (Figure 7 B). Thus *Smo*I digestion yields three bands for female birds (127, 73 and 51 bp) and two bands for male birds (73 and 51 bp), which can be more easily identified on a gel (Figure 7 B).

2.3.2 Primer Design & Restriction Site

Due to the large amount of fragmentation in the museum samples, amplification of the *ATP5A1* gene should target small amplicons. In addition, the selected fragment should have a unique restriction site on one of the two sex chromosomes as the length polymorphism might be too small to detect on a gel. Primers were designed based on the published sequence data of *Ara ararauna* (GenBank: AY004860.1, AY004859.1), *Aratinga mitrata*,(GenBank: AY004858.1, AY004857.2), *Ara macao* (GenBank: AOUJ01421624.1, AOUJ01207454.1) and *Psittacula krameri* (GenBank: AY004855.2, AY004854.1), whom are all members of the Psittacidae family. The sequences were aligned to find conserved regions for primer design. Additionally primers were checked for compatibly. Finally a suitable Z chromosome specific restriction site was identified. All analyses were performed in Geneious version 6 (http://www.geneious.com, Kearse et al., 2012).

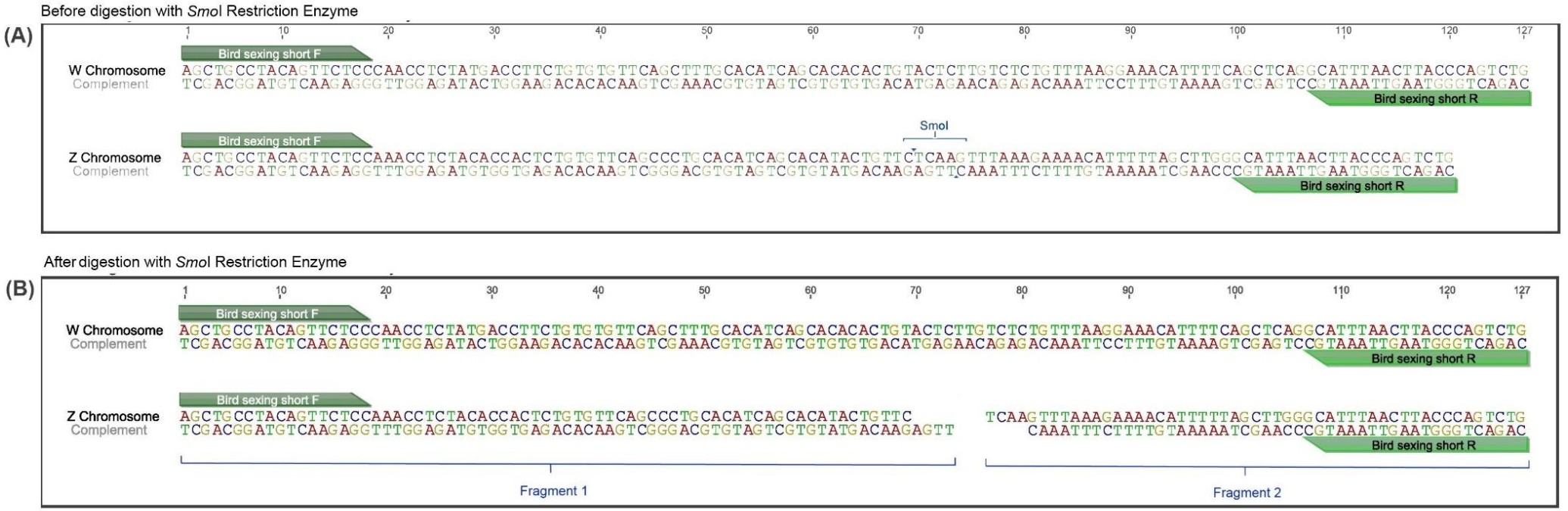

Figure 6: Target sequences for sex determination based on *Ara ararauna* sequence data. Primer sequences are indicated with overlaying green boxes. The *Smo*I cutting site is indicated with small blue arrows. A: the target sequence of the *ATP5A1* gene, which is present on both the Z and W sex chromosomes in birds. The PCR products are 127 bp and 120 bp for the W and Z chromosome respectively. B: the fragments after treatment with *Smo*I restriction enzyme. Fragment 1 of the Z chromosome is 73 bp, while fragment 2 is 51 bp. The fragment of the W chromosome remains unchanged after treatment, as the *Smo*I restriction site is not present on this sequence. Figures were created in Geneious version 6 (http://www.geneious.com, Kearse et al., 2012).

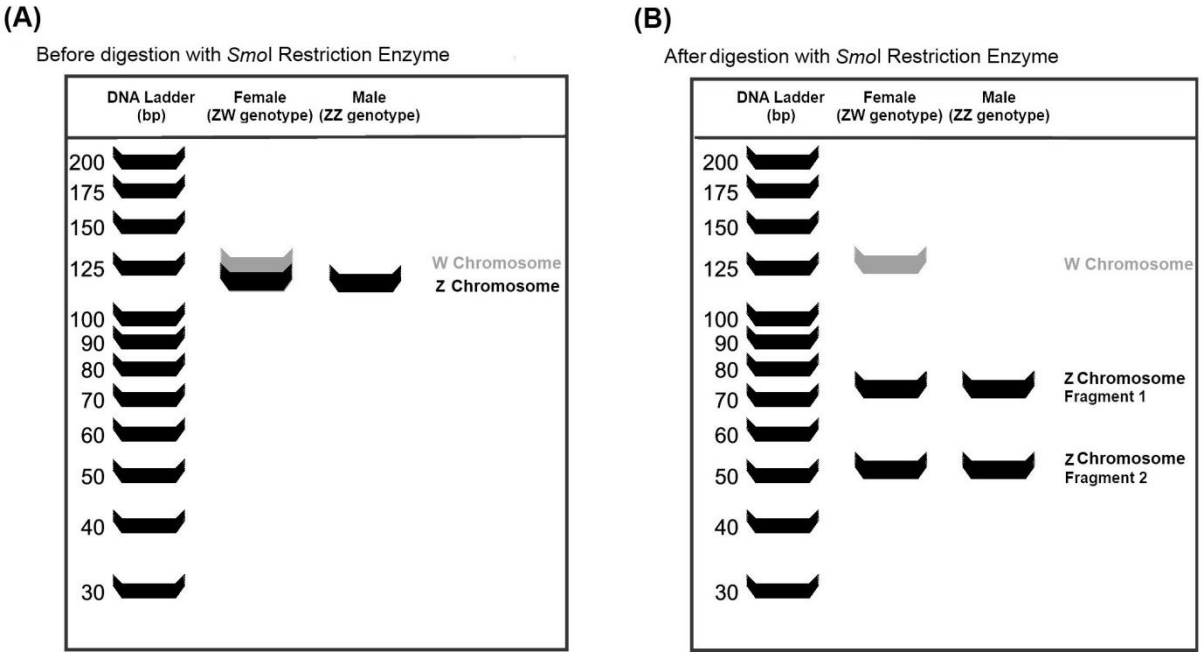

Figure 7: Predicted gel patterns for sex determination in *A. militaris* museum samples before (A) and after (B) digestion with *Smo*I restriction enzyme. *Smo*I only cuts the Z chromosome, resulting in 3 bands for female samples (ZW genotype) and two bands for male samples (ZZ genotype). Figures were created in Geneious version 6 (http://www.geneious.com**, Kearse et al., 2012).**

2.3.3 PCR RFLP

A segment of the *ATP5A1* gene was amplified by the newly designed primer pair *Bird sexing short F* and *Bird sexing short R* (Table 5). Each PCR was performed in 10 µL containing 1 µL sample DNA (approximately 5-30 ng), 100 ng of each primer, 0.2 mM of each dNTP, 0.5 µg of bovine serum albumin (BSA), 1.25 U of Ampli*Taq* Gold DNA Polymerase (Applied Biosystems), 1 µL of PCR Gold Buffer 10x (Applied Biosystems) and 3mM $MgCl_2$ (Applied Biosystems). Amplification started with denaturation at 94°C for 8 minutes, followed by 40 cycles of 94°C for 1 minute, 50.7°C for 30 seconds and 72°C for 30 seconds with a final extension step of 72 °C for 2 minutes (modified from Bantock *et al*, 2008). Amplification success was confirmed by running 2 µL of final amplicon on a 1.2% agarose gel for 30 minutes on 120 Volts.

Table 5: Primers used for bird sexing.

Primer	Direction	Sequence (5'-3')
Bird sexing short F	Forward	AGCTGCCTACAGTTCTCC
Bird sexing short R	Reverse	CAGACTGGGTAAGTTAAATGC

Digestion of the positive samples was performed in 16 µL containing 5 µL of the PCR mixture,1 µL *Smo*l (Thermo Scientific), 1 µL 10x Buffer Tango (Thermo Scientific) and 9 µL nuclease-free water. After incubating the mixture for 2 hours at 55 °C, *Smo*l was thermally inactivated by incubating the mixture for 20 minutes at 80 °C. To visualize the digested products, 8 µL of the digestion mixture was mixed with 1.6 µL loading dye and separated on a 2.4% agarose gel for 75 minutes on 80 Volts.

3. RESULTS

3.1 Genetic Characterization

3.1.1 Mitochondrial genome reconstruction

Before starting the actual preparation of all museum samples, two museum samples, samples 393 340 and 105 363, were run through a trial to validate the effect of the removal of deaminated cytosines by USER enzyme and the efficiency of the enrichment protocol as described by Maricic et al. (2010). While the USER enzyme treatment was inefficient *i.e.* the final number of sequences was greatly reduced (Lemmens, 2015 *unpublished data*), the enrichment protocol proved to be effective. Without enrichment, 0.49% and 0.31% of the reads of sample 393 341 and 105 363 respectively mapped to the military macaw mitochondrial genome, while after enrichment these values increased to 63% and 50.2% respectively (Figure 8).

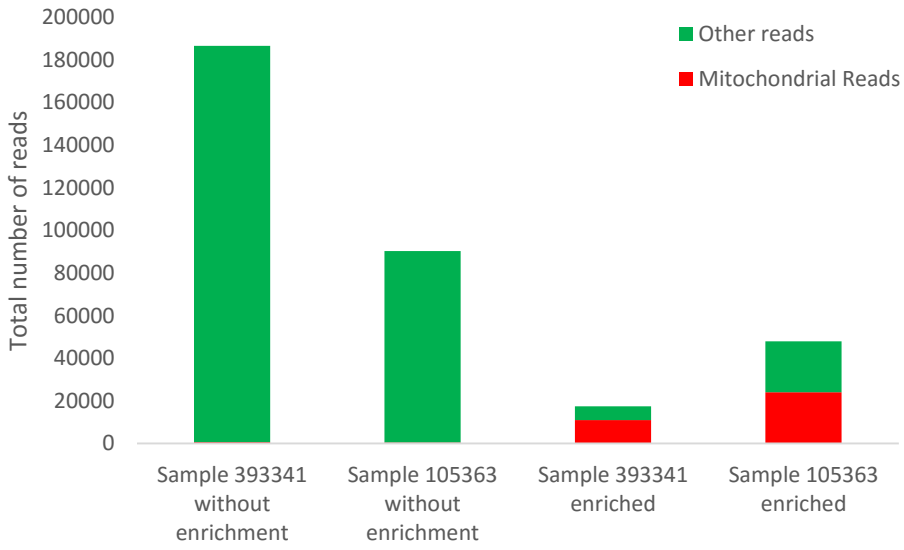

Figure 8: Number of reads sequenced and aligned to the mitochondrial genome for sample 393 341 and 105 363. Red bars represent the amount of sequences that mapped to the military macaw mitochondrial genome, while green bars represent sequences of other sources.

Subsequently the other 38 museum samples were successfully enriched for mitochondrial DNA, sequenced and quality filtered. The number of reads mapping to the military macaw mitochondrial reference sequence (GenBank: NC_027839.1) per individual varied between 978 and 352 758 (Table 6) and the median percentage of reads of each samples mapping to the mitochondrial genome was 30.6%. The average coverage per museum sample varied between 1.7- and 1157.2-fold (Table 6), indicating unbalanced library pools before sequencing, and the

median coverage of each sample was 29.8-fold. The coverage did not only vary between samples, but also between different sites of the same sample (Figure 9), with coverages ranging from 0- to 109-fold within a sample (*e.g.* Figure 9B). Overall, the regions of high and low coverage appear to be similar between different samples, irrespective of their average coverage across the mitochondrial genome (Figure 9).

Table 6: Summary of the sequencing and mapping results (continued on next page).

Library Pool	Sample ID	Number of reads in total flow cell (%)	Number of unique mapped reads against target mitochondrial genome	Number of unique mapped reads against human mitochondrial genome	Average read length (bp)	Average coverage
1	A7	2.46	118 794	1728	62.5 ± 26.9	379.8 ± 144.3
	A28	0.33	1652	1	58.6 ± 20.1	3.2 ± 3.0
	A19	0.73	10 985	128	54.8 ± 21.0	22.0 ± 11.5
	A11	7.33	352 758	4304	68.6 ± 24.3	1157.2 ± 448.4
	A26	0.40	9038	175	75.1 ± 28.3	23.5 ± 11.2
	A15	0.79	18 125	312	64.1 ± 23.1	43.0 ± 24.1
2	A17	0.47	5573	75	64.4 ± 21.9	12.4 ± 9.4
	A30	0.70	13 296	186	62.6 ± 21.3	28.1 ± 15.6
	A35	2.07	85 473	1116	66.7 ± 25.4	235.5 ± 106.1
	A25	1.5437	59 994	838	69.0 ± 25.1	168.2 ± 78.5
	A12	0.5586	6301	114	56.7 ± 18.5	11.5 ± 7.8
	A20	0.7631	21 083	361	66.6 ± 22.3	51.7 ± 23.7
3	A36	1.5376	57 394	850	84.0 ± 31.9	209.8 ± 85.3
	A23	0.6504	8014	124	67.1 ± 25.0	17.5 ± 9.2
	A16	0.5505	13 476	152	63.7 ± 23.9	31.5 ± 15.2
	A39	1.139	30 959	509	70.4 ± 26.9	80.9 ± 41.2
	A8	1.5615	72 301	814	72.8 ± 27.0	248.0 ± 90.9

Table 6 (continued): Summary of the sequencing and mapping results.

Library Pool	Sample ID	Number of reads in total flow cell (%)	Number of unique mapped reads against target mitochondrial genome	Number of unique mapped reads against human mitochondrial genome	Average read length (bp)	Average coverage
4	A22	0.3026	3526	20	59.9 ± 19.1	7.7 ± 5.7
	A27	0.2907	5388	94	66.8 ± 22.1	12.1 ± 7.0
	A32	0.5946	23 879	341	66.3 ± 23.9	67.9 ± 37.0
	A14	0.402	11 114	221	63.3 ± 22.3	27.5 ± 15.6
	A34	0.3844	9073	160	68.5 ± 25.5	24.7 ± 15.3
5	A38	0.5444	15 264	299	73.8 ± 28.9	39.4 ± 20.6
	A37	0.3926	10 050	206	70.3 ± 25.2	26.6 ± 15.7
	A5	0.4172	6271	4	76.1 ± 28.5	18.3 ± 9.8
	A6	0.4111	12 574	244	72.8 ± 26.3	34.6 ± 19.8
	A29	0.1993	3498	87	65.5 ± 23.2	7.7 ± 5.5
6	A24	0.1636	4312	82	69.6 ± 25.4	11.1 ± 6.8
	A21	0.0922	978	12	53.4 ± 25.0	1.7 ± 2.0
	A3	0.1879	3514	6	66.9 ± 23.4	9.3 ± 6.1
	A4	0.2113	5702	123	73.1 ± 28.9	14.9 ± 9.4
	A33	0.3078	7959	137	66.6 ± 24.0	19.9 ± 12.8
7	A1	6.6685	293 172	4416	70.9 ± 25.1	910.2 ± 402.7
	A10	1.5989	39 507	684	55.0 ± 24.5	75.9 ± 31.6
	A13	1.5977	40 836	728	64.2 ± 23.3	91.9 ± 41.2
	A2	1.3299	22 918	430	71.1 ± 23	56.7 ± 25.8
	A18	1.1179	20 864	302	63.8 ± 21.9	47.7 ± 24.8
	A40	0.6503	14 709	292	73.6 ± 27.8	38.9 ± 17.0

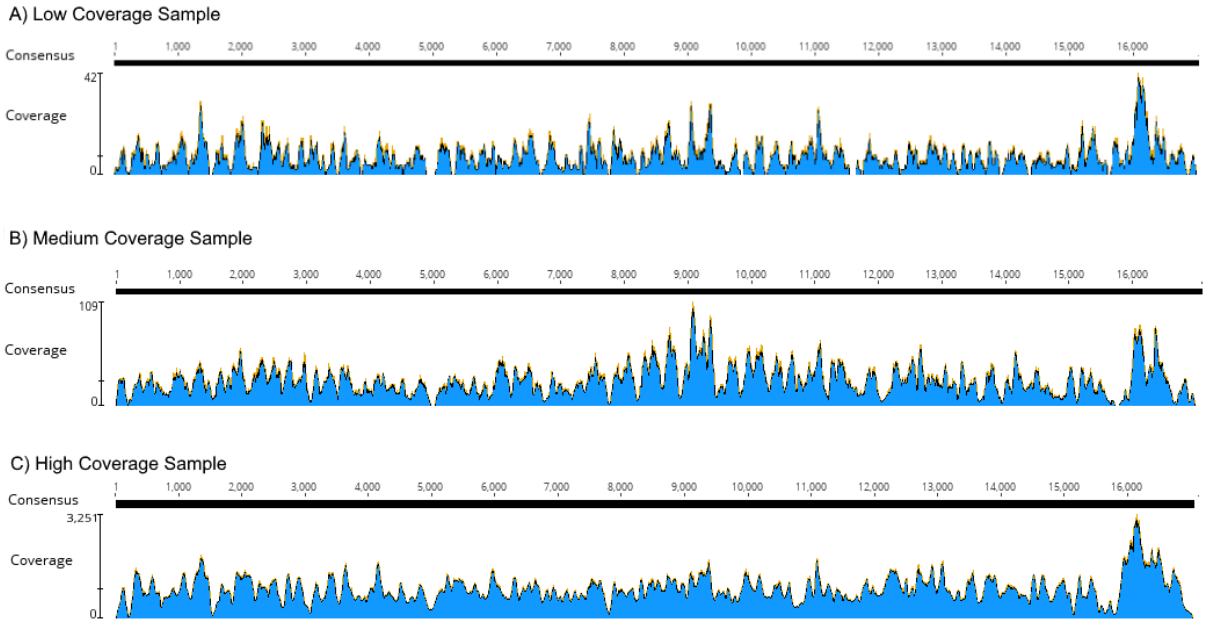

Figure 9: Coverage of each position across the mitochondrial genome for three different samples. (A) depicts the coverage profile of A22, a sample with a low average coverage. (B) depicts the coverage profile of A37, a sample with a medium average coverage. (C) depicts the coverage profile of A1, a sample with a high average coverage.

The average read length of the trimmed and aligned reads varied less between samples, ranging from 53.3 bp to 84.0 bp. A typical read length distribution is shown in Figure 10. The distribution is right-skewed with the number of reads abruptly increasing near 138 bp. This increase is caused by summation of fragments in the right-tail by that were initially longer than 140 bp, but were cut due to the read length of 150 bp and additional quality trimming (remember that in every read 6bp were clipped from both read ends decrease the amount of incorrect base-callings). Interestingly, the average read length is lower than what was initially measured with the Fragment Analyzer™ (Supplementary Material 4). This may points to either a bias towards shorter fragments in the enrichment protocol, a decrease in average fragment length to due absence of longer (>150 bp) fragments or less precise results by the Fragment Analyzer™.

Contamination remains a large risk when working with museum samples and NGS methods. However, the number of reads that preferentially mapped against the Cambridge reference sequence of the human mitochondrial genome (GenBank: NC_012920) was limited to 0.52 ± 0.29% on average for all reads. Furthermore, closer examination of these reads showed that they were mostly of bacterial origin.

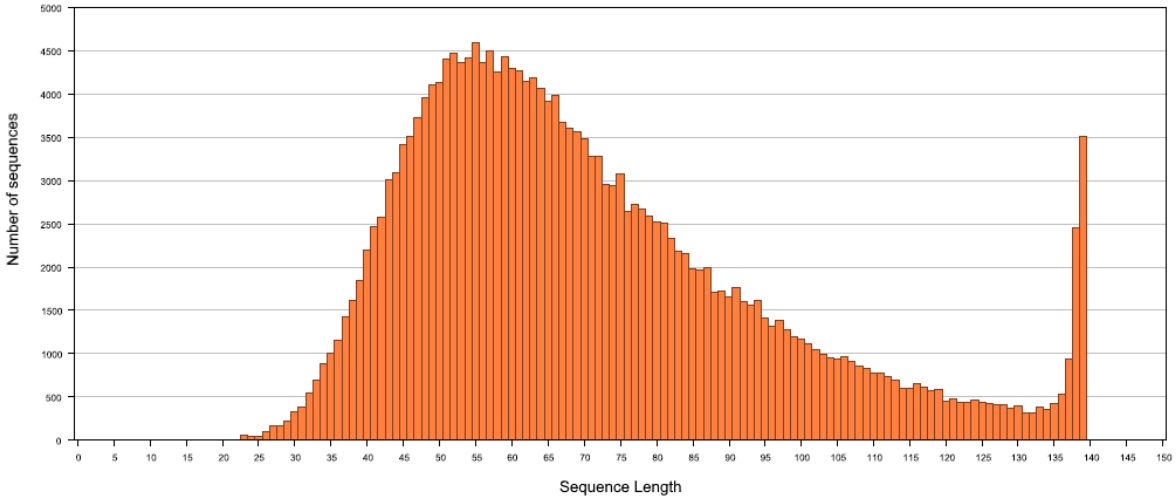

Figure 10: Length distribution of the reads of sample A1 after quality trimming. The sudden increase in number of sequences near the length of 140 bp is caused by reads that were originally longer than 150 reads, but were cut off due to the maximum read length of 150 bp and additional quality trimming.

3.1.2 Phylogeny

The best partitioning and evolutionary model schemes for the mitochondrial sequences were identified with Partitionfinder and resulted in 20 different partitions (Table 7). Using these partitioning and evolutionary model schemes, a Bayesian tree was constructed, which resulted in an highly supported tree topology with only two nodes with low support (<75% posterior probability) (Figure 11).

In agreement with a previous study (Eberhard et al., 2015), the phylogenetic analysis supports the monophyly of both the *Ara militaris* and *Ara ambiguus* clades. Within the military macaw clade, a next differentiation is found between the macaws from Mexico and those from South-America. Additional diversification within the Mexican clade does not seem to have a geographic structure. For example, sample A14 and A30 both originate from Las Penas but cluster in two different groups. Interestingly, in the South-American clade, three of the *A.m. militaris* samples (A17, A27 and A28) from Loreta cluster together with *A.m. bolivianus* samples. A fourth sample from Loreta however, clusters with the other *A.m. militaris* samples, indicating that at least in Loreta no clear differentiation for these two presumed subspecies exists. For the great green macaw clade, a clear differentiation exists for the samples from Ecuador, *Ara ambiguus guayaquilensis*, and those from Central-America, *Ara ambiguus ambiguus.*

Table 7: Best partitioning and evolutionary model schemes identified with Partitionfinder for the mitochondrial sequences of the 40 museum samples using models implemented in MrBayes only.

Partition	Evolutionary Model	Data Subset
1	HKY+I+G	tRNAs
2	GTR+I	12s RNA pos 1, 12s RNA pos 3
3	GTR+G	12s RNA pos 2, 16s RNA pos 1, 16s RNA pos 2, 16s RNA pos 3, ND5 pos 2
4	HKY+G	CytB pos 2, ND1 pos 1, ND3 pos 2
5	GTR+I	ATP6 pos 1, ATP8 pos 2, ND1 pos 2, ND2 pos 2
6	GTR	CytB pos 1, ND1 pos 3, ND2 pos 3
7	HKY	ND2 pos 1
8	GTR+G	COX1 pos 1
9	HKY+I	COX1 pos 2, COX2 pos 3, COX3 pos 1
10	GTR+I	COX1 pos3, COX2 pos 1, COX3 pos 2, CytB pos 3
11	GTR	ATP8 pos 3, COX2 pos 2, ND5 pos 1
12	GTR+I	ATP8 pos 1, ND3 pos 1, ND4L pos 2
13	GTR+I	ATP6 pos 2, ND6 pos 3
14	HKY+G	ATP6 pos 3, ND4L pos 3
15	HKY	COX3 pos 3
16	HKY+I	ND3 pos 3
17	GTR+I	ND4L pos 1, ND5 pos 3
18	HKY	ND6 pos 1
19	HKY+I	ND6 pos 2
20	HKY+I+G	Control region D-loop pos 1, Control region D-loop pos 2, Control region D-loop pos 3

HKY = Hasegawa, Kishino and Yano evolutionary model
GTR = Generalised time-reversible evolutionary model
+ I = A proportion of the sites are invariable
+ G = Gamma-distributed rates across sites
+ I + G = A proportion of the sites are invariable while the rate for the remaining sites are drawn from a gamma distribution.

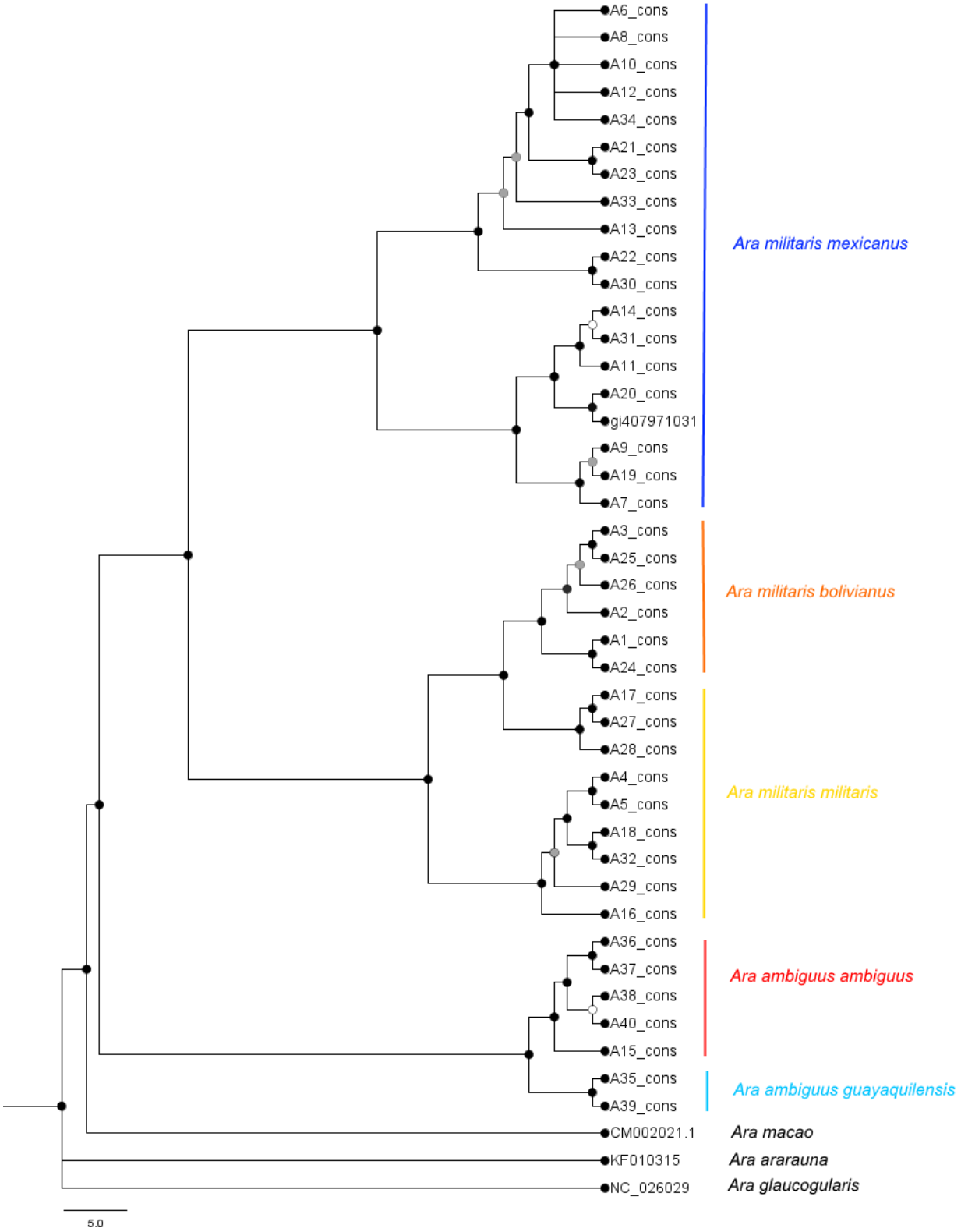

Figure 11: Bayesian phylogenetic tree of full mitochondrial sequences of green macaws. The color of the nodes represent posterior probabilities: well supported nodes are indicated in black (>95%), moderately supported nodes are indicated in grey (>70%) and notes that are not supported are indicated in white. Samples with an 'Axx_con' code represent sequences generated in this study and correspond to those listed in Table 3 , while other sample names indicate sequences downloaded from GenBank. The originally presumed subspecies of the samples, based on the sampling location, are indicated on the right. *Ara macao, Ara ararauna* and *Ara glaucogularis* were implemented as outgroup species. Figure from FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree**).**

In addition to the Bayesian tree, an additional UPGMA tree was reconstructed in Geneious version 6 (http://www.geneious.com, Kearse et al., 2012), combining both data from this study as from the study by Eberhard et al. (2015). Even though the data was limited to concatenated ND2-COI-ATPase 6 and 8 sequences and no partitioning schemes were implemented, a tree with a nearly identical topology (but with many low support values) as the one based on the complete mitochondrial sequences was generated (Figure 12). Moreover, an additional diversification between samples from Eastern Mexico, a region not covered in our own study, and Western Mexico was found. An important difference between the two trees however, is the placement of sample A15. In the tree based on full mitochondrial sequences, A15 is the sister species to all other *A.a. ambiguus* samples, whereas in the tree based on the concatenated sequences, A15 is placed basal as sister species to both the *A.a. ambiguus* and *A.a. guayaquilensis* samples. Furthermore, in contrast with Eberhard et al. (2015), samples from Ecuador (*A.a. guayaquilensis*) clearly cluster together and are the sister clade to the samples from Central-America (*A.a. ambiguus*). Finally, when trees based on just a single gene (*i.e.* ND2, COI or ATPase 6 and 8) were reconstructed, different topologies with low support were obtained (Supplementary Material 1,2 and 3), indicating that these regions alone are too short to reconstruct reliable phylogenies.

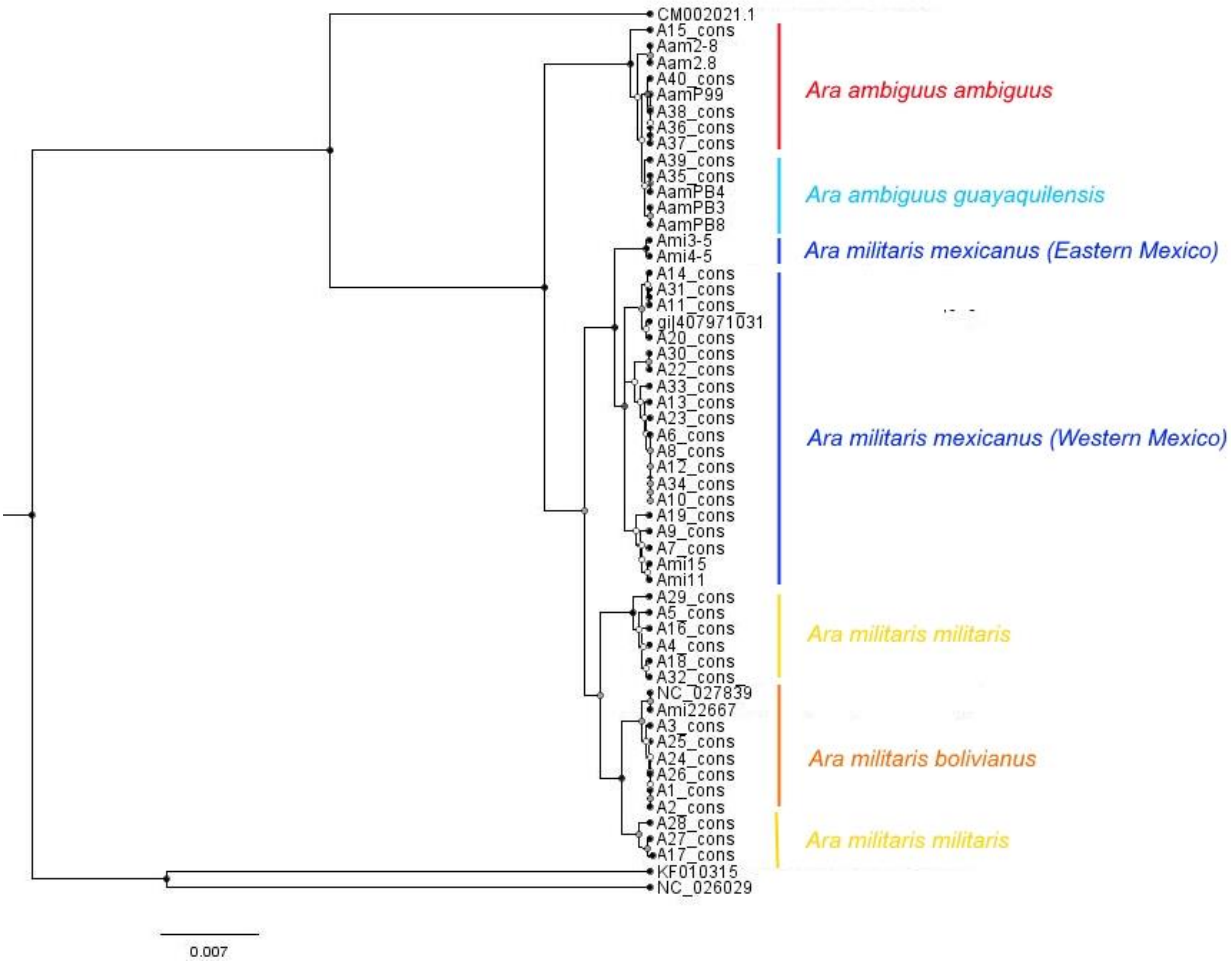

Figure 12: UPGMA phylogenetic tree based on concatenated ND2 – COI – ATPase 6 and 8 sequences from the mitochondrial genome. The color of the nodes represent posterior probabilities: well supported nodes are indicated in black (>95%), moderately supported nodes are indicated in grey (>70%) and notes that are not supported are indicated in white. Samples with an 'Axx_con' code represent sequences generated in this study and correspond to those listed in Table 3, samples with a 'Amixx' or 'AamPBxx' code correspond to data from Eberhard et al. (2015) and other sample names indicate sequences downloaded from GenBank. The originally presumed subspecies of the samples, based on their sampling location, are indicated in color on the right. Figure from FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree**).**

3.2 Sexing

To determine the sex of the museum samples, a small region of the *ATP5A1* gene was amplified and treated with *Smo*I restriction enzyme. Using this method, the sex of three museum samples was successfully determined (Figure 13). As expected, the newly designed primers produced fragment lengths of 120 and 127 bp for the W and Z chromosome respectively, although this difference is difficult to detect on a gel. For the positive controls an extra nonspecific band of approximately 200 bp was generated. This band wasn't affected by the *Smo*I treatment though and could be ignored in the later interpretation. The *Smo*I treatment successfully cut the Z fragment, while leaving the W fragment intact and the sex of the samples could therefore easily be determined by either the presence (female birds) or absence (male birds) of a band around

120 bp. Note that for the museum samples the second elution was used, as first elutions are too valuable to use while still optimizing the sexing method.

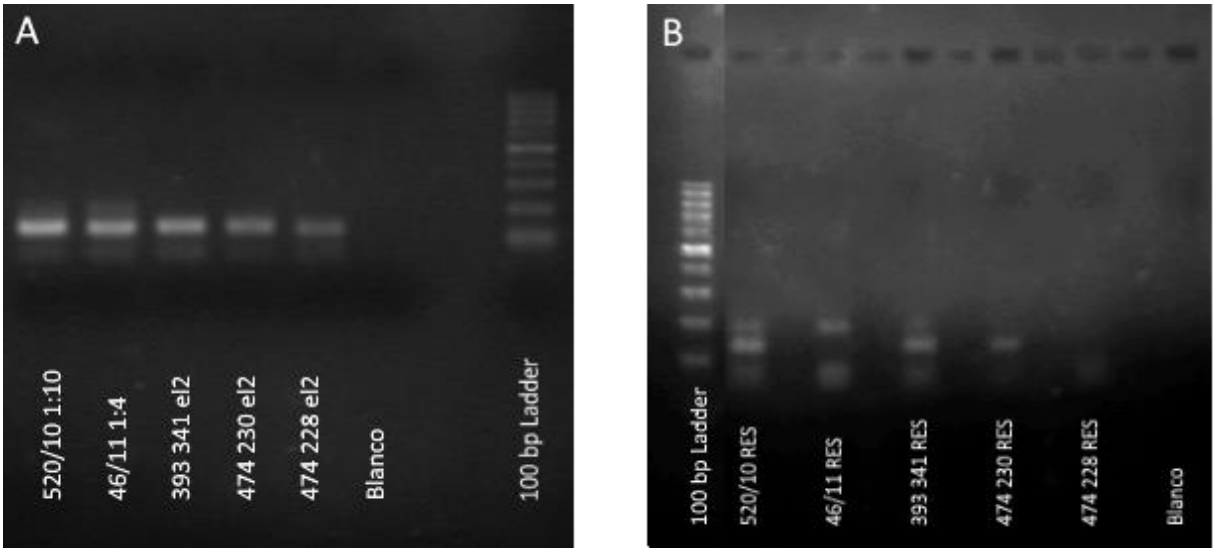

Figure 13: Gel results of the sex determination of 2 recent and 3 museum samples. (A) Result of the sexing PCR before digestion. Sample 520/10 represents a control of a known female individual, sample 46/11 a control from a known male individual. Sample 393 341 el2, 474 230 el and 474 228 el2 are the analyzed museum samples. All samples show a clear band near 120 bp. (B) Results of the sexing PCR after digestion. Female samples (520/10, 393 341 and 474 230) can be discriminated from male samples (46/11 and 474 228) by the presence of a band near 120 bp, the W fragment.

4. DISCUSSION

4.1 Phylogeny

In this study, we determined the phylogeny of military and great green macaws for a first time using a comprehensive dataset based on the mitochondrial genome and covering almost the complete species' distribution. Overall, the obtained topology agrees with an earlier genetic study with a limited sample size (Eberhard et al., 2015); the *militaris* and *ambiguus* clade represent two distinct species and additional diversification occurred between military macaws from Mexico and those South-America. However, by incorporating more samples from South-America, the relationship between *A.m. militaris* and *A.m. bolivianus* could be assessed. Remarkably, the analysis showed that mtDNA sequences are overlapping for the Loreta population (point 13 on Figure 1), with three samples (A17, A27 and A28) clustering together within the *A.m. bolivianus* clade, while the fourth sample (A16) is subdivided into the *A.m. militaris* clade, indicating that *A.m. bolivianus* is actually paraphyletic. Moreover, overall genetic distances observed between samples from *A.a. militaris* and *A.a. bolivianus* were similar to those within samples from Mexico. Altogether, this suggests that these two presumed subspecies are, atleast in Loreta, in contact after all. Data from nuclear regions is necessary though to determine the exact relationship of these two subspecies, but this nevertheless proves that the boundaries between subspecies are not as strict as initially thought.

For the Mexican *militaris* clade, no clear geologic structure was found in the mtDNA sequences and as such it can be assumed that there is gene flow across their complete distribution. Interestingly, the study of Eberhard et al., 2015 contained two samples from Eastern Mexico, a region that was not covered in our own study and when combining the two datasets, these samples were placed as sister clade of the Western Mexico samples. Additional sampling is necessary though to assess whether this two clades may be considered as two different subspecies.

In contrast to the study of Eberhard et al. (2015) who didn't find a significant phylogenetic structure within the *ambiguus* clade, a clear differentiation was found between *A.a. ambiguus* and *A.a. guayaquilensis*. Even when combining our own data and data generated by Eberhard et al. (2015), this differentiation still existed. However, based on the Bayesian tree (Figure 11),

the genetic distances between these subspecies are rather small and their differentiation probably only happened recently.

One important remark is that the reconstructed phylogenies in this study were strictly based on mitochondrial DNA, which is maternally inherited. As such, these phylogenies only reveal a part of the evolutionary history of a species (Avise, 2009). For example, in case of sex-biased dispersal *e.g.* when females are sedentary and males migrate between sites, significant differentiations may be found in the mitochondrial DNA, even while there is considerable exchange of genetic material among the populations (*e.g.* Lyrholm et al., 1999; Guschanski et al., 2013).

Another issue when working with only mitochondrial DNA is the fact that it does not exhibit recombination and therefore behaves as a single genetic unit. However, phylogenetic trees constructed from different genes may result in conflicting topologies (Degnan and Rosenberg, 2009). It is therefore advised to integrate as many independent loci as possible in a phylogenetic study in order to improve the reliability of the phylogenetic tree.

Lastly, Numts *i.e.* nuclear sequence of mitochondrial origin, may also impede the phylogenetic analysis. Numts represent translocations of mitochondrial sequences into the nuclear genome and may co-enrich with the mitochondrial DNA (Hazkani-Covo et al., 2010; Li et al., 2012), possible leading to erroneous base-callings at some sites. However, given the study species, the enrichment protocol and the quality control steps we used, it is unlikely that numts may have led to errors in our phylogenetic analyses. First, it has been proven that mitochondrial DNA preserves better in museum samples than nuclear DNA (Green et al., 2010). Moreover given the fact that multiple copies of mtDNA are present in the cell as opposed to only two nuclear copies, the captured DNA is expected to be mostly of mitochondrial origin. Second, the chances of having a numt-derived error has been estimated to be 1.6×10^{-5} in guenons, a genus of Old World monkeys (Guschanski et al., 2013). Although the number of numts in parrots has not yet been investigated, the overall number of numts in bird species is believed to be much lower than in mammals (Pereira and Baker, 2004; Qu et al., 2008) and as such the numt-derived error rate in green macaws is probably even lower than the one estimated for guenons. Finally, given the overall high alignment score of the consensus sequences after the quality filtering steps, the larger part of the reads is believed to be of mitochondrial origin.

4.2 Conservation

While resolving the green macaw's taxonomy can help unravelling the evolutionary history of the species and more broadly speciation patterns in Central-America, it also offers a unique opportunity for conservations purposes. Both military and great green macaws are endangered and once their taxonomy is unraveled, 'evolutionary significant units' (ESU's) can be defined. An ESU is a population that requires separate conservation, *i.e.* when it is genetically or ecologically irreplaceable by another population (Crandall et al., 2000). Given the observed differentiation, at least one of the presumed subspecies meets this requirement: *A.m. mexicanus*. The conservation status of *A.a. ambiguus* and *A.a. guayaquilensis* is less clear due to their small genetic distances. More specifically, the distance between populations from Ecuador and Central-America is smaller than between some of the Mexican samples from the same location. Both clades are reciprocally monophyletic though and it could be argued that differentiation only started recently. The addition of nuclear regions can hopefully provide more insights into the relation of these two populations. For the South-American clade, overlap in mtDNA sequences was found for *A.m. militaris* and *A.m. bolivianus* samples. These presumed subspecies could therefore be considered as one (sub)species and as such also as a single ESU. Again, nuclear regions should provide more definite answers. Lastly, since the two samples from Eastern Mexico generated by Eberhard et al. (2015) were placed as sister taxa to the other Western Mexico samples, it can be interesting to assess whether Eastern and Western Mexican populations should be managed as two separate ESU's. To date however, the available data is insufficient to make suggestions about the exact relationship of these two populations.

The current military macaw population at the Zoo of Antwerp (but also multiple other captive populations) consist of confiscated animals. As such, genetic screenings are necessary to ascertain the geographic origin of these animals before they can effectively participate into breeding programs. Many current techniques rely on the use of the mitochondrial cytochrome oxidase I (COI) gene to identify a species (Hebert et al., 2003). However, given the small genetic distances between the formulated subspecies, identification based on this single gene may not give reliable answers (Fišer Pečnikar and Buzan, 2014). Indeed, when an UPGMA tree was constructed based on a single gene (either COI, ND2 or ATPase 6 and 8) (Supplementary Material 1,2 and 3), some discordances with the original topology arose. As such, at least the full concatenated ND2-COI-ATPase 6 and 8 mitochondrial sequences are necessary to determine the identity of a sample.

4.3 Methods

Here, we have proved that museum samples offer a valuable source of DNA for phylogenetic studies. Although the amount of endogenous DNA is limited in museum samples, by using an enrichment strategy a mean coverage of 29.8-fold across the mitochondrial genome was obtained, which is comparable to previous studies using a similar strategy (Guschanski et al., 2013; Springer et al., 2015). Moreover, with some small adjustments, this protocol can also be applied to other species in the future.

The average coverage varied notable between and within library pools though, indicating that the indexed library pools were unbalanced before sequencing. This pinpoints one of the difficulties when working with sensitive next generation sequencers *i.e.* the necessity to exactly quantify the amount of DNA into each library before sequencing (Robin et al., 2016). While qPCR is an overall reliable method to quantify DNA, it is also prone to varying library fragment sizes, which may result in both an over-or underestimations of the DNA concentration in the samples (Sedlackova et al., 2013; Robin et al., 2016). Although varying fragment sizes cannot be fixed, pooling larger volumes and quantifying multiple replicates should help avoiding unreliable quantifications.

Lastly, contamination remains a challenge when working with ancient DNA and should be avoided. Specifically for our lab, where genetic projects including storks and vultures are conducted, contaminations from these species are most likely and should ideally be assessed. Unfortunately due to time constraints only human mitochondrial contamination was considered in this study. Overall, the human contamination was limited with only $0.51 \pm 0.29\%$ of the reads on average preferentially mapping to the human mitochondrial genome. Interestingly, most of these reads turned out to be of bacterial origin. To date however, to our knowledge, no study has been conducted to examine the percentage of bacterial contamination when using a target enrichment strategy with magnetic beads.

4.4 Sexing

In this study, new primers were designed to determine the sex of military macaw samples by amplifying a fragment of the *ATP5A1* gene. By following a restriction fragment length

polymorphism (RFLP) strategy, amplicon lengths even smaller than the ones proposed by Bantock et al. (2008) could be successfully discriminated. As such, this method proved to be a valuable option to determine the sex of degraded and fragmented DNA samples such as in museum samples and, with some adjustments, can be extended to other bird species in the future. Finally, determining the sex of the military macaw museum samples is an important step in resolving their evolutionary history, as it may reveal sex-biased dispersal patterns (see 4.1).

Although the sex of three museum samples and two positive controls was successfully determined using the proposed primers, additional optimization of the method is necessary. For example, a nonspecific band around 200 bp was generated for the positive controls. However, these bands probably arose due to suboptimal PCR-conditions and either increasing the annealing temperature (and thus decreasing non-specific binding of the primers) or reducing the initial template concentration should resolve this issue.

4.5 Future prospects

Now that the mitochondrial genomes have been successfully reconstructed for 40 macaw museum samples and a robust phylogenetic tree has been built, the next important step in this project will involve the sequencing of nuclear regions to assure whether the observed patterns can also be found in nuclear DNA, to evaluate possible sex-biased dispersal and to verify whether hybridization has occurred between populations (Avise, 2009). Additionally, more samples from Eastern-Mexico should be included to confirm their relationship to the other Mexican samples. Furthermore, by dating the mtDNA phylogenetic tree by applying a molecular clock, the diversification times of the different lineages can be estimated. These estimates can then be linked to geological events of known age and as such evolutionary patterns *e.g.* vicariance, radiation, dispersion can be inferred (*e.g.* Barber and Klicka, 2010; Guschanski et al., 2013*)*. Lastly, while the combination of ND2, COI and ATPase 6 and 8 seems promising to identify the origin of captive animals, this method relies on three different PCR's and as such it might be more favorable to look for a single region with sufficient differentiation to identify the species, which eventually will be more cost-and time efficient.

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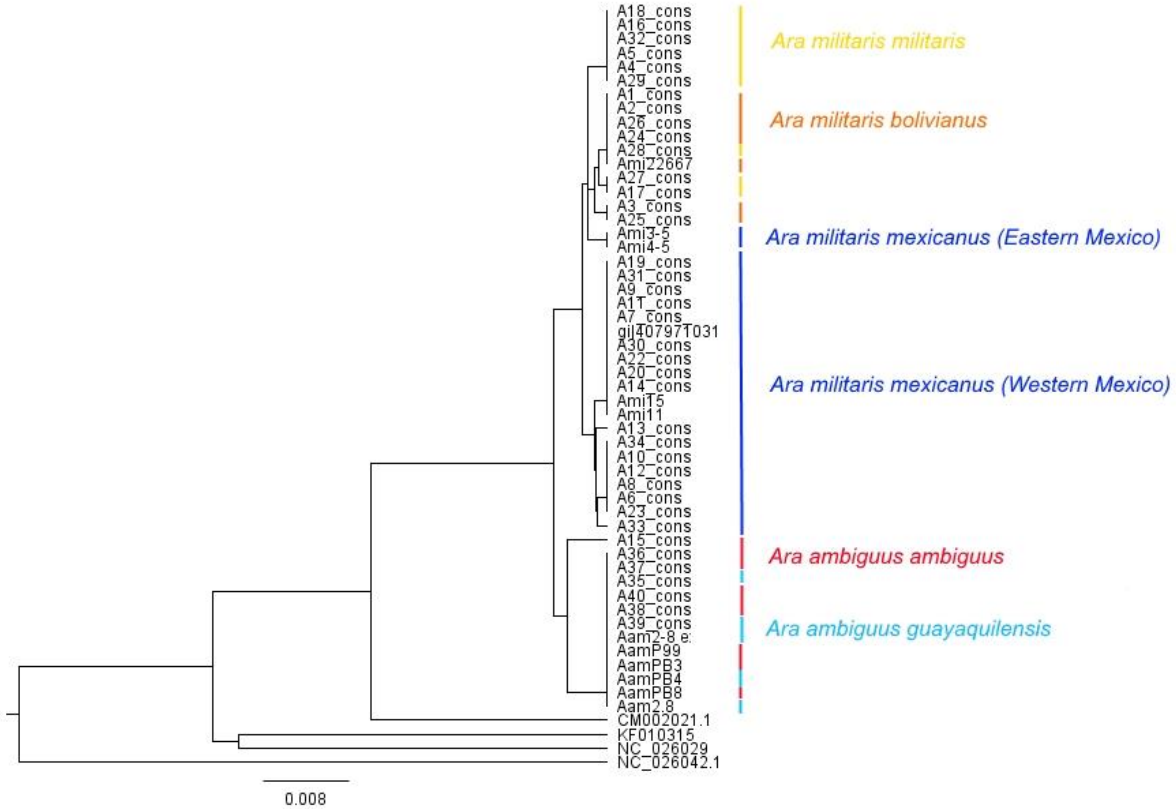
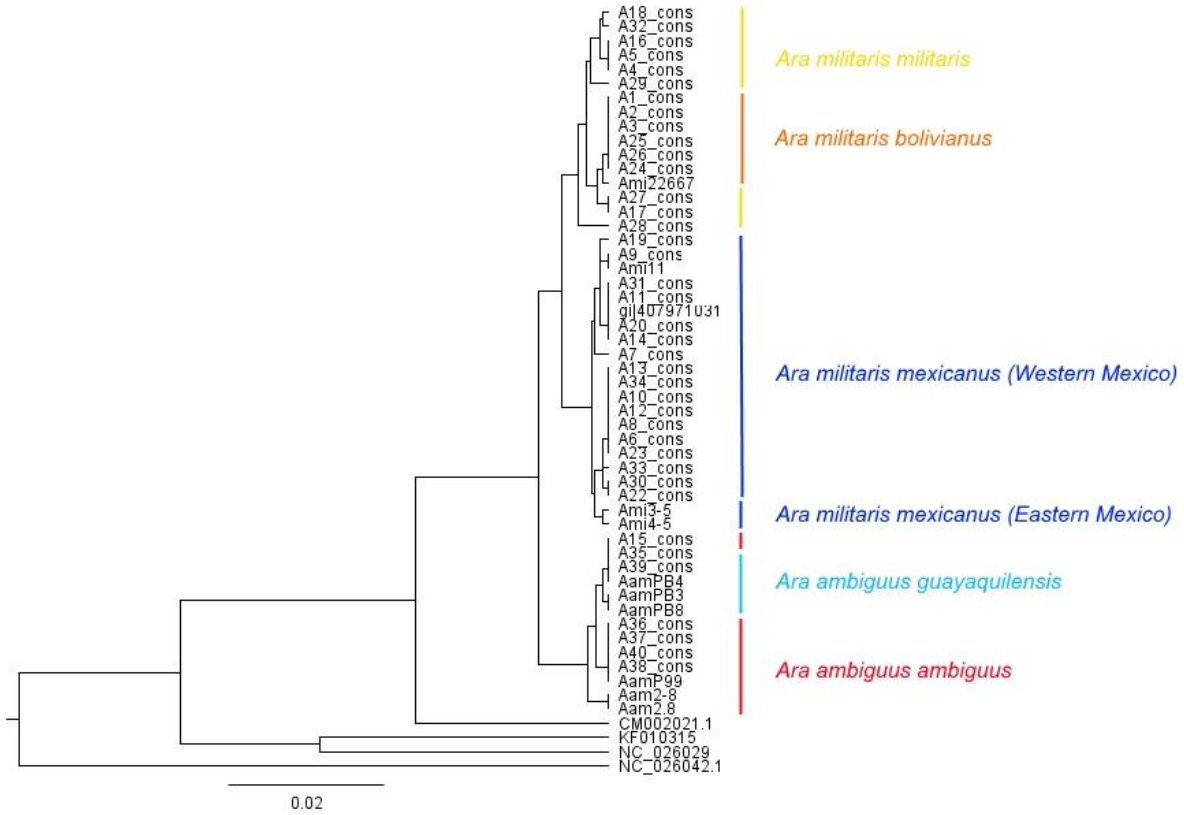
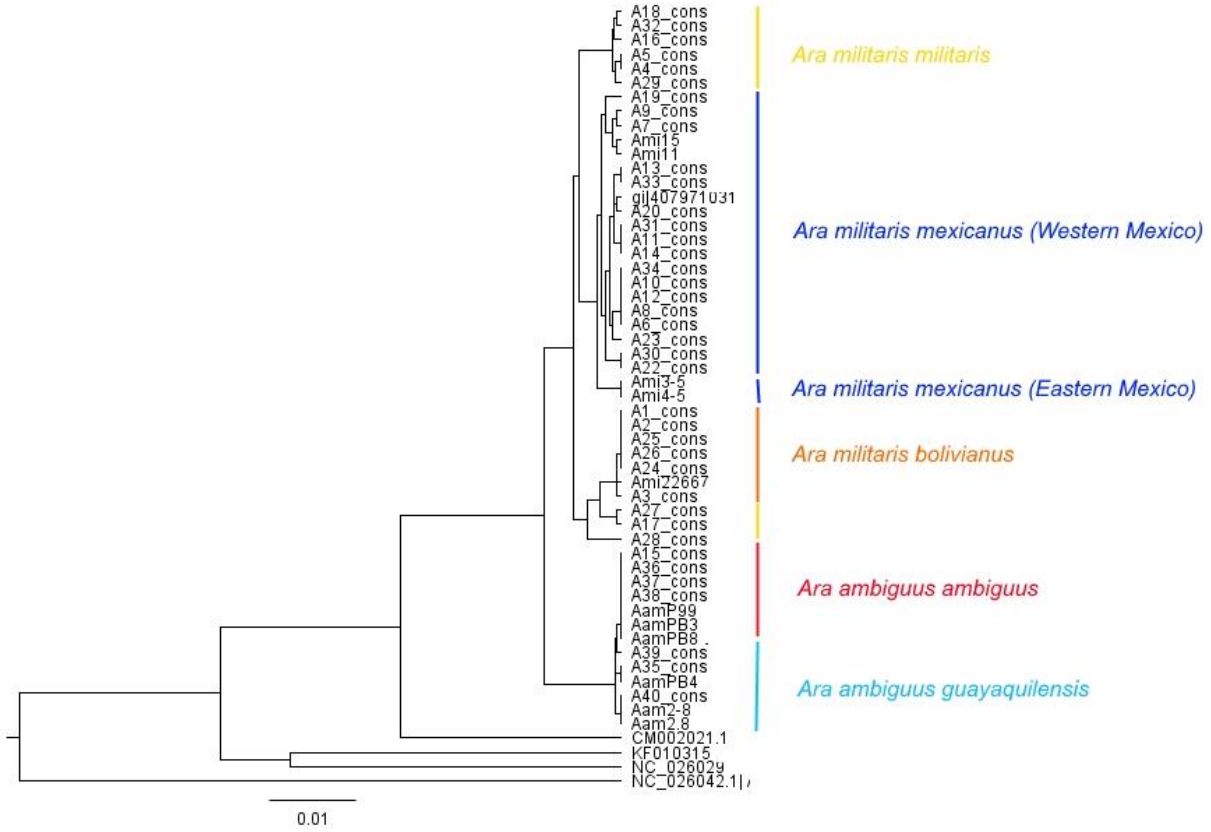
SUPPLEMENTARY MATERIAL



Supplementary Material 1: UPGMA phylogenetic tree based on COI gene sequences from the mitochondrial genome. Samples with an 'Axx_con' code represent sequences generated in this study and correspond to those listed in Table 3, samples with a 'Amixx' or 'AamPBxx' code correspond to data from Eberhard et al. (2015) and other sample names indicate sequences downloaded from GenBank. The originally presumed subspecies of the samples, based on their sampling location, are indicated in color on the right.



Supplementary Material 2: UPGMA phylogenetic tree based on ND2 gene sequences from the mitochondrial genome. Samples with an 'Axx_con' code represent sequences generated in this study and correspond to those listed in Table 3, samples with a 'Amixx' or 'AamPBxx' code correspond to data from Eberhard et al. (2015) and other sample names indicate sequences downloaded from GenBank. The originally presumed subspecies of the samples, based on their sampling location, are indicated in color on the right.



Supplementary Material 3: UPGMA phylogenetic tree based on ATPase 6 and 8 gene sequences from the mitochondrial genome. Samples with an 'Axx_con' code represent sequences generated in this study and correspond to those listed in Table 3, samples with a 'Amixx' or 'AamPBxx' code correspond to data from Eberhard et al. (2015) and other sample names indicate sequences downloaded from GenBank. The originally presumed subspecies of the samples, based on their sampling location, are indicated in color on the right.

Sample	Average Size (bp)
A18	94
A28	98
A20	122
A3	108
A18	77
A20	87
A3	95

Supplementary Material 4: Average size of the museum fragments, as measured with the Fragment Analyzer™.