Trends in Genetics

Rates of evolution of penguin mitochondrial genomes

Racial disparities in human genomics

How many pathways result in microcephaly?

Chromatin indexing in *Arabidopsis*

High mitogenomic evolutionary rates and time dependency

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Using entire modern and ancient mitochondrial genomes of Adélie penguins (*Pygoscelis adeliae*) that are up to 44,000 years old, we show that mitogenomic rates of evolution are two to six times higher than phylogenetic rate estimates. Although the rate at constrained sites including nonsynonymous positions and RNAs varies over two fold with time (between shallow and deep nodes), the rate of synonymous site evolution remains the same. The time independent neutral evolutionary rates reported here will be useful for the study of recent evolutionary events.

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1 **Rates of molecular evolution**

2 Rates of molecular evolution underpin much of modern evolutionary biology because
3 they can be used, for example, to estimate the time of divergence between
4 species/lineages and to estimate the age of morphological novelties. Generally
5 molecular rates have been estimated using phylogenetic comparisons among multiple
6 species, where levels of divergence have been calibrated using points in the fossil
7 record [1-4] or major biogeographic events [5]. Recent studies have shown a much
8 higher rate of evolution within species compared to the rate obtained using inter-
9 species comparisons [6, 7]. Based on these observation it has been suggested that
10 rates of molecular evolution are time dependent [8, 9]. The apparent time dependency
11 of evolutionary rates is potentially a result of two factors. First, the estimation of
12 inter-species divergence at neutral sites such as synonymous positions and the hyper-
13 variable region is likely to suffer from the saturation effects due to multiple
14 substitutions. This would result in an underestimation of molecular rates over long
15 time periods [10-12]. Second, the presence of short-lived slightly deleterious
16 mutations among the populations of a species would lead to an overestimation of the
17 actual substitutions over shorter time scales [12-14]. Although the rate estimations
18 based on within species comparisons are not influenced by saturation effects, the
19 presence of short-lived deleterious mutations still poses an obstacle to obtaining
20 unbiased estimates of evolutionary rates for single species. Furthermore,
21 phylogenetic methods cannot be used to infer intra-species rates due to the lack of
22 calibration points within a short timescale.

23 We have used serially preserved ancient DNA samples to resolve this impasse by
24 sequencing the mitochondrial genomes of 12 modern and eight ancient Adélie
25 penguins from Antarctica, including two samples dating back ~44,000 years [7, 15].

1 These sequences enabled us to estimate precisely the overall mitochondrial genomic
2 evolutionary rate and specific rates for ribosomal RNAs, transfer RNAs, synonymous
3 and nonsynonymous positions of the protein-coding regions. These specific rates
4 enabled us to examine the effects of time dependency on rates of molecular evolution.
5 We have used three different analytical methods to estimate these molecular rates in
6 mitochondrial genomes of Adélie penguins.

7

8 **Estimating genomic rates of evolution using ancient mitochondrial DNA**

9 We amplified and sequenced mitochondrial genomes using blood samples of modern
10 Adélie penguins and from the sub-fossil bones of these birds from Antarctica (Figure
11 1). The size of the genome recovered from each of these ancient samples was 12.5–
12 16.1 kb (Figure S1). The ages of these sub-fossil bones ranged from 250 to ~44,000
13 years, which were estimated using radiocarbon methods. The phylogenetic
14 relationship of modern and ancient populations of Adélie penguins is shown in
15 supplementary Figure S2. First, to estimate the neutral evolutionary rate we analyzed
16 only the synonymous positions (fourfold degenerate sites) of the mitochondrial
17 protein-coding regions, which are known to be largely free from selection [16]. We
18 used a Bayesian statistics-based Markov chain Monte Carlo (MCMC) method [17], as
19 well as Maximum Likelihood [18] approaches to estimate evolutionary rates. The
20 Bayesian MCMC analysis showed that the mitogenomic rate of evolution at
21 synonymous sites (μ_s) is 0.073 (HPD, 95% Highest Posterior Density intervals 0.025
22 – 0.123 substitutions per site per million years (s/s/My) (Figure 2). This rate estimate
23 was obtained using a general time-reversible model of evolution. To account for rate
24 variation among nucleotide positions we used a discrete gamma distribution of six
25 rate categories. The rate was then estimated assuming a constant population size and

a relaxed uncorrelated log-normal molecular clock. The estimates remained almost the same when modifying a range of prior assumptions. To avoid any methodological bias we also estimated the rate of evolution using a Maximum Likelihood method, which resulted in a rate of 0.062 s/s/My (CI 0.03-0.09). This rate is slightly less than, but comparable to that estimated using the Bayesian MCMC approach (Figure 2). Furthermore, a similar rate was obtained using a simple distance based serial UPGMA method [19] (0.072 s/s/My, CI 0.0 – 0.317).

Evolutionary rates from modern penguin samples

Next we estimated the rate of mitogenome evolution in Adélie penguins using only the modern samples. We calculated the ratio of the mean pair-wise divergences for noncoding sites at the HVR-I (d_H) and synonymous sites (d_S) using mitochondrial genomes recovered from 12 modern Adélie penguins. The rate for synonymous positions (μ_S) was estimated as $\mu_S = \mu_H \cdot (d_S / d_H)$, where μ_H is the neutral mutation rate at the HVR-I region, which was previously estimated as 0.55 s/s/My [20]. Our current analysis shows that the synonymous sites from modern Adélie penguin mitochondrial genomes evolve at a rate (μ_S) of 0.054 (CI 0.031 - 0.090) s/s/My (Figure 2). This rate is not significantly different from those obtained using ancient DNA methods as the confidence intervals are almost the same.

Inferring tempo of evolution at constrained sites in the mitochondrial genome

Estimation of evolutionary rates at constrained sites (nonsynonymous sites and RNAs) is prone to large stochastic errors owing to the low divergence in population data. However the average rate of evolution at constrained sites can be estimated

1 using the synonymous evolutionary rate. The neutral theory defines the rate of
2 evolution (K) at constrained sites as $K = \mu \cdot f_0$ where μ is the mutation rate and f_0 is the
3 fraction of neutral positions (or neutral mutations) [21]. Since the rate of evolution at
4 synonymous sites (μ_s) of mitochondrial genes is known to be similar to the mutation
5 rate (μ) and the divergence at these sites (d_s) largely reflects the accumulation of
6 neutral mutations over time [16], the evolutionary rates in the constrained sites can be
7 estimated as $K_X = \mu_s \cdot (d_X/d_s)$ where K_X and d_X are the rate of evolution and the
8 divergence in constrained sites respectively. The ratio (d_X/d_s) is actually the fraction
9 of neutrally evolving positions (f_0). Using this approach, we estimated that the
10 nonsynonymous rate of change for the mitochondrial genome of Adélie penguins is
11 0.005-0.007 s/s/My.

12 The synonymous molecular rate (0.054-0.073 s/s/My) differs from the
13 nonsynonymous rate by more than 10 times (Figure 2). The mitochondrial tRNAs
14 (0.015-0.020 s/s/Mya) evolve almost twice as fast as rRNAs (0.008-0.010 s/s/My).
15 The rate of evolution in the complete mitochondrial molecule (excluding the control
16 region) is 0.018-0.024 s/s/My, which is 1.8-2.4 times faster than the classical
17 phylogenetic rate of 0.01 s/s/My that has been used for several decades [1, 22]. A
18 previous study on the mitochondrial genomes of the extinct mammoth also suggests
19 that the rate based on internal calibrations (within mammoths) is ~1.6 times higher
20 than that obtained using external (mammoth-elephant) calibration [23].

21 By contrast, the synonymous substitution rate (0.054-0.073 s/s/My) estimated
22 here is five to seven times higher than previous phylogenetic rate estimates [1-4] and
23 significantly higher than those based on intra-specific comparisons within human
24 (0.048-0.052 s/s/My) [14] and Neanderthal (0.036-0.042 s/s/My) [24] populations.
25 These results clearly argue against the use of the classical 1% rate per lineage (or the

2% rule as it is commonly known) to study the evolution or genetics of the individual species.

Time dependency of molecular rates

Previously it has been suggested that rates of molecular evolution are time dependent and that rates estimated over short timescales are higher than long-term evolutionary rates [6, 9]. To detect any time dependency, we first determined the coalescence times of shallow and deep branches of modern Adélie penguin populations. Previously studies have shown the presence of two distinct lineages of Adélie penguins namely Antarctic (A) and Ross Sea (RS) lineages [7, 15]. Using the complete mitogenomic evolutionary rate, we estimated the coalescence time between the lineages (A/RS) to be 62,000 years (62 Ky) and the coalescence time within individual lineages are 18 Ky and 19 Ky for A and RS respectively. We then estimated the root heights of the most recent common ancestor (μ_{MRCAs} , substitutions per site) of A, RS and A/RS lineages using synonymous (third codon) positions and constrained (nonsynonymous sites+tRNAs+rRNAs) positions. The μ_{MRCAs} were estimated using the program MCMCcoal [25] and using the coalescence times, the rate of evolution at constrained- and at synonymous sites for inter- and intra-lineages of Adélie penguins were computed.

Our examination of the UPGMA trees (Supplementary Figure S3) reveals that the node-heights of A and RS lineages are much longer (with respect to the inter-lineage – A/RS height) for constrained sites (Figure S3b) than those constructed using synonymous sites (Figure S3a). The rate of evolution at constrained sites is more than two fold higher within A (0.02 s/s/My, HPD 0.014-0.033 s/s/My) and within RS (0.017 s/s/My, HPD 0.011-0.034 s/s/My) lineages, compared to that obtained for

1 between A and RS lineages (0.008 s/s/My, HPD 0.007-0.01 s/s/My) (Figure 3).
2 However the evolutionary rates at synonymous sites estimated for intra- and inter-
3 lineages are very similar (Figure 3). In a recent study a comparable pattern was
4 observed in human populations [14]. The rate of evolution at third codon positions
5 was found to be similar to that recorded within human populations (0.052 s/s/My) and
6 between humans and chimpanzees (0.048 s/s/My). By contrast, the nonsynonymous
7 rate of evolution within human populations (0.013 s/s/My) was 3.4 times higher than
8 that estimated between humans and chimpanzees (0.0038 s/s/My). The rate
9 difference (3.4 times) recorded for the hominid data is higher than that reported in this
10 study (2 times, Figure 3). This is because the hominid comparison is on a much
11 deeper time scale (between species) than our comparison involving two lineages of
12 the same species. Since multiple substitutions are not an issue for comparisons within
13 a species or for closely related species (particularly for synonymous sites), there is no
14 reason to expect any significant variation in the rate of neutral evolution at different
15 timescales. These results suggest that the time dependency of evolutionary rates
16 holds true only for the sites under selection and not for the neutral synonymous sites.

17 Furthermore we examined the effect of time dependency on the rate for the
18 mitochondrial hyper-variable region (HVR), which is also known to evolve under
19 neutrality. The rate of evolution between lineages (A/RS) (0.496 s/s/My, HPD 0.399-
20 0.615 s/s/My) was not significantly different from that within the Antarctic lineage
21 (A) (0.413 s/s/My, HPD 0.288-0.675 s/s/My), but the rate within Ross Sea lineage
22 (RS) (0.798 s/s/My, HPD 0.511-1.59 s/s/My) was higher than the inter-lineage rate.
23 Since the rate of evolution within the RS lineage is based on only three sequences (see
24 Supplementary Figure S3) as opposed to nine sequences for the A lineage, the higher
25 rate for the RS lineage could be simply due to stochastic errors. Furthermore the

1 well-known mutational hot spots in HVR could also influence the rate estimation.
2 For instance recently Howell *et al.* [26] have shown that, while the rate of evolution
3 of a large fraction of the HVR region is comparable to that for synonymous sites,
4 some other sites mutate an order of magnitude faster than the remainder. Therefore
5 the rate difference observed within A and within RS lineages is most likely a result of
6 mutational processes and not due to purifying selection or the effects of time
7 dependency.

8

9 **Concluding remarks**

10 For decades, phylogenetic estimates of rates of molecular change have been used
11 widely in evolutionary studies but the field has recently been characterized by debates
12 about the use of intra-specific rate estimates [6, 7]. Previous studies of the
13 mitochondrial hyper-variable regions (HVR) suggested much higher intra-specific
14 evolutionary rates in comparison to classical phylogenetic rates estimated using
15 multiple species. However HVR rates cannot be used to infer molecular rates for
16 mitochondrial genomes as this region evolves much faster than rest of the genome
17 [12, 27]. By contrast, the evolutionary rate for synonymous positions reported here is
18 useful to infer the rates of constrained protein coding sites, tRNA and rRNAs, which
19 will help to understand the patterns of purifying selection acting at the population
20 level. Furthermore we have shown that the high neutral rate reported in this study is
21 similar between shallow and deep branches. Therefore this time independent rate can
22 be used to study the recent evolutionary histories of many avian species.

23

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List of Figure Legends

Figure 1. The location of ancient and modern samples of Adélie penguin used in this study.

(a) Ross Sea Coast colonies and **(b)** Ross and Beaufort Island colonies sampled. Red indicates modern and black indicates ancient samples.

Figure 2. Mitogenomic rates of evolution in Adélie penguins.

Using synonymous positions (fourfold degenerate sites) of the mitochondrial genomes of eight ancient and 12 modern Adélie penguins, the evolutionary rate at synonymous sites (μ_S) was estimated by Bayesian statistic based MCMC (red column) and a Maximum likelihood approach (yellow column). In addition, using only the modern genomes the synonymous rate was also inferred using the ratio of the average pair-wise divergence in the synonymous positions (d_S) to that in the hyper-variable region (d_H) and using the mutation rate (μ_H) of the latter, as $\mu_S = \mu_H (d_S/d_H)$ where $\mu_H = 0.55$ (0.32-0.92) substitutions/site/million years as reported from a previous study [20] (blue column). The evolutionary rates in different mitochondrial regions (K_X) are estimated as $K_X = \mu_S (d_X/d_S)$ where d_X is the average pair-wise distance obtained for the given region X (e.g. tRNA). The error bars show 95% HPD intervals.

Figure 3. Rate of evolution for within and between the two lineages of modern Adélie penguins.

Evolutionary rates were estimated using synonymous (3rd codon) positions (a) and constrained positions (nonsynonymous sites + tRNAs + rRNAs) (b). The coalescence times for within and between lineages were estimated by the program BEAST [17] using the rate obtained for the complete mitochondrial genome. The evolutionary rates for A and R lineages and that between them (AR) were obtained using the respective coalescence times and the root heights of most recent common ancestor (μt_{MRCA}) estimated by MCMCcoal [25] using synonymous and constrained sites. The error bars are based on the 95% HPD intervals obtained from coalescence times estimation.

Figure 1. Subramanian et al. 2009

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Figure 2. Subramanian et al. 2009

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Figure 3. Subramanian et al. 2009

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High mitogenome-wide rates of evolution and time dependency

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Stratigraphy and ¹⁴C Dating

Sub-fossil Adélie penguin bones were collected from six locations in Antarctica (Figure 1) [1] Radiocarbon dates were supplied by Geochron Laboratory - Krueger Enterprise Inc., Cambridge, Massachusetts (conventional and AMS, GX-), IsoTrace Radiocarbon Laboratory, Toronto (AMS, TO-) and NOSAMS, Woods Hole Oceanographic Institute (AMS, OS-) and the New Zealand Institute of Geological and Nuclear Sciences, Lower Hutt, New Zealand (AMS, NZA-).

DNA extraction

Shavings of bone were taken from samples using a sterile surgical blade. About 200mg of bone material was transferred to a sterile Eppendorf tube. In between samples bench area and blade were cleaned using 10% bleach. After adding 1.5ml of 0.5M EDTA and 0.75mg of proteinase-K, the samples were rotated overnight at 56°C. Samples were then extracted once with Tris-saturated phenol, once with Tris-saturated phenol: chloroform: isoamyl-alcohol (25:24:1) and finally once with chloroform: isoamyl-alcohol (24:1). This extracted solution was then concentrated to 50µL on a Vivaspin-30 (Viva Science, U.K.) membrane. The sample was then purified using QIAGEN DNA mini kit eluting in 50µL 1xTE and stored at -20°C. All DNA extractions and setting up of PCR's from sub-fossil material were conducted in a dedicated ancient DNA laboratory, which underwent regular decontamination with

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UV-irradiation and hypochloride treatment. Mock DNA extractions and blank PCR's were continually screened for contamination.

Multiplex PCR and Sequencing

Modern samples were extracted using a 10% Chelex 100 (Sigma-Aldrich) solution in 100mM Tris pH8.8. Amplification of the mitochondrial genomes was performed using a high throughput plate set up. The set up of the multiplex PCR is as described in a previously published protocol (Römlper *et al.* 2006). All samples were amplified using a two stage multiplex PCR, using 51 overlapping primer pairs and another 51 nested primer pairs (Table S1). The first stage 50 μ l multiplex reaction contained final concentrations of: 0.4 μ M of each primer (two reactions with 25 or 26 pairs), 1x PCR Buffer, 2.5mM MgCl₂, 0.2mM dNTPs, 1mg/ml BSA, 1.25 units of Platinum Taq DNA polymerase (Invitrogen) and 1 μ l of DNA extract. The thermo cycling was performed as follows: 4min 95 °C, 35x (30 sec 95°C, 30sec 55°C, 45sec 72°C), 10min 72°C. The second stage 50 μ l simplex reaction contained final concentrations of: 0.25 μ M of each primer (51 reactions with one pair), 1x PCR Buffer, 2.5mM MgCl₂, 0.2mM dNTPs, 1mg/ml BSA, 1.25 units of Platinum Taq DNA polymerase and 1 μ l of 1:50 diluted 1st stage PCR product. The thermo cycling was performed as described for the first stage reaction.

PCR reactions were purified for sequencing using the AMPure kit according to manufacturer's protocol. Samples were then subjected to ethanol precipitation and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (ABI), according to manufacturer's protocol.

Evolutionary rate estimation

The length of Adélie penguin mitochondrial genomes recovered varies between 12.5 – 16.1 kb (Figure S1). Amino acid sequences of each protein coding gene and the nucleotide sequences of each tRNA and rRNA gene were aligned by CLUSTAL-W [2]. The cDNA alignments of protein coding genes were created using the alignments of amino acid sequences. We employed a complete site deletion method by excluding the nucleotide positions that contained an alignment gap in one or more sequences. Fourfold and zerofold degenerate sites were extracted from the cDNA alignments. Our final dataset consists of 1349 fourfold degenerate positions, 4860 zerofold degenerate positions, 1136 tRNA and 1089 rRNA sites from 12 modern and eight ancient Adélie penguins. Pair-wise distance estimations were performed using MEGA [3].

To estimate the neutral evolutionary rate, only the fourfold degenerate sites were used. First a simple distance based evolutionary rate was estimated using a serial UPGMA method employed in the software *Pebble* under a HKY+Gamma model of evolution [4]. Furthermore *Pebble* was used to construct an UPGMA tree of modern and ancient Adélie penguins by accounting for the ages of the ancient sequences (Figure S2). The program TipDate [5] implemented in PAML [6] was used to estimate the rate of evolution by the maximum likelihood method using a GTR+Gamma model of evolution and the UPGMA tree was used to provide the topology.

We also employed Bayesian statistics based on a Markov chain Monte Carlo approach using the software BEAST [7] assuming a relaxed molecular clock, constant population size and GTR+Gamma model of evolution. Wide uniform prior

distributions were used to avoid any bias due to restricted prior assumptions. The rate estimate and the highest posterior density intervals were similar when varying a number of *a priori* assumptions. The rate estimates were sampled once every 1000 cycles from the total MCMC chain length of 10,000,000 after discarding the initial 1,000,000 burn-in 1,000,000 steps. The posterior distributions were examined using the software TRACER [8] and the Effective Sample Sizes (ESS) for mean evolutionary rate, population size and posterior likelihood were found to be >100 for all the models used.

To estimate the root height (μ_{MRCA} , substitutions per site) of the most recent common ancestor the MCMCcoal was used [9]. μ_{MRCA} s were A, RS and A/RS lineages using synonymous (third) positions and also using constrained sites including nonsynonymous sites, tRNAs and rRNAs.

Table S1. External and Internal primers used to amplify complete Adélie penguin mitochondrial genomes. Plate = name of master primer plate. Row and Col depict location in master primer plates. Gapdh and Zenk are nuclear loci used to monitor the presence of nuclear DNA.

PLATE	ROW	COL	PRIMERNAME	PRIMER SEQ
Pa_mt_External	A	1	PaMTOF01	AACCAAAGCATGGCACTGAAGAT
Pa_mt_External	E	1	PaMTOF02	TTAACTGTCACACGGCGTAAAGA
Pa_mt_External	A	3	PaMTOF03	TCGCCTGAAAGAACAACAGTGA
Pa_mt_External	E	3	PaMTOF04	AACACAAAGCACTCAGCTTACACCT
Pa_mt_External	A	5	PaMTOF05	AGTGGGATGACTTGTTAGTAGAGGT
Pa_mt_External	E	5	PaMTOF06	AAGCGCAAGCTTACATCACCACATT
Pa_mt_External	A	7	PaMTOF07	ACTGTCTCTTGACAGACAATCAATG
Pa_mt_External	E	7	PaMTOF08	TTTACGACCTCGATGTTGGATCA
Pa_mt_External	A	9	PaMTOF09	ATCCCAATCCTAGTCGCAGT
Pa_mt_External	E	9	PaMTOF10	TATGATCAGGGTGGGCCTCAAA
Pa_mt_External	A	11	PaMTOF11	GCAGGTCCATTGCTTTATTCTT
Pa_mt_External	E	11	PaMTOF12	CAACCCTCTCATTTCCTGAGATACA
Pa_mt_External	B	1	PaMTOF13	TTCACAGGACAGTGAGACATCA
Pa_mt_External	F	1	PaMTOF14	CCCAAACCTTACCCTATTAACCTTCTACC
Pa_mt_External	B	3	PaMTOF15	AGTTGCCATCCTTACCTCCTTAT
Pa_mt_External	F	3	PaMTOF16	AAAGGACTACAGCCTAACGCCT
Pa_mt_External	B	5	PaMTOF17	CCCGACATAGCATTTCCT
Pa_mt_External	F	5	PaMTOF18	ACCGAAACCTAAACACTACCT
Pa_mt_External	B	7	PaMTOF19	TGGGCTTCATCTTCCTCTTCACTAT
Pa_mt_External	F	7	PaMTOF20	ATTCATCATCTGAGAAGCCCT
Pa_mt_External	B	9	PaMTOF21	AATTCGGATTCCAAGATGCCT
Pa_mt_External	F	9	PaMTOF22	ACATAATCCCAACAACAGACC
Pa_mt_External	B	11	PaMTOF23	AATCGTAGTAGAATCTGCCC
Pa_mt_External	F	11	PaMTOF24	AGGAATTCCGCTAATCCTACT
Pa_mt_External	C	1	PaMTOF25	AATCGAAACCACTAGCCTACTCA
Pa_mt_External	G	1	PaMTOF26	TAACCCTTGGCCTACTTTCCATA
Pa_mt_External	C	3	PaMTOF27	AATCCACGCACTAACCCTAACAA
Pa_mt_External	G	3	PaMTOF28	AATCACATTCACTCACCCTCTCC
Pa_mt_External	C	5	PaMTOF29	ATGAGTGAACACAAGGAGGCCTAGA
Pa_mt_External	G	5	PaMTOF30	ATACTAGTTGCCTCAACCCGAA

Pa_mt_External	C	7	PaMTOF31	AAACCACCTTCAACACGAACCT
Pa_mt_External	G	7	PaMTOF32	AGCCCTACTAACAGCATTCATAGT
Pa_mt_External	C	9	PaMTOF33	TCCGGTGCAATGATCCTCATAAT
Pa_mt_External	G	9	PaMTOF34	ATACTACTAACAAACCCAACGAGG
Pa_mt_External	C	11	PaMTOF35	AACCAACAAGAGCTGCTAACT
Pa_mt_External	G	11	PaMTOF36	GCATTACATCCTACTGAGAGTGA
Pa_mt_External	D	1	PaMTOF37	TACAACCGAATCGGAGACATT
Pa_mt_External	H	1	PaMTOF38	TAATAACCCAACTGCCCTCA
Pa_mt_External	D	3	PaMTOF39	AGACCTCATTATCGAAAGCCT
Pa_mt_External	H	3	PaMTOF40	ACCTAAACTTCTCCTCCTCCTTA
Pa_mt_External	D	5	PaMTOF41	TGCCTGACTACACAAATCCTGA
Pa_mt_External	H	5	PaMTOF42	ATATATTGGCCAAACCCTCGT
Pa_mt_External	D	7	PaMTOF43	AACAAACTGGGAGGTGTCCTA
Pa_mt_External	H	7	PaMTOF44	TGGTCTTGTAAGCCAAAGAGT
Pa_mt_External	D	9	PaMTOF45	AACCCAACAACAAGCACCAAGAT
Pa_mt_External	H	9	PaMTOF46	AAGTCATAGCAATTCGCGCTT
Pa_mt_External	D	11	PaMTOF47	CATTTAATGTACGTACTAGGAC
Pa_mt_External	H	11	PaMTOF48	GGACTGTCATGTAACAAACGACT
Pa_mt_External	A	2	PaMTOR01	ACATCTTGGGCTTATGACAGCTT
Pa_mt_External	E	2	PaMTOR02	AAATGTAGCCCATTCTTCCACC
Pa_mt_External	A	4	PaMTOR03	TTGGGCTAGAGTTTGGCTTCAA
Pa_mt_External	E	4	PaMTOR04	TTCATTTACAGGCAACCAGCTA
Pa_mt_External	A	6	PaMTOR05	TGGGCTAACAGGGCAAGTTTAAT
Pa_mt_External	E	6	PaMTOR06	TTTAAGTTCCACAGGGTCTTCTCGT
Pa_mt_External	A	8	PaMTOR07	AATCGTTGAACAAACGAGCCCTT
Pa_mt_External	E	8	PaMTOR08	TTTCGGGCTTGTATGTAGCTT
Pa_mt_External	A	10	PaMTOR09	AAATAGTTTGTGCTACTGCCCGT
Pa_mt_External	E	10	PaMTOR10	TGGGTTGAGAAACAGAATGGT
Pa_mt_External	A	12	PaMTOR11	TTGATTTCTTCTGCGTAGGTTG
Pa_mt_External	E	12	PaMTOR12	ATTGCAATTGCTGCTGTGAGT
Pa_mt_External	B	2	PaMTOR13	TCTTGTTGTGTTGAGTGTGAGA
Pa_mt_External	F	2	PaMTOR14	TCCTAAGTTTCTAGGTGGTTGCT
Pa_mt_External	B	4	PaMTOR15	TGCCGATATCTTTGTGGTTGGTT
Pa_mt_External	F	4	PaMTOR16	TTCTACTGTGGATGAGGCTAGT
Pa_mt_External	B	6	PaMTOR17	AGAATCAGAAGAGGTGTTGGT
Pa_mt_External	F	6	PaMTOR18	ATGTCCAATGAGGAGTTTGCT
Pa_mt_External	B	8	PaMTOR19	TCATTCAATGTTGGTGGCAGT
Pa_mt_External	F	8	PaMTOR20	TGCAGATTGCTAGGGCAACTATT
Pa_mt_External	B	10	PaMTOR21	TAACAACACGGTGGTCTACTT
Pa_mt_External	F	10	PaMTOR22	AGGCTAGTGCTGTTTCATAGCTT
Pa_mt_External	B	12	PaMTOR23	ACGGTTGGTAATTCATCGGTT
Pa_mt_External	F	12	PaMTOR24	TTAGTTGAATGAGTAGGTGGCCT
Pa_mt_External	C	2	PaMTOR25	TGGCCTTGAAATGTGCTTTCT
Pa_mt_External	G	2	PaMTOR26	AGCAATTGAGAATGGTGCTTCGT
Pa_mt_External	C	4	PaMTOR27	TGGCTTGTGCCAATCAGAAGTTT
Pa_mt_External	G	4	PaMTOR28	ATGGTCTGTTGAGCCGAAATCAA
Pa_mt_External	C	6	PaMTOR29	TGGTGGGAAGGAGTATGATTGTT
Pa_mt_External	G	6	PaMTOR30	TGAGGAAGGGTTGGATTGTGATT
Pa_mt_External	C	8	PaMTOR31	AGATCCTGCAATTGGTGCTTCTA
Pa_mt_External	G	8	PaMTOR32	AAGAATTCGGGTGTGTGTTTCGTT
Pa_mt_External	C	10	PaMTOR33	AGGTGTTCTCGTGTGTTTGAGTT
Pa_mt_External	G	10	PaMTOR34	ACTTGATTTGCACCAAGATG

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Pa_mt_External	C	12	PaMTOR35	TCATGTTACGAACAGTGCGAT
Pa_mt_External	G	12	PaMTOR36	AGGTGTTTATTGTTGAGGCGA
Pa_mt_External	D	2	PaMTOR37	ACATGTAGCGGCAAATAGTGT
Pa_mt_External	H	2	PaMTOR38	ATAGGTTGCGGTGAATGATGT
Pa_mt_External	D	4	PaMTOR39	TTTGTCCGCTGTTTAGTAGGT
Pa_mt_External	H	4	PaMTOR40	ATTGTACATCTCGGCATGTGT
Pa_mt_External	D	6	PaMTOR41	AGGAAGTGTAAGTGCAGAAGAAT
Pa_mt_External	H	6	PaMTOR42	TTGTGCGCTGTTTAGACTTGT
Pa_mt_External	D	8	PaMTOR43	AAATGCCAGCTTTGGGAGTTGGATA
Pa_mt_External	H	8	PaMTOR44	TCTTTGGCAGCGGATTTGTTT
Pa_mt_External	D	10	PaMTOR45	TTCCTGTGGTTGAAGTAACAACG
Pa_mt_External	H	10	PaMTOR46	TACTCGTTTAATCCGAAGGGA
Pa_mt_External	D	12	PaMTOR47	GTGTTCAAGCTCTGCCGTACC
Pa_mt_External	H	12	PaMTOR48	AAAGGGAACCAACAGTGCCAAA

Pa_mt_Internal	A	1	PaMTIF01	ACTGAAGATGCCAAGATGATGCT
Pa_mt_Internal	A	2	PaMTIR01	CATCTTGGGCTTATGACAGCTTAG
Pa_mt_Internal	A	3	PaMTIF03	CCTGAAAGAACAACAGTGAGCAT
Pa_mt_Internal	A	4	PaMTIR03	TAGAGTTTGGCTTCAAGGCGAT
Pa_mt_Internal	A	5	PaMTIF05	TGGGATGACTTGTTAGTAGAGGTGA
Pa_mt_Internal	A	6	PaMTIR05	GCAAGTTTAATATTTGGCTTGTGGG
Pa_mt_Internal	A	7	PaMTIF07	CAATGAAATTGATCTTCCTGTGC
Pa_mt_Internal	A	8	PaMTIR07	ATCGTTGAACAAACGAGCCCTTA
Pa_mt_Internal	A	9	PaMTIF09	ATCCTAGTCGCAGTCGCCTACC
Pa_mt_Internal	A	10	PaMTIR09	TTTGTGCTACTGCCCCGTAGTG
Pa_mt_Internal	A	11	PaMTIF11	GGTCCATTGCTTTATTCTTCTTAGC
Pa_mt_Internal	A	12	PaMTIR11	TTGATTTCTTCTGCGTAGGTTGAC
Pa_mt_Internal	B	1	PaMTIF13	ACAGTGAGACATCACCCAATAA
Pa_mt_Internal	B	2	PaMTIR13	GGTTGTGTTGAGTGTGAGAAATACTG
Pa_mt_Internal	B	3	PaMTIF15	TTGCCATCCTTACCTCCTTATCA
Pa_mt_Internal	B	4	PaMTIR15	GCCGATATCTTTGTGGTTGGTTGAGAAT
Pa_mt_Internal	B	5	PaMTIF17	CCCGTATAAATAACATAAGC
Pa_mt_Internal	B	6	PaMTIR17	AGAAGAGGTGTTGGTATAGG
Pa_mt_Internal	B	7	PaMTIF19	TCATCTTCCTCTTCACTATCG
Pa_mt_Internal	B	8	PaMTIR19	TCAATGTTGGTGGCAGTTAGT
Pa_mt_Internal	B	9	PaMTIF21	ATTCCAAGATGCCTCATCTCCATT
Pa_mt_Internal	B	10	PaMTIR21	TAACAACACGGTGGTCTACTTCT
Pa_mt_Internal	B	11	PaMTIF23	CCCACTCACCCACTTCGAGA
Pa_mt_Internal	B	12	PaMTIR23	AGACGGTTGGTAATTCATCGGTTGT
Pa_mt_Internal	C	1	PaMTIF25	TCGAAACCACTAGCCTACTCAT
Pa_mt_Internal	C	2	PaMTIR25	TGGCCTTGAAATGTGCTTCTCGTA
Pa_mt_Internal	C	3	PaMTIF27	TCCACGCACTAACCCTAACAAT
Pa_mt_Internal	C	4	PaMTIR27	GGCTTGTGCCAATCAGAAGTTTA
Pa_mt_Internal	C	5	PaMTIF29	TGAACACAAGGAGGCCTAGAGTG
Pa_mt_Internal	C	6	PaMTIR29	AAGGAGTATGATTGTTGGGAGGA
Pa_mt_Internal	C	7	PaMTIF31	TTCAACACGAACCTCCAACACGAA
Pa_mt_Internal	C	8	PaMTIR31	ATCCTGCAATTGGTGCTTCTAC
Pa_mt_Internal	C	9	PaMTIF33	CGGTGCAATGATCCTCATAATCT
Pa_mt_Internal	C	10	PaMTIR33	GGTGTTCTCGTGTGTTTGAGTTT
Pa_mt_Internal	C	11	PaMTIF35	AACAAGAGCTGCTAACTCCTGCAT
Pa_mt_Internal	C	12	PaMTIR35	TCATGTTACGAACAGTGCGATG
Pa_mt_Internal	D	1	PaMTIF37	AACCGAATCGGAGACATTGG
Pa_mt_Internal	D	2	PaMTIR37	ATGTAGCGGCAAATAGTGTGGAT
Pa_mt_Internal	D	3	PaMTIF39	ATCGAAAGCCTAAATACCTCC
Pa_mt_Internal	D	4	PaMTIR39	TTGTCCGCTGTTTAGTAGGTT
Pa_mt_Internal	D	5	PaMTIF41	TGACTACACAAATCCTGACC
Pa_mt_Internal	D	6	PaMTIR41	TGTAGTGCGAAGAATCGGGTTA
Pa_mt_Internal	D	7	PaMTIF43	AAACTGGGAGGTGTCCTAGCCTTA
Pa_mt_Internal	D	8	PaMTIR43	AGCTTTGGGAGTTGGATATGGAGGTTT
Pa_mt_Internal	D	9	PaMTIF45	CCCAACAACAAGCACCAAGATAA
Pa_mt_Internal	D	10	PaMTIR45	TTGAAGTAACAACGGCGGCTT
Pa_mt_Internal	D	11	PaMTIF47	GTAAGTACTGTTATTGTATTATCGGGC
Pa_mt_Internal	D	12	PaMTIR47	TGTTCAAGCACTGCCGTACCTATTT
Pa_mt_Internal	E	1	PaMTIF02	TGTCACACGGCGTAAAGAGT
Pa_mt_Internal	E	2	PaMTIR02	ATAGGCTATACCTTGACCTGTCTTGT
Pa_mt_Internal	E	3	PaMTIF04	CACTCAGCTTACACCTGAGAGATA
Pa_mt_Internal	E	4	PaMTIR04	ATTCACAGGCAACCAGCTATCA

Pa_mt_Internal	E	5	PaMTIF06	GCGCAAGCTTACATCACCACATTAT
Pa_mt_Internal	E	6	PaMTIR06	AGGGTCTTCTCGTCTTATGGGTTTA
Pa_mt_Internal	E	7	PaMTIF08	ATGTTGGATCAGGACACCCTAATG
Pa_mt_Internal	E	8	PaMTIR08	TTCGGGCTTGTATGTAGCTTAGGA
Pa_mt_Internal	E	9	PaMTIF10	AGGGTGGGCCTCAAACCTCAA
Pa_mt_Internal	E	10	PaMTIR10	GGGTTGAGAAACAGAATGGTAGT
Pa_mt_Internal	E	11	PaMTIF12	CAACCCTCTCATTTCTGAGATACAA
Pa_mt_Internal	E	12	PaMTIR12	AATTGCTGCTGTCAGTAGGAGGCA
Pa_mt_Internal	F	1	PaMTIF14	CCAAACTTACCCTATTAACCTTCTACCT
Pa_mt_Internal	F	2	PaMTIR14	AGTTTCTAGGTGGTTGCTAGGATT
Pa_mt_Internal	F	3	PaMTIF16	TACAGCCTAACGCCTCGACA
Pa_mt_Internal	F	4	PaMTIR16	ATGAGGCTAGTAGCAGTAGGAA
Pa_mt_Internal	F	5	PaMTIF18	ACCTAAACACTACCTTCTTCG
Pa_mt_Internal	F	6	PaMTIR18	TGTCCAATGAGGAGTTTGCTA
Pa_mt_Internal	F	7	PaMTIF20	TCTGAGAAGCCCTTACATCAA
Pa_mt_Internal	F	8	PaMTIR20	GCAGATTGCTAGGGCAACTATTA
Pa_mt_Internal	F	9	PaMTIF22	ATAATCCCAACAACAGACCTTCC
Pa_mt_Internal	F	10	PaMTIR22	AGGCTAGTGCTGTTTCATAGCTTCT
Pa_mt_Internal	F	11	PaMTIF24	GAATTCCGCTAATCCTACTATCA
Pa_mt_Internal	F	12	PaMTIR24	TTAGTTGAATGAGTAGGTGGCCTG
Pa_mt_Internal	G	1	PaMTIF26	TTGGCCTACTTTCCATACTCCT
Pa_mt_Internal	G	2	PaMTIR26	GCAATTGAGAATGGTGCTTCGTA
Pa_mt_Internal	G	3	PaMTIF28	ACATTCATACTCACCTCTCCCT
Pa_mt_Internal	G	4	PaMTIR28	TGTTGAGCCGAAATCAACTGTCT
Pa_mt_Internal	G	5	PaMTIF30	ACTAGTTGCCTCAACCCGAACCT
Pa_mt_Internal	G	6	PaMTIR30	TGAGGAAGGGTTGGATTGTGATTA
Pa_mt_Internal	G	7	PaMTIF32	CCCTACTAACAGCATTATAGTAAA
Pa_mt_Internal	G	8	PaMTIR32	GGGTGTGTGTTTCGTTGTAATTT
Pa_mt_Internal	G	9	PaMTIF34	TACTAACAAACCCAACGAGGGA
Pa_mt_Internal	G	10	PaMTIR34	TTGGATTTGCACCAAGATGG
Pa_mt_Internal	G	11	PaMTIF36	ACATCCTACTGAGAGTGAA
Pa_mt_Internal	G	12	PaMTIR36	GTGTTTATTGTTGAGGCGAGT
Pa_mt_Internal	H	1	PaMTIF38	ATAACCCAACTGCCCTCACCTAT
Pa_mt_Internal	H	2	PaMTIR38	TTGCGGTGAATGATGTGGCTA
Pa_mt_Internal	H	3	PaMTIF40	TCCTCCTCCTTAGGATACTTCAA
Pa_mt_Internal	H	4	PaMTIR40	ATTGTACATCTCGGCATGTGTG
Pa_mt_Internal	H	5	PaMTIF42	ATATTGGCCAAACCTCGTAGAA
Pa_mt_Internal	H	6	PaMTIR42	TTGTGCGCTGTTTAGACTTGTGGA
Pa_mt_Internal	H	7	PaMTIF44	TGTAAGCCAAAGAGTGAAGAC
Pa_mt_Internal	H	8	PaMTIR44	TTTGGCAGCGGATTTGTTTC
Pa_mt_Internal	H	9	PaMTIF46	ATAGCAATTCCCGCTTGGCTTT
Pa_mt_Internal	H	10	PaMTIR46	TTTAATCCGAAGGGATTAGGAGCTGGT
Pa_mt_Internal	H	11	PaMTIF48	CTGTCATGTAACAAACGACTATCTCC
Pa_mt_Internal	H	12	PaMTIR48	AAAGGGAACCAACAGTGCCAAAC

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PRIMERNAME	PRIMER SEQ
PaMTOF49	GGTTGGCTATAGTTCACCAT
PaMTOR49	ACGGTAAGGTGATAGCAACAA
PaMTIF49	TGGCTATAGTTCACCATTT
PaMTIR49	CGGTAAGGTGATAGCAACAAA
PaMTOF50	TCGATACCCATTGCATTCT
PaMTOR50	TTAAGCGGTTACGTGGTAGTT
PaMTIF50	ACCCATTGCATTCTAATTTCTAC
PaMTIR50	TAAGCGGTTACGTGGTAGTTT
PaMTOF51	TCGATACCCATTGCATTCT
PaMTOR51	CGCTCCCTTTGTCTAGTTAAT
PaMTIF51	CCCATTGCATTCTAATTTCTAC
PaMTIR51	CCCTTTGTCTAGTTAATAAGAGCG
PaZenKOF1	ACTGCCACCAATAACCTACACG
PaZenKOR1	ATCATTGGCACCTGAAAGTTGGT
PaZenKIF1	TGCCACCAATAACCTACACGG
PaZenKIR1	ATTGGCACCTGAAAGTTGGTCTT
PaZenKOF2	TCCAATTTATTCGGCTGCACCA
PaZenKOR2	TTGATCAGCTGGGACTGATAGTT
PaZenKIF2	AATTTATTCGGCTGCACCAACTTTCCC
PaZenKIR2	AGCTGGGACTGATAGTTAGTGTT
PaGapdhOF1	TTTGTCAAGCTGGTTTCCTGGT
PaGapdhOR1	TTCCCATCCACTTCCAGTAAAGA
PaGapdhIF1	TGTCAAGCTGGTTTCCTGGTA
PaGapdhIR1	CCACTTCCAGTAAAGAAAGTAGGAAGCA
PaGapdhOF2	TTATATGTAGACCGCTGCTGAGT
PaGapdhOR2	AGAAACAGAAAGTGCTGTCAGGAT
PaGapdhIF2	ATATGTAGACCGCTGCTGAGTC
PaGapdhIR2	AACAGAAGTGCTGTCAGGATTGA

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Figure S1. Percent recovery of sequences from different regions of mitochondrial genomes obtained from individual samples. Total numbers of base pairs recovered are given in parentheses.

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Figure S2. Serial UPGMA tree based on the complete mitogenomes from ancient and modern populations of Adelie penguins. This tree was constructed by using the software *Pebble* that accounts for the ages of the ancient sequences (Drummond and Rodrigo, 2000). Carbon dated ages of ancient sub-fossil bones are given in the box.

Figure S3. UPGMA trees of modern Adeline penguins. Trees were constructed using
a. synonymous sites b. constrained sites (nonsynonymous sites + tRNAs + rRNAs).
The nodes show the two distinct Antarctic (A) and Ross sea (R) lineages, which
coalesce at the root (A-R).

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