

Supplementary Material 3. PCR amplification of mitochondrial genes

We conducted PCR amplification of four mitochondrial genes (*16S*, *COX1*, *COX3*, and *NAD3*) using TaKaRa Ex Taq polymerase (Takara) or KOD FX Neo polymerase (Toyobo) in Life Touch or Life ECO thermal cyclers (Biore). The PCR primer sequences are as follows (see main text for the references):

***16S* primers**

5'-ATTAAAGTATGGCAAAAGGAACTCG-3'

5'-AAACTTCCTCTTTCAGCAGTCCT-3'

***COX1* primers**

5'-GGTCAACAAATCATAAAGATATTGG-3'

5'-TAAACTTCAGGGTGACCAAAAAATCA-3'

Degenerate *COX1* primers

5'-CCTGTTYTRCTTTAYGGKAARCGRGAG-3'

5'-CACAAAACATGRGAAATTATYCCAAA-3'

K = G/T; R = A/G; and Y = C/T

***COX3* primers**

5'-TGTCTTCCAAACAAAAGGTATCC-3'

5'-CATAATCTTCGACATCAACAAAGC-3'

***NAD3* primers**

5'-GCATAAAAGTTTTTGGTGCTT-3'

5'-TCGAAACCTAAATTCGACGTA-3'

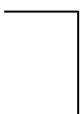
1. PCR conditions (15 µl volume)—TaKara Ex Taq—

1-1. PCR mixture

10×Ex Taq buffer	1.5 µl
dNTPs (2 mM each)	1.5 µl (200 µM each)
Primer 1 (5 pmol/µl)* ¹	0.5 µl (167 nM)
Primer 2 (5 pmol/µl)* ¹	0.5 µl (167 nM)
Taq polymerase (5 units/µl)	0.2 µl
DNA (≥ 0.5 ng/µl)	1.0 µl
Nuclease-free sterilized water	9.8 µl

*¹In PCR amplification using the degenerate *COX1* primers, the concentration of the primers was increased fourfold (final concentration of 667 nM). The total reaction volume was adjusted to 15 µl by decreasing the amount of water.

1-2. PCR cycles

98°C	10 sec		45–48 cycles
48°C* ²	30 sec		
72°C	1 min		
72°C	5 min (final elongation)		
10°C	Hold		

*²Annealing temperature was lowered to 42°C–45°C for poorly amplified specimens. In PCR amplification with the degenerate *COXI* primers, the annealing temperature was increased to 58°C.

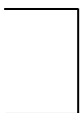
2. PCR conditions (15 µl volume)—KOD FX Neo—

2-1. PCR mixture

2×KOD FX Neo buffer	7.5 µl
dNTPs (2 mM each)	1.5 µl (200 µM each)
Primer 1 (5 pmol/µl)* ¹	0.5 µl (167 nM)
Primer 2 (5 pmol/µl)* ¹	0.5 µl (167 nM)
Taq polymerase (1 unit/µl)	0.2 µl
DNA (≥ 0.5 ng/µl)	1.0 µl
Nuclease-free sterilized water	3.8 µl

*¹The concentration of the degenerate *COXI* primers was increased fourfold as with the Ex Taq PCR.

2-2. PCR cycles

98°C	2 min (preheating)		
98°C	10 sec		45–48 cycles
48°C* ²	30 sec		
68°C	1 min		
72°C	5 min (final elongation)		
10°C	Hold		

*²Annealing temperature was lowered to 42°C–45°C for poorly amplified specimens. In PCR amplification with the degenerate *COXI* primers, the annealing temperature was set at 58°C.