GAPDH as a control gene to estimate genome copy number in Great Tits, with cross-amplification in Blue Tits

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Estimating the number of genome copies in a tissue sample can serve various purposes. For example, such an estimate serves as a scaling variable when measuring telomeres with quantitative PCR. We describe the primer development and evaluation for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in the Great Tit *Parus major*, as a control gene to estimate genome copy number. We demonstrate specific amplification with negligible variation in 48 Great Tits and cross-amplification in 53 Blue Tits *Cyanistes caeruleus*. We conclude this primer set to be reliable for amplification of GAPDH as a reference gene for quantitative PCR analysis in Great and Blue Tits.

Key words: reference gene, glyceraldehyde-3-phosphate dehydrogenase, qPCR, telomeres, avian, passerine

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An invariant control gene, which estimates genome copy number, is used in molecular studies to correct for variation in sample loading or reaction efficiencies (Sturzenbaum & Kille 2001). To serve this purpose, all individuals need to have the same number of copies of the control gene per genome. Secondly, there needs to be negligible variation between individuals in the amplified region of the control gene to ensure that they are multiplied at comparable rates in samples of different individuals. A control gene used for qPCR studies of telomeres in birds is the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Primers are designed for the Zebra Finch *Taeniopygia guttata*, and telomere lengths measured with qPCR compared with those of terminal restriction fragment analysis by in-gel hybridization as a reference. A strong correlation between both methods was found, and cross-amplification in the Alpine Swift *Apus melba* (Criscuolo *et al.* 2009).

Telomeres, tandem repeats at the ends of chromosomes, have been proposed as an indicative measure for past life stress in humans (Boonekamp *et al.* 2013) and birds (Monaghan & Haussmann 2006, Bauch *et al.* 2013). This view is supported by studies demonstrating links between telomere length and survival (e.g. Haussmann *et al.* 2005, Bize *et al.* 2009, Salomons *et al.* 2009). Several techniques are available to measure telomeres (Aubert *et al.* 2012), of which quantitative PCR (Cawthon 2002, O'Callaghan *et al.* 2008) has the advantages that it is relatively less labour intensive, and requires only small quantities of DNA. Recently this method was also introduced to measure telomeres in birds (Criscuolo *et al.* 2009, Barrett *et al.* 2012).

The Zebra Finch GAPDH primers are to an unknown extent species specific and our preliminary results showed that they are not invariant in Great Tits *Parus major* and Blue Tits *Cyanistes caeruleus*. We therefore derived the sequence of the GAPDH gene from a de novo assembly of Great Tit Illumina sequences, a platform for next-generation sequencing, and designed and validated new primers on part of the GAPDH gene in the Great Tit. We further show that these primers cross-amplify in Blue Tits.

Methods

Blood sampling

We collected blood samples of Great and Blue Tit populations on Vlieland, an island in the Dutch Wadden Sea

(Verhulst & van Eck 1996). For both species, age at which blood samples were taken ranged from one to five years (Great Tit age = 1.7 ± 1.13; Blue Tit age = 1.7 ± 1.16; averages ± SD). Blood samples were collected in 2% EDTA, stored at 4–7°C for up to three weeks, and subsequently stored at –80°C after being snap-frozen in a 40% glycerol buffer.

Molecular analysis

Whole blood was centrifuged for 4 minutes at 3500 rpm and DNA was extracted from 3 μl red blood cells using the innuPREP Blood DNA Mini Kit (Analytik Jena) following the manufacturer's protocol. Concentrations and purity (A_{260}/A_{280} and A_{230}/A_{260}) were quantified using a NanoDrop 2000C Spectrophotometer (Thermo Scientific). Aliquots of DNA were diluted to 1.67 ng/μl and stored at –20°C for all downstream applications.

We used a fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a control gene. The starting point was the GAPDH sequence of the Zebra Finch (Criscuolo *et al.* 2009). We located this sequence in the Great Tit by performing a local NCBI BLAST search of the Zebra Finch GAPDH sequence using Blastn with default settings and an E-value cutoff of $1.0 \times e^{-25}$, against contigs produced from assembled Illumina by next-generation sequencing of Great Tit sequences (van Bers *et al.* 2010, 2012). The 2180 bp long sequence consisting of two contigs aligned 99% with the Zebra Finch, with E-values ranging between $5.0 \times e^{-37}$ and $3.0 \times e^{-167}$. We designed primers that amplified an 80 bp fraction of an exon on the GAPDH gene in the Great Tit using Primer3 (Rozen & Skaletsky 2000).

DNA of 52 individual Great Tits was subjected to PCR to test for the specificity of the primers and variation between individuals. Primer concentrations were 100 nM for both the forward and reverse primer in a total volume of 10.5 μl. Reaction conditions were: 1μl 10x PCR + Mg buffer (Roche), 200 μM of each dNTP, 0.075 μl Taq polymerase (5 U/μl, Roche). Between 10 and 100 ng of DNA was used as template. The PCR reaction was performed in a TC-512 thermal cycler (Techne). After initial denaturation at 95°C for 10 seconds, 40 cycles of 1 minute at 95°C, 1 minute annealing at 60°C and 1 minute at 72°C were completed, followed by a final extension of 5 minutes at 72°C. The PCR product was subjected to electrophoresis for 90 minutes at 5.45 V/cm in a 2.5% agarose gel with ethidium bromide and compared with a 100 bp size standard. DNA of 24 Blue Tits was subjected to the same procedure.

To test the quality of the primer set with higher resolution, we amplified DNA of 48 Great Tits and 53 Blue Tits in a quantitative PCR reaction (qPCR), with each species analysed on separate plates. The variation in cycle quantification value (Cq) and melting temperature or lack thereof will give more details about similarity between individuals and the amount of different amplicons respectively. As a negative, no-template control we used AccuGENE Molecular Biology Water (Lonza). Primer concentrations were 100 nM for both the forward and reverse primer in a final volume of 15 μl containing 7.5 μl of iQ SYBR Green Supermix (BioRad). Each reaction contained 10 ng of DNA. The qPCR reactions were performed in a CFX96 (Bio-Rad) instrument. Thermal cycles were: 15 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C, 30 seconds annealing at 60°C for GAPDH and 30 seconds extending at 72°C. Data were collected during the extension phase. We made a dilution curve of a serial dilution of Great Tit DNA, when amplified with GAPDH primers. We diluted a sample consisting of a mix of DNA samples of 4 Great Tits two times in 4 subsequent steps with a starting concentration of 3.33 ng/μl. A log linear curve would confirm specific amplification in the GAPDH region of the DNA, independent of DNA concentration. Calculations of amplification efficiencies and cycle threshold values were made with the program LinRegPCR 12.13 (Ruijter *et al.* 2009). The effects of age at which blood samples were taken and species on Cq values were evaluated with a GLM in the program JMP 7.0 (SAS Inst., Marlow, UK).

As a final evaluation we established a qPCR-coupled High Resolution Melting-curve (HRM) analysis in 25 Great and 24 Blue Tit DNA samples. This method is able to detect point mutations and is therefore more sensitive to detect variation between individuals. As positive controls for resolution of this method we used a synthetic oligomer (A) of the 80 bp GAPDH amplicon and this same oligomer (B) with a point mutation (A-G at position of 31 bp) (See Appendix 1 for details). We used the same reaction volumes as in the qPCR analysis, but as fluorescent dye EvaGreen 2x Epitect HRM PCR master mix (Qiagen). The qPCR-coupled HRM was performed in a Rotor-Gene Q (Qiagen) instrument (5 minutes at 95°C followed by 55 cycles of 10 seconds at 95°C, 30 seconds annealing at 60°C and 15 seconds extending at 72°C succeeded by HRM from 65°C rising with 0.1°C each step till 95°C were reached). Data were normalised in the program Rotor-Gene Q 2.0.3 and melt peaks compared in Statistix 8.0.

Furthermore, we made calculations for absolute telomere length as done recently in birds (Barrett *et al.*

2012) comparing calculations with standard curves of oligomers A and B in order to estimate the error in telomere length calculations when there would be a point mutation (A-G) in GAPDH. The weight of the oligo standards compared to the amplicon in the birds is important for our methods, as this is used to calculate genome copy numbers. The weight difference is highest in a transition of purines (A-G). Even though a transversion from C-G would lead to the biggest weight difference, we did not use this because transversions are less common mutations. As a standard curve for telomere length we used results obtained in Zebra Finches in our lab, as this should provide a realistic proxy for calculation purposes. In short, to calculate the amount of telomere (Kb) and GAPDH (number of diploid genomes), we used the equation:

$$X0 = {E_{AMP_s}}^{b_a \times \log E_{AMP_s} (E_{AMP_a}) - C_{q_s}}$$

(Gallup & Ackermann 2008), where E_{AMP_s} is the amplification efficiency of the individual sample as given by LinRegPCR and E_{AMP_a} is the amplification efficiency of the standard curve as calculated by:

$$E_{AMP} = 10^{\left(\frac{-1}{m}\right)},$$

and b and m are the intercept and slope of the standard curve. This way we could correct for differences in efficiencies between the sample and the synthetic oligomers. Telomere length (Kb) was divided by the number of GAPDH genome copies to calculate telomere length per diploid genome.

Results and Discussion

The average DNA concentration we obtained was 45.5 ng/μl in the Great Tit and 33.8 ng/μl in the Blue Tit. Purities, estimated as the ratio of the intensity of absorption at 260/280 nm and 230/260 nm, were all close to the (normal) range of respectively 1.8–2.0 and 2.0–2.2. The primers we designed,
Forward 5'-TGTGATTTCAATGGTGACAGC-3' and
Reverse 5'-AGCTTGACAAAATGGTCGTTC-3',
resulted in amplification of an 80 bp product including primers, as estimated from the 100 bp size standard after electrophoresis. There was no distinguishable variation in product size between individuals of either species.

Average efficiency of the qPCR was at 2.02 ± 0.01, exactly the same for Great Tits (range 1.85–2.15) and Blue Tits (range 1.91–2.19). There was no amplification in our no-template control. The dissociation curves showed a single peak, indicating one product was amplified (Figure 1). Melting temperature was 81.5°C for all tested individuals in both Great Tits and Blue Tits, except for one Blue Tit with a peak at 81.0°C (temperature was changed in 0.5°C steps). Thus a similar product was amplified in both species. The Cq value (mean ± SD) was 22.2 ± 0.25 (range 21.7–22.8) in Great Tits and 22.3 ± 0.22 (range 21.6–22.8) in Blue Tits. Given the small standard deviation of the Cq value in both species (CV = 1.1%) we conclude that there is negligible variation in amplification between individuals. Moreover, in a GLM we found no effect of species (n = 101; P = 0.58) nor age (n = 64; P = 0.58) on Cq value. Because Cq value and melting temperature were indistinguishable from those obtained in Great Tits, we conclude the primer set cross-amplifies in the Blue Tit. Secondly, we met the requirement for a control gene in telomere analyses with qPCR, that there is no effect of age at which the blood sample was taken on the length of the amplified product. We made a serial dilution standard curve of Great Tit DNA with R^2 = 0.999, and Blue Tit DNA with R^2 = 0.985. This confirms specific amplification in the GAPDH region of the DNA, independent of DNA concentration.

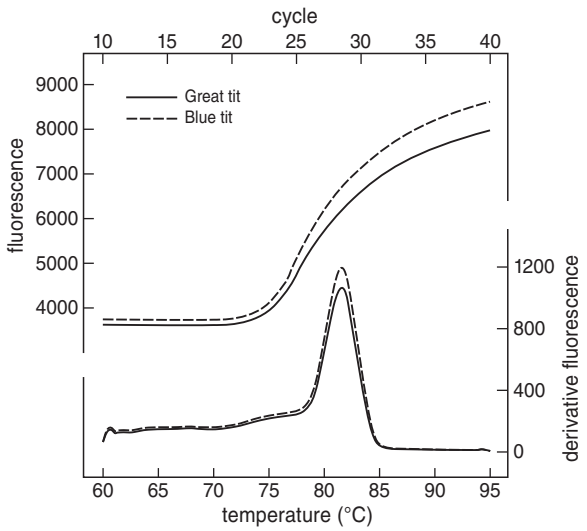

Figure 1. Amplification (top) and dissociation (bottom) curves of GAPDH as averaged over all tested individuals. Cq Great Tit = 22.2, Cq Blue Tit = 22.3; Tm Great Tit = 81.5°C, Tm Blue Tit = 81.5°C.

When analysing the data with HRM we found two clusters of Great Tits which were distinguished by a small shift of the melt curve (Figure 2). One group overlapped with oligomer A (n = 5), the other was right shifted (n = 20). Blue Tits all overlapped with the right shifted group, indicating they had the same

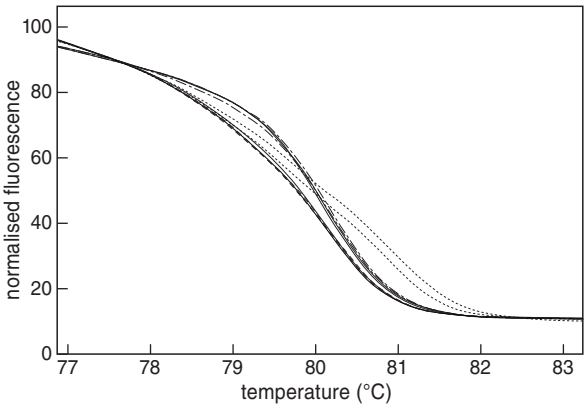

Figure 2. qPCR-coupled High Resolution Melting-curve (HRM) analysis depicted by two representative individuals for each cluster. Great Tit (both clusters): solid lines, Blue Tit: dashed-dotted lines, oligo A: dashed lines, oligo B: dotted lines. Values on the y-axis are normalized fluorescence in Rotor-Gene Q allowing all curves to be compared with the same starting and ending fluorescent signal level.

sequence. The difference between oligomers A and B, caused by an A to G mutation, is larger than the difference between the clusters of birds. Also an analysis of the melt peak temperatures showed a significant difference between oligomer B and all other clusters, and between the two clusters of Great Tits (Tukey pairwise comparison, Appendix 2). However, since the difference in melting temperature between the clusters of birds was smaller than 0.2°C, it cannot be detected in a standard qPCR as commonly used for telomere length analyses. Also Cq values of the two clusters of Great Tits were similar (left group 22.1 ± 0.21; right shifted group 22.3 ± 0.18). We calculated hypothetical telomere length based on oligomer A and B, in order to determine the absolute effect of an A to G mutation on telomere estimates (Appendix 3). For the minimum and maximum Cq values obtained for GAPDH in Great Tits and assuming Cq = 11 for telomere length, this gave a negligible difference of 0.04% in telomere length.

Conclusion

We designed primers for an invariant control gene, GAPDH, to estimate genome copy number in the Great Tit and showed its cross-amplification in the Blue Tit. Amongst other things, this opens up the possibility of telomere analysis with qPCR in these species. Further applications for these primers may be in determining the number of nuclei in tissue samples and the cross-amplification in a larger set of passerines. The GAPDH protein is involved in many cellular functions and is highly conserved (Sirover 2011). When performing a BLAST search for the 80 bp GAPDH amplicon in the Red Jungle Fowl (*Gallus gallus*, GenBank ref. NW_003763490.1), Turkey (*Meleagris gallopavo*, NW_003432340.1) and Zebra Finch (*Taeniopygia guttata*, NW_002197702.1) we find one product with a >96% match in the GAPDH part of the sequence in these species. Although in the red jungle fowl potentially a second product (NW_003763646.1) is amplified, this might give scope for usage of this primer set as a control gene in other bird species.

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Samenvatting

Het kan om allerlei redenen nodig zijn om het aantal genoomkopieën in een weefselmonster te schatten, bijvoorbeeld wanneer telomeren gemeten worden door middel van een quantitative PCR (qPCR). Telomeren zijn aaneengeschakelde herhalingen aan de uiteinden van de chromosomen met een beschermende werking op chromosomen. Bij zowel mensen als vogels is aangetoond dat telomeren gebruikt kunnen worden als een maat voor stress die door een organisme ervaren is. Van de technieken die gebruikt kunnen worden om telomeren te meten is het voordeel van qPCR dat het relatief weinig werk is en dat er kleine hoeveelheden DNA voor nodig zijn. Deze methode is onlangs aangepast vanuit gebruik bij mensen naar gebruik bij vogels. Voor deze aanpassing is het vinden van een geschikt controle gen zonder variatie tussen individuen essentieel. Helaas is het tot nu bij vogels gebruikte fragment DNA variabel bij Koolmees *Parus major* en Pimpelmees *Cyanistes caeruleus* en dus niet geschikt als controle gen bij deze soorten. In dit artikel beschrijven we de ontwikkeling en evaluatie van een nieuw fragment in het glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gen, geschikt als controle gen om het aantal kopieën van het genoom in een weefselmonster te schatten. We laten zien dat de primers die we ontworpen hebben om dit fragment te amplificeren (vermenigvuldigen), specifiek binden bij Koolmezen en de variatie tussen individuen in het fragment verwaarloosbaar is. Daarnaast wordt eenzelfde fragment bij Pimpelmezen geamplificeerd door deze primers, zonder enige variatie tussen individuen. We concluderen daarom dat deze primer-set betrouwbaar is om te gebruiken voor GAPDH als controle gen in qPCR analyses bij Koolmezen en Pimpelmezen.

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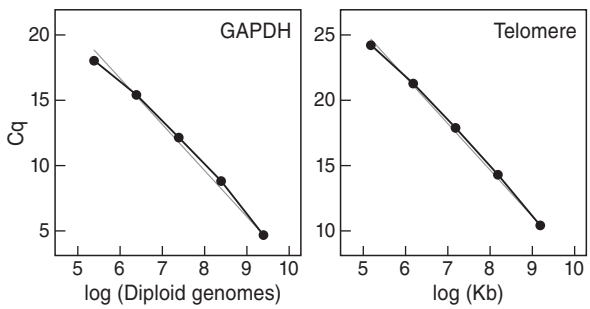
Appendix 1. Standards used in a qPCR-coupled High Resolution Melting-curve (HRM) analysis: synthetic oligomer (A) of the 80 bp GAPDH amplicon and the same oligomer (B) with a point mutation A-G at position of 31 bp.

Name	Purpose	Sequence	Molecular weight (u)
A Oligomer	Positive control; amplicon obtained from Great Tit sequence	5′ –TGT GAT TTC AAT GGT GAC AGC CAT TCC TCC **A**CC TTT GAT GCG GGT GCT GGC ATT GCA CTG AAC GAC CAT TTT GTC AAG CT–3′	24593
B Oligomer	Positive control; amplicon with point mutation	5′ –TGT GAT TTC AAT GGT GAC AGC CAT TCC TCC **G**CC TTT GAT GCG GGT GCT GGC ATT GCA CTG AAC GAC CAT TTT GTC AAG CT–3′	24609



Standard curves of synthetic oligomer standards, GAPDH oligomer A and B and Telomere (TTAGGG repeated 14 times). Oligomer A: R^2 = 0.9937, slope = –3.3175, intercept = 36.320; Oligomer B: R^2 = 0.9937, slope = –3.3175, intercept = 36.319; Telomere: R^2 = 0.9976, slope = –3.4833, intercept = 42.711.

Appendix 2. Tukey pairwise comparison of melt peaks for groups.

Group	Mean	BT	GT1	GT2	Oligomer A
BT	80.186				
GT1	80.257	0.071			
GT2	80.133	0.053*	0.123*		
Oligomer A	80.217	0.031	0.040	0.083	
Oligomer B	81.000	0.814*	0.743*	0.867*	0.783*

Alpha	0.05
Critical Q Value	4.009
Error term used	Error, 48 DF

Appendix 3. Input for calculations of telomere length.

Telomere					**GAPDH**					
Cq	b_a	E_{AMPa}	E_{AMPs}	X_0	*Cq*	b_a	E_{AMPa}	E_{AMPs}	X_0	*TL/ Dipl.genome*
11.0	42.711	1.937	1.900	2299.040	21.78	A:36.320	2.002	1.980	0.866	2654.20
11.0	42.711	1.937	1.900	2299.040	22.76	A:36.320	2.002	1.980	0.443	5184.00
11.0	42.711	1.937	1.900	2299.040	21.78	B:36.319	2.002	1.980	0.866	2655.28
11.0	42.711	1.937	1.900	2299.040	22.76	B:36.319	2.002	1.980	0.443	5186.11