Rapid estimation of species-specific DNA digestibility based on differential qPCR

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Abstract:
Metagenomic analysis of fecal DNA has been gaining increasing attention in studying feeding ecology of various organisms in various habitats. This method, however, is based on an assumption that DNA digestibility is similar among various prey species, which has been questioned by some researchers. Thus, the present study was conducted to estimate/compare DNA digestibility of selected prey species using a novel qPCR-based methodology. Digestibility of prey DNA was estimated by an in vivo feeding trial using six test diets. Each diet contained one of the following: (A) goldfish, (B) goldfish +citric acid, (C) goldfish +$CaCO_3$, (D) shrimp, (E) snail, and (F) goldfish +shrimp +snail. The diets were fed to rainbow trout and fecal samples were collected. Mitochondrial DNA was extracted from both diet and fecal samples, and quantified by qPCR using two sets of species-specific primers for each prey species. The first set of primers amplified a short stretch of DNA (51-80 bp), whereas another set amplified a longer stretch (126-162 bp). DNA digestibility was estimated based on the ratio between short and long amplicons using the following formula: Digestibility (%) = $\log_{((Mt\text{-}S)/(Mt\text{-}L))}$ ([S]/[L])/(0.01 × Mt), where Mt:16K (bp), S:length of short amplicon (bp), L:length of long amplicon (bp), [S]:relative quantity of short amplicon, [L]:relative quantity of long amplicon. DNA extracted from the Diets A-F were successfully quantified for all species tested by both long and short PCR. Fecal DNA, however, were more difficult to amplify, especially by long PCR. Calculated fecal digestibility of goldfish DNA was 2.07% (A), 1.12% (B), 1.95% (C), and -1.05% (F). Digestibility of shrimp DNA was 10.79% (D) and 12.61% (F). Digestibility of snail DNA was 1.88% (E) and 2.06% (F). These results suggest that the digestibility of dietary DNA may be estimated based on the ratio between long and short fragments.

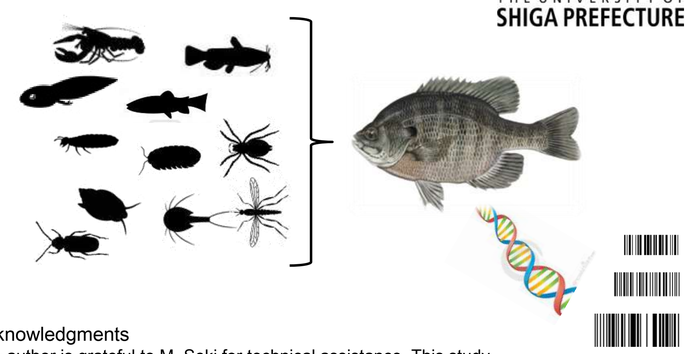

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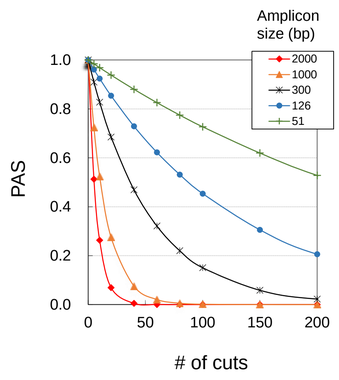
Table 1 qPCR primers used for fecal and diet analyses

Prey items	Sequences (5'-3')	Annealing temp (ºC)	Extension time (sec)	Amplicon size (bp)
Goldfish, short	F: cccacaacctaaatatcgttacc R: tctttcctttgttgcactcc	60.2	20	51
Shrimp, short	F: aagttactttagggataacagcgt R: taattcaacatckaggtcgc	62.0	20	80
Snail, short	F: taccnbagggataacagcataat R: tartccaacatcgaggtcaya	57.5	20	76
Goldfish, long	F: caacccaagagagcaatgtg R: tgtgtttgccgagttccttc	60.3	25	126
Shrimp, long	F: tgaccgtgcaaaggtagc R: tagggtcttatcgtccccttag	58.6	30	148
Snail, long	F: agttggggcgactaaggaac R: caacatcgaggtcacaaccc	61.3	30	162
All species*	F: darytacynyrgggataacag R: rrtycaacatcgaggtmryaa	55.3	50	79

* universal degenerate primers

Table 2 Theoretical relationship among PCR amplicon size, # of cuts (endonuclease digestion), and probability of amplification success (PAS)

# of cuts [1]	Digesti-bility [2]	PAS [3] Amplicon size (bp)				51 / 126 bp
		1000	*300*	*126*	*51*	ratio
0	0.00	1.000	1.000	1.000	1.000	1.00
10	0.06	0.524	0.828	0.924	0.969	1.05
100	0.63	0.002	0.151	0.454	0.727	1.60
300	1.88	0.000	0.003	0.093	0.384	4.11
800	5.00	0.000	0.000	0.002	0.078	43.4
2000	12.50	0.000	0.000	0.000	0.002	12421



1 Number of endonuclease cleavage within mtDNA sequence
2 Digestibility (%) = # of cuts x 100 / 16K (16K is the full length of mtDNA)
3 PAS (probability of amplification success) = [(16K – Amplicon size)/16K]^ # of cuts. PAS were calculated assuming there was no exonuclease digestion. Amplicon sizes of 126 and 51 represent goldfish long and short PCR, respectively.

Table 3 DNA digestibility estimated by qPCR

Sample[1]	Digestibility (%)[2] Goldfish	Shrimp	Snail	Conc.[3]
Diet A	0.93 ± 0.49 [a] (S 3/3, L 3/3)	---	---	22.2 ± 17.5
Feces A	*2.07 ± 0.39* [a] (S 5/5, L 0/5)	---	---	0.17 ± 0.10
Diet B	0.07 ± 0.20 [a] (S 3/3, L 3/3)	---	---	1.83 ± 1.33
Feces B	1.12 ± 0.67 [a] (S 5/5, L 2/5)	---	---	6.78 ± 5.69
Diet C	0.93 ± 0.40 [a] (S 3/3, L 3/3)	---	---	0.77 ± 0.59
Feces C	*1.95 ± 0.24* [a] (S 5/5, L 0/5)	---	---	0.55 ± 0.36
Diet D	---	12.45 ± 1.12 [b] (S 3/3, L 3/3)	---	0.11 ± 0.03
Feces D	---	10.79 ± 1.80 [b] (S 5/5, L 1/5)	---	0.02 ± 0.01
Diet E	---	---	-1.46 ± 0.18 [a] (S 3/3, L 3/3)	0.26 ± 0.12
Feces E	---	---	1.88 ± 0.00 [a] (S 1/5, L 1/5)	0.02 ± 0.01
Diet F	-0.66 ± 1.83 [a] (S 3/3, L 3/3)	13.72 ± 1.22 [b] (S 3/3, L 2/3)	0.00 ± 1.46 [a] (S 3/3, L 3/3)	0.25 ± 0.02
Feces F	*-1.05 ± 1.06* [a] (S 3/5, L 0/5)	12.61 ± 1.29 [b] (S 4/5, L 1/5)	*2.06 ± 0.19* [a] (S 1/5, L 0/5)	0.06 ± 0.02

1 Diet A: goldfish; Diet B: goldfish +citric acid; Diet C: goldfish +CaCO3; Diet D: shrimp; Diet E: snail; Diet F: goldfish +shrimp +snail. Feces A-F correspond to the feces of trout fed Diets A-F, respectively
2 Values are mean ± SD (n = 3 for diets, n = 5 for feces). Values in italic are approximate values (for non-amplified samples). PCR success rates are indicated in parentheses, where "S" and "L" denote short and long PCR, respectively. The fractions indicate the number of samples amplified / the number of samples analyzed by PCR. Dash lines indicate "non-applicable". Values with different superscript letters are significantly different (Scheffe's test, $p < 0.05$)
3 Relative DNA concentration of extracted solution (arbitrary unit). Values are mean ± SD (n = 3 for diets, n = 5 for feces)

Results

Quantification of prey DNA in diets and feces using long and short PCR
DNA extracted from the Diets A-F were successfully quantified for all species tested by both long and short PCR (Table 3). Fecal DNA, however, were more difficult to amplify, especially by long PCR. Fecal goldfish DNA was successfully quantified by short PCR. By long PCR, however, fecal goldfish DNA was not amplified after 40 PCR cycles, except two samples of Feces B (Table 3). Fecal shrimp DNA was successfully quantified by short PCR. However, by long PCR, it was not amplified except one sample of Feces D and one from Feces F. Fecal snail DNA was amplified by short PCR for only two samples (one from Feces E and one from Feces F) out of 10 samples analyzed (Table 3). By long PCR, only one sample (Feces E) was amplified.

Digestibility of prey DNA
Digestibility of DNA was calculated for both diets and feces. Digestibility of goldfish DNA in the Diets A, B, C and F was less than 1.0%. Digestibility of goldfish DNA in feces ranged from -1.05% (Feces F) to 2.07% (Feces A) (Table 3). Digestibility of shrimp DNA in diets was 12.45% for Diet D and 13.72% for Diet F. Digestibility of shrimp DNA in feces was similar to that of the diets (10.79% for Feces D, 12.61% for Feces F). Digestibility of snail DNA in diets ranged from -1.46% to 0.00% (Table 3). Digestibility of snail DNA in feces was 1.88% (Feces E) and 2.06% (Feces F). Digestibility of shrimp DNA was higher (Scheffe's test, $p < 0.05$) than that of goldfish and snail DNA (Feces F). Overall, fecal digestibility was only slightly higher than the corresponding diet digestibility. Citric acid and CaCO3 had little observable effect on DNA digestibility.

Concentration of DNA in extracted solutions
Using universal degenerate primers, DNA was successfully amplified for all diet and fecal samples (Table 3). Relative DNA concentrations determined using universal primers, however, were unrelated to species-specific concentrations of DNA for both fecal and diet samples: r = 0.32 (n = 15) with fecal DNA quantified by goldfish short PCR, r = -0.24 (n = 10) with dietary DNA quantified by goldfish short PCR, r = -0.23 (n = 10) with dietary DNA quantified by goldfish long PCR.

Introduction

Previously, we studied prey items of largemouth bass and bluegill based on visual inspection of stomach contents as well as fecal DNA analyses based on clone-library and group-specific real-time PCR (qPCR). However, in some cases, prey profiles estimated by the two different approaches disagreed considerably. For example, some molluscan DNA was detected in largemouth bass feces, whereas few molluscan species were identified by stomach content inspection. These observations prompted the author to estimate/compare digestibility of different prey species.

But, how can DNA digestibility differ among prey items? First, DNA is more stable at alkaline pH compared to acidic pH. Thus, calcium carbonate brought by molluscan shells, for example, could counteract DNA degradation by neutralizing gastric acid in predator's stomach. Second, dietary DNA is digested by pancreatic deoxyribonucleases (DNases) and intestinal polynucleotidases. Some prey organisms, e.g., shrimps, have high endogenous DNase activity, resulting in enhanced autolytic digestion. Also, DNase activities are affected by luminal Mg, Ca, Mn and Co concentrations. Third, a matrix effect increases the stability of dietary DNA. For example, plant DNA is more or less protected from acid and enzymatic degradation, and therefore more stable than naked DNA (e.g., plasmid DNA, PCR products) in the GI tract. Prey remains such as fish bones could have a similar sheltering effect, protecting DNA within the bone matrix.

Determination of nutrient digestibility generally involves chemical analyses of nutrients of interest and an inert marker (e.g., Cr), in the diet and feces. However, such a conventional approach is not only laborious and time-consuming, but also does not allow determination of DNA digestibility for each prey species on wild-caught predator fish such as largemouth bass and bluegill. The present study was conducted to estimate DNA digestibility of selected prey species using a novel qPCR-based methodology.

Materials and Method

Test diets
Prey items tested were (1) goldfish *Carassius auratus* standard length ~50 mm, (2) shrimp *Palaemon paucidens* body length ~40 mm , and (3) snail *Bellamya* (*Sinotaia*) *quadrata histrica* shell height ~20 mm. In addition, effects of citric acid and $CaCO_3$ on DNA digestibility were determined. Further, the mixture of all three species was tested. Thus, the following six diets were prepared: Diet A goldfish, Diet B goldfish +citric acid (5% per diet), Diet C goldfish +$CaCO_3$ (5% per diet), Diet D shrimp, Diet E snail, and Diet F goldfish +shrimp +snail. Each prey item was homogenized, mixed with a casein-gluten basal diet at a 1:9 ratio (prey 1: basal diet 9), and cold-extruded to make moist pellets. The moist-pellets were stored at 0-4ºC, and used within 23 days.

Fish rearing and fecal collection
Rainbow trout (mean body weight 38.4g) were used in the feeding trial. Each tank (50l) was continuously supplied with filtered lake water (7-13ºC) at 2l / min. Fish were hand-fed once daily with respective diets for 19 days at 1.3% (dry weight basis) of their body weight daily for all groups. Feces were collected once from each trout by stripping. Five fecal samples were collected for each dietary group. Fecal samples were homogenized and stored at -20ºC until analyses.

DNA extraction from diet and fecal samples
Fecal or diet sample (~2 mg) was placed in a 0.2ml-PCR tube, to which 20µl of DNA-extraction reagent (Prepman Ultra) was added, heated (99ºC, 10 min), cooled down, 60µl of distilled water was added, vortexed vigorously, and centrifuged (3000 × g, 1 min). The supernatant was further diluted (× 20) with distilled water, and used as a template for qPCR.

Primer design and optimization of PCR conditions
Prey DNA concentration was determined on DNA templates prepared above using two sets of species-specific primers (per prey species). One set of primers amplified a short stretch of DNA (subsequently called "short PCR"), whereas another set of primers amplified a longer stretch of DNA (subsequently called "long PCR") (Table 1). The primers were carefully designed to anneal specifically the target sequences within the 16S rRNA (mtDNA). All the primers were tested thoroughly, adjusted to their optimal PCR conditions, and verified their performances using templates of known compositions.

Quantification of prey DNA in fecal and diet samples
The qPCR reaction mix was as follows: distilled water 4.75µl, forward primer (100 pmole/µl) 0.25µl, reverse primer (100 pmole/µl) 0.25µl, SYBR Premix Ex Taq II (Takara Bio) 6.25µl, and DNA template (prepared above) 1µl. The qPCR conditions were: initial denaturation 95ºC -15 sec, followed by PCR amplification 95ºC -10 sec and XºC -Y sec (X and Y depend on respective primer sets, see Table 1) x 40 cycles. All the qPCR reactions were verified by thermal dissociation analysis as well as agarose gel electrophoresis. When non-specific or unintended PCR products appeared, the PCR was rerun. The amounts of initial template DNA were calculated using the following equation (*Eq* 1):

$$[\mathrm{DNA}]_0 = \frac{Th}{(1+e)^c} \qquad \cdots\cdots\cdots\cdots\cdots\cdots \textit{Eq 1}$$

where [DNA]0 is the initial template amount, Th is the threshold line (arbitrarily set at 4), e is the efficiency of PCR amplification (e=1 at 100% efficiency), and c is the Ct value (threshold cycle).

Calculation of DNA digestibility
In this study, the digestibility of DNA is defined as follows. When the full stretch of mtDNA (~16 kb) is intact, this is 0% digestibility, and when the DNA is completely broken down to nucleotides, this state is 100% digested (100% digestibility). Theoretically, the PCR amplification fails progressively as the template DNA degrades and as the length of PCR product increases (Table 2). Thus, by determining the ratio between short and long PCR products, the degree of DNA degradation (i.e., digestibility) can be estimated. First, probability of amplification success (PAS) represents the copy number that can be amplified out of 100 copies (1.0 = 100%), which is expressed as follows (*Eq* 2):

$$\mathrm{PAS} = \left(\frac{Mt - Amplicon\ size}{Mt}\right)^x \qquad \cdots\cdots\cdots\cdots\cdots\cdots \textit{Eq 2}$$

where Mt is 16,000 (bp, approximate full length of mtDNA), and x is the number of cuts (endonuclease digestion) within the full stretch of mtDNA sequence. PAS is calculated assuming there is no exonuclease digestion. Hence, DNA digestibility is calculated using the following formulae (*Eq* 3, 4).

$$\frac{\left\{\frac{Mt-S}{Mt}\right\}^x}{\left\{\frac{Mt-L}{Mt}\right\}^x} = \frac{[S]}{[L]} \qquad \cdots\cdots\cdots\cdots\cdots\cdots \textit{Eq 3}$$

$$\mathrm{Digestibility\ (\%)} = \frac{x}{Mt} \times 100 \qquad \cdots\cdots\cdots\cdots\cdots\cdots \textit{Eq 4}$$

where S is the length of PCR product (bp) amplified by short PCR, L is the length of PCR product (bp) amplified by long PCR, [S] is the initial template amount ([DNA]0) quantified by short PCR, and [L] is the initial template amount ([DNA]0) quantified by long PCR. From *Eq* 3 and *Eq* 4, Digestibility (%) is expressed as follows (*Eq* 5).

$$\mathrm{Digestibility\ (\%)} = \frac{\log_{\left(\frac{Mt-S}{Mt-L}\right)}\left(\frac{[S]}{[L]}\right)}{0.01 \times Mt} \qquad \cdots\cdots\cdots\cdots\cdots\cdots \textit{Eq 5}$$

When fecal DNA was not amplified after 40 PCR cycles, the DNA quantity was tentatively calculated assuming Ct=40 (i.e., amplified at 40th PCR cycle) in order to calculate the "minimum" digestibility (If true Ct is higher than 40, the actual digestibility will be higher than the "minimum" digestibility).

Correction of initial DNA concentrations
The initial (total) DNA concentration was thought to be varied greatly from sample to sample due to different DNA extraction efficiency and different DNA contents among samples. Hence, relative DNA concentrations of all template solutions were examined using universal degenerate primers (Table 1) that could amplify all Animalia species in the extracted solution. The primers were designed to amplify a highly conserved 16S rRNA coding region of mtDNA.

Statistical methods
Digestibility values were compared among treatments based on one-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test. Differences were considered significant at p <0.05. Correlation coefficients (r) were calculated between relative (total) DNA concentrations and species-specific template concentrations. Statistical calculations were performed using statistical software (Ekuseru-toukei 2012, SSRI Co. Ltd., Tokyo).

Discussion

In this study, amplification of fecal DNA by long PCR was not very successful. In some cases, none of the five replicate samples was amplified by long PCR. Since the control and diet samples were successfully amplified by long PCR, the unsuccessful amplification of fecal DNA is unlikely due to primer dysfunction. Hence, the problem could be solved by making the PCR amplicon shorter (e.g., <100 bp). However, when the amplicon sizes of short and long PCRs are similar, the digestibility may not be estimated accurately. Also, serial qPCR amplifying prey DNA of 3-5 fragment sizes may not be feasible in this study because of the apparent absence of long DNA fragments in feces.

The present method of digestibility calculation does not require adjusting or correcting the total DNA concentrations of the extracted solutions among samples. However, the DNA concentrations of the extracted solutions were determined in order to verify that non-amplification (with species-specific primers) was not due to low total DNA concentrations. Results showed no correlation between DNA concentrations and the amplification success (by species-specific long PCR), suggesting DNA extraction / dilution protocols were not the major reasons for non-amplification. The present method of quantifying DNA concentration (using universal degenerate primers) is affected by the integrity of template DNA (i.e. degree of DNA fragmentation). So, the measured concentrations should be construed as relative, and compared only among fecal samples or among diet samples.

Numerous studies evaluated the effects of various food processing conditions on the survival of DNA in foods, especially genetically modified (GM) foods, using long and short PCR including quantitative competitive PCR (QC-PCR) and qPCR. These studies, however, did not calculate quantitative indices such as digestibility or degradability. Several researchers determined the degree of DNA damage to evaluate genotoxic effects of various chemicals, oxidations, and radiations based on qPCR. They amplified long DNA sequences (3-18 kb) to detect DNA damage and short DNA sequences (100-250 bp) to quantify DNA template concentrations (assuming that the short sequences are undamaged). Others calculated the degree of DNA damage using fecal or artificially digested samples and amplifying several different sizes of DNA fragments. Their values (i.e., ~0.001-0.02) are similar to those in the present study, but their protocols are more elaborate.

The present calculation is based on an assumption that there is little exonuclease digestion (i.e., DNA is digested mostly by endonucleases), and that the sites of endonuclease digestion follows a random (Poisson) distribution. Dietary DNA is digested in the intestine by pancreatic DNases and intestinal polynucletidases. However, the percentage of DNA digested by endonucleases is largely unknown. If exonucleases play a significant role in DNA digestion, then the present calculation may involve that much error. Because of this, in vitro digestion trials (using e.g., DNase I) will not verify the accuracy of the present method. The process of in vivo digestion of dietary DNA is complicated and is not well-characterized. This study reports one potential method that may be useful to study factors affecting DNA degradation, including in vivo digestion and various processing / preserving conditions of foods or GM foods.

The data reported herein show overall low digestibility of dietary DNA. However, this does not indicate that dietary DNA is mostly unabsorbed. Numerous studies showed that dietary DNA can be absorbed as macromolecules, and dietary DNA of as large as 500bp can be found in various tissues of animals including fishes. This suggests that, despite low calculated digestibility, substantial portions of dietary DNA may be absorbed. If the intestinal absorption of short to medium length DNA (50-150 bp) is substantial and the absorption (disappearance) rate is higher for shorter fragments, then the ratio of short to medium DNA in feces will not accurately reflect the extent of dietary DNA fragmentation. Obviously, the rate of DNA absorption in relation to its fragment size needs to be studied in the future.