Research Article **Open Access**

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DNA barcoding increases resolution and changes structure in Canadian boreal shield lake food webs

DOI 10.1515/dna-2015-0005
Received December 12, 2014; accepted June 8, 2015

Abstract: Food webs are important in understanding the structure, function, and behaviour of ecosystems, but, due to methodological limitations, are often poorly resolved in ways that impact food-web properties. Although DNA barcoding has proven useful in determining the diet of consumers, few studies have used this technique to determine food-web structure. These studies report mixed impacts on various food-web properties, but are limited by their taxonomic focus and their failure to evaluate DNA barcoding for both diet analysis and food-web structure. In this study, we show that, when compared to a morphological approach, DNA barcoding increases food-web resolution by increasing the number and frequency of prey species identified in the stomach contents of eight species of Canadian boreal shield predatory fishes. In addition, we observed differences in food-web structure, such as increased generalism, habitat coupling, and omnivory, that have strong implications for food-web stability and dynamics. We conclude that DNA barcoding is a powerful tool to evaluate how resolution impacts food-web properties and can help further our understanding of how food webs are structured by identifying feeding interactions in an unprecedented and highly detailed manner.

Keywords: COI, diet analysis, feeding links, morphology, omnivory, predatory fish, prey species, resolution, stomach contents, trophic interactions

1 Introduction

The dynamics and functioning of whole ecosystems depend intimately on the transfer of energy and nutrients. Feeding interactions are the primary vector for the transfer of energy and nutrients in ecosystems, and consequently play a major role in both dictating the structure and dynamics of communities and determining whole-ecosystem responses to natural and human-induced perturbations [1]. Despite the fundamental need to identify feeding interactions, empirical food webs—descriptions of feeding interactions in an ecosystem (Table 1)—have historically been poorly resolved and constructed using a variety of methodologies [2]. Nonetheless, empirical food webs have been exhaustively analyzed in an effort to identify universal structural patterns [3]. The poor quality of food-web data has raised questions about how both the resolution and methods used to construct food webs influence perception of food-web structure (non-random patterns in food webs, Table 1) within and across ecosystems [3, 4, 5].

Not surprisingly, food-web resolution can affect inferences about various food-web properties [5, 6, 7], threatening the meaningful documentation of empirical food webs. For example, food web resolution impacts numerous patterns in the topology of food webs thought to relate to their stability, including food-chain length (Table 1) [6], the number of links per species [6] (linkage density), the degree of feeding at multiple trophic levels [6] (omnivory, Table 1), the ratio of the number of realized

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Table 1. Definitions of key terms.

Term	Definition
Food-chain length	The number of sequential links that connect a basal resource to a top predator [69]. Because food webs often have multiple pathways between resource and a top predator, several metrics are used, including maximum, minimum, and mean food-chain length [70].
Food web	A description of feeding interactions between species in an ecosystem [71]. Also, a type of ecological network that emphasizes consumptive connections.
Food-web resolution	The amount of detail included in a food web, usually referring to the degree of aggregation of organisms. Traditional food webs are highly aggregated (e.g., [72]) and thus poorly resolved [2,6]. Recently, researchers have been pushing to increase food-web resolution by including more species rather than higher level taxa (less aggregation) in food webs [9].
Food-web structure	The non-random patterns in a food web [73]. A food web has two major structural components: (1) the topology (who eats whom, see [2]), and (2) the strength of each interaction (which can be defined in various ways, see [74,75]).
Generalism	The dietary breadth of a consumer, often measured as dietary species richness [11, 76].
Habitat coupling	A process in which discrete habitats are connected through the movement of energy and nutrients [39]. In the context of food webs, it often occurs via foraging by a mobile consumer and connects spatially distinct habitats [77].
Omnivory	Feeding on species from more than one level in a food chain by a consumer [69,78]; that is, when a predator eats the prey of its prey.

links to the number of possible links [8] (connectance, although see Martinez [6]), and the presence of distinct subwebs [9] (compartmentation). Many observed patterns in food webs may be caused by poor resolution, implying a need for improved data and the development of new methods for evaluating food webs [2, 10].

Food-web resolution has largely been limited by methodological constraints. Accurate identification of the many feeding interactions that comprise food webs is difficult, especially in systems where direct observation of feeding by predators is impractical or nearly impossible. Morphological identification of prey from the stomach contents or feces of consumers has traditionally been used to describe detailed food-web structure. The constituent species in these morphology-based food-web datasets are often aggregated into taxonomic or trophic groups because of the considerable time, expense, and difficulty associated with identifying the vast number of species and feeding interactions present in ecosystems (e.g., [11]). This has led to alternative methods for establishing food-web structure. For example, stable isotopes of carbon and nitrogen have been widely used because they can infer the trophic position and determine the carbon sources of consumers [12]. As a result, stable-isotope analysis provides a useful broad metric of food-web structure because it can identify the presence of major energy pathways [13] and shifts in the feeding habits of key species [14]; however, stable-isotope analysis does not provide the highly refined picture of feeding interactions required to address issues surrounding food-web resolution.

DNA barcoding is increasingly recognized as an effective means for identifying trophic interactions [15, 16, 17], making it a potentially powerful tool to parse out food-web structure [18, 19]. Barcoding uses a short, standardized DNA sequence and a molecular reference library (i.e., the Barcode of Life Data Systems, BOLD [20]) to identify species [21]. The efficacy of barcoding to establish feeding interactions comes in large part from its proven utility in identifying animal tissues when little or no morphological information is available [22, 23]. Many studies have now confirmed the value of barcoding in identifying prey in the stomach contents or feces of certain predators, such as bats [24], beetles [25], marine invertebrates [26, 27], seabirds [28], sharks [29], and other marine fishes [28, 30]. Additionally, some researchers have used taxon-specific approaches that rely on the DNA barcode region to identify feeding interactions of interest in bats [31] and insects [32], but very few studies have used barcoding at the scale of whole food webs.

A small number of studies have used barcoding to establish feeding links in a whole food web, rather than the identification of prey species for a single consumer or prey taxon of interest [33, 34, 35]. These studies have consistently demonstrated that barcoding increases species diversity [33, 34], reveals more feeding links (about three times as many in Wirta *et al.* [35]), changes identifications (31% of

individuals in Kaartinen *et al*. [33]), and identifies cryptic taxa [33, 34] in their respective food webs; however, these studies do not show consistent changes to a number of other food-web properties. For example, Kaartinen *et al*. [33] and Smith *et al*. [34] found increased specialization and connectance using barcoding, but Wirta *et al*. [35] found increased generalism and decreased connectance. Similarly, Kaartinen *et al*. [33] found small decreases in linkage density—the average number of prey species per predator—and the average number of predator species per prey when barcoding data was incorporated in their food web, although Wirta *et al*. [35] found increases in all of these properties.

To date, the studies that evaluate barcoding for establishing food-web structure have two important limitations. Firstly, none of these studies has explicitly compared the resolution provided by barcoding and morphology when characterizing stomach content items, individuals, predator species, and food webs. This comparison is required to understand how an increase in the resolution of stomach-content identifications translates into an increase in food-web resolution; more resolved identifications of stomach-content items increase the resolution of prey identification for both individuals and species, altering the pattern of feeding interactions that we term food-web structure. Secondly, studies to date that evaluate how barcoding influences our understanding of food-web structure afford limited generality because they focus on only terrestrial food webs dominated by insects, parasitoids, and dietary specialists [33, 34, 35] rather than aquatic systems or systems comprised of generalist or vertebrate predators. Researchers observed that barcoding frequently identified the presence of cryptic diversity and host specificity among insects (e.g., Hebert *et al*. [36] and Smith *et al*. [37]). Thus, they expected and later confirmed that examining insect food webs dominated by parasitoids and dietary specialists with barcoding would result in higher parasitoid diversity and increased dietary specialization [33, 34, 35]. However, using barcoding for the dietary analysis generalist, vertebrate predators with well-studied taxonomies is likely to increase prey diversity without revealing cryptic predator diversity, resulting in different predicted impacts on food-web structure. These impacts are of wide concern because such generalist, vertebrate predators that variably feed on a heterogeneous landscape are theorized to be common and important stabilizing factors in ecosystems [1].

The food webs of boreal shield lakes in Canada offer a good system to evaluate the relative utility of barcoding for dietary analysis of generalist, vertebrate predators and the construction of aquatic food webs. These lakes contain several native, predatory fish species, including lake trout (*Salvelinus namaycush*), walleye (*Sander vitreus*), and northern pike (*Esox lucius*), as well as invasive smallmouth bass (*Micropterus dolomieu*). In these systems, there is evidence of food-web variability across lakes [38], omnivory by top predators [13, 38], and the coupling of spatially distinct near-shore and off-shore habitats that are believed to represent food-web compartments [39]. Stable isotopes of carbon and nitrogen have been widely used as time-integrated metrics to infer the general feeding habits of predatory boreal shield fishes (e.g [12, 13, 14, 38, 39, 40]). Although the diets of fish predator species in boreal shield lakes have been extensively studied using morphology [41], molecular analyses have not previously been applied to this system.

In this study, we use predatory fish from Canadian boreal shield lakes to evaluate barcoding as a tool to increase food-web resolution by identifying stomach contents, establishing feeding interactions, and determining food-web structure. We evaluate five predictions about how barcoding and morphology compare for diet analysis. We predict that barcoding will: (i) identify stomach-content items to a lower taxonomic level than morphology; (ii) increase the diversity and frequency of prey recovery; (iii) increase the average number of prey identified per stomach sampled; (iv) increase the average number of prey identified for boreal shield predatory fish species; and (v) increase the number of predator species identified per prey species. We evaluate seven additional predictions about how barcoding impacts the resolution and structure of boreal shield food webs. We predict that, when compared to food webs constructed using morphology, those constructed using barcoding will have: (i) more feeding links; (ii) higher linkage density; (iii) higher connectance; (iv) a higher percentage of possible feeding links; (v) higher rates of omnivory; (vi) increased maximum food-chain length; and (viii) more food-web links that couple habitats.

2 Materials and Methods

2.1 Specimen collection

The Ontario Ministry of Natural Resources collected fish from two lakes for standardized fish surveys using a combination of gill netting (following Sandstrom *et al*. [42]) and angling in Ontario, Canada in July of 2009. Richardson Lake (50°10'01"N, 92°03'54"W) and Delaney Lake (50°05'26"N, 94°03'00"W) have similar physical characteristics and predator species compositions,

including lake whitefish (*Coregonus clupeaformis*), northern pike (*Esox lucius*), burbot (*Lota lota*), smallmouth bass (*Micropterus dolomieu*), lake trout (*Salvelinus namaycush*), and walleye (*Sander vitreus*). In addition, Delaney Lake contained muskellunge (*E. masquinongy*) and Richardson Lake contained rock bass (*Ambloplites rupestris*). The number of fish collected in each lake can be found in Supplemental Table 1. We removed the gastrointestinal tracts (esophagus to anus) from each of 81 individuals and preserved each in 95% ethanol. The species identity of each predator fish was confirmed through barcoding based on a sample (at least 10mm^3) of epaxial muscle tissue taken from each individual. We placed all samples on ice for up to 24 hours and stored them at -20°C until analysis.

2.2 Stomach content subsampling

We rinsed prey remains from each stomach using 95% ethanol and isolated all discrete stomach content items (between one and 23 per stomach) for morphological identification and tissue subsampling for barcoding. TJB (who has fish identification training from the Ontario Ministry of Natural Resources and invertebrate identification training from the University of Guelph) morphologically identified each stomach content item to the lowest possible taxonomic level using various keys and identification guides [41 and 43 for fishes; 44 for mammals; 45, 46, 47 for insects and other invertebrates]. The shape of exposed bony structures, such as vertebrae and otoliths, was not used to identify stomach content items because we could not be certain whether or not shape was unaltered by digestion. We considered samples that could not be assigned to any taxonomic group to have no morphological identification.

2.3 DNA barcoding

We collected tissue subsamples of approximately 5 mm^3 from each stomach content item that was isolated for morphological analysis, rinsed them thoroughly in 95% ethanol, and stored them individually in 95% ethanol at -20°C until analysis. We placed tissue subsamples into 45µl of lysis buffer and 5µl of proteinase K, incubated at 56°C for 18 hours, and then extracted DNA using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. We amplified the DNA barcode region, approximately 650 base pairs at the 5′ end of the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene [21] using three primer sets: LCO1490/ HCO2198 [48], C_VF1LFt1/C_VR1LRt1 [49] with M13 tails [50], and C_FishF1t1/C_FishR1t1 [49] with M13 tails [50]. Our thermocycler conditions followed Braid *et al*. [27]. We sequenced PCR products in the forward direction using BigDye v3.1 and LCO1490 or M13F primers and manually edited sequences using Sequencher 4.0.5. We uploaded sequences to the Barcode of Life Data System (BOLD, www.boldsystems.org [20]) as a dataset: [DS-BSLFW] Constructing Boreal Shield Lake Food Webs Using DNA Barcoding (DOI: 10.5883/DS-BSLFW).

2.4 Sequence identification

We attempted to identify each sequence using reference barcodes in the BOLD species ID engine [20] in February of 2014. We categorized a sequence as having a species-level match if it met one of the following criteria: a) it was at least 99% similar to a single, known species [24]; b) it was at least 99% similar to multiple, known species and all but one of those species could be excluded based on known geographic distributions; or c) it was assigned to a BIN (Barcode Index Number [51]) that was comprised of a single species. We categorized a sequence as having a species-complex match when it was either: a) 99% similar to two or more species with overlapping geographic distributions; or b) was assigned to a BIN that contained two or more species. We categorized sequences assigned to BINs with no species-level identifications as belonging to the lowest level taxonomic group given to the associated BIN. We considered sequences that were both not assigned to a BIN and that were less than 99% similar to any taxon to have no DNA barcode identification. We grouped all species-complex-level identifications as species-level identifications for all analyses. We considered cases in which the DNA sequence matched the identity of the predator separately from other species-level identifications because we were not able to distinguish between self-contamination by the predator and cannibalism.

2.5 Analyses

We compared the ability of morphology and barcoding to identify stomach contents by counting the number and taxonomic level of stomach-content items that were identified to a lower taxonomic level (i.e., closer to species and thus higher resolution) using each technique. For example, a stomach-content item that was identified to family using morphology and to species using DNA barcoding was identified to a lower taxonomic level using DNA barcoding, and was thus counted for that technique. We compared the average number of

prey taxa (all taxonomic levels) and prey species per predator species in each lake using analysis of variance (ANOVA) and Tukey honest significant differences (Tukey's HSD) tests. We compared the average number of prey taxa (including species and all other taxa) per stomach, the average number of prey species (excluding taxa other than species) identified per stomach, the frequency each prey species was detected, and the number of predator species per prey species using non-parametric methods (Kruskal-Wallis and Pairwise Wilcoxon rank sum tests) because the data did not meet the assumptions of normality required for ANOVA, even after transformation. We performed all statistical tests in R v 3.1.0, and information for each statistical test can be found in Supplemental Table 2. Each statistical test compared data for morphology, barcoding, and the two techniques combined to determine whether the techniques provided supplementary information, even though the data for the techniques combined may not be considered independent of the morphology and DNA barcoding groups (post-hoc comparisons of morphology and barcoding can be found in Supplemental Table 2). We performed tests for taxa of all levels and for only species-level matches to determine the impact of excluding higher-level taxonomic groups. We constructed rarefaction curves with 95% confidence intervals for both the number of prey species and food-web links (unique combinations of predator species and prey species, e.g., smallmouth bass and yellow perch (*Perca flavescens*)) using EstimateS 9.1.0 [51]. Statistical analysis and rarefaction excluded stomachs that did not contain identifications for either morphology or barcoding (i.e., had no dietary information).

We calculated linkage density as L/N, where L is the number of observed food web links, and N is the number of taxa (including prey and predator species). We calculated connectance as $L/(N(N\text{-}1))$ to exclude cannibalistic feeding links as they were not distinguishable from self-contamination using barcoding and no such links were identified using morphology. We calculated the percentage of possible links as $L/((N\text{-}1)^{*}P)$, where P is the number of predators from which stomach contents were taken. A species was considered an omnivore if it consumed at least one of the other predator fish species. We calculated the maximum food-chain length as the maximum number of trophic steps from a prey to a predator species in each lake. We evaluated the coupling of near-shore and off-shore habitats by assigning every prey taxon as near-shore or off-shore. We did not consider higher-level prey taxa with members that could exist in both habitats (e.g., Diptera) for the evaluation of habitat coupling.

3 Results

3.1 Stomach contents

We evaluated a total of 537 stomach-content items using morphology and barcoding. Using morphology, we were able to identify 27 stomach-content items to species and 300 to a taxonomic level higher than species. We could not identify 210 stomach content items to any taxonomic level using morphology. Using barcoding, we produced at least one sequence from 492 of the 537 stomach content items. From those sequences, we identified 394 samples to species, 121 of which we matched to the identity of the predator. Based on their barcodes, we identified ten stomach-content items to a species complex, and 61 to higher taxonomic levels. We found no barcode identification for the remaining 72 stomach-content items. We could not identify 42 stomach content items using either technique. Of the 81 fish stomachs sampled, we found no identifiable stomach-content items in 21 stomachs using morphology and 17 stomachs using DNA barcoding. We did not identify any stomach-content items to species from 71 stomachs using morphology and 23 stomachs using DNA barcoding. We could not identify any stomach content items from 10 stomachs using either technique, and did not include these stomachs in any further analyses.

We identified more than 80% of stomach-content items to a lower taxonomic level with barcoding than with morphology (Figure 1). Of these, we identified nearly 88% to the species level, and we matched approximately 25% of the sequences to the predator fish species from which they came. Using morphology, we identified only about 5% of stomach-content items to a lower taxonomic level than barcoding, and we found that all but one of these identifications were at the family level or higher. We identified less than 15% of stomach-content items to the same taxonomic level using both morphology and barcoding (i.e., the techniques tied). Overall, we identified four prey species and eight taxa to family level or higher using morphology, whereas we identified 25 prey species or species complexes (including all species identified by morphology), one prey genus, and two prey families using barcoding (Figure 2a). We also identified one parasite species (*Leptorhynchoides thecatus*) in the stomach contents of five smallmouth bass using barcoding.

We found a significant difference in the frequency of prey recovery between the techniques (Kruskal-Wallis rank sum test, $H(2) = 40.90$, $p < 0.001$), with prey species significantly less often with morphology than with either

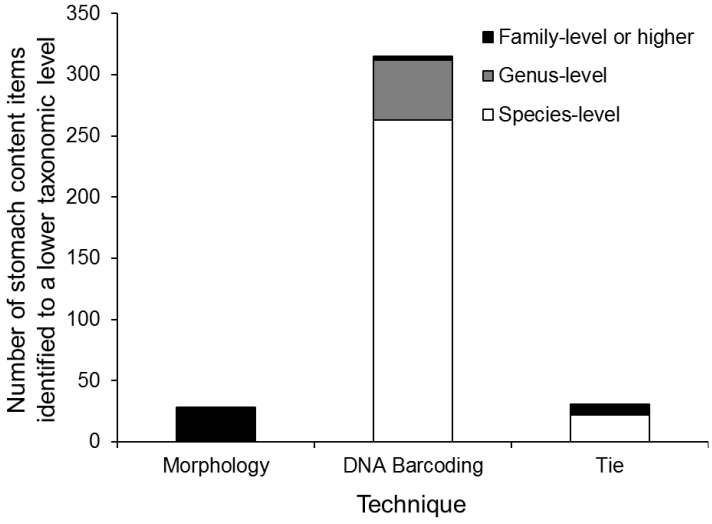

Figure 1. The number of stomach-content items recovered from boreal shield lake predatory fishes for which the highest resolution identification was made using morphology or DNA barcoding, and where the techniques had equally resolved identifications (the techniques tied). For each technique, the number of identifications to the level of species, genus, and family or higher is indicated. Stomach-content items that were identified with DNA barcoding to the same species as the predator from which they were sampled are not included (see Supplemental Table 1).

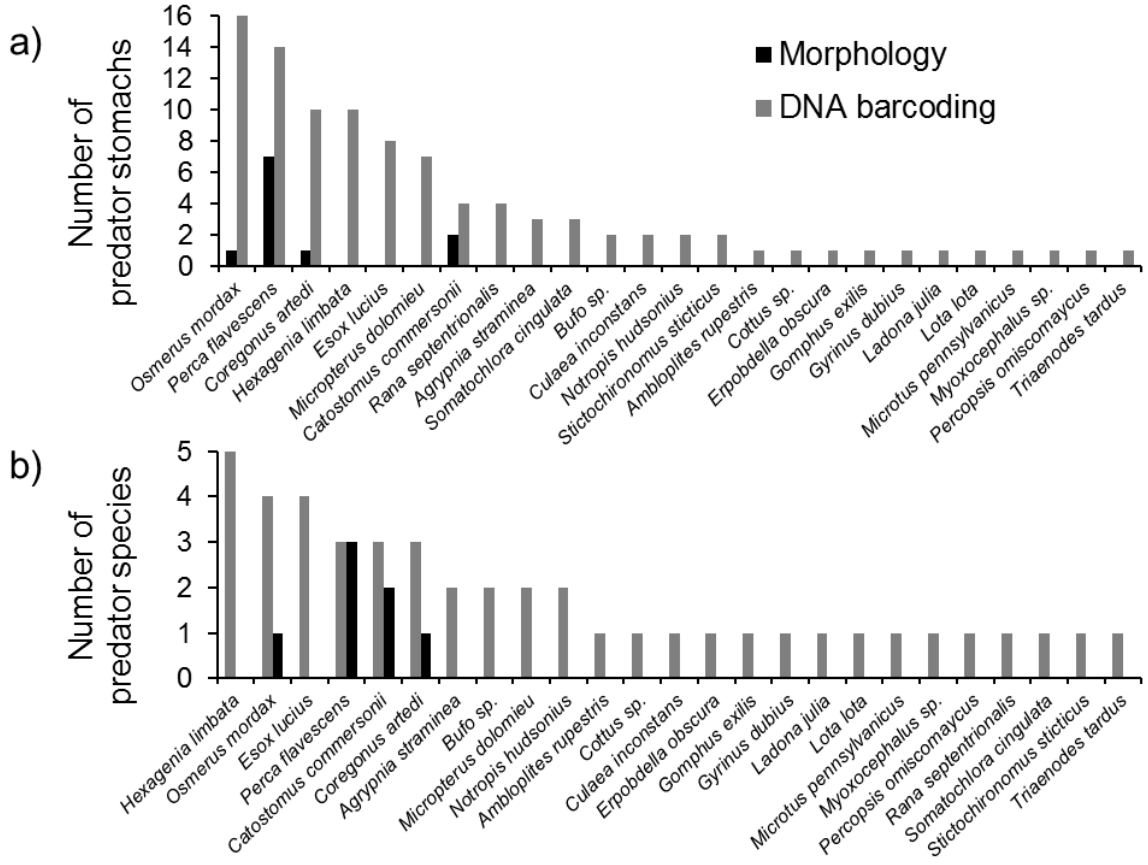

Figure 2. Frequency of prey-species identifications from eight boreal shield fish predators using morphology and DNA barcoding: (a) the number of individual predators that were found to be feeding on each prey species (Kruskal-Wallis rank sum test, $H(2) = 40.90$, $p < 0.001$); (b) the number of predator species that were found to be feeding on each prey species identified (Kruskal-Wallis rank sum test, $H(2) = 42.43$, $p < 0.001$).

barcoding or a combination of barcoding and morphology but no difference in the frequency of prey-species recovery using barcoding alone and that of the techniques combined (Supplemental Table 2). Similarly, we found that the average number of predator species per prey species differed significantly with technique (Kruskal-Wallis rank sum test, H (2) = 42.43, p < 0.001); the average number of predator species per prey species was significantly lower with morphology than with barcoding or the techniques combined, but there was no such difference between barcoding and the techniques combined (Supplemental Table 2). Stomach-content identification data for the various predator species and lakes can be found in Supplemental Table 1.

3.2 Individual predators

We found that the number of prey taxa and species identified per stomach differed significantly between techniques (Kruskal-Wallis ran sum tests, for all taxa, H (2) = 92.74, p < 0.001, and for species, H (2) = 20.93, p < 0.001). We identified significantly fewer prey taxa and prey species per stomach using morphology than with either barcoding or the techniques combined, but we found no such difference in the number of prey taxa and prey species recovered using barcoding alone and that of morphology in combination with barcoding (Supplemental Table 2). When including all prey taxa or only species, respectively, we found an average of 1.09 ± 0.080 and 0.154 ± 0.048 prey per stomach using morphology, 1.68 ± 0.150 and 1.42 ± 0.134 prey per stomach using DNA barcoding, and 1.92 ± 0.140 and 1.62 ± 0.140 prey per stomach using the techniques combined. Using morphology, we did not identify any stomach content items to the species level for the majority of predator stomachs. In contrast, we identified at least one species in the majority of stomachs using barcoding. In addition, rarefaction curves indicated that barcoding alone and in combination with morphology identified significantly more species than morphology alone, but barcoding alone and in combination with morphology did not differ significantly (Figure 3). Rarefaction data for the number of prey species identified can be found in Supplemental Table 3.

3.3 Predator species

We found a marginally significant difference between techniques in the number of prey taxa identified per predator species (ANOVA, F (2,21) = 2.76, p = 0.0862). We found an average of 2.88 ± 0.611 prey taxa per predator species using morphology, 5.75 ± 1.01 prey taxa per predator species using DNA barcoding, and 6.25 ± 1.10 prey taxa per predator species using the techniques combined. We identified significant differences in the number of prey species per predator species between techniques (ANOVA, F (2,21) = 6.01, p = 0.00863), with a significantly larger number of prey species per predator species using both barcoding and the techniques combined when compared to morphology, but no such difference between barcoding and the techniques combined (Supplemental Table 2). We found an average of 0.750 ± 0.250 prey species per predator species using morphology, 5.00 ± 0.824 prey species per predator species using both DNA barcoding and the techniques combined.

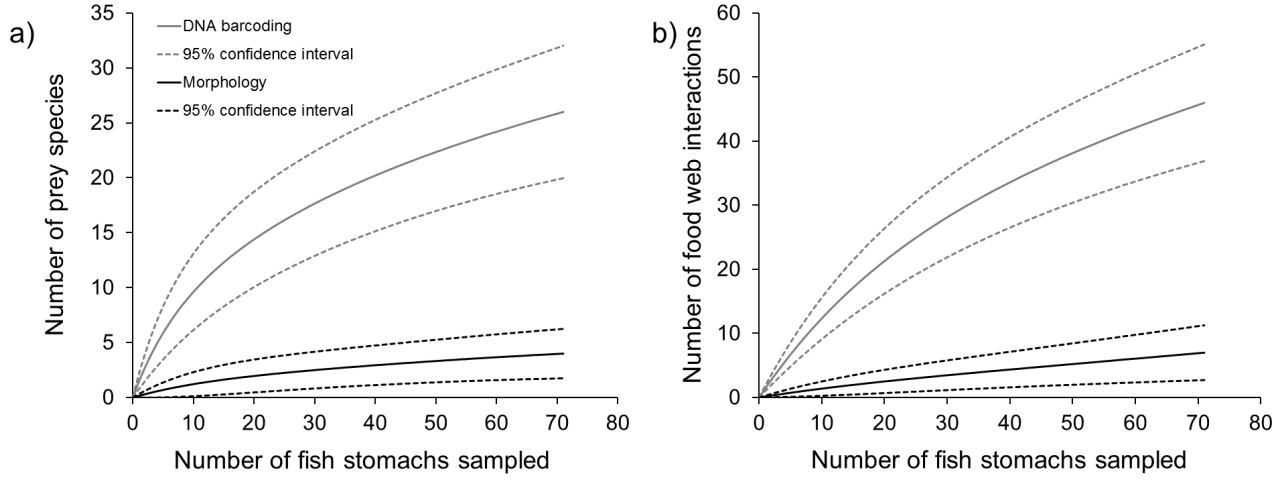

Figure 3. The rarefaction curves and 95% confidence intervals for stomach contents of eight boreal shield lake fish predators identified using morphology and DNA barcoding: (a) the number of prey species; or (b) feeding links (unique prey and predator species combinations). Data (including those for the techniques combined) can be found in Supplemental Tables 3 and 4. Rarefaction curves and 95% confidence intervals were calculated using EstimateS 9.1.0 [52].

For all species of predatory fish, we identified a higher proportion of prey species using barcoding than morphology (Supplemental Table 1). We found only four cases where the techniques combined found more prey taxa for a given species of predator than barcoding alone. In all of these cases, the prey found using morphology was an invertebrate identified to the family level or higher, where no corresponding taxon was found using barcoding. In addition, we found a single case in which a prey species was identified for a predator species using morphology that was not found using barcoding. However, we identified no prey species or other taxon using morphology that was not also identified using barcoding across all predator species in each lake.

3.4 Food-web properties

We identified more feeding links using barcoding alone or the techniques combined than using morphology alone in both Delaney and Richardson Lakes (Table 2). Using rarefaction curves, we found significantly more food-web interactions using barcoding alone and the techniques combined than morphology alone, but barcoding alone and the techniques combined found a similar number of food-web interactions (Figure 3) Rarefaction data for the number of food-web interactions can be found in Supplemental Table 4. When considering all taxonomic levels, we found similar linkage density and the percentage of possible links between techniques in Delaney Lake, but we found these values differed between techniques in Richardson Lake (Table 2). When considering only species-level identifications in both lakes, we found that linkage density and the percentage of possible links were higher in food webs constructed using barcoding alone and the techniques combined than they were when using morphology alone. However, we found no consistent difference in the connectance between food webs constructed with barcoding and those constructed with morphology. Using barcoding, we identified feeding interactions between predator fish species (i.e., omnivory), but no such interactions were identified using morphology. As a result, we identified an increased maximum food-

Table 2. Food-web parameters calculated for Delaney Lake and Richardson Lake based on the eight predatory fish species' stomach contents identified using morphology, DNA barcoding, and both techniques combined from eight boreal shield lake fish predators. Metrics were calculated for both all prey taxa and for only species-level prey.

Food Web Parameter	Lake Name	All Taxa			Species Only		
		Morphology	DNA Barcoding	Combined	Morphology	DNA Barcoding	Combined
Number of taxa	Delaney	15	22	24	9	20	20
	Richardson	16	24	26	8	20	20
Number of links	Delaney	14	23	26	6	21	22
	Richardson	15	36	38	2	30	30
Linkage density	Delaney	0.933	1.05	1.08	0.667	1.05	1.10
	Richardson	0.938	1.50	1.46	0.250	1.50	1.50
Connectance	Delaney	0.0667	0.0498	0.0471	0.0833	0.0553	0.0579
	Richardson	0.0625	0.0652	0.0585	0.0357	0.0790	0.0790
Percentage of possible links	Delaney	16.7	18.3	18.8	12.5	18.4	19.3
	Richardson	16.7	26.1	25.3	4.76	26.3	26.3
Omnivorous predators	Delaney	0	2	2	0	2	2
	Richardson	0	5	5	0	5	5
Maximum food chain length	Delaney	1	3	3	1	3	3
	Richardson	1	4	4	1	4	4
Number of links that couple habitats	Delaney	1	2	2	1	2	2
	Richardson	0	4	4	0	4	4

chain length using barcoding than morphology (Table 2). In addition, we found that walleye in Delaney Lake (node 3, Figure 4a,b) and lake trout in Richardson Lake (node 2, Figure 4c,d) were isolated from the majority of the food web constructed using only morphology. However, we established additional links using barcoding that were incorporated into a single food web (Figure 4).

Overall, we identified more feeding links that indicate the coupling of near-shore and off-shore habitats using barcoding than when using morphology (Table 2). In Delaney Lake, we identified an additional feeding habitat for both lake trout (e.g., node 2 and node 12, Figure 4a,b) and northern pike (e.g., node 5 and node 1 in Figure 4a,b) using barcoding. Likewise, in Richardson lake, we established that lake whitefish (e.g., node 1 and node 20, Figure 4c,d), lake trout (e.g., node 2 and node 13, Figure 4c,d), and smallmouth bass (e.g., node 6 and node 9, Figure 4c,d) were feeding in both near-shore and off-shore habitats when using barcoding, but morphology only found that they were feeding in one of those habitats. In addition, we found that walleye (node 3, Figure 4c,d) in Richardson Lake had feeding links in both near-shore (e.g., node 20) and offshore (e.g., node 11) habitats using barcoding, but morphology did not identify any taxon that could be assigned to either habitat.

4 Discussion

In this study, we used predatory fish from Canadian boreal shield lakes to evaluate whether DNA barcoding increases food-web resolution by increasing the number or frequency of recovery for prey species found in stomachs, and if such differences affect food-web structure. Although a handful of previous studies used barcoding to elucidate food-web structure [33, 34, 35], our study is unique in two important ways. Ours is the first study to demonstrate that explicitly increasing dietary resolution maps to meaningful changes to our understanding of food-web structure. In addition, this is the first study to focus on an aquatic food web and a food web of generalist, vertebrate predators. Our

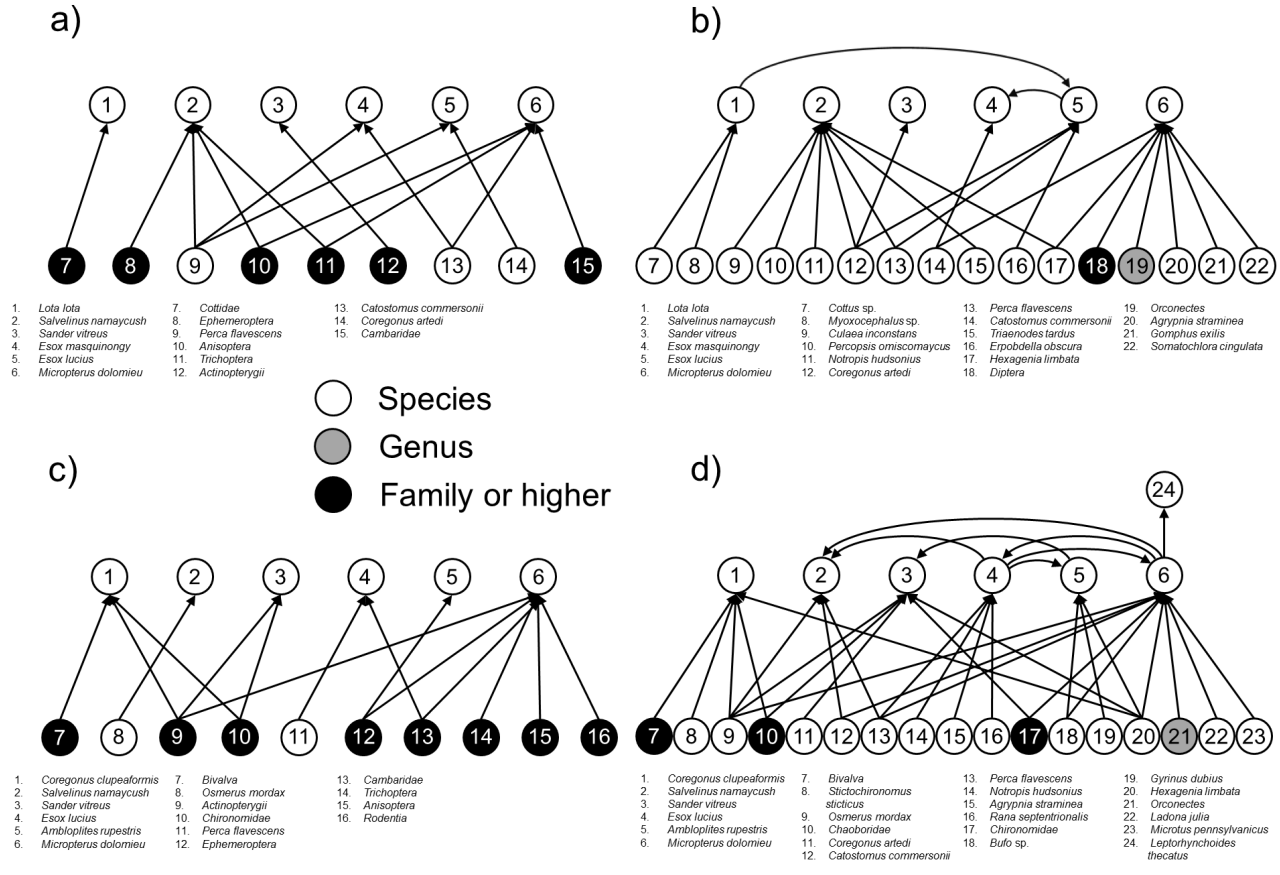

Figure 4. Food web diagrams for Delaney Lake ((a) and (b)) and Richardson Lake ((c) and (d)) constructed using morphology ((a) and (c)) and DNA barcoding ((b) and (d)) based on the stomach contents of eight boreal shield lake fish predators. White nodes indicate species-level identifications, grey nodes indication genus-level identifications, and black nodes indicate family-level and higher-level identifications. Node 24 in (d) indicates a parasite (*Leptorhynchoides thecatus*) that was identified in the stomachs of smallmouth bass (*Micropterus dolomieu*, node 6).

expectations for boreal shield lake food webs differed from previous studies because barcoding has already been used to examine diversity and look for cryptic species for the predator species that are the focus of this study [52], and because dietary analysis using barcoding is likely to increase the diversity of prey, therefore indicating more generalised feeding habits and making our study relevant to a wide variety of ecosystems.

We found that barcoding offered more resolved identifications for a large majority of stomach-content items in predatory boreal shield fish, and, unlike morphology, it often provided species-level identifications. The strong performance of barcoding resulted from its ability to identify degraded tissue remains with few or no discernable morphological characteristics [22, 23]; the physical characteristics required for morphological identifications were degraded or absent from many stomach-content items in our study. Our results support the conclusions of many studies indicating that barcoding is highly effective for determining predator diet from stomach contents or feces [24-30]. For a small number of stomach-content items, we found that barcoding could only identify prey to a taxon above the species level but still outperformed morphology. In all of these cases, the DNA barcode sequences from these stomach-content items were long and of good quality, and matched to a BIN (Barcode Index Number [51]), but the species-level coverage of the reference database was incomplete, suggesting that additional barcode identifications are possible with future database development through campaigns such as FISH-BOL [53]. It is possible that the performance of morphology relative to DNA barcoding is dependent on the level of training and expertise for any individual making morphological identifications as well as the keys and characteristics they employ; however, if the consistency and accuracy of morphological identifications depend heavily on the individual performing them, DNA barcoding may provide a more reliable method for identifying stomach contents for most individuals because of limited access to taxonomic experts and training and the standardized nature of DNA barcoding [21]. Future studies that do not rely on comparing identifications of individual stomach-content items could benefit greatly from combining next-generation sequencing approaches and barcoding because such approaches may not require detailed dissections and may be able to detect prey in mixed remains that we could not identify using Sanger sequencing, further improving the performance of a DNA barcoding approach [54].

DNA barcoding increased the number and frequency of prey-species recovery, which has implications for our understanding of interaction strengths in food webs. Although the frequency of prey-species recovery from stomach contents is not equivalent to interaction strength *per se*, such an increase suggests that morphology-based estimates of interaction strength may be misleading in two important ways. Firstly, using barcoding, we identified more than half of species from one stomach, but we did not identify any of these species using morphology. This implies that morphology underestimates both the number of interactions and the prevalence of weak interactions, and that barcoding has the potential to reveal these weak interactions. Rarefaction curves suggest that increased sampling effort using barcoding will yield further increases in the number of prey species and food-web links, but such efforts with morphology are less likely to produce such increases. Secondly, we identified all the prey species found by morphology more frequently with barcoding. This suggests that interaction strength is underestimated when based on morphology. These underestimations are concerning because accurate estimations of interaction strength and the identification of weak interactions are essential to accurately evaluate food-web stability; unstable dynamics are typical of systems dominated by strong interactions [55], and weak interactions are thus expected to be common in food webs [56]. It seems that barcodes, in combination with highly quantitative molecular tools such as qPCR [54,57], could be particularly useful in furthering our understanding of patterns of interaction strength in food webs if current issues with these methods can be resolved [57].

We identified a more generalised diet using barcoding, as well as prey species that demonstrate the coupling of near-shore and off shore habitats by predators, indicating that morphology fails to capture both key food-web links and the dietary breadth of predatory boreal shield fish and so underestimates the degree to which they act as dietary generalists. Similar to other studies (e.g., Clare *et al*. [24]), our result that barcoding increases the number of prey species detected suggests that it is a useful technique for determining dietary generalism in both individuals and species of vertebrate predators. We found that increased prey diversity through barcoding revealed a number of feeding links that indicate the coupling of near-shore and off-shore habitats. For example, smallmouth bass (*Micropterus dolomieu*) and lake whitefish (*Coregonus clupeaformis*), which have traditionally been considered to feed primarily in one habitat [41], may have more general diets that couple near-shore and off-shore habitats. An increase in generalism and the coupling of spatially distinct habitats both have strong implications for food-web dynamics because such flexibility in feeding

habits is strongly linked to food-web stability [58, 59]. In addition, increased generalism and habitat coupling have important implications when considering the impacts of invasive smallmouth bass on native lake trout (*Salvelinus namaycush*) [14] and could impact the ways that multispecies fisheries are managed. We also found that barcoding identified feeding links that incorporated unconnected species, such as walleye in Delaney Lake and lake trout in Richardson Lake, into the food web. An increase in the number of predator species that couple habitats, combined with the highly resolved nature of dietary data through barcoding, suggests that barcoding is a potent tool to tease out compartments in food webs and the linking of these compartments by mobile generalist predators.

We found that using barcoding to construct food webs changed various food-web metrics that have been previously examined for sensitivity to food-web resolution. In barcode-based food webs, we found a higher number of feeding links, resulting in a higher linkage density, and a higher percentage of possible feeding links than in morphology-based webs. However, we found that connectance varied inconsistently between food webs constructed using morphology and barcoding. Previous studies have reported similarly conflicting impacts of barcoding on connectance [34, 35]. Changes to our values of connectance were likely attributable to the methodological constraints of having dietary data for only a small, fixed subset of all species in the system. Because of this, the proportion of possible links, which showed a consistent increase with the incorporation of barcode-based dietary information, is likely more meaningful for our dataset. Our connectance values were similar to those of previous studies using barcoding to determine food-web structure [34, 35], which are considerably lower than the connectance of traditional food webs [60, 61]. This disparity is likely in part a result of an increase in prey-species diversity with the use of barcoding.

The differences in connectance between barcode-based food webs, such as ours, and traditional food webs are likely attributable in part to differences in the methodologies for how the feeding links were established. Traditional food webs are often constructed cumulatively, including all feeding interactions believed to be occurring across space and time (e.g. Martinez [5]), whereas molecular food webs to date represent more of a 'snapshot' based on a discrete series of observations [33, 34, 35]. Here, we have demonstrated that the 'snapshot' DNA approach results in differences in food-web structure. Even though we have not presented an exhaustive representation of all feeding interactions that may occur in our study lakes, rarefaction data (Figure 3) indicate that larger sampling efforts are likely to increase the disparity between morphology-based and barcode-based food webs. This indicates that barcoding can indeed uncover how food webs vary in space and time, which is particularly important because of growing evidence that variation in food webs is fundamental to their stability and function [1]. The results of our study, taken together with those of previous barcode-based and traditional food webs, suggest that barcoding should be considered by future researchers as a tool to increase the detail included in food webs now that it has revealed significant changes in common food-web metrics [34, 35, 60, 61].

DNA barcoding revealed several features in food-web topology not found through morphology, but which are strongly believed to influence the stability and dynamics of food webs. Morphology did not identify any omnivorous feeding interactions, but, as predicted, barcoding identified the presence of omnivory for multiple species in each lake (for one example, see Figure 4(d), nodes 3,5 and 20), increasing maximum food-chain length. Omnivory in these cases is likely ontogenetically driven; juveniles of one species are being consumed by adults from another, and more information is required to fully characterize the feeding relationships between these species. The identification of omnivorous links changes the bipartite food web structure established by morphology and produces a significantly more complex topology that has implications for the dynamics of the predator species because omnivory can be stabilizing or de-stabilizing [62]. In addition, the more complex food-web topologies more accurately reflect the diversity of feeding habits exhibited by generalist predators, which have been observed using stable isotopes [12, 13, 14, 38, 39, 40]. In any molecular dietary analysis, there is the potential risk of cross-contamination between predators even if every conceivable precaution is employed [15], and although unlikely, it is possible that some of these results represent contamination because we identified no omnivory using morphology.

The use of barcoding could be particularly useful in identifying other food-web motifs, in addition to omnivory, that are expected to be common in food webs, but for which there has been poor or conflicting evidence [63, 64, 65]. For example, barcoding indicated the presence of a 'diamond' motif (Figure 4(d), nodes 2, 4, 6 and 13), which has been empirically shown to impart stability when interaction strengths are asymmetrical [66]. There is also an example of mutual predation (Figure 4(d), nodes 4 and 6). However, it is important to note that we were unable to unambiguously detect some potentially important food-

web properties, such as cannabilism, using barcoding. We attribute two factors to our relatively high rate of matches to the DNA of the corresponding predator species (approximately 22% of our stomach-content items, compared to only 3% in [24]). Firstly, self-contamination of stomach contents with the DNA of the predatory fish species is very likely because the primers selected have a universal design to target as many prey species as possible. Secondly, cannibalism likely occurs in these lakes because it has been well documented in several predatory boreal shield fishes, such as lake trout and northern pike (*Esox lucius*) [41]. Although cytochrome c oxidase subunit I (COI) was selected as the DNA barcoding region due to its ability to differentiate species rather than individuals, it would be possible to detect cannibalism through small differences between CO1 sequences. However, this would rely heavily on high-quality sequence data that would be difficult to produce through unidirectional sequencing of degraded tissue remains, and thus we did not consider barcodes that matched the predator species' DNA in our analyses.

Combining barcoding and morphology did not appreciably change food-web resolution when compared with barcoding alone, but did change compared to morphology alone, suggesting that the techniques do not complement one another or provide different types of dietary information. Although, for a few stomach-content items, morphology provided more highly resolved identifications than barcoding due to presence of persistent hard parts and a failure to recover DNA, all except one of these identifications were to the family level or higher. Thus, morphology was still able to provide some additional dietary information even though barcoding was usually more effective for identifying specific stomach-content items. However, we found that barcoding alone had comparable results to the techniques combined for identifying the number of prey per stomach and per species, while morphology alone found less prey diversity than the combination of these techniques. This suggests that the majority of prey diversity was identified using barcoding, and that including morphological results overall provides little supplementary information to barcoding when examining food-web structure. Thus, morphology is of little additional value in producing highly resolved whole food webs when barcoding is employed. Our finding differs considerably from the complementarity between molecular and morphological techniques reported by Wirta *et al.* [35], likely due to differences in methodology, as Wirta *et al.* [35] used rearing and other methods not relevant to a boreal shield lake system. The non-complementary performance of morphology and the effectiveness of barcoding indicate that a barcode-based approach to collecting dietary information would reduce catch requirements, which is consistent with animal use policies and conservation practices.

DNA barcoding had some unanticipated but interesting results which suggest that the application of barcoding to diet analysis may provide data useful for ecological questions in addition to establishing food-web structure. For example, the parasite *Leptorhynchoides thecatus* was identified from the stomachs of smallmouth bass. Wood [67] suggested that barcoding could be useful in the identification of parasites in food webs, and our results support this notion. Barcoding could be a valuable and simple way to address the concern that food webs that lack parasites are incomplete [68]. In addition, we detected predation of *Cottus* sp. and *Myoxocephalus* sp. by burbot (*Lota lota*), and brook stickleback (*Culaea inconstans*) by lake trout in Delaney lake using barcoding, but these prey species were not reported in the netting surveys for the lakes from which we collected samples. This suggests that diet data from barcoding might be an effective alternative way to examine species diversity in a food web or find prey species of interest in ecosystems.

DNA barcoding is a powerful technique that can increase food-web resolution and allow ecologists to examine and understand species interactions in an unprecedented and highly detailed manner. Highly resolved food webs are required to settle long-standing questions about how the quality of food-web data impacts our understanding of food webs. More importantly, increased food-web resolution through barcoding helps ecologists meet their fundamental need to identify feeding interactions, giving them the potential to address many fundamental questions in ecology, such as how food webs are structured in nature, how structure influences the dynamics and behaviour of communities, and the how diversity influences ecosystems. Further studies seeking to observe real-world food webs would benefit from employing DNA barcoding to observe food webs in a comprehensive and highly resolved manner.

Acknowledgements: This work was supported by the National Science and Engineering Research Council of Canada through a Discovery Grant to K.S.M., a Strategic Grant to K.S.M., B.J.S. and N.P.L., a Strategic Grant to R.H.H., and a Canada Graduate Scholarship to T.J.B. We thank K. Armstrong, J. Wright and many other biologists and technical staff at the Ontario Ministry of Natural Resources for assistance with sample collection, as well as T. Tunney, A. Naaum, R. Saint, and K. Leung for their assistance with fieldwork and sample processing. We

thank J. Gross and the Genomics Facility at the University of Guelph for DNA sequencing services. We also thank our anonymous reviewers for their helpful feedback.

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Supplemental Material: The online version of this article (DOI: 10.1515/dna-2015-0005) offers supplementary material.