Electronic Supplementary Material

DNA metabarcoding and the cytochrome *c* oxidase subunit I marker: not a perfect match

(A) Example metabarcoding studies using COI and alternative markers

Recent examples in which COI is used or promoted as an animal metabarcoding marker:

Geller J., Meyer C., Parker M., Hawk H. 2013 Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Mol Ecol Resour* **13**(5), 851-861. (doi:10.1111/1755-0998.12138). (COI metabarcoding)

Gibson J., Shokralla S., Porter T.M., King I., van Konynenburg S., Janzen D.H., Hallwachs W., Hajibabaei M. 2014 Simultaneous assessment of the macrobiome and microbiome in a bulk sample of tropical arthropods through DNA metasystematics. *Proc Natl Acad Sci USA* **111**(22), 8007-8012. (doi:10.1073/pnas.1406468111). (COI metabarcoding; also bacterial 16S and protozoa 18S rRNA)

Hajibabaei M., Spall J.L., Shokralla S., van Konynenburg S. 2012 Assessing biodiversity of a freshwater benthic macroinvertebrate community through non-destructive environmental barcoding of DNA from preservative ethanol. *BMC Ecol* **12**. (doi:10.1186/1472-6785-12-28). (COI metabarcoding)

Ji Y., Ashton L., Pedley S.M., Edwards D.P., Tang Y., Nakamura A., Kitching R., Dolman P.M., Woodcock P., Edwards F.A., et al. 2013 Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. *Ecol Lett* **16**(10), 1245-1257. (doi:10.1111/ele.12162). (COI metabarcoding)

Leray M., Yang J.Y., Meyer C.P., Mills S.C., Agudelo N., Ranwez V., Boehm J.T., Machida R.J. 2013 A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Front Zool* **10**, e34. (doi:10.1186/1742-9994-10-34). (COI metabarcoding)

Liu S., Li Y., Lu J., Su X., Tang M., Zhang R., Zhou L., Zhou C., Yang Q., Ji Y., et al. 2013 SOAPBarcode: revealing arthropod biodiversity through assembly of Illumina shotgun sequences of PCR amplicons. *Methods Ecol Evol* **4**(12), 1142-1150. (doi:10.1111/2041-210X.12120). (COI metabarcoding)

Meusnier I., Singer G.A.C., Landry J.F., Hickey D.A., Hebert P.D.N., Hajibabaei M. 2008 A universal DNA mini-barcode for biodiversity analysis. *BMC Genomics* **9**. (doi:10.1186/1471-2164-9-214). (COI metabarcoding)

Tang C.Q., Leasi F., Obertegger U., Kieneke A., Barraclough T.G., Fontaneto D. 2012 The widely used small subunit 18S rDNA molecule greatly underestimates true diversity in biodiversity surveys of the meiofauna. *Proc Natl Acad Sci USA* **109**(40), 16208-16212. (doi:10.1073/pnas.1209160109). (COI metabarcoding)

Yu D.W., Ji Y., Emerson B.C., Wang X., Ye C., Yang C., Ding Z. 2012 Biodiversity soup: metabarcoding of arthropods for rapid biodiversity assessment and biomonitoring. *Methods Ecol Evol* **3**(4), 613-623. (doi:10.1111/j.2041-210X.2012.00198.x). (COI metabarcoding)

Diet metabarcoding studies using the COI primers from Zeale *et al.* [1]. These primers (highlighted in Figure 1) were widely adopted after initial results suggested “this primer set is capable of detecting the complete range of arthropod prey in diets”

Zeale M.R.K., Butlin R.K., Barker G.L.A., Lees D.C., Jones G. 2011 Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. *Mol Ecol Resour* **11**(2), 236-244. (doi:10.1111/j.1755-0998.2010.02920.x). (COI primers – development of Zeale *et al.* primers)

Alberdi A., Garin I., Aizpurua O., Aihartza J. 2012 The Foraging Ecology of the Mountain Long-Eared Bat *Plecotus macrobullaris* Revealed with DNA Mini-Barcodes. *PLoS One* **7**(4), e35692. (doi:10.1371/journal.pone.0035692). (COI - Zeale *et al*. primers)

Bohmann K., Monadjem A., Noer C.L., Rasmussen M., Zeale M.R.K., Clare E., Jones G., Willerslev E., Gilbert M.T.P. 2011 Molecular diet analysis of two African free-tailed bats (Molossidae) using high throughput sequencing. *PLoS One* **6**(6), e21441. (doi:10.1371/journal.pone.0021441). (COI - Zeale *et al*. primers)

Burgar J.M., Murray D.C., Craig M.D., Haile J., Houston J., Stokes V., Bunce M. 2013 Who's for dinner? High-throughput sequencing reveals bat dietary differentiation in a biodiversity hotspot where prey taxonomy is largely undescribed. *Mol Ecol* **23**(15), 3605–3617. (doi:10.1111/mec.12531). (COI - Zeale *et al*. primers)

Clare E.L., Symondson W.O.C., Fenton M.B. 2013 An inordinate fondness for beetles? Variation in seasonal dietary preferences of night-roosting big brown bats (*Eptesicus fuscus*). *Mol Ecol* **23**(15), 3633–3647. (COI - Zeale *et al*. primers)

Clare E.L., Symondson W.O.C., Broders H., Fabianek F., Fraser E.E., MacKenzie A., Boughen A., Hamilton R., Willis C.K.R., Martinez-Nuñez F., et al. 2013 The diet of *Myotis lucifugus* across Canada: assessing foraging quality and diet variability. *Mol Ecol* **23**(15), 3618–3632. (doi:10.1111/mec.12542). (COI - Zeale *et al*. primers)

Emrich M.A., Clare E.L., Symondson W.O.C., Koenig S.E., Fenton M.B. 2013 Resource partitioning by insectivorous bats in Jamaica. *Mol Ecol* **23**(15), 3648–3656. (doi:10.1111/mec.12504). (COI - Zeale *et al*. primers)

Goerlitz H.R., ter Hofstede H.M., Zeale M.R.K., Jones G., Holderied M.W. 2010 An aerial-hawking bat uses stealth echolocation to counter moth hearing. *Curr Biol* **20**(17), 1568-1572. (doi:10.1016/j.cub.2010.07.046). (COI - Zeale *et al*. primers)

Gonsalves L., Bicknell B., Law B., Webb C., Monamy V. 2013 Mosquito consumption by insectivorous bats: does size matter? *PLoS One* **8**(10), e77183. (doi:10.1371/journal.pone.0077183). (COI - Zeale *et al*. primers)

Hamad I., Delaporte E., Raoult D., Bittar F. 2014 detection of termites and other insects consumed by african great apes using molecular fecal analysis. *Sci Rep* **4**, 4478. (doi:10.1038/srep04478). (COI - Zeale *et al*. primers)

Hope P., Bohmann K., Gilbert M.T., Zepeda-Mendoza M., Razgour O., Jones G. 2014 Second generation sequencing and morphological faecal analysis reveal unexpected foraging behaviour by *Myotis nattereri* (Chiroptera, Vespertilionidae) in winter. *Front Zool* **11**(1), 39. (COI - Zeale *et al*. primers)

Krüger F., Clare E.L., Greif S., Siemers B.M., Symondson W.O.C., Sommer R.S. 2013 An integrative approach to detect subtle trophic niche differentiation in the sympatric trawling bat species *Myotis dasycneme* and *Myotis daubentonii*. *Mol Ecol* **23**(15), 3657–3671. (doi:10.1111/mec.12512). (COI - Zeale *et al*. primers)

Piñol J., San Andrés V., Clare E.L., Mir G., Symondson W.O.C. 2014 A pragmatic approach to the analysis of diets of generalist predators: the use of next-generation sequencing with no blocking probes. *Mol Ecol Resour* **14**(1), 18-26. (doi:10.1111/1755-0998.12156). (COI - Zeale *et al*. primers)

Razgour O., Clare E.L., Zeale M.R.K., Hanmer J., Schnell I.B., Rasmussen M., Gilbert T.P., Jones G. 2011 High-throughput sequencing offers insight into mechanisms of resource partitioning in cryptic bat species. *Ecol Evol* **1**(4). (doi:10.1002/ece3.49). (COI - Zeale *et al*. primers)

Rolfe A.K., Kurta A., Clemans D.L. 2014 Species-level analysis of diets of two mormoopid bats from Puerto Rico. *J Mammal* **95**(3), 587-596. (doi:10.1644/13-mamm-a-190). (COI - Zeale *et al*. primers)

Sedlock J.L., Krüger F., Clare E.L. 2014 Island Bat Diets: Does it Matter More Who You Are or Where You Live? *Mol Ecol* **23**(15), 3684–3694. (doi:10.1111/mec.12732). (COI - Zeale *et al*. primers)

Vesterinen E.J., Lilley T., Laine V.N., Wahlberg N. 2013 Next generation sequencing of fecal DNA reveals the dietary diversity of the widespread insectivorous predator Daubenton's bat

(*Myotis daubentonii*) in southwestern Finland. *PLoS One* **8**(11), e82168. (doi:10.1371/journal.pone.0082168). (COI - Zeale *et al*. primers)

Recent examples where rRNA markers are used or promoted as animal metabarcoding markers:

Andersen K., Bird K.L., Rasmussen M., Haile J., Breuning-Madsen H., KjÆR K.H., Orlando L., Gilbert M.T.P., Willerslev E. 2012 Meta-barcoding of 'dirt' DNA from soil reflects vertebrate biodiversity. *Mol Ecol* **21**(8), 1966-1979. (doi:10.1111/j.1365-294X.2011.05261.x). (MtDNA 16S metabarcoding)

Bienert F., De Danieli S., Miquel C., Coissac E., Poillot C., Brun J.-J., Taberlet P. 2012 Tracking earthworm communities from soil DNA. *Mol Ecol* **21**(8), 2017-2030. (doi:10.1111/j.1365-294X.2011.05407.x). (MtDNA 16S metabarcoding)

Bik H.M., Porazinska D.L., Creer S., Caporaso J.G., Knight R., Thomas W.K. 2012 Sequencing our way towards understanding global eukaryotic biodiversity. *Trends Ecol Evol* **27**(4), 233-243. (doi:10.1016/j.tree.2011.11.010). (Nuclear 18S metabarcoding)

Chariton A.A., Court L.N., Hartley D.M., Colloff M.J., Hardy C.M. 2010 Ecological assessment of estuarine sediments by pyrosequencing eukaryotic ribosomal DNA. *Front Ecol Environ* **8**(5), 233-238. (doi:10.1890/090115). (Nuclear 18S metabarcoding)

Clarke L.J., Soubrier J., Weyrich L.S., Cooper A. 2014 Environmental metabarcodes for insects: *in silico* PCR reveals potential for taxonomic bias. *Mol Ecol Resour*, *In press*. (doi:10.1111/1755-0998.12265). (Comparison of mtDNA 16S and COI)

Coissac E., Riaz T., Puillandre N. 2012 Bioinformatic challenges for DNA metabarcoding of plants and animals. *Mol Ecol* **21**(8), 1834-1847. (doi:10.1111/j.1365-294X.2012.05550.x). (Comparison of various markers)

Creer S., Fonseca V.G., Porazinska D.L., Giblin-Davis R.M., Sung W., Power D.M., Packer M., Carvalho G.R., Blaxter M.L., Lambshead P.J.D., et al. 2010 Ultrasequencing of the meiofaunal biosphere: practice, pitfalls and promises. *Mol Ecol* **19**, 4-20. (doi:10.1111/j.1365-294X.2009.04473.x). (Nuclear 18S metabarcoding)

De Barba M., Miquel C., Boyer F., Mercier C., Rioux D., Coissac E., Taberlet P. 2014 DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: application to omnivorous diet. *Mol Ecol Resour* **14**(2), 306-323. (doi:10.1111/1755-0998.12188). (MtDNA 16S, 12S and plant metabarcoding)

Deagle B.E., Kirkwood R., Jarman S.N. 2009 Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. *Mol Ecol* **18**(9), 2022-2038. (doi:10.1111/j.1365-294X.2009.04158.x). (MtDNA 16S and nuclear 18S, 28S)

Fonseca V.G., Carvalho G.R., Sung W., Johnson H.F., Power D.M., Neill S.P., Packer M., Blaxter M.L., Lambshead P.J.D., Thomas W.K., et al. 2010 Second-generation environmental sequencing unmasks marine metazoan biodiversity. *Nat Commun* **1**, e98. (doi:10.1038/ncomms1095). (Nuclear 18S metabarcoding)

Hirai J., Kuriyama M., Ichikawa T., Hidaka K., Tsuda A. 2014 A metagenetic approach for revealing community structure of marine planktonic copepods. *Mol Ecol Resour*, In press. (doi:10.1111/1755-0998.12294). (Nuclear 28S metabarcoding)

Jarman S.N., McInnes J.C., Faux C., Polanowski A.M., Marthick J., Deagle B.E., Southwell C., Emmerson L. 2013 Adélie penguin population diet monitoring by analysis of food DNA in scats. *PLoS One* **8**(12), e82227. (doi:10.1371/journal.pone.0082227). (Nuclear 18S metabarcoding)

Murray D.C., Haile J., Dortch J., White N.E., Haouchar D., Bellgard M.I., Allcock R.J., Prideaux G.J., Bunce M. 2013 Scrapheap Challenge: A novel bulk-bone metabarcoding method to investigate ancient DNA in faunal assemblages. *Sci Rep* **3**, 3371. (doi:10.1038/srep03371). (MtDNA 16S, 12S metabarcoding)

Porazinska D.L., Giblin-Davis R.M., Faller L., Farmerie W., Kanzaki N., Morris K., Powers T.O., Tucker A.E., Sung W., Thomas W.K. 2009 Evaluating high-throughput sequencing as a method for metagenomic analysis of nematode diversity. *Mol Ecol Resour* **9**(6), 1439 - 1450. (doi:10.1111/j.1755-0998.2009.02611.x). (Nuclear 18S metabarcoding)

Shehzad W., Riaz T., Nawaz M.A., Miquel C., Poillot C., Shah S.A., Pompanon F., Coissac E., Taberlet P. 2012 Carnivore diet analysis based on next-generation sequencing: application to the leopard cat (*Prionailurus bengalensis*) in Pakistan. *Mol Ecol* **21**(8), 1951-1965. (doi:10.1111/j.1365-294X.2011.05424.x). (MtDNA 12S metabarcoding)

Zhan A., Bailey S.A., Heath D.D., Macisaac H.J. 2014 Performance comparison of genetic markers for high-throughput sequencing-based biodiversity assessment in complex communities. *Mol Ecol Resour, In press*. (doi:10.1111/1755-0998.12254). (Comparison of nuclear 18S, mtDNA 16S and COI)

(B) Comments on the use of variable primers in metabarcoding

To encompass variation observed in primer binding regions primer cocktails (a mixture of several distinct primer variants) and/or degenerate primers (those synthesised with some positions having several possible bases) are often used. This is expected to enable the primers to bind to a wider range of templates and allow their amplification. It has been shown to improve the range of species recovered when amplifying DNA from single species templates [e.g. 2, 3]. However, in metabarcoding studies primer mixes have not allowed recovery of all expected species. For example, when Yu *et al.* [4] tested fully degenerate primers for bulk sequencing of COI from mixed arthropod DNA they found between 24% (>2 read threshold) and 36% (>5 read threshold) of the species known to be in their mixtures were not recovered. There are likely a few reasons why using variable primers can be less than ideal. First, the number of primers required to account for variability increases exponentially as the number of variable sites goes up. Geller *et al*. [2] calculated that >3 billion primer sequence permutations would result if they created a fully degenerated consensus of one binding site they targeted in marine invertebrates. This is an extreme case, but the number of primers created when incorporating degenerate sites commonly found in COI alignments quickly climbs into 100's or 1000's of sequences. Even if you incorporate all variation in binding sites, some oligonucleotide primers will be better at annealing to priming sites than others, some will self anneal (i.e. form secondary structure which prevents PCR priming) or will bind with other primers in the mixture. The incorporation of inosine (an artificial base that can bind to all natural DNA bases) has been suggested [2], but we are not aware of this being tested in metabarcoding analysis. The success rate of the [2] marine invertebrate primers using inosine was ~75% in single template PCR and is likely to be lower in a competitive PCR amplification. Inosine also has different thermal stability when bounded to the four natural DNA bases [5] and may not provide unbiased recovery. In summary, there are several potential difficulties in the recovery of all DNA sequences present in mixtures and this includes variation in primer binding between species. For the most robust protocols, we feel that the preferred option is to target markers with less variation in the primer binding sites rather than trying to incorporate variation into primers.

(C) Additional Details for Figure 1

This figure shows a comparison of sequence variability of four markers in representative insects and highlights the variation in associated primers (primers in Table S1a). Variation in the three mitochondrial markers are shown for 40 insects (representing 25 orders) extracted from complete mtDNA sequences present in GenBank; comparable nuclear 18S rRNA (SSU) sequences were also examined (species listed in Table S2 and S3 respectively; sequences available as Electronic Supplementary Data). To visualize information content, and presence/absence of conserved regions, entropy values were calculated for positions across the sequences (calculated in Bioedit [6] using aligned sequences with gaps removed). Schematic of representative primers designed to amplify DNA from a broad range of insects highlight variability in potential primer binding sites. The forward 18S rRNA primer is near the start of most sequences in GenBank and a few base pairs of sequence are missing the 5' regions of some primers (positions coloured white).

The COI primers were developed as arthropod mini-barcodes for use in a bat diet metabarcoding study [1]. Despite considerable variability in targeted taxa they have been used as the single primer set to recover arthropod markers in over a dozen published papers including diet studies on primates, spiders and bats (see list of references above in Electronic Supplementary Material A).The mitochondrial 16S and 12S rRNA genes have been targeted in various metabarcoding studies (see list of references above in Electronic Supplementary Material A) although we are not aware if these specific primers have been used. The primers for nuclear 18S small subunit (nSSU) have been applied in several metabarcoding studies [e.g. 7, 8].

(D) Additional Details for Figure 2

Sequences of PCR primers that amplify regions of two mitochondrial genes were extracted from complete mtDNA sequences from vertebrates (listed in Table S1b; Sequences available as Electronic Supplementary Data). The summary shown in the figure is based on pairwise distances at primer binding sites between taxa for various taxonomic ranks. For example, Family level comparisons include pairwise distances between taxa in different families but in the same Order. Comparisons below class level are based on fish sequences (Actinopterygii; n=135 sequences; Table S4; variability in these primers is shown in Figure S1). Representative Vertebrata were considered between class comparisons (n=20 sequences; Table S5).

Table S1: Primers considered in the current paper

(a) Primer sets shown in Figure 1. This includes three mtDNA markers and one from the nuclear genome. In the paper's colour schematic, reverse complement of reverse primers is shown.

Gene	Name	Sequence (5' - 3')	Reference
COI	ZBJ-ArtF1c	AGATATTGGAACWTTATATTTTATTTTTGG	[1]
	ZBJ-ArtR2c	WACTAATCAATTWCCAAATCCTCC	[1]
16s rRNA	LR-J-13017*	TTACGCTGTTATCCCTAA	[9]
	LR-N-13398* (16Sar-5')†	CACCTGTTTAACAAAAACAT	[9] and [10]
12s rRNA	SR-J-14199*	TACTATGTTACGACTTAT	[9]
	SR-N-14594*	AAACTAGGATTAGATACCC	[9] and [10]
18s rRNA	SSU_FO4	GCTTGTCTCAAAGATTAAGCC	[8]
	SSU_R22	GCCTGCTGCCTTCCTTGGA	[8]

(b) Primer sets analysed in Figure 2 of paper.

Gene	Name	Sequence (5' - 3')	Reference
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	[11]
	mlCOIintF	GGWACWGGWTGAACWGTWTAYCCYCC	[3]
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	[11]
16s rRNA	16Sar-5'* (LR-N-13398)†	CGCCTGTTTATCAAAAACAT	[12] and [10]
	Chord_16S_F*	CGAGAAGACCCTRTGGAGCT	[13]
	16Sbr-3'* (LR-J-12887)†	CCGGTCTGAACTCAGATCACGT	[12] and [10]

*Note that the standard sequence direction provided in GenBank for insect mitochondrial rRNA genes is the reverse complement of that for vertebrates. This is because the mtDNA strand which serves as template for transcription of these genes varies between animal groups. It can be confusing when aligning sequences across groups, and also means the "reverse" primer in insects can becomes a "forward" primer in vertebrates (e.g. LR-N-13398 in figures 1 and 2).

†These are alternative names for primers binding to the same site.

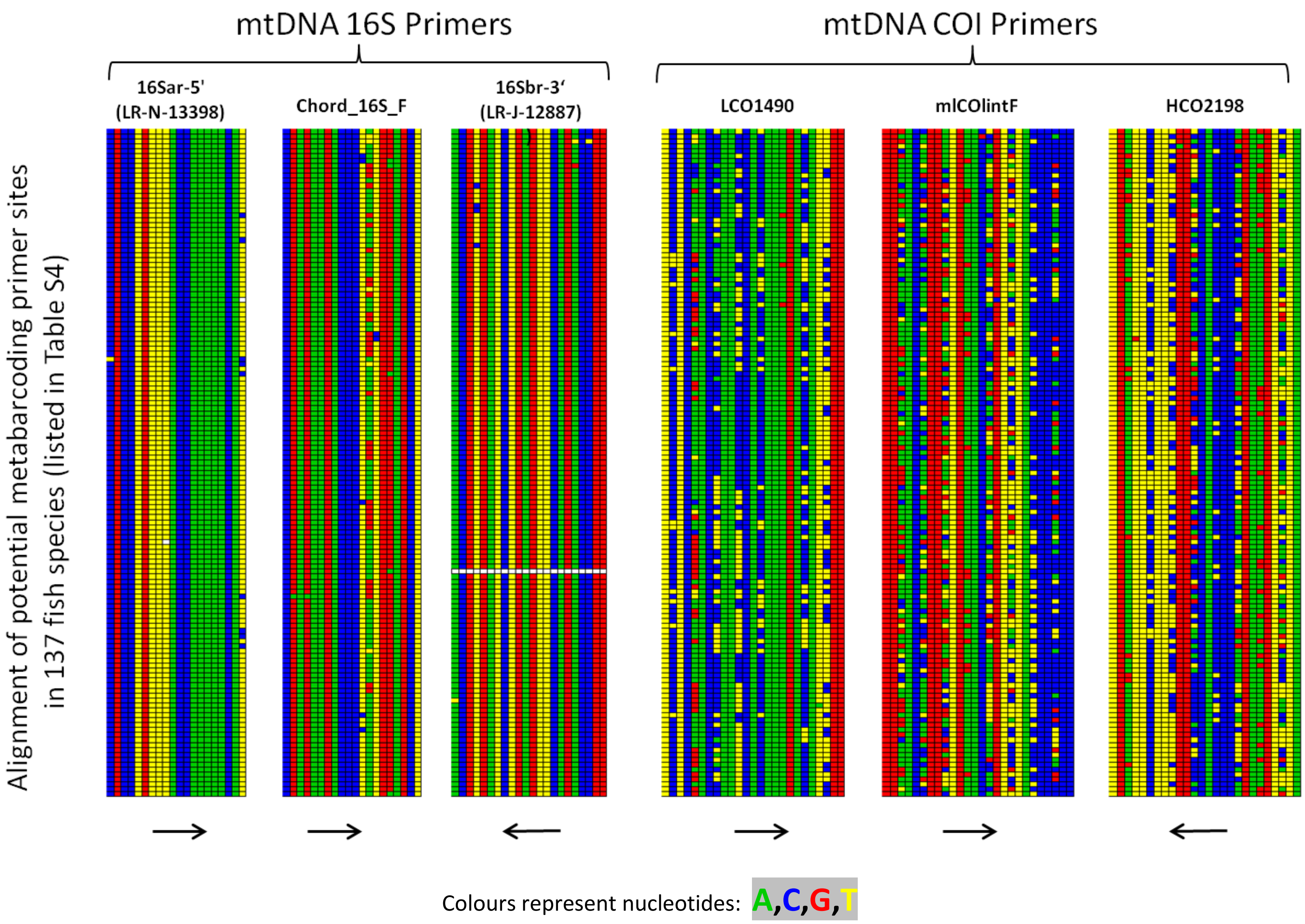

Figure S1: Differences in primer binding regions across 135 fish sequences in two mtDNA markers (primers are those used in construction of Figure2).

Table S2: List of insects and associated mtDNA sequence reference used for construction of figure 1. Sequences from each of the three regions (COI, 16S and 12S) are available as Electronic Supplementary Data.

Order	Species	Common name	Accession #
Odonata	*Pseudolestes mirabilis*	Damselfly	gi|470229443
	Davidius lunatus	Dragonfly	gb|EU591677.1|
Orthoptera	*Locusta migratoria*	Locus	gb|JN858206.1|
	Teleogryllus emma	Field cricket	gi|219524229
Hemiptera	*Lycorma delicatula*	Plant hopper	gb|EU909203.1|
	Acyrthosiphon pisum	Aphid	gi|213948225
	Nezara viridula	Stink bug	gb|EF208087.1|
Plecoptera	*Dinocras cephalotes*	Stonefly	gb|KF484757.1|
	Pteronarcys princeps	Stonefly	gb|AY687866.1|
Hymenoptera	*Apis florea*	Honey bee	gb|JX982136.1|
	Pristomyrmex punctatus	Ant	gi|322422364
	Cephus cinctus	Sawfly	gb|FJ478173.1|
Coleoptera	*Trachypachus holmbergi*	Carabid beetle	gi|208433759
	Rhagophthalmus lufengensis	Firefly	gi|190349565
	Tribolium castaneum	Flour beetle	gi|133755325
Mecoptera	*Neopanorpa pulchra*	Scorpion fly	gi|256985280
Diptera	*Anopheles oryzalimnetes*	Mosquito	gb|HQ335345.1|
	Chironomus tepperi	Crane fly	gb|JN861749.1|
	Drosophila melanogaster	Fruit fly	gb|JQ686694.1|
Lepidoptera	*Biston panterinaria*	Moth	gi|435855846
	Papilio machaon	Swallowtail Butterfly	gi|391224048
	Bombyx mori	Silkmoth	gb|GU966630.1|
Siphonaptera	*Jellisonia amadoi*	Flea	gi|556506033
Ephemeroptera	*Ephemera orientalis*	Oriental mayfly	gi|229324789
	Baetis sp.	Mayfly	gb|GU936204.1|
Blattaria	*Periplaneta Americana*	Cockroach	gi|378829200
	Cryptocercus relictus	Relic cockroach	gi|394831453
Isoptera	*Reticulitermes flavipes*	Termite	gb|EF206317.1|
Mantodea	*Tamolanica tamolana*	Mantid	gi|84488776
Dermaptera	*Challia fletcheri*	Earwig	gi|403531341
Phasmatodea	*Phraortes illepidus*	Walking stick	gi|313199763
Thysanoptera	*Thrips imaginis*	Thrip	gi|25057389
Phthiraptera	*Psococerastis albimaculata*	Lice	gi|511347991
Megaloptera	*Corydalus cornutus*	Dobson fly	gb|FJ171323.1|
Neuroptera	*Chrysopa pallens*	Lace wing	gi|426406212
Raphidioptera	*Mongoloraphidia harmandi*	Snakeflies	gi|258649574
Psocoptera	*Psococerastis albimaculata*	Barklice	gi|511347991
Strepsiptera	*Mengenilla moldrzyki*	Twisted wing	gi|403531501
Mantophasmatodea	*Sclerophasma paresisense*	Gladiator	gi|84488734
Thysanura	*Thermobia domestica*	Silverfish	gb|AY639935.1|

Table S3: List of insects and associated nuclear 18S SSU rRNA sequence references used for construction of figure 1. Sequences are available as Electronic Supplementary Data.

Order	Species	Accession #
Odonata	*Pseudolestes_mirabilis*	gi|158264680
	Davidius_fruhstorferi	gi|260207002
Orthoptera	*Locusta_migratoria*	gi|15986534
	Gryllus_assimilis	gi|46405911
Hemiptera	*Fulgoridae_sp.*	gi|449084459
	Acyrthosiphon_pisum	gi|886117
	Rhaphigaster_nebulosa	gi|1150554
Plecoptera	*Dinocras_cephalotes*	gi|157417136
	Pteronarcys_sachalina	gi|157417155
Hymenoptera	*Apis_mellifera*	gi|51873301
	Proceratium_stictum	gi|51873307
	Cephus_pygmaeus	gi|300391083
Coleoptera	*Trachypachus_holmbergi*	gi|6578779
	Rhagophthalmus_sp.	gi|68301644
	Tribolium_castaneum	gi|299152224
Mecoptera	*Neopanorpa_sp.*	gi|64483043
Diptera	*Anopheles_gambiae*	gi|103421630
	Acricotopus_lucens	gi|38422424
	Drosophila_melanogaster	gi|425869670
Lepidoptera	*Aglaope_infausta*	gi|110289912
	Papilio_troilus	gi|11095947
	Bombyx_mori	gi|84310305
Siphonaptera	*Monopsyllus_anisus*	gi|83282942
Ephemeroptera	*Ephemera_simulans*	gi|239736518
	Baetis_sp.	gi|34223577
Blattaria	*Blattidae_sp.*	gi|46405869
	Pelmatosilpha_guyanae	gi|163017888
Isoptera	*Reticulitermes_flavipes*	gi|126678839
Mantodea	*Mantis_religiosa*	gi|126678840
Dermaptera	*Tagalina_sp.*	gi|46405880
Phasmatodea	*Carausius_morosus*	gi|1149618
Thysanoptera	*Sericothrips_staphylinus*	gi|449084537
Phthiraptera	*Pedicinus_badii*	gi|219881925
Megaloptera	*Sialis_hamata*	gi|46405906
Neuroptera	*Kempynus_kimminsi*	gi|219398629
Raphidioptera	*Mongoloraphidia_martynovae*	gi|219398632
Psocoptera	*Cerastipsocinae_sp.*	gi|402170151
Mantophasmatodea	*Sclerophasma_paresisense*	gi|46405904
Thysanura	*Thermobia_domestica*	gi|15986531

Table S4: List of fish (Actinopterygii) and associated mtDNA sequence reference used for construction of figure 2 in comparisons below class level. Most sequences were taken from a collection of 100 complete mtDNA sequences [14]. Sequences from additional species with complete mtDNA sequences (shown in bold) were taken from GenBank to increase the number of comparisons at lower taxonomic levels. Sequences from the two regions analysed (COI, 16S) are available as Electronic Supplementary Data.

Order	Family	Genus_species	Accession
Clupeiformes	Clupeidae	*Sardinops_melanostictus*	AB032554
	Clupeidae	***Clupea_harengus***	***gi|148922530***
	Clupeidae	***Clupea_pallasii***	***gi|148922558***
	Clupeidae	***Sardina_pilchardus***	***gi|148922544***
	Clupeidae	***Sprattus_sprattus***	***gi|148922628***
Cypriniformes	Cyprinidae	*Cyprinus_carpio*	X61010
	Cyprinidae	***Danio_rerio***	***gi|15079186***
	Cobitidae	*Crossostoma_lacustre*	M91245
Salmoniformes	Salmonidae	*Oncorhynchus_mykiss*	L29771
	Salmonidae	***Oncorhynchus_nerka***	***gb|EF055889.1|***
	Salmonidae	***Oncorhynchus_tshawytscha***	***gi|14647165***
	Salmonidae	***Oncorhynchus_keta***	***gi|385843132***
	Salmonidae	***Oncorhynchus_masou***	***gi|120586724***
	Salmonidae	*Coregonus_lavaretus*	AB034824
	Salmonidae	***Salmo_salar***	***gb|KF792729.1|***
	Salmonidae	***Salmo_trutta***	***gb|JQ390057.1|***
Stomiiformes	Gonostomatidae	*Diplophos_sp*	AB034825
	Gonostomatidae	*Sigmops_gracile*	AB016274
	Stomiidae	*Chauliodus_sloani*	AP002915
Ateleopodiformes	Ateleopodidae	*Ateleopus_japonicus*	AP002916
	Ateleopodidae	*Ijimaia_dofleini*	AP002917
Aulopiformes	Aulopodidae	*Aulopus_japonicus*	AB047821
	Chlorophthalmidae	*Chlorophthalmus_agassizi*	AP002918
	Synodontidae	*Harpadon_microchir*	AP002919
	Synodontidae	*Saurida_undosquamis*	AP002920
Myctophiformes	Neoscopelidae	*Neoscopelus_microchir*	AP002921
	Myctophidae	*Myctophum_affinec*	AP002922
	Myctophidae	*Diaphus_splendidusc*	AP002923
Lampridiformes	Lamprididae	*Lampris_guttatusc*	AP002924
	Trachipteridae	*Trachipterus_trachypterusc*	AP002925
	Trachipteridae	*Zu_cristatusc*	AP002926
Polymixiiformes	Polymixiidae	*Polymixia_japonica*	AB034826
	Polymixiidae	*Polymixia_lowei*	AP002927
Percopsiformes	Aphredoderidae	*Aphredoderus_sayanus*	AP004403
	Percopsidae	*Percopsis_transmontana*	AP002928
Ophidiiformes	Carapidae	*Carapus_bermudensis*	AP004404
	Ophidiidae	*Bassozetus_zenkevitchi*	AP004405
	Ophidiidae	*Lamprogrammus_niger*	AP004410
	Ophidiidae	*Sirembo_imberbisc*	AP004406

	Bythitidae	*Cataetyx_rubrirostris*	AP004407
	Bythitidae	*Diplacanthopoma_brachysoma*	AP004408
Gadiformes	Macrouridae	*Caelorinchus_kishinouyei*	AP002929
	Macrouridae	***Squalogadus_modificatus***	***gi|109689665***
	Macrouridae	***Trachyrincus_murrayi***	***gi|109689637***
	Moridae	*Physiculus_japonicus*	AP004409
	Bregmacerotidae	*Bregmaceros_nectabanusc*	AP004411
	Gadidae	*Lota_lota*	AP004412
	Gadidae	*Gadus_morhua*	X99772
	Gadidae	***Gadus_ogac***	***gi|225622210***
	Gadidae	***Merlangius_merlangus***	***gb|DQ020496.1|***
	Gadidae	***Pollachius_virens***	***gi|322422606***
	Gadidae	***Pollachius_pollachius***	***gi|322422647***
Lophiiformes	Lophiidae	*Lophius_americanus*	AP004413
	Lophiidae	*Lophius_litulonc*	AP004414
	Chaunacidae	*Chaunax_abei*	AP004415
	Chaunacidae	*Chaunax_tosaensis*	AP004416
	Caulophrynidae	*Caulophryne_pelagica*	AP004417
	Melanocetidae	*Melanocetus_murrayi*	AP004418
Mugiliformes	Mugilidae	*Mugil_cephalus*	AP002930
	Mugilidae	*Crenimugil_crenilabisc*	NC_017884
Atheriniformes	Melanotaenidae	*Melanotaenia_lacustris*	AP004419
	Atherinidae	*Hypoatherina_tsurugae*	AP004420
Beloniformes	Adrianichthyidae	*Oryzias_latipes*	AP004421
	Scomberesocidae	*Cololabis_saira*	AP002932
	Exocoetidae	*Exocoetus_volitans*	AP002933
Cyprinodontiformes	Poeciliidae	*Gambusia_affinis*	AP004422
	Poeciliidae	***Xiphophorus_hellerii***	***gb|FJ226476.1|***
Stephanoberyciformes	Melamphaidae	*Scopelogadus_mizolepisc*	AP002934
	Melamphaidae	*Poromitra_oscitansc*	AP002935
	Rondeletiidae	*Rondeletia_loricata*	AP002937
	Cetomimidae	*Cetostoma_regani*	AP004423
	Cetomimidae	*Danacetichthys_galathenus*	AP002936
	Mirapinnidae	*Parataeniophorus_sp.*	AP004424
Beryciformes	Anoplogastridae	*Anoplogaster_cornuta*	AP004425
	Diretmidae	*Diretmoides_veriginaec*	AP004426
	Diretmidae	*Diretmus_argenteusc*	AP004427
	Anomalopidae	*Anomalops_katoptronc*	AP004428
	Monocentridae	*Monocentris_japonicus*	AP004429
	Trachichthyidae	*Hoplostethus_japonicus*	AP002938
	Berycidae	*Beryx_splendens*	AP002939
	Berycidae	*Beryx_decadactylus*	AP004430
	Berycidae	***Beryx_mollis***	***gi|288904147***
	Holocentridae	*Myripristis_berndti*	AP002940
	Holocentridae	*Ostichthys_japonicus*	AP004431
	Holocentridae	*Sargocentron_rubrum*	AP004432
Zeiformes	Parazenidae	*Parazen_pacificus*	AP004433
	Macrurocyttidae	*Zenion_japonicum*	AP004434
	Zeidae	*Zeus_faber*	AP002941
	Zeidae	*Zenopsis_nebulosus*	AP002942

	Oreosomatidae	*Allocyttus_niger*	AP004435
	Oreosomatidae	*Neocyttus_rhomboidalis*	AP004436
	Caproidae	*Antigonia_capros*	AP002943
Gasterosteiformes	Hypoptychidae	*Hypoptychus_dybowskii*	AP004437
	Gasterosteidae	*Gasterosteus_aculeatusc*	AP002944
	Gasterosteidae	***Gasterosteus_wheatlandi***	***gi|212725466***
	Gasterosteidae	***Pungitius_kaibarae***	***gb|EU332749.1|***
	Gasterosteidae	***Pungitius_pungitius***	***gi|212725480***
	Gasterosteidae	***Culaea_inconstans***	***gi|212725564***
	Gasterosteidae	***Apeltes_quadracus***	***gi|212374495***
	Indostomidae	*Indostomus_paradoxusc*	AP004438
Synbranchiformes	Synbranchidae	*Monopterus_albus*	AP002945
	Synbranchidae	*Synbranchus_marmoratusc*	AP004439
	Mastacembelidae	*Mastacembelus_favus*	AP002946
Scorpaeniformes	Dactylopteridae	*Dactyloptena_tiltoni*	AP004440
	Dactylopteridae	*Dactyloptena_peterseni*	AP002948
	Scorpaenidae	*Helicolenus_hilgendorfi*	AP002947
	Triglidae	*Satyrichthys_amiscus*	AP004441
	Cottidae	*Cottus_reinii*	AP004442
	Cottidae	***Cottus_poecilopus***	***gb|EU332750.1|***
	Cottidae	***Cottus_hangiongensis***	***gb|EU332751.1|***
	Cottidae	***Trachidermus_fasciatus***	***gi|408772194***
	Cottidae	***Clinocottus_analis***	***gi|288903365***
	Cyclopteridae	*Aptocyclus_ventricosusc*	AP004443
Perciformes	Carangidae	*Carangoides_armatus*	AP004444
	Carangidae	*Caranx_melampygus*	AP004445
	Carangidae	***Caranx_ignobilis***	***gi|560877732***
	Carangidae	***Seriola_dumerili***	***gi|375267163***
	Carangidae	***Seriola_lalandi***	***gi|375267149***
	Carangidae	***Trachurus_trachurus***	***gi|38603494***
	Emmelichthyidae	*Emmelichthys_struhsakeri*	AP004446
	Lutjanidae	*Pterocaesio_tile*	AP004447
	Zoarcidae	*Lycodes_toyamensis*	AP004448
	Pholidae	*Enedrias_crassispina*	AP004449
	Blennidae	*Petroscirtes_breviceps*	AP004450
	Blennidae	*Salarias_fasciatus*	AP004451
	Gobiesocidae	*Arcos_sp.*	AP004452
	Gobiesocidae	*Aspasma_minimac*	AP004453
	Rhyacichthyidae	*Rhyacichthys_aspro*	AP004454
	Eleotridae	*Eleotris_acanthopoma*	AP004455
Pleuronectiformes	Paralicthyidae	*Paralicthys_olivaceus*	AB028664
	Pleuronectidae	*Platichthys_bicoloratusc*	AP002951
	Pleuronectidae	***Hippoglossus_hippoglossus***	***gi|154090893***
	Pleuronectidae	***Colistium_nudipinnis***	***gi|584593531***
Tetraodontiformes	Balistidae	*Sufflamen_fraenatus*	AP004456
	Monacanthidae	*Stephanolepis_cirrhiferc*	AP002952

Table S5: Representative Vertebrata species used in figure 2 for comparisons of primer divergence between classes. These are from complete mtDNA sequences in GenBank and two sequence regions were analysed (COI, 16S).

Class	Genus_species	Common name	Accession
Myxini (hagfish)	*Myxine_glutinosa*	Hagfish	gi|12084808
Hyperoartia (lampreys)	*Petromyzon_marinus*	Lamprey	gb|U11880.1|
Sarcopterygii	*Latimeria_chalumnae*	Coelacanth	gi|121308881
(lobe-finned fish)	*Protopterus_annectens*	Lungfish	gb|JX568887.1|
Chondrichthyes	*Squalus_acanthias*	Dogfish shark	gi|5835708
(cartilaginous fishes)	*Carcharodon_carcharias*	Great-white shark	gi|545600999
	Neotrygon_kuhlii	Stingray	gi|525341128
Osteichthyes	*Hippoglossus_hippoglossus*	Halibut	gi|154090893
(bony fishes)	*Culaea_inconstans*	Stickleback	gi|212725564
	Salmo_trutta	Trout	gb|JQ390057.1|
Amphibia (amphibians)	*Bombina_maxima*	Toad	gi|194246883
	Ranodon_sibiricus	Salamander	gi|21450026
	Xenopus_laevis	Frog	gi|5834981
Reptilia (reptiles)	*Boa_constrictor*	Snake	gi|74310505
	Phoenicolacerta_kulzeri	Lizard	gi|215399126
	Crocodylus_porosus	Crocodile	gi|108802842
Aves (birds)	*Accipiter_gentilis*	Hawk	gi|219524160
	Alectura_lathami	Turkey	gi|71658022
	Ambystoma_californiense	Parrot	gi|62161196
Mammalia (mammals)	*Rattus_rattus*	Rat	gi|225622213
	Connochaetes_gnou	Wildebeest	gi|470233679
	Hydrosaurus_amboinensis	Seal	gi|296940265

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