Supplementary Material

Table S1. Geographic locations of eDNA studies collated in our review of the literature.

Country	No. of Studies
USA	44
Canada	11
Japan	11
France	9
Norway	9
Russia	9
Germany	8
China	5
New Zealand	5
Sweden	5
Australia	4
Denmark	4
Switzerland	4
Great Britain	4
Costa Rica	3
Czech Republic	3
Finland	2
Spain	2
Turkey	2
Argentina	1
Austria	1
Ivory Coast	1
Iceland	1
India	1
Israel	1
Italy	1
Kenya	1
Madagascar	1
The Netherlands	1
Panama	1
Philippines	1
Poland	1
Thailand	1
Vietnam	1
French Guyana	1
Taiwan	1
Greenland	1

Table S2. Numbers of environmental DNA studies out of 167 reviewed papers that targeted species that are invasive, rare, or both.

Taxa	Invasive	Rare	Both
Fish	13	3	
Amphibians	1	8	1
Reptiles	2		
Invertebrates	5		1
Plants	1	1	
Fungi	1		
Oomycetes	1		
Total	24	12	2

Table S3. Habitats and sample media analysed in 167 research studies on environmental DNA.

Habitat	Sample Media	Number of studies
Terrestrial	Soil	32
	Sediment	2
	Midden	1
	Permafrost	1
	Stone	1
Freshwater	Water	59
	Sediment	14
	Biofilm	2
Marine	Sediment	18
	Water	17
	Biofilm	1
	Hydrothermal vent	1
	Phytosphere	1
Animal material	Gut contents and faeces	8
	Malaise-trapped invertebrates	4
	Other invertebrate samples	4
	Mammalian tissue	1
Plant material	Bark and twigs	3
	Roots	1
	Leaves	2
Other	Geothermal sediment	3
	Hypersaline pond water	1
	Industrial bioreactor	1
Total[1]		179

[1]The total number of sample media exceeds the number of studies due to the analysis of multiple sample media in a number of studies.

Table S4. Summary of extraction kits used for the analysis of eDNA from different sample media collated in our review of the literature. Manual methods refer to DNA extraction approaches that do not use commercially available kits. Extraction and media combinations used in more than ten studies are shown in bold font.

DNA extraction kit	Media from which DNA is extracted							
	Water	**Soils and sediments**	**Animal tissue**	**Plant material**	**Gut contents and faeces**	**Biofilm**	**Other substrates**	**Total**
DNeasy Blood & Tissue (Qiagen)	**28**		4	1	2			35
DNeasy PowerSoil (Qiagen)	2	**29**		1				32
DNeasy PowerWater (Qiagen)	**13**							13
QIAmp (Qiagen)	4	2	2	2	1			11
DNeasy PowerMax (Qiagen)	2	8						10
DNeasy Ultraclean Soil (Mo BIO)	2	5	1					8
FastDNA Spin (MP Biomedicals)	1	6					1	8
Nucleospin (Macherey-Nagel)		4				1		5
DNeasy Plant (Qiagen)				3				3
EZNA Soil (Omega Biotek)		1		1				2
Gentra Puregene Tissue (Qiagen)	2							2
Animal tissue kit (Mole Genetics)	1							1
Axygen Biosciences					1			1
DNA Isolation Kit for Water (Epicentre Biotechnologies)	1							1
EZNA Plant (Omega Biotek)	1							1
FastPrep Lysing Matrix (MP Biomedicals)		1						1
GeneMatrix Stool DNA (EURx)			1					1
ISOPLANT II (Nippon Gene)					1			1
DNeasy PowerBiofilm (Qiagen)						1		1
ISOIL (Nippon Gene)		1						1
ZR Soil Microbe Miniprep (Zymo Research)		1						1
Manual methods	**25**	**12**	1		2	1	2	43
Unclear		3			1			4
Total	82	73	9	8	8	3	3	186

Table S5. Primers used to amplify DNA barcodes from prokaryotes.

Example citation	Primer pair	Region amplified	Specificity to prokaryotes	Inclusivity among prokaryotes
Parada et al. 2015	515F/806RB	V3/V4; 301 bp	Chloroplast/mitochondrial DNA and a few eukaryotic 18S rRNA genes	Most bacterial 16S rRNA gene sequences detected
Parada et al. 2015	515F/926R	V3/V4; 421 bp	Chloroplast/mitochondrial DNA detected Many eukaryotic 18S rRNA genes	Most bacterial and archaeal 16S rRNA gene sequences detected
Klindworth et al. 2013	S-D-Bact-0341-b-5-17/S-D-Bact-0785-a-A-21	V3/V4; 460 bp	Chloroplast/ mitochondrial DNA detected	Mainly bacterial 16S rRNA gene sequences detected

Appendix S6. PCR protocol for prokaryote DNA.

The following protocol is partly extracted from www.earthmicrobiome.org/emp-standard-protocols/16s/. Users of this approach are directed to the Earth Microbiome project (EMP) for more information and may refer to Caporaso et al. (2012) regarding use of the Illumina platform to sequence DNA amplified using the Bacteria/Archaea specific primers 515f/806r.

An important distinction between the protocol described by the EMP and the protocol provided below is that here we recommend the use of a two-step PCR approach for multiplex indexing. This means that only two primers (one forward and one reverse) are normally required for amplification, rather than a different primer combination for every sample if using pre-barcoded PCR indexing primers (as recommended in the current EMP protocol). The recommended primer pair is shown below.

Updated primers

The 515f-806r primer pair originally used in Caporaso et al. (2012) have been modified by Apprill et al. (2015) to remove known biases against Crenarachaeota/Thaumarchaeota (515f modification) and the marine and freshwater Alphaproteobacterial clade SAR11 (806r modification).

515f - 5' **GTGYCAGCMGCCGCGGTAA** 3'
806RB - 5' **GGACTACNVGGGTWTCTAAT** 3'

Illumina Nextera adaptor overhang sequences (underlined) are added to the gene-specific primer sequences (bold) following the guidelines presented in Illumina (undated).

515f 5'<u>TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAG</u> **GTGYCAGCMGCCGCGGTAA** 3'
806RB 5'<u>GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAG</u> **GGACTACNVGGGTWTCTAAT** 3'

Note: The approach outlined here is for sequencing using an Illumina MiSeq DNA sequencing platform. Sequencing on MiniSeq, NextSeq and HiSeq 300/400 systems requires the use of a different dual indexing workflow.

Conditions for 96 well thermocycler

1. 94°C 3 minutes
2. 94°C 45 seconds
3. 55°C 45 seconds
4. 72°C 60 seconds
5. Repeat steps 2–4 30 times
6. 72°C 10 minutes
7. 4°C HOLD

Additional protocol details

Samples should usually be amplified in triplicate, meaning each sample will be amplified in three replicate 25 µL PCR reactions. DO NOT combine amplicons from different samples at this point.

Following amplification, we recommend that the approach of Illumina (undated) is followed. Briefly, this includes the following steps. First, purify each amplicon mixture using AMPure XP beads, following the manufacturer's recommendations. Second, verify the size and purity of PCR products using an Agilent Bioanalyser 1000 DNA chip. The expected band size for 515F/806RB is roughly 300–350 bp. Third, attach dual multiplex barcode indices and Illumina sequencing adapters using the Nextera XT Index kit. Finally, additional purifications followed by quantification, normalisation and sample pooling steps are carried out as detailed in Illumina (undated), before submission for DNA sequencing.

If available, the Pippin Prep (Sage Science) can be used as an alternative to purification by AMPure XP beads, and Labchip (PerkinElmer) can be used in the place of the Bioanalyzer and quantification methods. Where large numbers of samples require purification, use of 96 well SequalPrep™ Normalisation Plate kits (Invitrogen Ltd.) or similar product may be considered. Each well of a SequalPrep Normalisation plate can bind and elute ~25 ng of PCR amplicon via a limited binding capacity solid phase. Additional DNA concentration normalisation is not required as excess DNA is washed away. However, for successful normalisation at least 250 ng product is required per well. Harris et al. (2010) found this quantitative binding approach sufficient to process very large multiplex pools with hundreds and perhaps thousands of amplicons included. However, we are currently unable to recommend the use of SequalPrep kits to process small numbers of samples since the kit is currently available in a 10 x 96-well plate format with a shelf life of only six months. Regardless of the approach used, it is important to document the chosen method.

Note: The addition of multiplex barcode indices and sequencing adaptors and subsequent steps is offered by some DNA sequencing service providers, such as New Zealand Genomics Limited.

Table S7. Primers used to amplify DNA barcodes from eukarya.

Example citation	Primer pair	Region amplified	Specificity to eukaryotes	Inclusivity among eukaryotes
Drummond et al. 2015	#3, #5RC; Machida & Knowlton 2012	18S V7–V8; 330 bp	A limited number of Bacteria and Archaea detected	Fungi, metazoans, protists, plants detected
Ramirez et al. 2014	1391F, EukBr; Amaral-Zettler et al. 2009	18S V9; 150 bp		
Bates et al. 2013	F515, R1119; Bates et al. 2012	18S V4–V5; 600 bp		
Meadow & Zabinski 2012	F-907, R-1428; Troedsson et al. 2008	18S V5–V7; 535 bp		74% of Silva database sequences
Fonseca et al. 2010; Creer et al. 2010	SSUF04, SSUR22; Creer et al. (2010)	18S V1–V2 450 bp		'Meiofauna'
Creer et al. 2010	NF1, 18Sr2b; Porazinska et al. 2009	18S V7–V8; 320 bp		'Meiofauna'. Possibly omits major fungal taxa.
Chariton et al. 2010	All18SF, All18SR; Chariton et al. 2010	18S V7; 140 bp		
Nolte et al. 2010	Unnamed primers; Nolte et al. 2010	18S V3; 180–200 bp		
Stoeck et al. 2009	1389F, 1390F, 1510R; Amaral-Zettler et al. 2009	18S V9; 150 bp	13% Archaea, Bacteria and other non rRNA gene sequences detected	All major eukaryote taxa detected.

Appendix S8. PCR Protocol for Eukaryote DNA.

The following protocol is partly extracted from Machida and Knowlton (2012). An important distinction between the protocol described by Machida and Knowlton (2012) and later users of these primers (Drummond et al. 2015) is that the latter used a two-step PCR approach for multiplex indexing, which is the approach that we recommend. This means that only two primers (one forward and one reverse) are normally required for amplification of multiple samples.

18S gene-specific primers:

#3 - 5' **GYGGTGCATGGCCGTTSKTRGTT** 3'

#5RC - 5' **GTGTGYACAAAGGBCAGGGAC** 3'

Illumina Nextera adaptor overhang sequences (underlined) are added to the gene-specific primer sequences (bold) following the guidelines presented in Illumina (undated).

#3 - 5'
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
GYGGTGCATGGCCGTTSKTRGTT 3'

#5RC - 5'
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
GTGTGYACAAAGGBCAGGGAC 3'

Note: The approach outlined here is for sequencing using an Illumina MiSeq DNA sequencing platform. Sequencing on MiniSeq, NextSeq and HiSeq 300/400 systems requires the use of a different dual indexing workflow.

Conditions for 96 well thermocycler

1. 94°C 3 minutes
2. 94°C 30 seconds
3. 58°C 30 seconds
4. 72°C 45 seconds
5. Repeat steps 2–4 30 times
6. 72°C 10 minutes
7. 4°C HOLD

Additional protocol details

Samples should usually be amplified in triplicate, meaning each sample will be amplified in three replicate 25 µL PCR reactions. DO NOT combine amplicons from different samples at this point.

Following amplification, we recommend that the approach of Illumina (undated) is followed. Briefly, this includes the following steps. First, purify each pooled amplicon using AMPure XP beads, following the manufacturer's recommendations. Second, verify the size and purity of PCR products using an Agilent Bioanalyser 1000 DNA chip. The expected band size for #3/#5RC is roughly 350 bp. Third, attach dual multiplex barcode indices and Illumina sequencing adapters using the Nextera XT Index kit. Finally, additional purifications followed by quantification, normalisation and sample pooling steps are carried out as detailed in Illumina (undated), before submission for DNA sequencing.

If available, the Pippin Prep (Sage Science) can be used as an alternative to purification by AMPure XP beads, and Labchip (PerkinElmer) can be used in the place of the Bioanalyzer and quantification methods. Where large numbers of samples require purification, use of 96 well SequalPrep™ Normalisation Plate kits (Invitrogen Ltd.) or similar product may be considered. Each well of a SequalPrep Normalisation plate can bind and elute ~25 ng of PCR amplicon via a limited binding capacity solid phase. Additional DNA concentration normalisation is not required as excess DNA is washed away.

For successful normalisation however at least 250 ng product is required per well. Harris et al. (2010) found this quantitative binding approach sufficient to process very large multiplex pools with hundreds and perhaps thousands of amplicons included. However, we are currently unable to recommend the use of SequalPrep kits to process small numbers of samples since the kit is currently available in a 10 x 96-well plate format with a shelf life of only six months. Regardless of the approach used, it is important to document the chosen method.

Note: The addition of multiplex barcode indices and sequencing adaptors and subsequent steps is offered by some DNA sequencing service providers, such as New Zealand Genomics Limited.

Table S9. Primers used to amplify DNA barcodes from Chromista.

Example citation	Primer pair	Region amplified	Specificity to Chromista	Inclusivity among Chromista
Lentendu et al. 2014	Cer2F–1R; Lentendu et al. 2014	18S rRNA gene (average of 560 bp)	Unclear	Targets only Cercozoa
Arjen de Groot et al. 2016	25 F–1256R (Bass & Cavalier-Smith 2004) followed by 1256R and PreV4	18S rRNA gene (*500 –700 bp)	Not demonstrated	Targets only Cercozoa.
Vannini et al. 2013	ITS6+7; Cooke et al. 2000	ITS1 (~300 bp)		Mostly found Phytophthora and a few Pythium
Coince et al. 2013; Sapkota & Nicolaisen 2015	Semi-nested: ITS6+ITS4 (White et al. 1990); followed by ITS6+7 (Cooke et al. 2000)	ITS1 (350–400 bp)	Highly variable from >90% non-target (Coince et al. 2013) to <10% non-target amplification	A few mismatches to some Aphanomyces

*these primers have been used for 454 pyrosequencing and are not currently suitable for use on sequencing platforms such as the Illumina MiSeq because of the long fragment length.

Appendix S10. PCR protocol for Chromista DNA.

The following protocol is partly extracted from Sapkota and Nicolaisen (2015). DNA amplification is completed using a semi-nested approach to target the ITS1 intergene region. The ITS1 region is first amplified using ITS4 and ITS6 primers for 15 cycles of PCR. This PCR product is diluted 1:10 before being used as template for a second round of PCR using the primers ITS6 and ITS7 for 25 cycles (Cooke et al. 2000).

Key reference: Sapkota R, Nicolaisen M 2015. An improved high throughput sequencing method for studying oomycete communities. Journal of Microbiological Methods 110: 33–39.

An important distinction between the protocol described by Sapkota & Nicolaisen (2015), which was designed for use with a 454 sequencing platform is that here we recommend the use of a two-step PCR approach for multiplex indexing on the Illumina MiSeq platform. This means that only two primers (one forward and one reverse) are normally required for amplification, rather than a different primer combination for every sample if using barcoded PCR primers.

Primers

Semi-Nested PCR 1:
ITS6: 5′ GAAGGTGAAGTCGTAACAAGG 3′
ITS4: 5′ TCCTCCGCTTATTGATATGC 3′
Semi-Nested PCR 2:
ITS6: 5′ GAAGGTGAAGTCGTAACAAGG C 3′
ITS7: 5′ AGCGTTCTTCATCGATGTGC 3′

Illumina Nextera adaptor overhang sequences (underlined) are added to the gene specific primer sequences (bold) used in the final round of PCR, following the guidelines presented in Illumina (undated).

Semi-Nested PCR 1:
1TS6: 5′ GAAGGTGAAGTCGTAACAAGG 3′
ITS4: 5′ TCCTCCGCTTATTGATATGC 3′
Semi-Nested PCR 2:
ITS6adapt: 5′
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
GAAGGTGAAGTCGTAACAAGG 3′.
ITS7adapt: 5′
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
AGCGTTCTTCATCGATGTGC 3′

Note: The approach outlined here is for sequencing using an Illumina MiSeq DNA sequencing platform. Sequencing on MiniSeq, NextSeq and HiSeq 300/400 systems requires the use of a different dual indexing workflow.

Conditions for 96 well thermocycler

Nested PCR 1:
For use with primers ITS6 and ITS4
1. 94°C 3 minutes
2. 94°C 30 seconds
3. 55°C 30seconds
4. 72°C 60 seconds
5. Repeat steps 2–4 15 times
6. 72°C 10 minutes
7. 4°C HOLD

Nested PCR 2:
For use with primers ITS6adapt and ITS7adapt, a 1:10 dilution of the PCR product generated from 'semi-nested PCR 1' is used as the DNA template.

1. 94°C 3 minutes
2. 94°C 30 seconds
3. 59°C 30seconds
4. 72°C 60 seconds
5. Repeat steps 2–4 25 times
6. 72°C 10 minutes
7. 4°C HOLD

Additional protocol details

Samples should usually be amplified in triplicate, meaning each sample will be amplified in three replicate 25 μL PCR reactions. DO NOT combine amplicons from different samples at this point.

Following amplification, we recommend that the approach of Illumina (undated) is followed. Briefly, this includes the following steps. First, purify each pooled amplicon using AMPure XP beads, following the manufacturer's recommendations. Second, verify the size and purity of PCR products using an Agilent Bioanalyser 1000 DNA chip. The expected band size for ITS6adapt/ITSF7adapt is roughly 350–450 bp. Third, attach dual multiplex barcode indices and Illumina sequencing adapters using the Nextera XT Index kit. Finally, additional purifications followed by quantification, normalisation and sample pooling steps are carried out as detailed in Illumina (undated), before submission for DNA sequencing.

If available, the Pippin Prep (Sage Science) can be used as an alternative to purification by AMPure XP beads, and Labchip (PerkinElmer) can be used in the place of the Bioanalyzer and quantification methods. Where large numbers of samples require purification, use of 96 well SequalPrep™ Normalisation Plate kits (Invitrogen Ltd.) or similar product may be considered. Each well of a SequalPrep Normalisation plate can bind and elute ~25 ng of PCR amplicon via a limited binding capacity solid phase. Additional DNA concentration normalisation is not required as excess DNA is washed away. For successful normalisation however at least 250 ng product is required per well. Harris et al. (2010) found this quantitative binding approach sufficient to process very large multiplex pools with hundreds and perhaps thousands of amplicons included. However, we are currently unable to recommend the use of SequalPrep kits to process small numbers of samples since the kit is currently available in a 10 x 96-well plate format with a shelf life of only six months. Regardless of the approach used, it is important to document the chosen method.

Note: The addition of multiplex barcode indices and sequencing adaptors and subsequent steps is offered by some DNA sequencing service providers, such as New Zealand Genomics Limited.

Appendix S11. PCR protocol for plant DNA.

These reaction conditions are specified by Chen et al. (2010). The number of PCR cycles may be reduced if sufficient product can be obtained with less, thereby minimising the likelihood of PCR bias.

Primers

S2F: ATGCGATACTTGGTGTGAAT

S3R: GACGCTTCTCCAGACTACAAT

Illumina Nextera adaptor overhang sequences (underlined) are added to the gene-specific primer sequences (bold) following the guidelines presented in Illumina (undated).

S2F:
5'<u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u>
ATGCGATACTTGGTGTGAAT 3'
S3R
5'<u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG</u>
GACGCTTCTCCAGACTACAAT 3'

Note: The approach outlined here is for sequencing using an Illumina MiSeq DNA sequencing platform. Sequencing on MiniSeq, NextSeq and HiSeq 300/400 systems requires the use of a different dual indexing workflow.

1. 94° 5 minutes
2. 94° 30 seconds
3. 56° 30 seconds
4. 72° 45 seconds
5. Repeat steps 2–4 40 times
6. 72° 10 minutes
7. 4°C HOLD

Additional protocol details

Samples should usually be amplified in triplicate, meaning each sample will be amplified in three replicate 25 μL PCR reactions. DO NOT combine amplicons from different samples at this point.

Following amplification, we recommend that the approach of Illumina (undated) is followed. Briefly, this includes the following steps. First, purify each pooled amplicon using AMPure XP beads, following the manufacturer's recommendations. Second, verify the size and purity of PCR products using an Agilent Bioanalyser 1000 DNA chip. The expected band size for S2F/S3R is roughly 160–320 bp. Third, attach dual multiplex barcode indices and Illumina sequencing adapters using the Nextera XT Index kit. Finally, additional purifications followed by quantification, normalisation and sample pooling steps are carried out as detailed in Illumina (undated), before submission for DNA sequencing.

If available, the Pippin Prep (Sage Science) can be used as an alternative to purification by AMPure XP beads, and Labchip (PerkinElmer) can be used in the place of the Bioanalyzer and quantification methods. Where large numbers of samples require purification, use of 96 well SequalPrep™ Normalisation Plate kits (Invitrogen Ltd.) or similar product may be considered. Each well of a SequalPrep Normalisation plate can bind and elute ~25 ng of PCR amplicon via a limited binding capacity solid phase. Additional DNA concentration normalisation is not required as excess DNA is washed away. However, for successful normalisation at least 250 ng product is required per well. Harris et al. (2010) found this quantitative binding approach sufficient to process very large multiplex pools with hundreds and perhaps thousands of amplicons included. However, we are currently unable to recommend the use of SequalPrep kits to process small numbers of samples since the kit is currently available in a 10 x 96-well plate format with a shelf life of only 6 months. Regardless of the approach used, it is important to document the chosen method.

Note: The addition of multiplex barcode indices and sequencing adaptors and subsequent steps is offered by some DNA sequencing service providers, such as New Zealand Genomics Limited.

Table S12. Primers used to amplify DNA barcodes from fungi.

Example citation	Primer pair	Region amplified	Specificity to fungi	Inclusivity among fungi
McGuire et al. 2013	ITS1F+ITS2; Gardes & Bruns 1993	ITS1 (~350 bp)	ITS1F highly specific to fungi (Bellemain et al. 2010)	Many mismatches to fungi in ITS1F (Bellemain et al. 2010)
Schmidt et al. 2013	ITS1FI2 (Schmidt et al. 2013) + ITS2 (Gardes & Bruns 1993)	ITS1 (~350 bp)	Unknown	Unknown
Clemmensen et al. 2013	gITS7 (Ihrmark et al. 2012) + ITS4 (White et al. 1990)	ITS2 (184 –396 bp)	15% of sequences non-target in Clemmensen et al. (2013)	
Clemmensen et al. 2013	*fITS9 (Ihrmark et al. 2012) + ITS4 (White et al. 1990)	ITS2 (228–440)	2% of sequences non-target in Clemmensen et al. (2013)	

*The alternative primer fITS7 differs to gITS7 in one base pair, excluding more plants but also some fungi (Mucormycotina; Bokulich & Mills, 2013).

Appendix S13. PCR protocol for fungal DNA.

The following protocol is partly extracted from Ihrmark et al. (2012). An important distinction between the protocol described by Ihrmark et al. (2012) is that here we recommend the use of use of a two-step PCR approach for multiplex indexing using the Illumina MiSeq DNA sequencing platform. This means that only two primers (one forward and one reverse) are normally required for amplification, rather than a different primer combination for every sample if using barcoded PCR primers. We recommend fITS7 for most samples where plant DNA is abundant, with the caveat that gITS7 may be preferable where plant DNA is low and greater inclusivity of fungi is a priority. This is suggested as Ihrmark et al. (2012) identified that the single base mismatch between the forward primers fITS7 and gITS7 allows primer gITS7 to readily amplify plant DNA. ITS4 is recommended as the reverse primer.

Primers

fITS7 5' GTGARTCATCGAATCTTTG 3' (Ihrmark et al. 2012)
gITS7 5' GTGARTCATCGARTCTTTG 3' (Ihrmark et al. 2012)
ITS4 5' TCCTCCGCTTATTGATATGC 3' (White et al. 1990)

Illumina Nextera adaptor overhang sequences (underlined) are added to the gene-specific primer sequences (bold) following the guidelines presented in Illumina (undated).

fITS7 5'CGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGARTCATCGAATCTTTG** 3'
gITS7 5'CGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGARTCATCGARTCTTTG** 3'
ITS4 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TCCTCCGCTTATTGATATGC** 3'

Note: The approach outlined here is for sequencing using an Illumina MiSeq DNA sequencing platform. Sequencing on MiniSeq, NextSeq and HiSeq 300/400 systems requires the use of a different dual indexing workflow.

Conditions for 96 well thermocycler:

1. 94°C 5 minutes
2. 94°C 30 seconds
3. 52°C 30 seconds
4. 72°C 45 seconds
5. Repeat steps 2-4 30 times
6. 72°C 10 minutes
7. 4°C HOLD

Note**:** Thermocycling conditions are based on Ihrmark et al. (2012), but with modifications to the annealing temperature to cater for the single base degeneracy and an longer extension time.

Additional protocol details

Samples should usually be amplified in triplicate, meaning each sample will be amplified in three replicate 25 µL PCR reactions. DO NOT combine amplicons from different samples at this point.

Following amplification, we recommend that the approach of Illumina (undated) is followed. Briefly, this includes the following steps. First, purify each pooled amplicon using AMPure XP beads, following the manufacturer's recommendations. Second, verify the size and purity of PCR products using an Agilent Bioanalyser 1000 DNA chip. The expected band size for (fITS7 or gITS7) / ITS4 varies between 122–245 bp. Third, attach dual multiplex barcode indices and Illumina sequencing adapters using the Nextera XT Index kit. Finally, additional purifications followed by quantification, normalisation and sample pooling steps are carried out as detailed in Illumina (undated), before submission for DNA sequencing.

If available, the Pippin Prep (Sage Science) can be used as an alternative to purification by AMPure XP beads, and Labchip (PerkinElmer) can be used in the place of the Bioanalyzer and quantification methods. Where large numbers of samples require purification, use of 96 well SequalPrep™ Normalisation Plate kits (Invitrogen Ltd.) or similar product may be considered. Each well of a SequalPrep Normalisation plate can bind and elute ~25 ng of PCR amplicon via a limited binding capacity solid phase. Additional DNA concentration normalisation is not required as excess DNA is washed away. For successful normalisation however at least 250 ng product is required per well. Harris et al. (2010) found this quantitative binding approach sufficient to process very large multiplex pools with hundreds and perhaps thousands of amplicons

included. However, we are currently unable to recommend the use of SequalPrep kits to process small numbers of samples since the kit is currently available in a 10 x 96-well plate format with a shelf life of only 6 months. Regardless of the approach used, it is important to document the chosen method.

Note: The addition of multiplex barcode indices and sequencing adaptors and subsequent steps is offered by some DNA sequencing service providers, such as New Zealand Genomics Limited.

Table S14. Primers used to amplify DNA barcodes from Glomeromycota.

Example citation	Primer pair	Region amplified	Specificity to Glomeromycota
Opik et al. 2009; *Dumbrell et al. 2011	NS31 (Simon et al. 1992) /AM1 (Helgason et al. 1998)	18S rRNA gene (~260 bp)	In some cases get high amplification of plant DNA (Van Geel et al. 2014)
Davison et al. 2012, 2015; Martinez-Garcia et al. 2015	†NS31 (Simon et al. 1992) / AML2 (Lee et al. 2008)	18S rRNA gene (~560 bp)	
Lumini et al. 2010; Cui et al. 2016	‡AMV4.5NF (Lumini et al. 2010) / AMDGR (Sato et al. 2005)	18S rRNA gene (~258 bp)	50–76% of sequences Glomeromycota

*Dumbrell et al. (2011) use a semi-nested protocol, replacing NS31 with an internal primer to shorten read lengths and add multiplex identifiers.
†Sometimes used in a nested PCR (e.g. Liu et al. 2011) but can be used directly.
‡Highly biased towards Glomeraceae at expense of other families (Van Geel et al. 2014).

Table S15. Primers used to amplify COI DNA barcodes from animals.

Example citation	Primer pair	Region amplified	Specificity to animals	Inclusivity among animals
Elbrecht & Leese 2015	LCO1490 / HCO2198; Folmer et al. 1994	COI (658 bp)		Animals
Yu et al. 2012	Fol-degen-for / Fol-degen-rev; Yu et al. 2012	COI (658 bp)	Amplifies bacteria from soil DNA	Arthropods
Brandon-Mong et al. 2015	LepF1 (Hebert et al. 2004) / MlepF1-Rev (Brandon-Mong et al. 2015)	COI (450 bp)		Insects (98% amplification success when tested on 80 spp. of insect and close allies).
Leray et al. 2013	mlCOIintF / jgHCO2198; Leray et al. 2013; Geller et al. 2013	COI (313 bp)	Amplifies fungi (mainly Ascomycota) as well as animals from soil DNA	Animals
Gibson et al. 2014	11 different primers with degeneracy: ArF1, 2, 3, 4, 5, 10 / ArR2, 3, 5, 6, 7, 9.	COI (310 bp)		Arthropods
Salinas-Ramos et al. 2015	ZBJ-ArtF1c/ZBJ-ArtR2c; Zeale et al. 2011	COI (175 bp)		Insects
Brandon-Mong et al. 2015	Uni-MinibarR1 / UniMinibarF1; Meusnier et al. 2008	COI (130 bp)		Developed for Eukarya (63% amplification success when tested on 80 spp. of insect and close allies.)

Table S16. Primers used to amplify mitochondrial 16S rRNA gene barcodes from animals.

Example citation	Primer pair	Region amplified / target length	Specificity to animals	Inclusivity among animals
Kartzinel & Pringle 2015	IN16STK-F/ IN16STK-1R; Kartzinel & Pringle 2015	16S rRNA gene (107 bp)	Unknown	Arthropods
Pansu et al. 2015	ewD/ewE; Bienert et al. 2012	16S rRNA gene (c. 70 bp)	Unknown	Earthworms
Boyer et al. 2013	nz worm 16S int F/nz worm 16S int R; Boyer et al. 2012	16S rRNA gene (134 bp)	Amplifies NZ native earthworms but not exotic earthworms or molluscs	Amplifies all NZ native earthworms tested

Appendix S17. PCR protocol for animal DNA.

The following protocol is partly extracted from Leray et al. (2013), which was designed for use with 454 sequencing platforms. Here, we recommend the use of a two-step PCR approach for multiplex indexing on the Illumina MiSeq platform. This means that only two primers (one forward and one reverse) are normally required for amplification, rather than a different primer combination for every sample if using barcoded PCR primers.

Primers

Forward mlCOIintF:
[GGWACWGGWTGAACWGTWTAYCCYCC]
Reverse jgHCO2198:
[TAIACYTCIGGRTGICCRAARAAYCA]

Note: Only the animal specific primer sequences are shown above. Additional primer sequence including primer barcode and sequence specific sequence are required for metabarcoded DNA sequences.

Illumina Nextera adaptor overhang sequences (underlined) are added to the gene-specific primer sequences (bold) following the guidelines presented in Illumina (undated).

mlCOIintF 5'
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
GGWACWGGWTGAACWGTWTAYCCYCC 3'
jgHCO2198 5'
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
TAIACYTCIGGRTGICCRAARAAYCA 3'

Conditions for 96 well thermocycler

Because of the high degeneracy of these primers it is recommended to follow a touchdown PCR approach, to minimise non-specific amplification as per the protocol proposed by Leray et al. (2013)

1. 95°C 3 minutes
2. 95°C 30 seconds
3. 62°C 60 seconds
4. 72°C 60 seconds
5. Repeat steps 2–4, 16 times

Followed by

6. 95°C 30 seconds
7. 46°C 30 seconds
8. 72°C 60 seconds
9. Repeat steps 6–8, 25 times
10. 72°C 10 minutes
11. 4°C HOLD

Additional protocol details

Samples should usually be amplified in triplicate, meaning each sample will be amplified in three replicate 25 μL PCR reactions. DO NOT combine amplicons from different samples at this point.

Following amplification, we recommend that the approach of Illumina (undated) is followed. Briefly, this includes the following steps. First, purify each pooled amplicon using AMPure XP beads, following the manufacturer's recommendations. Second, verify the size and purity of PCR products using an Agilent Bioanalyser 1000 DNA chip. The expected band size for mlCOIinfF/jgHCO2198 is roughly 300 bp. Third, attach dual multiplex barcode indices and Illumina sequencing adapters using the Nextera XT Index kit. Finally, additional purifications followed by quantification, normalisation and sample pooling steps are carried out as detailed in Illumina (undated), before submission for DNA sequencing.

If available, the Pippin Prep (Sage Science) can be used as an alternative to purification by AMPure XP beads, and Labchip (PerkinElmer) can be used in the place of the Bioanalyzer and quantification methods. Where large numbers of samples require purification, use of 96 well SequalPrep™ Normalisation Plate kits (Invitrogen Ltd.) or similar product may be considered. Each well of a SequalPrep Normalisation plate can bind and elute ~25 ng of PCR amplicon via a limited binding capacity solid phase. Additional DNA concentration normalisation is not required as excess DNA is washed away. For successful normalisation however at least 250 ng product is required per well. Harris et al. (2010) found this quantitative binding approach sufficient to process very large multiplex pools with hundreds and perhaps thousands of amplicons included. However, we are currently unable to recommend the use of SequalPrep kits to process small numbers of samples since the kit is currently available in a 10 x 96-well plate format with a shelf life of only 6 months. Regardless of the approach used, it is important to document the chosen method.

Note: The addition of multiplex barcode indices and sequencing adaptors and subsequent steps is offered by some DNA sequencing service providers, such as New Zealand Genomics Limited.

Table S18. Primers used to amplify DNA barcodes from terrestrial vertebrates.

Taxa	Example citation	Primer pair	Region amplified
Vertebrates	Riaz et al. 2011	12SV5F (TAGAACAGGCTCCTCTAG) 12SV5R (TTAGATACCCCACTATGC)	12S rRNA gene (73–110 bp)
Amphibians	As above	As above	As above
Reptiles	As above	As above	As above
Birds	Haile et al. 2007	12Se (CCCACCTAGAGGAGCCTGTTC) 12Sh (CCTTGACCTGTCTTGTTAGC)	12S rRNA gene (~125 bp)
	Oskam et al. 2010	12sf5 (CTAACAAGACAGGTCAAGGTAT) 12sr4 (CCTATTTTACTGCTAAATCCG)	12S rRNA gene (~125 bp)
	Oskam et al. 2010	12sa (CTGGGATTAGATACCCCACTAT) 12sh (CCTTGACCTGTCTTGTTAGC)	12S rRNA gene (~250 bp)
	Patel et al. 2010	COI primers	COI (130–328 bp)
Mammals	Andersen et al. 2012	16S A&M Fv2 69 (CCCCGAAACCAGACGAGCTA) 16S A&M Rv2 short (TCACTATTTTGCNACATAGA)	16S rRNA gene *(23–31 bp)
	Boessenkool et al. 2012	16Smam1 (CGGTTGGGGTGACCTCGGA) 16Smam2 (GCTGTTATCCCTAGGGTAACT)	16S rRNA gene (~120 bp)
	Ushio et al. 2016	MiMammal-U 12S rRNA gene primers	12S rRNA gene (~170 bp)

*The short length of this DNA fragment makes the use of these primers perhaps best suited for the analysis of low quality, or degraded DNA.

Table S19. Primers used to amplify DNA barcodes from fish.

Example citation	Primer pair	Region amplified	Sequencing platform
Miya et al. 2015	*MiFish-U/E	12S rRNA gene (163–185 bp)	Illumina MiSeq
Valentini et al. 2016	†teleo_F, L1848, ACACCGCCCGTCACTCT teleo_R, H1913, CTTCCGGTACACTTACCATG	12S rRNA gene (65 bp)	Illumina
Port et al. 2016	‡F-5′ ACTGGGATTAGATACCCC and R-5′ TAGAACAGGCTCCTCTAG	12S rRNA gene (106 bp)	Illumina
Kelly et al. 2014	AKA F1 and R1		
Thomsen et al. 2012	Fish2bCBR GATGGCGTAGGCAAACAAGA Fish2CBL ACAACTTCACCCCTGCAAAC	cytochrome *b* (80 bp)	454
Thomsen et al. 2012	Fish2degCBL ACAACTTCACCCCTGCRAAY Fish2CBR GATGGCGTAGGCAAATAGG	cytochrome *b* (80 bp)	454
Deagle et al. 2009	Chord_16S_F_TagB, Primer A GATCGAGAAGACCCTRTGGAGCT Ceph _16S_F Primer A, GACGAGAAGACCCTAWTGAGCT Chord_16S_R Primer B, GGATTGCGCTGTTATCCCT Ceph _16S_R Primer B AAATTACGCTGTTATCCCT	16S rRNA gene (~260–310 bp)	454
Deagle et al. 2009	Chord_16S_F_TagA Primer A ATGCGAGAAGACCCTRTGGAGCT Chord_16 s_R_Short Primer B CCTNGGTCGCCCCAAC	16S rRNA gene (~150 bp)	454
Deagle et al. 2009	Primer B AGAGGTGAAATTSTTGGAYCG	Nuclear 18S (285 bp)	454
	Primer A CCTTTAAGTTTCAGCTTTGCA		

*resolves many species but not some closely related species.
†lower resolution than Miya et al. (2015) primers.
‡low false negative rate for bony fish but high false negative rate for cartilaginous fish.

Appendix S20. PCR protocol for fish DNA.

The following protocol is partly extracted from Miya et al. (2015). An important distinction between the protocol described by Miya et al. (2015) and the protocol provided below is that that former includes six random hexamers in their primer design to enhance cluster separation on MiSeq flowcells during initial base calibrations. We have chosen not to include these six hexamers in the primer design shown below to encourage standard approaches for DNA amplification and sequencing to be adopted in multi-taxa studies.

Users of this protocol should refer to and cite the following article regarding use of the Illumina platform to sequence DNA amplified using the fish specific primers MiFish-U-F and MiFish-U-R:

Miya M, Sato Y, Fukunaga T, Sado T, Poulsen JY, Sato K, Minamoto T, Yamamoto S, Yamanaka H, Araki H, Kondoh M, Iwasaki W 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. Royal Society Open Science 2: 150088.

Primers

The MiFish-U-F and MiFish-U-R primer pair are based on the design of Miya et al. (2015) and configured to be used with the Illumina platform.

MiFish-U-F: 5' GTCGGTAAAACTCGTGCCAGC 3'
MiFish-U-R: 5'
CATAGTGGGGTATCTAATCCCAGTTTG 3'

Illumina Nextera adaptor overhang sequences (underlined) are added to the gene-specific primer sequences (bold) following the guidelines presented in Illumina (undated).

MiFish-U-F
5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
GTCGGTAAAACTCGTGCCAGC 3'
MiFish-U-R
5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
CATAGTGGGGTATCTAATCCCAGTTTG 3'

Note: The approach outlined here is for sequencing using an Illumina MiSeq DNA sequencing platform. Sequencing on MiniSeq, NextSeq and HiSeq 300/400 systems requires the use of a different dual indexing workflow.

Conditions for 96 well thermocycler

1. 95°C 3 minutes
2. 98°C 20 seconds
3. 65°C 15 seconds
4. 72°C 15 seconds
5. Repeat steps 2–4 35 times
6. 72°C 5 minutes
7. 4°C HOLD

Note: The protocol we outline differs slightly from the approach used by Miya et al. (2015). Therefore, further testing of these amplification conditions outlines above is recommended.

Additional protocol details

Samples should usually be amplified in triplicate, meaning each sample will be amplified in three replicate 25 μL PCR reactions. DO NOT combine amplicons from different samples at this point.

Following amplification, we recommend that the approach of Illumina (undated) is followed. Briefly, this includes the following steps. First, purify each pooled amplicon using AMPure XP beads, following the manufacturer's recommendations. Second, verify the size and purity of PCR products using an Agilent Bioanalyser 1000 DNA chip. The expected band size for MiFish-U-F and MiFish-U-R is roughly 170 bp. Third, attach dual multiplex barcode indices and Illumina sequencing adapters using the Nextera XT Index kit. Finally, additional purifications followed by quantification, normalisation and sample pooling steps are carried out as detailed in Illumina (undated), before submission for DNA sequencing.

If available, the Pippin Prep (Sage Science) can be used as an alternative to purification by AMPure XP beads, and Labchip (PerkinElmer) can be used in the place of the Bioanalyzer and quantification methods. Where large numbers of samples require purification, use of 96 well SequalPrep™ Normalisation Plate kits (Invitrogen Ltd.) or similar product may be considered. Each well of a SequalPrep Normalisation plate can bind and elute ~25 ng of PCR amplicon via a limited binding capacity solid phase. Additional DNA concentration normalisation is not required as excess DNA is washed away. For successful normalisation however at least 250 ng product is required per well. Harris et al. (2010) found this quantitative binding approach sufficient to process very large multiplex pools with hundreds and perhaps thousands of amplicons included. However, we are currently unable to recommend the use of SequalPrep kits to process small numbers of samples since the kit is currently available in a 10 x 96-well plate format with a shelf life of only 6 months. Regardless of the approach used, it is important to document the chosen method.

Note: The addition of multiplex barcode indices and sequencing adaptors and subsequent steps is offered by some DNA sequencing service providers, such as New Zealand Genomics Limited.

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