

Supplementary Table S1. Metabarcoding markers and gene regions, references, primer sequences, approximate target sequence length, and PCR parameters

Metabarcoding marker name (gene region)	Marker and reference(s)	Forward primer	Reverse primer	Target sequence length (median) (<i>N</i> nucleotides)	PCR anneal temperature (°Celsius)	<i>N</i> PCR cycles
LrCOI (mitochondrial <i>cytochrome c</i> <i>oxidase I</i>)	Leray <i>et al.</i> (2013) [48]; Geller <i>et al.</i> (2013) [49]	GGWACWGGWTGAACW GTWTAYCCYCC	TAIACYTCIGGRTGICCRAARA AYCA	313	55	35
Cop16S (mitochondrial 16S <i>ribosomal</i> RNA)	Clarke <i>et al.</i> (2017) [54]	TAAGGTAGCATARTAAT TWG	TAATTCAACATCGAGGTC	301	45	35
Mol16S (mitochondrial 16S <i>ribosomal</i> RNA)	Klymus <i>et al.</i> (2017) [36]	RRWRGACRAGAAGACC CT	ARTCCAACATCGAGGT	210 *196 with fish blocker	58	35
MiFish12S (mitochondrial 12S <i>ribosomal</i> RNA)	Miya <i>et al.</i> (2015)_ [58]	GTCGGTAAAACCTCGTGC CAGC	CATAGTGGGGTATCTAATCC CAGTTTG	172	65	35
FishCytb (mitochondrial <i>cytochrome b</i>)	Snyder and Stepien (2020) [61]; Snyder <i>et al.</i> (2020) [62]	GCCTACGCYATYCTHCG MTCHATYCC	GGTGTTTCNACNGGYATNC CNCCAATTCA	154	50	35

Supplementary Table S2. Total number of reads (pairs and single reads post-merge) at each step in the quality control pipeline.

Marker and Sample	Raw read pairs	Cutadapt adaptor trim pairs	DADA2 quality filter pairs	DADA2 merged reads	DADA2 non-chimeric reads	% Reads retained
LrCOI_1804_P22_v_BSsG	150,873	149,582	135,975	121,502	115,368	76.5
LrCOI_1804_P4_v_BSsG	101,257	100,201	90,265	85,884	79,220	78.2
LrCOI_1804_P8_o_BSsG	108,646	107,803	95,616	88,954	85,871	79.0
LrCOI_1804_P28_v_BSsG	156,378	154,821	139,414	129,924	125,243	80.1
LrCOI_1804_P38_o_BSsG	133,564	132,184	119,713	112,939	107,897	80.8
LrCOI_1804_P402_v_BSsG	121,640	120,730	116,599	111,413	89,640	73.7
LrCOI_1804_P12_v_BSsG	144,158	142,748	129,061	124,594	119,720	83.0
LrCOI_1809_P22_v_BSsG	127,444	126,318	109,545	102,749	96,069	75.4
LrCOI_1809_P8_o_BSsG	87,162	86,450	76,048	71,877	67,898	77.9
LrCOI_1809_P38_o_BSsG	122,167	121,178	111,784	105,531	101,428	83.0
Cop16S_1809_P22_v_BSsF	137,205	136,193	133,616	131,032	128,862	93.9
Cop16S_1809_P8_v_BSsF	202,613	201,185	197,483	190,517	187,912	92.7
Cop16S_1809_P28_v_BSsF	90,101	89,543	88,037	86,085	85,810	95.2
Cop16S_1809_P38_v_BSsF	174,617	173,419	169,535	165,757	164,272	94.1
Cop16S_1809_P38_o_BSsF	168,079	165,725	158,603	156,161	155,122	92.3
Cop16S_1809_P402_v_BSsF	96,453	95,784	93,519	92,727	92,666	96.1
Cop16S_1809_P12_v_BSsF	138,550	137,047	133,197	132,113	131,319	94.8

Mol16S_1804_P22_v_BS20	340,156	318,984	307,240	303,699	289,206	85.0
Mol16S_1804_P4_v_A_KAsH	88,068	54,754	48,913	44,689	42,708	48.5
Mol16S_1804_P4_v_BS20	210,286	198,775	187,630	175,458	164,106	78.0
Mol16S_1804_P8_v_BS20	273,904	256,405	246,976	242,168	225,155	82.2
Mol16S_1804_P28_v_BS20	172,836	163,125	154,473	150,052	134,945	78.1
Mol16S_1804_P402_v_BS20	411,653	391,621	382,218	380,908	377,995	91.8
Mol16S_1804_P12_v_BS20	281,659	264,924	245,339	237,723	234,648	83.3
Mol16S_1809_P4_v_BSsF	75,496	74,452	72,799	72,481	72,194	95.6
Mol16S_1809_P4_v_BS20	120,707	112,828	104,191	102,302	101,328	83.9
Mol16S_1809_P8_v_BS20	261,369	245,141	235,881	233,083	224,636	85.9
Mol16S_1809_P8_o_BSsF	107,398	105,762	104,281	103,382	102,581	95.5
Mol16S_1809_P28_v_BS20	284,093	264,123	252,036	248,801	241,705	85.1
Mol16S_1809_P38_v_BSsF	54,579	53,981	53,073	51,898	51,156	93.7
Mol16S_1809_P38_v_BS20	371,415	347,162	329,333	325,487	315,458	84.9
Mol16S_1809_P38_o_BSsF	193,581	191,306	187,602	182,944	179,128	92.5
Mol16S_1809_P402_v_BSsF	107,610	106,323	103,254	102,650	102,441	95.2
Mol16S_1809_P402_v_BS20	146,364	136,669	128,173	126,414	121,454	83.0
Mol16S_1809_P12_v_BSsF	69,173	68,317	67,517	67,084	66,351	95.9
Mol16S_1809_P12_v_BS20	265,703	240,887	212,225	207,240	197,702	74.4
Mol16S_FB_1804_P22_v_BS20	243,775	228,289	220,530	218,162	212,268	87.1

Mol16S_FB_1804_P4_v_BS20	175,305	165,465	157,998	150,335	143,039	81.6
Mol16S_FB_1804_P8_v_BS20	168,262	157,248	152,275	149,901	142,989	85.0
Mol16S_FB_1804_P28_v_BS20	127,931	120,053	114,586	113,152	107,279	83.9
Mol16S_FB_1804_P38_v_BSsH	132,895	131,252	127,581	126,125	116,578	87.7
Mol16S_FB_1804_P402_v_BS20	219,543	208,861	204,141	203,563	202,573	92.3
Mol16S_FB_1804_P12_v_BS20	209,625	197,201	185,006	183,493	183,029	87.3
Mol16S_FB_1809_P22_v_BSsH	122,139	120,675	119,194	118,594	116,908	95.7
Mol16S_FB_1809_P22_v_BS20	273,612	257,489	248,794	245,575	229,427	83.9
Mol16S_FB_1809_P4_v_BS20	266,993	249,868	237,842	232,855	221,668	83.0
Mol16S_FB_1809_P8_v_BS20	188,850	176,448	165,853	162,941	156,469	82.9
Mol16S_FB_1809_P8_o_BSsH	127,489	124,953	113,484	111,308	109,637	86.0
Mol16S_FB_1809_P28_v_BS20	296,595	278,120	268,091	265,814	262,380	88.5
Mol16S_FB_1809_P38_v_BS20	177,721	166,656	157,995	155,850	151,412	85.2
Mol16S_FB_1809_P38_o_BSsH	114,588	113,016	109,954	108,149	106,240	92.7
Mol16S_FB_1809_P402_v_BSsH	98,063	96,476	93,600	92,706	91,669	93.5
Mol16S_FB_1809_P402_v_BS20	167,697	157,135	148,533	146,509	140,963	84.1
Mol16S_FB_1809_P12_v_BSsH	52,628	51,961	50,322	49,928	48,421	92.0
Mol16S_FB_1809_P12_v_BS20	211,086	194,094	186,050	183,867	174,837	82.8
MiFish12S_1804_P22_v_BS20	161,912	144,423	57,662	56,608	56,224	34.7
MiFish12S_1804_P4_v_BS20	206,536	193,698	155,051	152,901	151,255	73.2

MiFish12S_1804_P8_v_BS20	270,810	247,369	84,739	83,850	83,815	30.9
MiFish12S_1804_P28_v_BS20	181,119	169,663	135,577	134,064	130,997	72.3
MiFish12S_1804_P38_v_BS20	197,727	185,713	158,503	157,094	155,507	78.6
MiFish12S_1804_P402_v_BS20	180,170	166,603	86,145	85,308	85,284	47.3
MiFish12S_1804_P12_v_BS20	147,083	138,556	124,580	124,145	123,226	83.8
MiFish12S_1809_P4_v_BS20	260,375	236,628	49,458	49,401	49,398	19.0
MiFish12S_1809_P8_o_BSsE	49,389	49,182	48,840	48,773	48,773	98.8
MiFish12S_1809_P38_v_BS20	230,787	218,826	176,369	175,660	174,276	75.5
MiFish12S_1809_P38_o_BSsE	107,889	107,356	106,671	105,563	103,768	96.2
FishCytb_1804_P22_v_BSsH	72,705	72,058	71,004	70,429	69,597	95.7
FishCytb_1804_P22_v_BS20	256,483	247,444	130,668	127,102	125,388	48.9
FishCytb_1804_P4_v_BSsH	73,304	72,706	72,310	71,687	70,225	95.8
FishCytb_1804_P4_v_BS20	266,189	260,623	256,740	252,260	245,441	92.2
FishCytb_1804_P8_v_BS20	284,845	276,670	268,384	261,365	242,182	85.0
FishCytb_1804_P8_o_BSsH	97,103	96,300	95,464	94,176	93,542	96.3
FishCytb_1804_P28_v_BSsH	55,917	55,440	55,049	54,720	54,047	96.7
FishCytb_1804_P28_v_BS20	279,533	273,164	257,561	254,781	250,962	89.8
FishCytb_1804_P38_o_BSsH	179,803	178,414	177,440	176,088	171,735	95.5
FishCytb_1804_P402_v_BS20	176,161	172,383	168,248	165,780	164,975	93.7
FishCytb_1804_P12_v_BSsH	81,579	80,989	80,581	80,406	80,397	98.6

FishCytb_1804_P12_v_BS20	217,277	211,658	208,411	207,696	207,654	95.6
FishCytb_1809_P28_v_BS20	103,187	100,581	87,654	85,888	85,218	82.6
FishCytb_1809_P38_v_BSsH	86,832	86,101	85,563	85,312	84,324	97.1
FishCytb_1809_P38_v_BS20	132,817	130,377	128,086	126,772	125,063	94.2
FishCytb_1809_P38_o_BSsH	62,214	61,682	61,217	61,051	61,013	98.1

Supplementary Table S5. Species studied in the laboratory and whether or not they were detected in this metabarcoding study.

Species name and authority	Common name	Detected in this study
<i>Clupeonella cultriventris</i> (von Nordmann, 1840)	Black Sea sprat	yes
<i>Dreissena rostriformis bugensis</i> (Andrusov, 1897)	Quagga mussel	no
<i>Dreissena polymorpha</i> (Pallas, 1771)	Zebra mussel	yes
<i>Etheostoma blennioides</i> Rafinesque, 1819	Greenside darter	no
<i>Gymnocephalus cernua</i> (Linnaeus, 1758)	Eurasian ruffe	yes
<i>Hypophthalmichthys molitrix</i> (Valenciennes, 1844)	Silver carp	no
<i>Micropterus dolomieu</i> Lacepède, 1802	Smallmouth bass	no*
<i>Neogobius melanostomus</i> (Pallas, 1814)	Round goby	no
<i>Perca flavescens</i> (Mitchill, 1814)	Yellow perch	no
<i>Perca fluviatilis</i> Linnaeus, 1758	European perch	no
<i>Percina caprodes</i> (Rafinesque, 1818)	Common logperch	no
<i>Sander vitreus</i> (Mitchill, 1818)	Walleye	yes*

*= Known from freshwaters and/or estuaries in the Salish Sea and/or NE Pacific region (USGS NAS 2023 [50])

Supplementary Table S6. Assessment of assigned sequence variants (ASVs) to putative nonindigenous species. *Best percent identity (PID) to putative nonindigenous species. Range shown for multi-ASV taxa. FB=results with fish blocker primer.
N/A=Not Available.

Marker: Putative nonindigenous species	N ASVs	Total Reads (Rel. Abund. Range)	N Unique Samples	PID* to putative nonindigenous species	Next closest hit species name (PID)	Potential local species (marker genes available for comparison)	Confidence (reason)
LrCOI: <i>Proceraea okadai</i>	1	14 (0.02)	1	99.7	<i>Proceraea nigropunctata</i> (86.6)	<i>Proceraea hanssoni</i> (16S, COI), <i>Proceraea nigropuncatata</i> (16S, COI)	High (High PID match, no close hit to local species)
Mol16S: <i>Proceraea okadai</i>	1	7 (0.01)	1	98.9	<i>Proceraea nigropunctata</i> (91.6)		
Mol16S_FB: <i>Proceraea okadai</i>	1	11 (0.01)	1	98.9	<i>Proceraea nigropunctata</i> (91.6)		
FishCytb: <i>Hediste diadroma</i>	1	12 (0.01)	1	100	<i>Hediste japonica</i> (84.3)	<i>Hediste limnicola</i> (16S, <u>no cytb</u>)	High (High PID match, no close hit for local species)
Mol16S: <i>Hediste diadroma</i>	1	12 (<0.01)	2	100	<i>Hediste japonica</i> (96.1)		
Mol16S_FB: <i>Hediste diadroma</i>	1	89 (<0.01- 0.03)	3	100	<i>Hediste japonica</i> (96.1)		
Cop16S: <i>Leonardsaxius amurensis</i>	1	36 (0.02)	1	97.6	<i>Eiconaxius borradailei</i> (83.3)	<i>Leonardsaxius spinulicauda</i> (<u>no 16S</u>)	Low (Low rRNA PID match, no marker reference for local species)
Mol16S: <i>Leonardsaxius amurensis</i>	1	24 (0.01)	1	97.1	<i>Pugnatrypaea pugnatrix</i> (91.5)		
LrCOI: <i>Crangon crangon</i>	1	30 (0.04)	1	97.3	<i>Crangon septemspinosa</i> (94.0)	<i>Crangon alaskensis</i> (COI, <u>no 16S</u>), <i>Crangon alba</i> (<u>no COI or 16S</u>), <i>Crangon dalli</i> (<u>no COI or 16S</u>), <i>Crangon franciscorum</i> (COI, <u>no 16S</u>), <i>Crangon nigricauda</i> (<u>no COI or 16S</u>), <i>Crangon holmesii</i> (<u>no COI or 16S</u>), <i>Crangon lockingtonii</i> (<u>no COI or</u>	Low (High PID match, no 16S and limited COI reference for local species)
Cop16S: <i>Crangon crangon</i>	2	185 (<0.01- 0.18)	4	99.1	<i>Neocrangon sagamiensis</i> (93.4)		

						<u>16S</u>), <i>Crangon nigromaculata</i> (<u>no COI or 16S</u>), <i>Crangon capensis</i> (<u>no COI or 16S</u>)	
Mol16S: <i>Lucinoma borealis</i>	3	2399 (0.53-1.28)	3	97.1-97.8	N/A	<i>Lucinoma annulata</i> (<u>no 16S</u>)	Low (Low rRNA PID match, no marker ref. for local species)
Mol16S_FB: <i>Lucinoma borealis</i>	2	2914 (0.37-2.15)	2	97.8	N/A		
Mol16S: <i>Dreissena polymorpha</i>	3	500 (<0.01-0.46)	6	99.4-100	<i>Dreissena presbensis</i> (96.1)	No local species, invasive throughout much of North America, multiple introductions from the Ponto-Caspian region [50]	High (High PID match, no known local species)
Mol16S_FB: <i>Dreissena polymorpha</i>	7	8682 (0.05-7.46)	8	98.3-100	<i>Dreissena presbensis</i> (96.1)		
FishCytb: <i>Alosa sapidissima</i>	1	129 (0.06)	1	100	<i>Alosa alabamae</i> (99.4)	No known local species, introduced to San Francisco Bay and Salish Sea [50]	High (High PID match, no known local species)
FishCytb: <i>Liparis ochotensis</i>	2	462 (0.01-0.55)	3	98.7-99.4	<i>Liparis liparis</i> (94.8)	<i>Liparis adiastol</i> (<u>no cytb</u>), <i>Liparis callyodon</i> (<u>no cytb</u>), <i>Liparis cyclopus</i> (<u>no cytb</u>), <i>Liparis dennyi</i> (cytb), <i>Liparis florae</i> (cytb), <i>Liparis fucensis</i> (<u>no cytb</u>), <i>Liparis greeni</i> (<u>no cytb</u>), <i>Liparis mucosus</i> (cytb), <i>Liparis pulchellus</i> (cytb)	Low (High PID match, only 44% of potential local species have marker reference sequences)
Mol16S: <i>Cryptacanthodes bergi</i>	1	376 (0.16-0.25)	2	99.3	<i>Cryptacanthodes giganteus</i> (98.6)	<i>Cryptacanthodes aleutensis</i> (<u>no 16S</u>), <i>Cryptacanthodes giganteus</i> (16S)	Low (High PID match, only one of the two local species has marker reference)
Mol16S_FB: <i>Cryptacanthodes bergi</i>	1	98 (0.07)	1	99.3	<i>Cryptacanthodes giganteus</i> (98.6)		

Supplementary Figures

Supplementary Figure S1. Taxonomic and read count/relative abundance information viewed through Krona plot hierarchical data browsers. doi:[10.6084/m9.figshare.21791738](https://doi.org/10.6084/m9.figshare.21791738)).

Supplementary Figure S2A. Decapoda Crustacea species resolved by morphology and/or four metabarcoding markers.

Filled circles denote presence, empty circles indicate that the taxon was not detected, and missing circles show lack of resolution for that method.

Supplementary Figure S2B. Cnidaria species resolved by morphology and/or four metabarcoding markers.

Filled circles denote presence, empty circles indicate that the taxon was not detected, and missing circles show lack of resolution for that method.

Supplementary Figure S2C. Echinodermata species resolved by morphology and/or three invertebrate metabarcoding markers.

Filled circles denote presence, empty circles indicate that the taxon was not detected, and missing circles show lack of resolution for that method.

Supplementary Figure S2D. Gastropoda Mollusca species resolved by morphology and/or three metabarcoding markers.

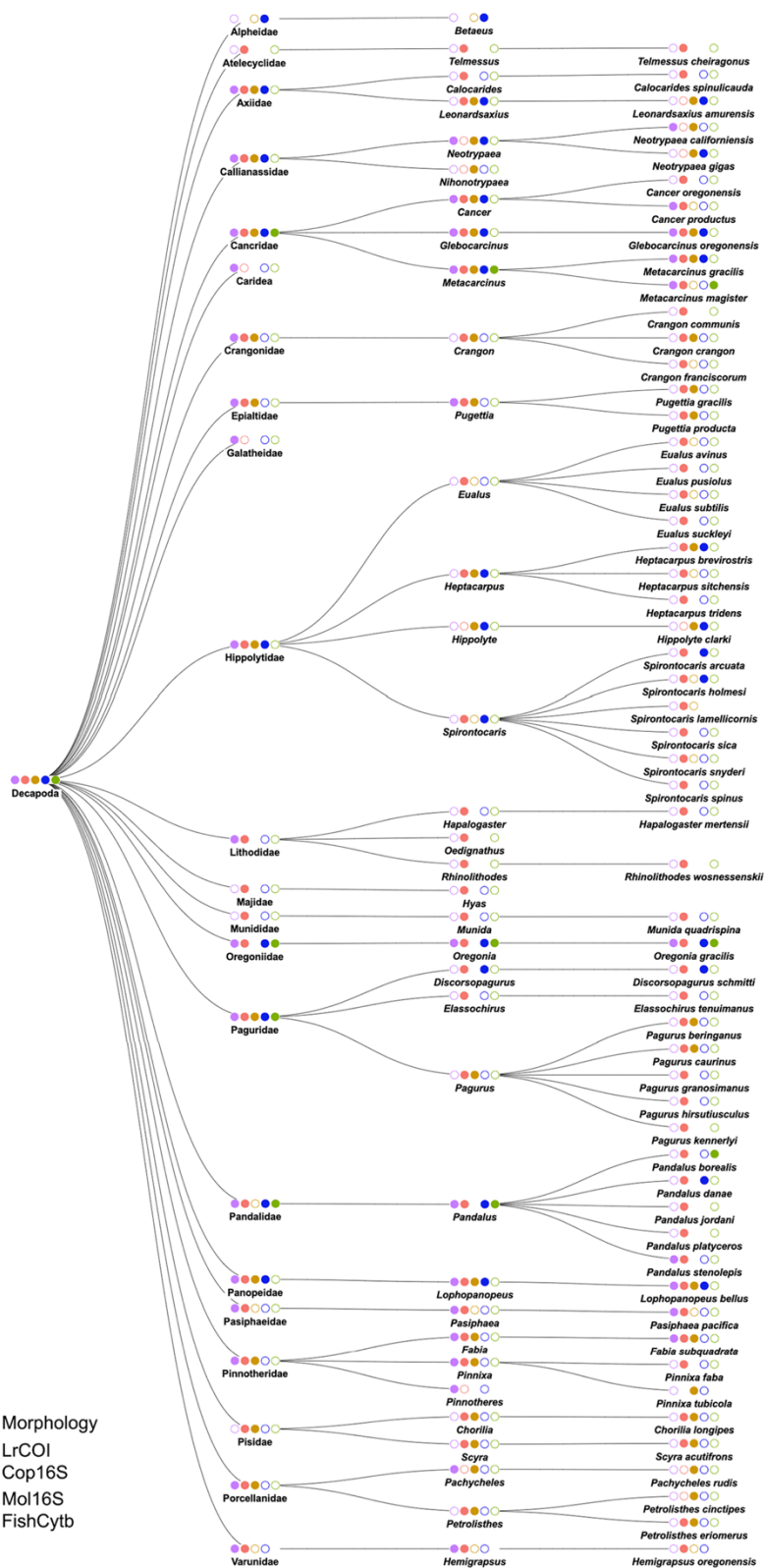
Filled circles denote presence, empty circles indicate that the taxon was not detected, and missing circles show lack of resolution for that method.

Supplementary Figure S2E. Actinopterygii (Teleostei) fish species resolved by morphology and/or four metabarcoding markers.

Filled circles denote presence, empty circles indicate that the taxon was not detected, and missing circles show lack of resolution for that method.

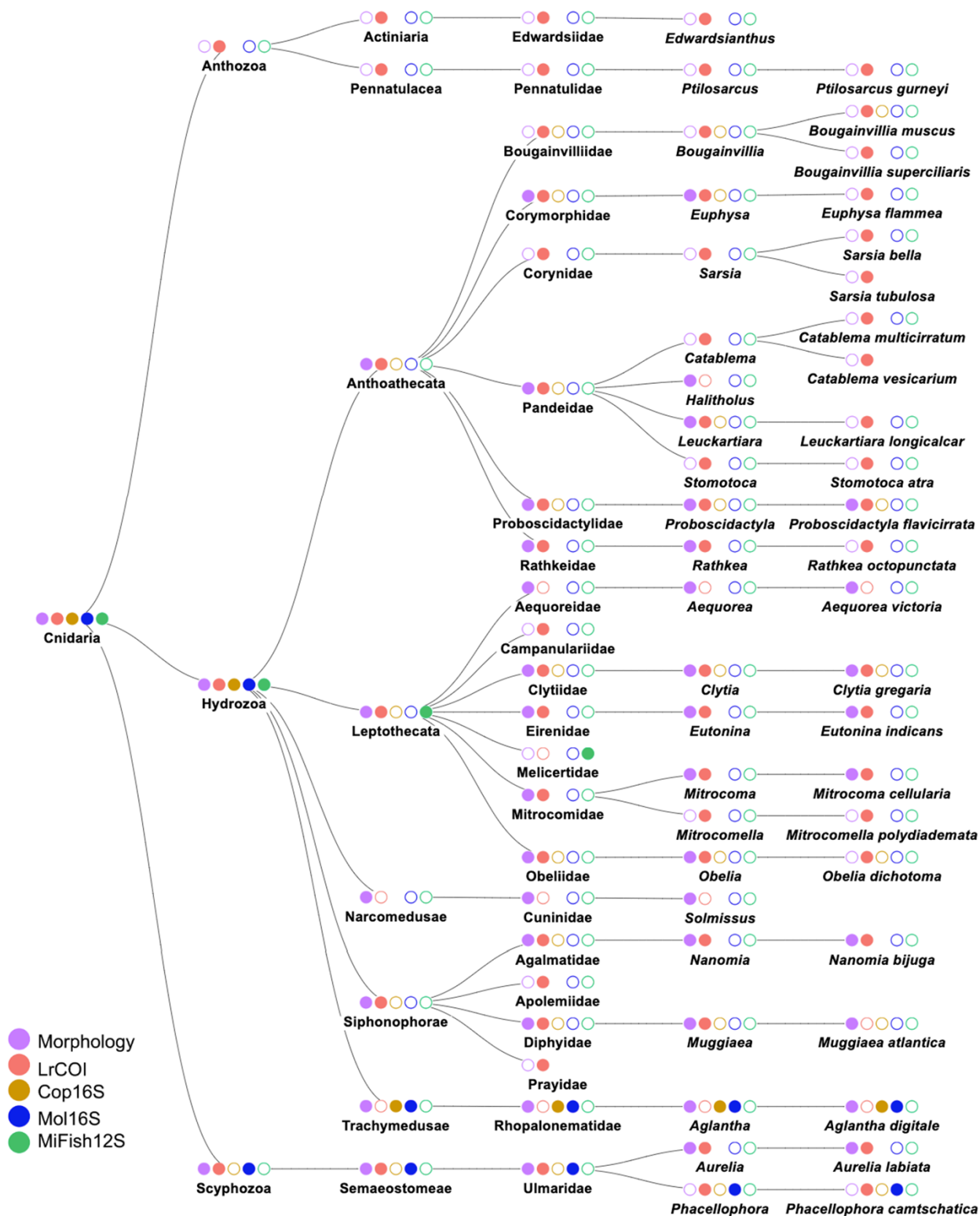
Supplementary Figure S3. Proportion of Arthropoda species carbon biomass versus proportion of Arthropoda sequence reads from metabarcoding marker LrCOI

Regression fit R^2 , with adjusted p -values for multiple comparisons [per 92], and Pearson correlation (r) coefficients indicated.



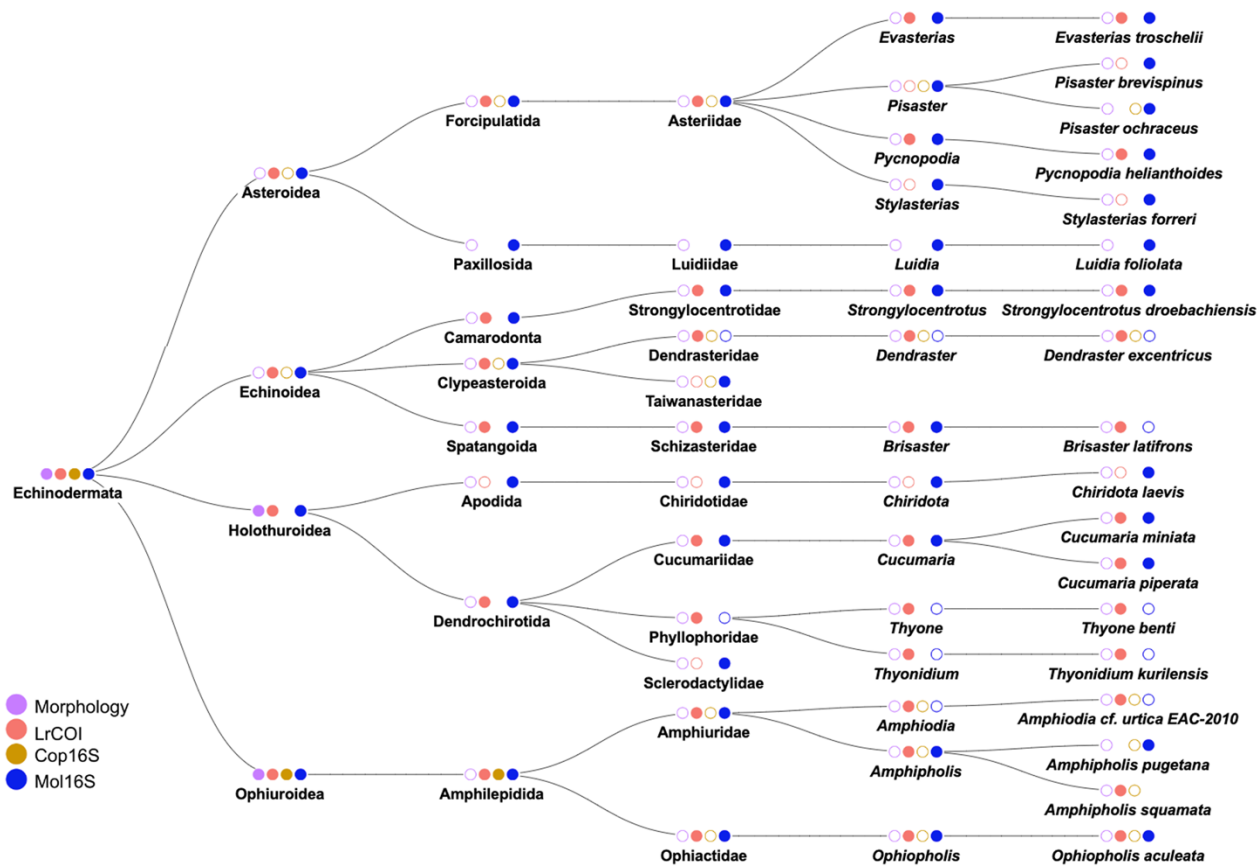
Supplementary Figure S2A. Decapoda Crustacea species resolved by morphology and/or four metabarcoding markers.

Filled circles denote identification, empty circles indicate that the taxon was not detected, and missing circles show lack of resolution for that method.



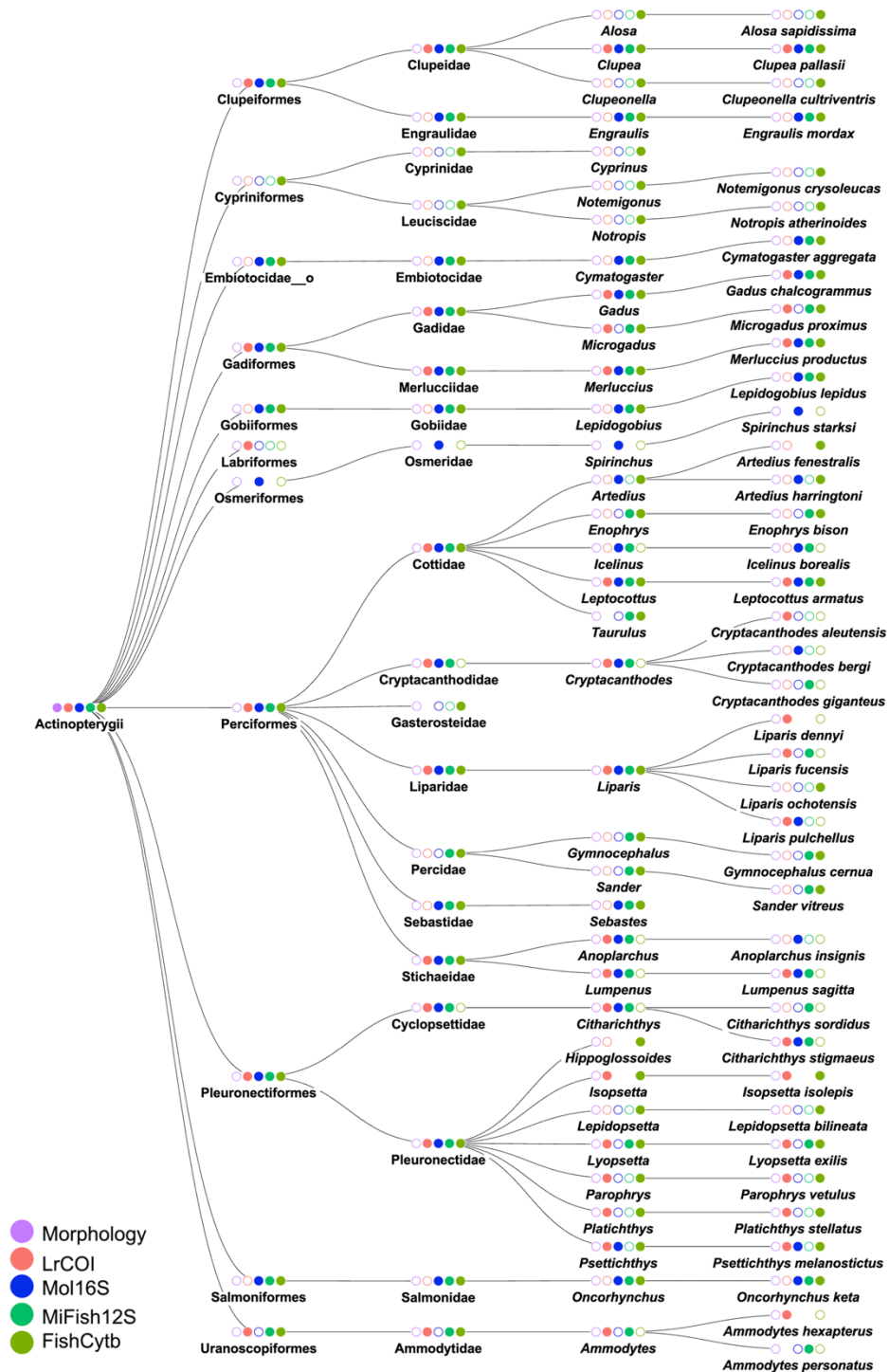
Supplementary Figure S2B. Cnidaria species resolved by morphology and/or four metabarcoding markers.

Filled circles denote identification, empty circles indicate it was not detected, and missing circles show lack of resolution by that method.



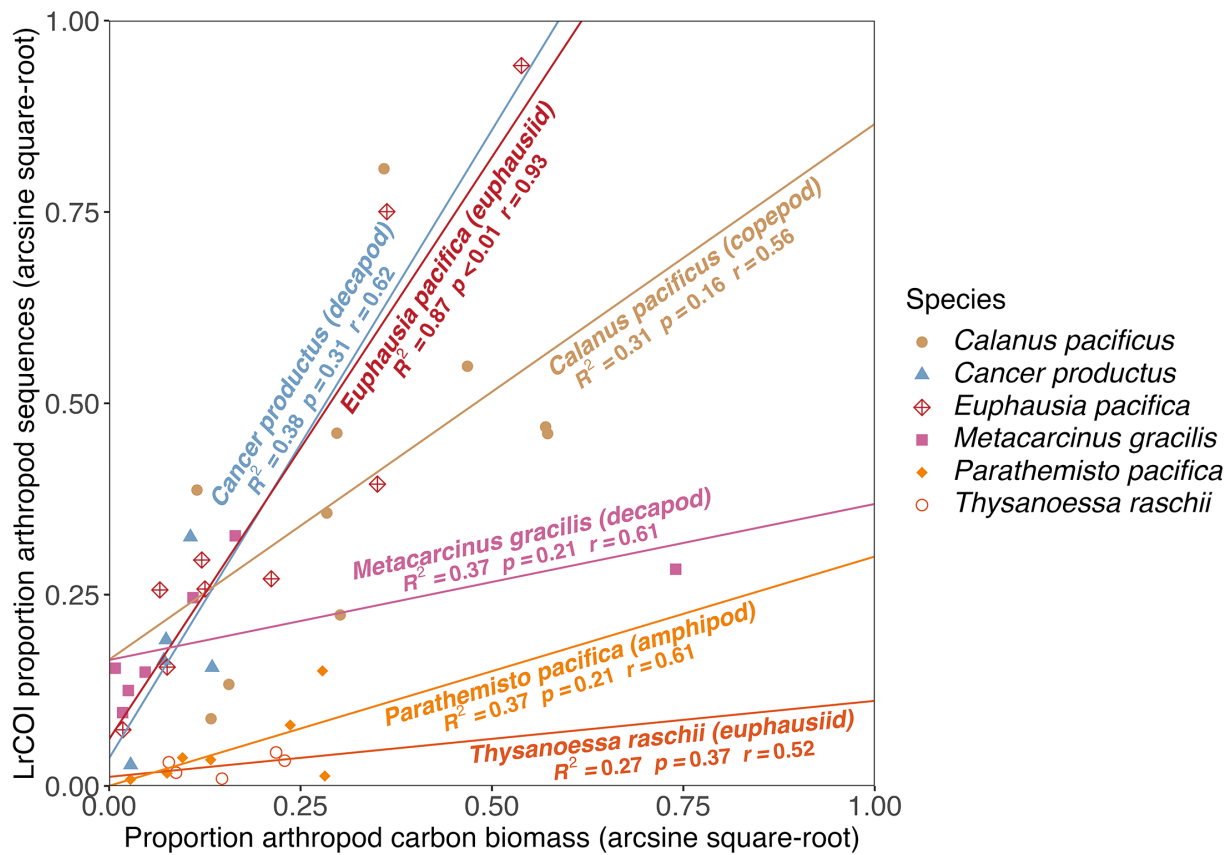
Supplementary Figure S2C. Echinodermata species resolved by morphology and/or three invertebrate metabarcoding markers.

Filled circles denote presence, empty circles indicate that the taxon was not detected, and missing circles show lack of resolution for that method.



Supplementary Figure S2E. Actinopterygii (Teleostei) fish species resolved by morphology and/or four metabarcoding markers.

Filled circles denote presence, empty circles indicate that the taxon was not detected, and missing circles show lack of resolution for that method.



Supplementary Figure S3. Proportion of Arthropoda species carbon biomass versus proportion of sequence reads (ASVs merged per species) from metabarcoding marker LrCOI.

Regression fit R^2 , with adjusted p -values for multiple comparisons [per 92], and Pearson correlation (r) coefficients indicated.