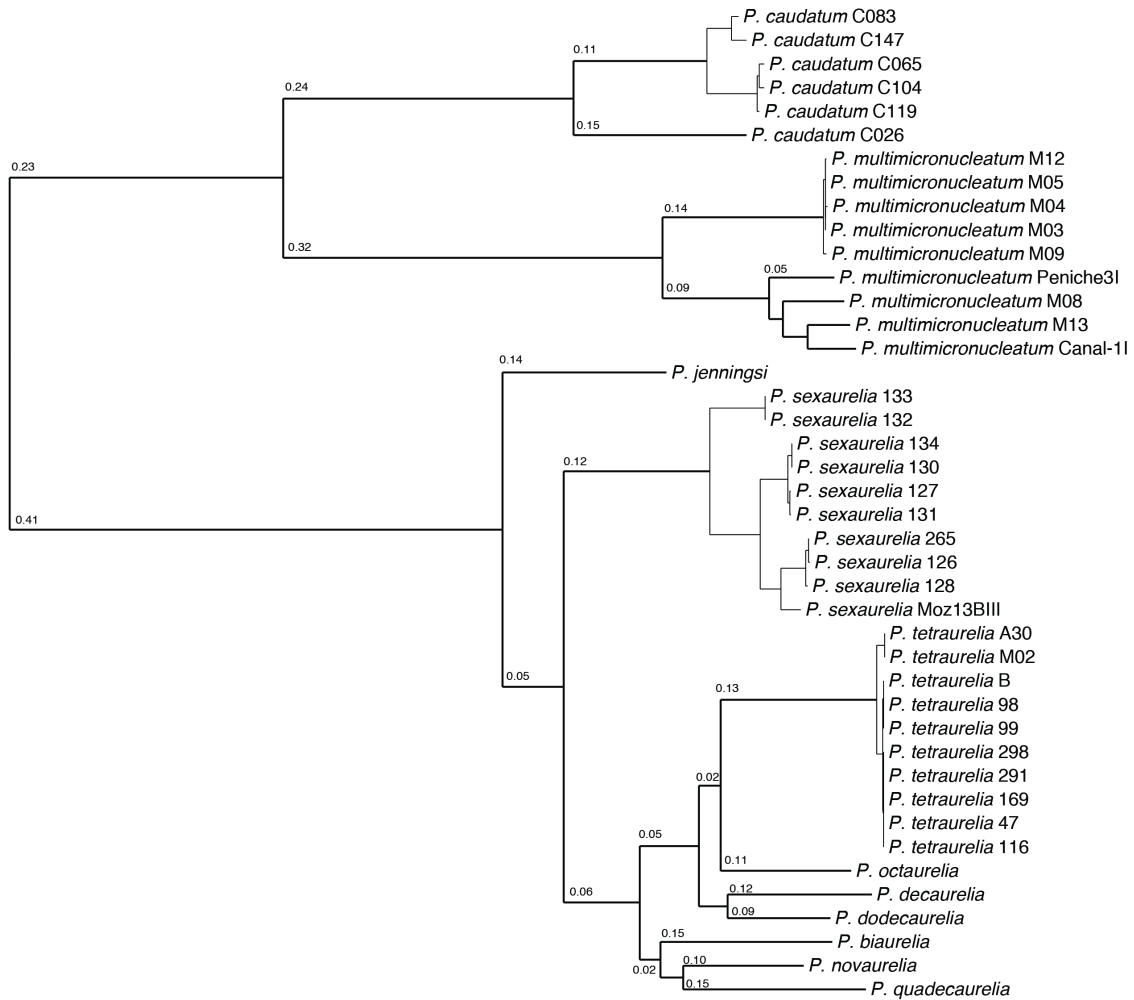# Supplementary Materials

Supplementary Figures

Supplementary Figure 1: Mitochondrial phylogeny of the Paramecium genus including individual isolates within each species. The phylogeny is built based on sequence variants identified by mapping reads to the reference genome for each species (rather than using *de novo* assemblies), and was built using RAxML (version 8.2.11), under the substitution model GTRGAMMA. Numbers on branches indicate total number of substitutions per site.
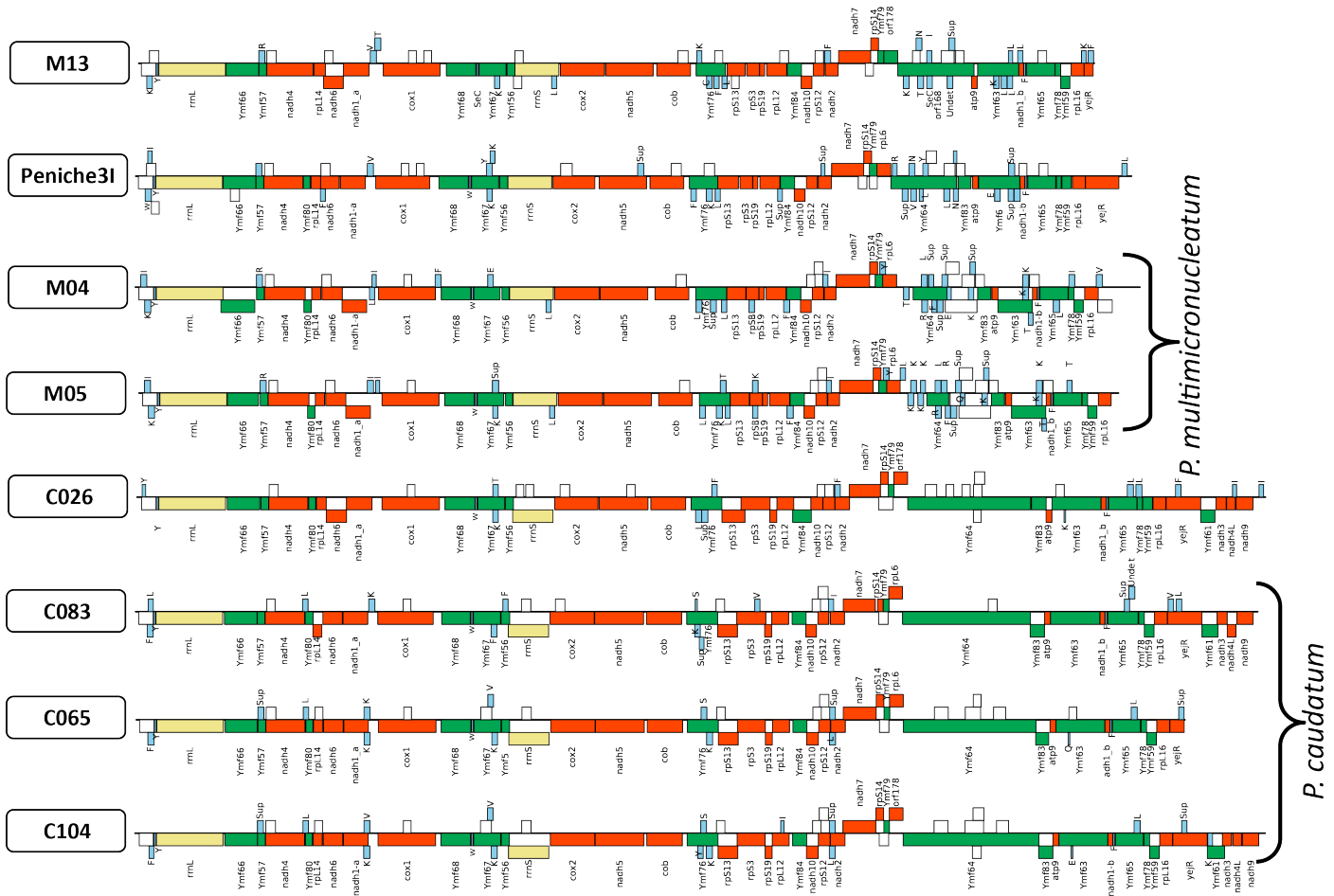
Supplementary Figure 2: Mitochondrial genomes of isolates belonging to the *P. caudatum* and *P. multimicronucleatum* outgroup lineages. Red: protein coding genes; white: novel predicted ORFs longer than 100aa; yellow: ribosomal RNAs; blue: tRNAs. Top layers: forward-strand genes; bottom layers: reverse-strand genes.
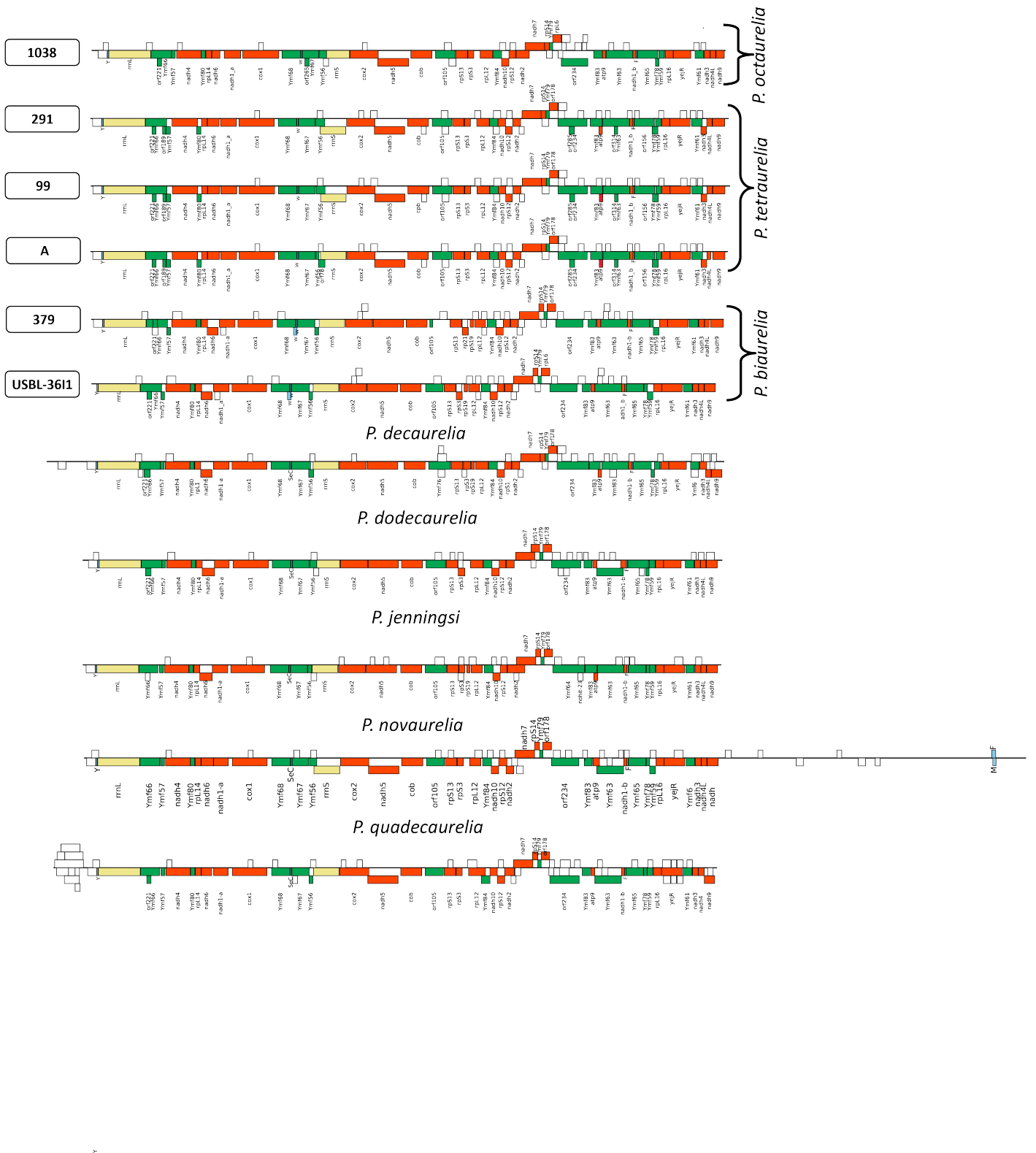
Supplementary Figure 3: Mitochondrial genomes of isolates belonging to the _P. aurelia_ species complexes (except for _P. sexaurelia_, shown in the next figure). Red: protein coding genes; white: novel predicted ORFs longer than 100aa; yellow: ribosomal RNAs; blue: tRNAs. Top layers: forward-strand genes; bottom layers: reverse-strand genes.
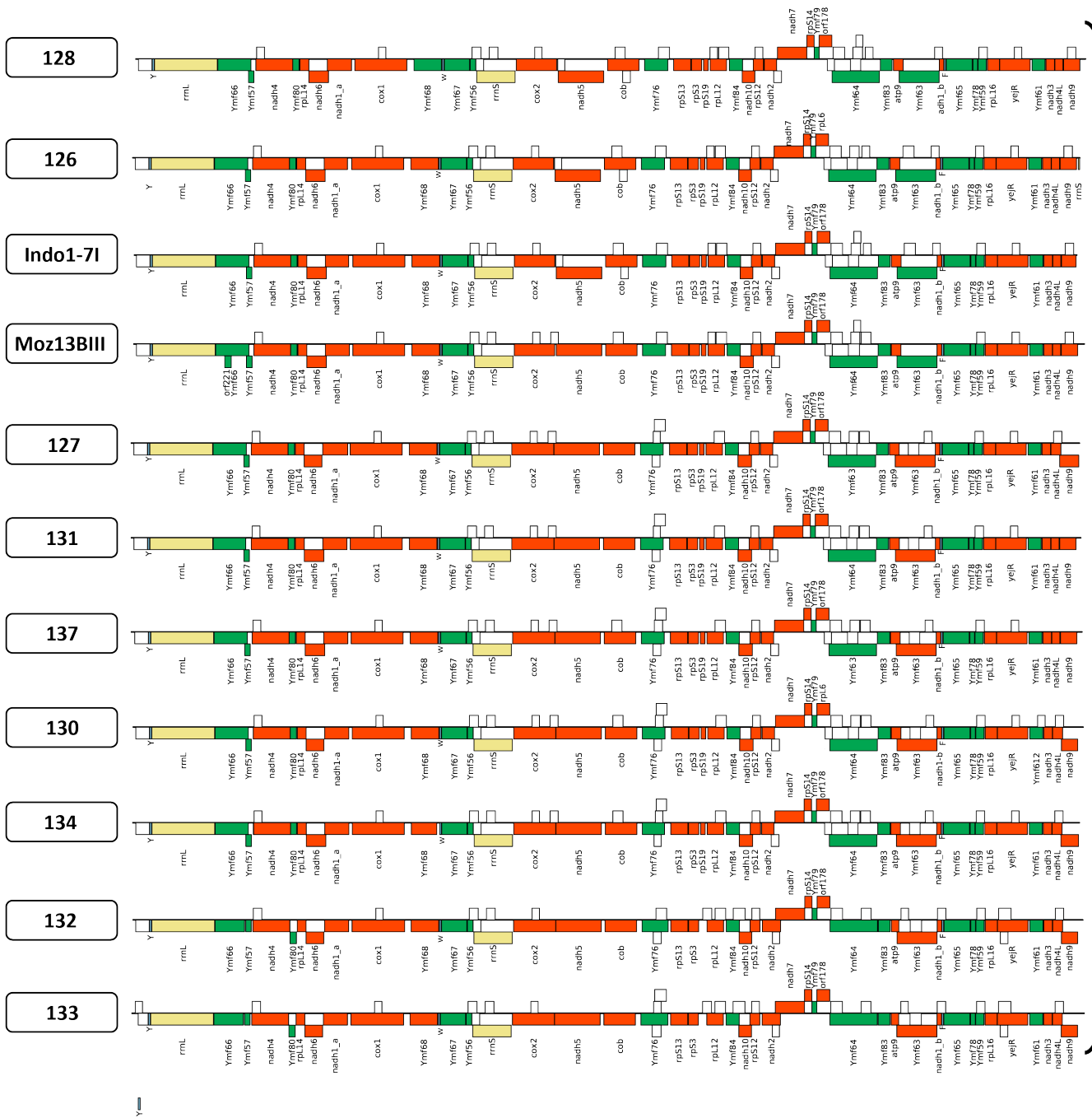
Supplementary Figure 4: Mitochondrial genomes of isolates belonging to *P. sexaurelia*. Red: protein coding genes; white: novel predicted ORFs longer than 100aa; yellow: ribosomal RNAs; blue: tRNAs. Top layers: forward-strand genes; bottom layers: reverse-strand genes.
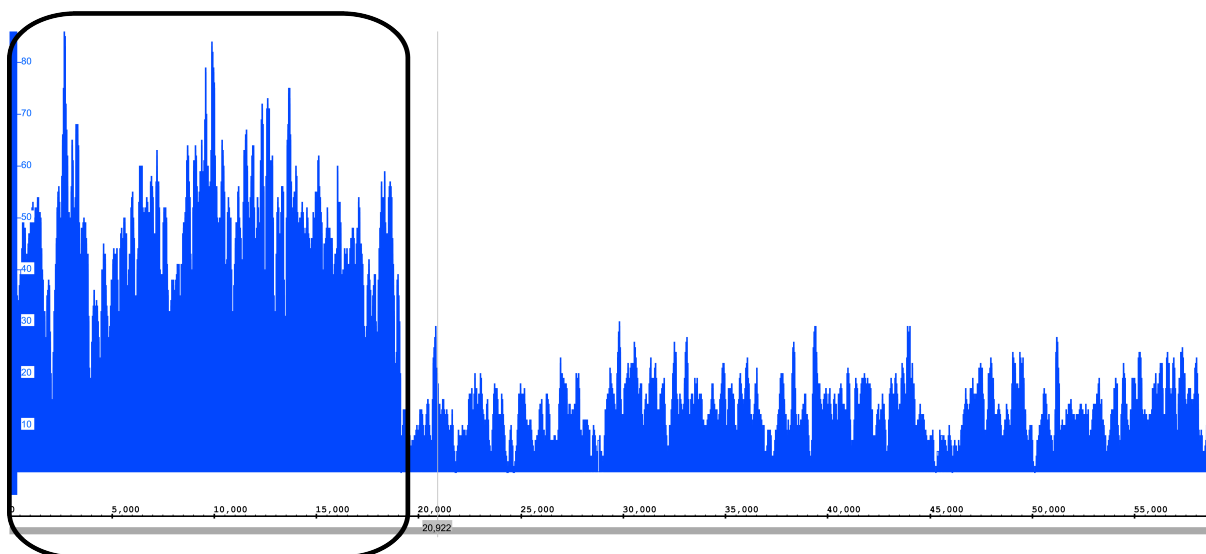
Supplementary Figure 5: Raw mitogenome assembly and sequencing read coverage for *P. novaurelia*. An ~19kb 3′ extension is present in the raw assembly. However, read coverage over that region is significantly higher than what is observed over the rest of the mitochondrial genome, thus the two regions most likely exist in different copy numbers and the extension might be the result of missassembly. See also Figure 3. Raw sequencing reads were mapped to the assembled mitogenome using Bowtie (Langmead et al. 2009).
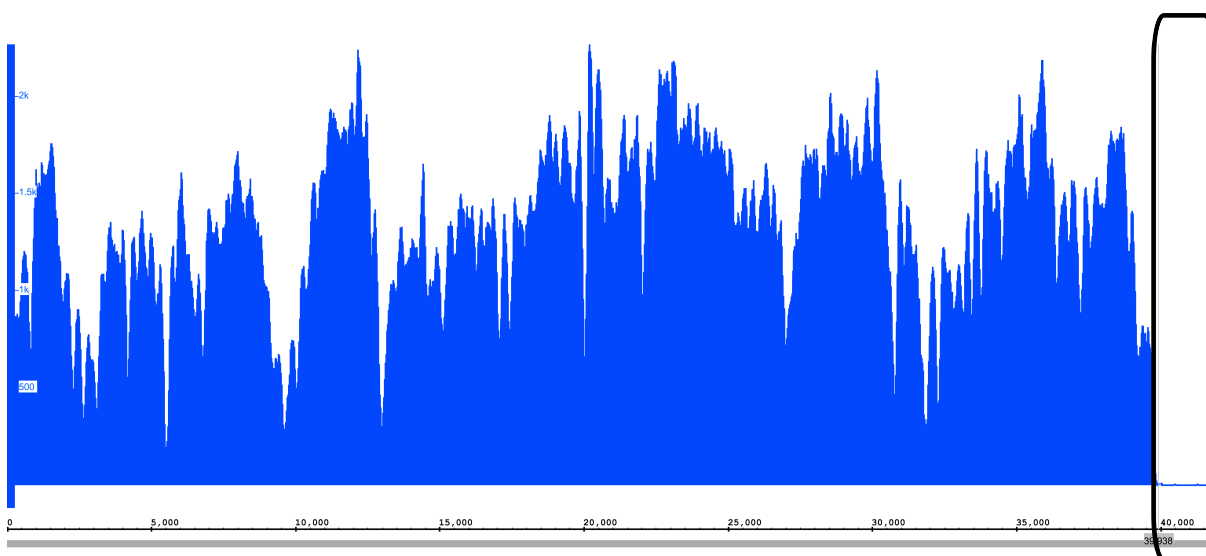
Supplementary Figure 6: Raw mitogenome assembly and sequencing read coverage for *P. quadecaurelia*. An small 5′ extension is present in the raw assembly. However, read coverage over that region is very low relative to what is observed over the rest of the mitochondrial genome, thus the extension is probably be the result of missassembly. See also Figure 3. Raw sequencing reads were mapped to the assembled mitogenome using Bowtie (Langmead et al. 2009).
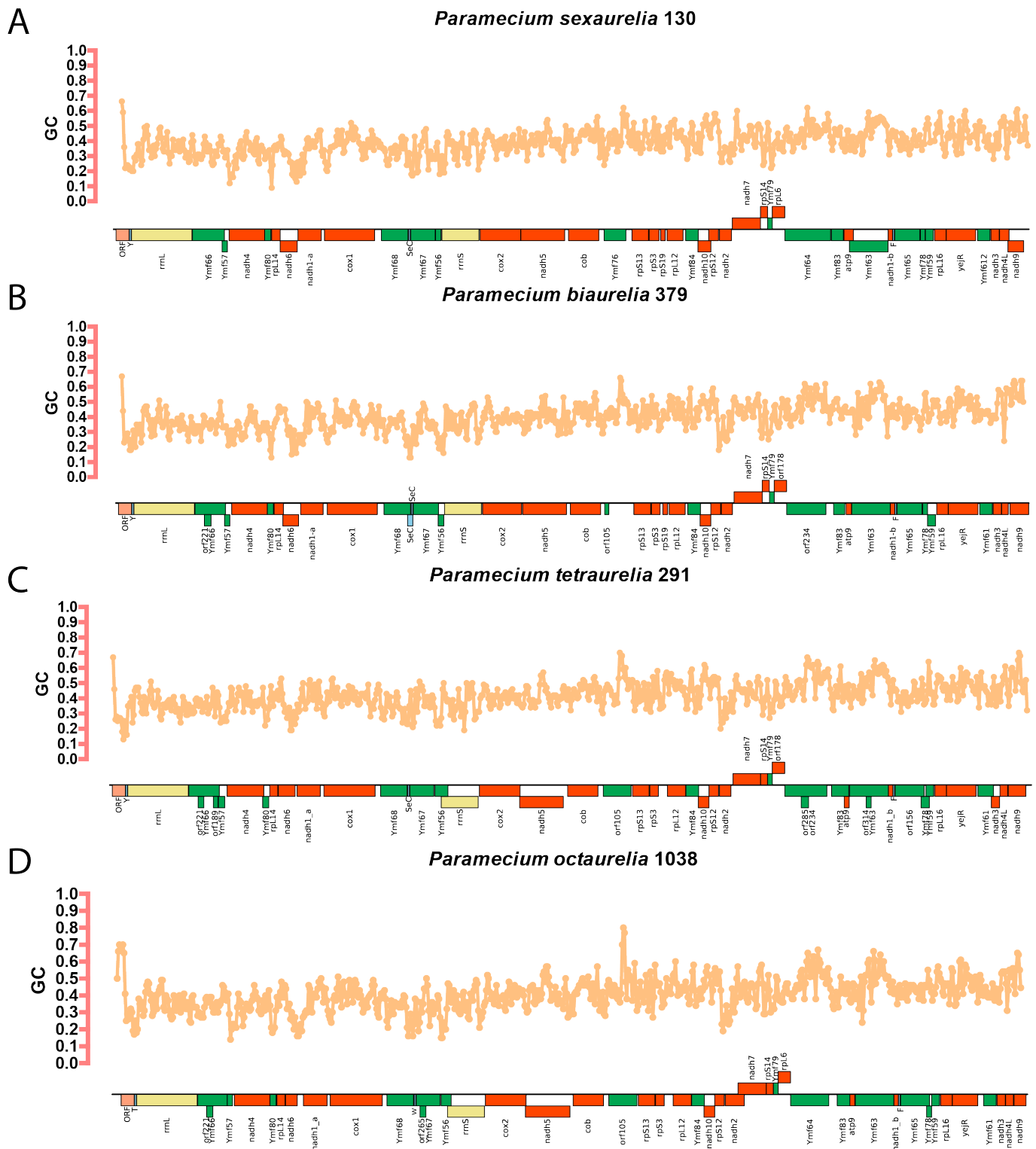
Supplementary Figure 7: GC content in *P. aurelia* species. Shown is the 100bp average of the GC content along the mitochondrial chromosome.

Supplementary Figure 8: GC content in *P. caudatum* and related species. Shown is the 100bp average of the GC content along the mitochondrial chromosome.

Supplementary Figure 9: GC content in *P. multimicronucleatum* and related species. Shown is the 100bp average of the GC content along the mitochondrial chromosome.

| Protein			P. multimicronucleatum			P. caudatum			P. biaurelia		P. tetraurelia			P. sexaurelia																
	M13	Peniche3l	M04	M05	C026	C065	C083	C104	379	USBL-36l1	291	99	A	126	127	128	130	131	132	133	134	137	Indo1-7l	Moz13BIII	P. octaurelia	P. novaurelia	P. decaurelia	P. dodecaurelia	P. quadecaurelia	P. jenningsi
atp9	75	75	80	80	78	77	77	77	78	78	72	72	72	136	136	136	136	136	104	104	136	136	136	136	72	136	75	86	80	85
cob	444	444	444	444	457	466	463	466	442	442	442	442	430	442	442	442	442	442	442	442	442	442	442	442	442	442	442	442	442	442
cox1	747	709	746	747	757	757	728	757	729	729	746	746	746	729	729	729	729	729	739	739	729	729	729	729	813	729	733	748	728	711
cox2	579	550	582	582	567	584	580	584	567	567	594	594	594	567	591	567	591	591	591	591	591	591	567	567	592	591	567	567	613	567
nadh10	141	141	141	141	157	157	141	157	156	156	156	156	156	178	189	178	189	189	177	177	189	189	178	189	156	156	156	156	156	156
nadh1-a	338	327	325	325	336	323	323	323	328	328	336	336	336	327	352	339	339	339	338	338	339	339	327	339	345	307	330	310	303	326
nadh1-b	63	63	61	61	64	63	63	63	64	64	64	64	64	63	63	63	63	63	63	63	63	63	63	63	63	64	64	64	63	63
nadh2	170	170	154	154	191	186	186	191	162	162	166	166	166	173	173	173	173	173	257	257	173	173	173	173	276	162	162	162	205	367
nadh3	nd	nd	nd	nd	134	nd	132	120	120	120	120	120	120	120	120	120	120	120	120	120	120	120	120	120	120	120	145	120	120	120
nadh4	607	512	536	536	512	512	518	512	517	514	534	534	534	514	517	517	517	517	517	517	517	517	517	517	517	517	517	517	512	512
nadh4L	nd	nd	nd	nd	115	nd	115	115	96	113	113	113	78	113	139	113	139	113	139	139	139	113	113	113	113	113	113	113	122	113
nadh5	622	622	627	627	641	641	641	641	638	638	637	637	637	638	638	638	638	638	638	638	638	638	638	638	648	638	638	638	638	638
nadh6	259	243	266	266	267	318	256	256	230	230	245	245	245	275	249	275	249	275	249	249	275	275	275	275	170	254	238	263	221	262
nadh7	423	423	442	442	411	433	423	433	411	411	400	400	400	411	411	411	411	411	411	411	411	411	411	411	411	411	411	400	411	411
nadh9	nd	nd	nd	nd	223	nd	215	215	270	229	256	256	256	229	269	229	229	229	229	229	249	229	205	229	175	229	218	229	218	229
rpL12	262	262	262	262	223	223	223	223	232	232	265	265	265	232	232	232	232	232	232	232	232	232	232	232	265	232	265	265	356	232
rpL14	150	125	119	119	128	119	119	119	134	134	119	119	119	125	125	125	125	125	125	125	125	125	125	125	119	119	125	128	119	125
rpL16	168	168	178	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168	168
orf178/rpL6	181	181	182	182	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181	181
rpS12	144	144	149	149	156	153	143	153	143	143	139	139	139	143	143	143	143	143	139	139	143	143	143	143	139	174	143	139	157	143
rpS13	237	271	249	249	302	261	261	261	244	244	241	241	241	241	241	241	241	241	241	241	241	241	241	241	241	241	241	241	241	241
rpS14	103	103	103	103	103	103	76	103	105	105	102	102	102	103	103	103	103	103	103	103	103	103	103	103	102	105	102	183	115	105
rpS19	68	68	68	68	92	92	92	92	69	69	-	-	-	66	66	66	66	66	-	66	66	66	66	-	-	66	-	-	66
rpS3	166	166	165	165	381	368	382	368	127	127	125	125	125	144	144	144	144	144	141	141	144	144	144	144	125	127	146	139	127	127
yejR	120	439	nd	nd	453	190	452	452	432	432	429	429	429	429	429	429	429	429	429	429	429	429	429	429	370	429	370	370	433	429
Ymf56	105	107	94	94	106	106	114	106	82	82	189	189	63	78	78	78	78	78	78	78	78	78	78	78	146	150	103	146	78	82
Ymf57	100	100	100	100	100	100	100	100	76	76	93	93	93	77	76	77	77	76	77	77	77	77	77	80	77	80	80	80	80	80
Ymf59	122	122	122	122	128	128	128	128	116	116	113	113	113	116	116	116	116	116	116	116	116	116	116	116	116	116	116	123	116	116
Ymf61	nd	nd	nd	nd	186	nd	176	227	185	185	228	228	228	182	182	182	182	182	185	185	182	182	182	182	182	182	182	185	185	179
Ymf63	538	540	450	450	642	641	647	641	561	561	563	563	563	561	561	561	561	561	561	561	561	561	561	561	561	561	561	559	559	551
Ymf64/orf234	1,000	863	442	288	1,614	1,742	1,679	1,783	572	572	613	613	613	666	666	666	666	666	666	666	666	666	666	666	557	572	668	613	619	671
Ymf65/orf156	374	365	370	370	371	366	397	367	365	365	423	423	423	365	365	365	365	365	365	365	365	365	365	365	422	365	385	434	365	365
Ymf66	428	428	447	409	427	419	427	427	445	445	445	445	445	466	466	466	466	456	413	413	466	466	466	466	429	413	428	428	398	398
Ymf67	354	354	344	344	354	354	354	354	366	378	348	348	348	355	355	355	355	355	355	355	355	355	355	355	345	345	402	345	362	355
Ymf68	387	387	387	387	395	389	389	389	387	387	387	387	387	387	387	387	387	387	387	387	357	387	387	387	387	387	390	387	387	387
Ymf76/orf105	385	385	405	405	405	405	405	405	61	369	405	405	405	324	321	327	317	321	366	380	310	320	326	324	410	372	453	409	379	441
Ymf78	72	63	73	73	69	73	73	73	72	72	122	122	122	66	66	66	66	66	66	66	66	66	66	66	72	72	72	70	66	66
Ymf79	78	99	104	104	75	75	75	75	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70	70
Ymf80	-	89	96	96	90	92	104	90	89	89	89	89	89	89	89	89	89	89	89	89	89	89	89	89	89	89	89	89	89	89
Ymf83	-	167	172	172	192	172	188	187	166	166	250	250	250	166	166	166	175	166	166	166	175	175	166	175	183	166	233	166	166	250
Ymf84	188	188	188	188	245	186	186	186	185	185	185	185	185	185	185	185	185	185	185	185	185	185	185	185	185	185	185	185	186	188
orf189	-	-	-	-	-	-	-	-	-	-	67	67	67	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
orf221	-	-	-	-	-	-	-	-	97	97	84	84	84	-	-	-	-	-	-	-	-	-	-	92	90	-	129	116	90	-
orf265	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	88	-	-	-	-	-
orf285	-	-	-	-	-	-	-	-	-	-	108	108	108	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
orf314	-	-	-	-	-	-	-	-	-	-	73	73	73	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
orf78	-	-	-	-	-	-	-	-	-	-	-	-	138	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
newORF	191	191	192	192	191	191	191	186	191	-	191	191	191	191	191	191	191	191	191	132	191	191	191	191	191	191	191	191	191	-

Supplementary Figure 10: Presence/absence of proteins encoded in mitochondrial genomes in species and individual isolates of species of the *Paramecium* genus. The numbers indicate the length of each protein in amino acids.

mean_dS	bi	tet	sex	oct	nov	dec	dodec	quadec	jenningsi	caudatum	caudatum C026	multimicronucleatum
bi	-	-	-	-	-	-	-	-	-	-	-	-
tet	1.22	-	-	-	-	-	-	-	-	-	-	-
sex	1.31	1.39	-	-	-	-	-	-	-	-	-	-
oct	1.17	0.93	1.42	-	-	-	-	-	-	-	-	-
nov	1.07	1.18	1.41	1.20	-	-	-	-	-	-	-	-
dec	1.32	0.99	1.27	1.12	1.11	-	-	-	-	-	-	-
dodec	1.20	0.94	1.30	1.04	1.23	0.85	-	-	-	-	-	-
quadec	1.19	1.27	1.29	1.20	1.06	1.24	1.21	-	-	-	-	-
jenningsi	1.42	1.38	1.37	1.41	1.26	1.31	1.27	1.28	-	-	-	-
caudatum	2.30	2.56	2.45	2.39	2.77	2.56	2.44	2.62	2.61	-	-	-
caudatum C026	4.86	2.48	2.37	2.53	2.48	2.46	5.05	2.48	2.45	1.36	-	-
multimicronucleatum	2.75	2.69	2.80	2.71	2.85	2.77	2.73	2.71	2.76	7.69	5.12	-
multimicronucleatum Peniche3I	2.67	2.88	2.79	2.57	2.84	2.86	2.69	2.76	2.79	4.95	2.45	1.97

Supplementary Figure 11: Mean pairwise dS between all *Paramecium* species. Species pairs with $dS < 1.0$ are shown in shaded boxes.
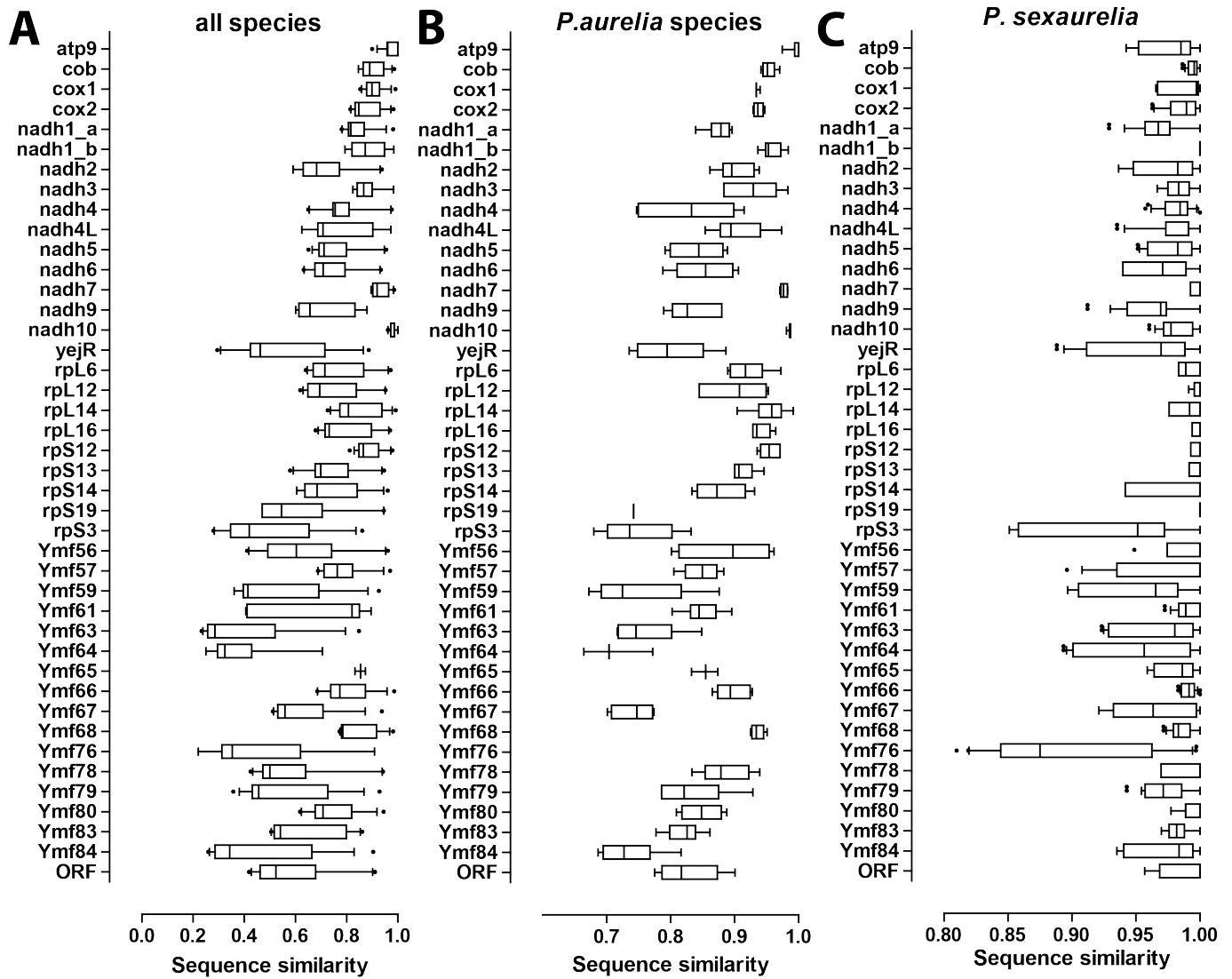
Supplementary Figure 12: Conservation and divergence of mitochondrial protein sequences in the *Paramecium* genus. Shown is the percent sequence identity for each protein within all species included in this study (A), within the *P. aurelia* species complex (B), and between the 11 individual *P. sexaurelia* isolates (C). Only one isolate per species was included in (A) and (B). The whiskers of the box plot correspond to the 5–95 percentile range.
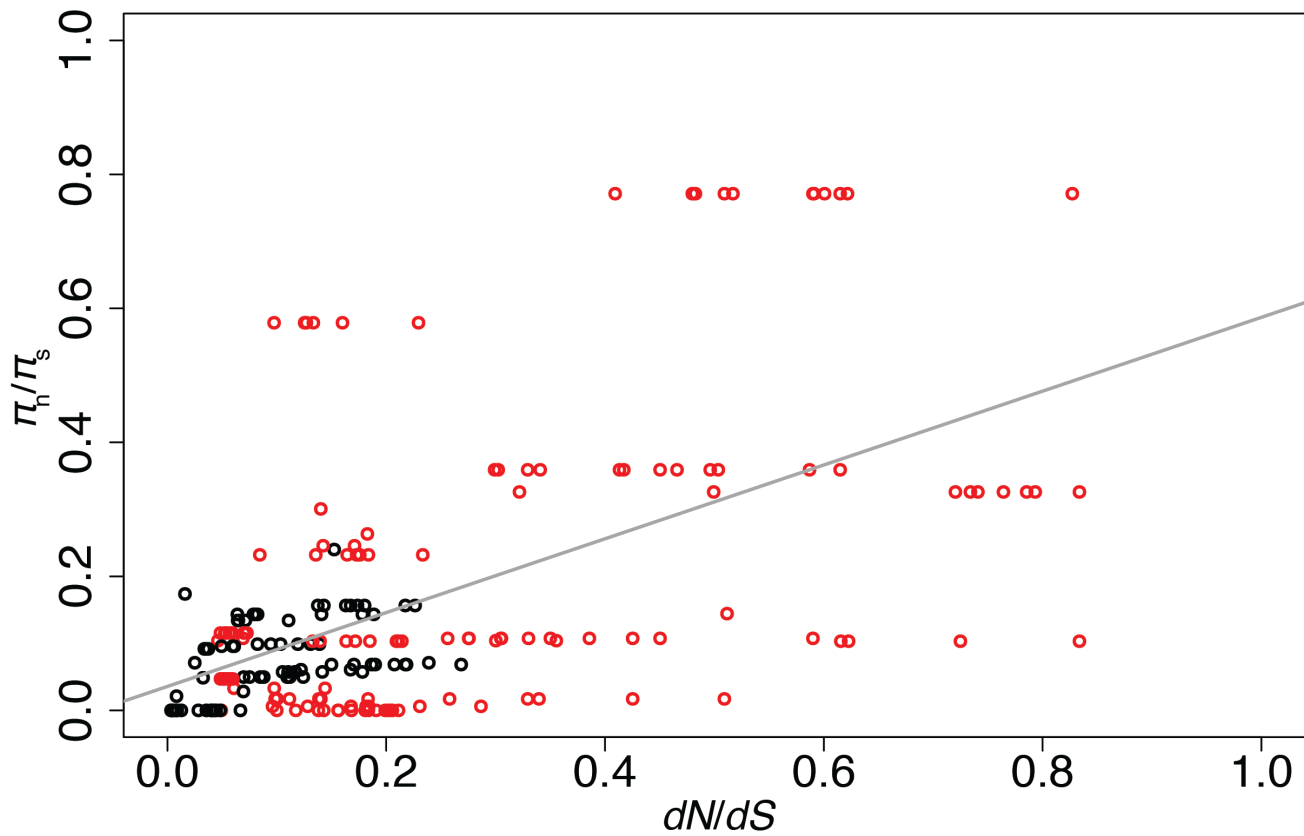
Supplementary Figure 13: Relationship between observed π_n/π_s and pairwise dN/dS with respect to the closest outgroup species. This analysis was performed for all protein-coding genes, with the conserved protein coding genes in black and the divergent *Ymf* genes indicated in red, for *P. tetraurelia*, *P. sexaurelia*, *P. caudatum* and *P. multimicronucleatum*. Only genes with $dS < 1.0$ and $\pi_s > 1$, where π_s is the total number of polymorphic sites at synonymous positions, were used in this analysis, resulting in 208 total pairs of observations. This filter was executed in order to reduce errors in π_n/π_s due to very low values of synonymous polymorphisms in a gene.

AA	codon	P. multimicronucleatum					P. caudatum			P. octaurelia	P. tetraurelia			P. biaurelia		P. sexaurelia											P. decaurelia	P. dodecaurelia	P. jenningsi	P. novaurelia	P. quadecaurelia
		M13	Peniche3I	M04	M05	C026	C083	C065	C104	1038	291	99	A	379	USBL-36I	126	128	Indo1-7I	Moz13BIII	127	131	137	130	134	132	133	223	274	M	TE	N1A
A	GCA	0.45	0.45	0.44	0.44	0.41	0.41	0.41	0.39	0.21	0.21	0.21	0.21	0.20	0.21	0.21	0.21	0.22	0.21	0.21	0.21	0.21	0.20	0.21	0.22	0.22	0.20	0.26	0.21	0.22	0.19
	GCC	0.02	0.02	0.01	0.01	0.03	0.03	0.03	0.04	0.38	0.38	0.38	0.38	0.38	0.38	0.36	0.35	0.36	0.35	0.37	0.38	0.38	0.37	0.37	0.37	0.37	0.37	0.36	0.36	0.34	0.34
	GCG	0.01	0.01	0.01	0.01	0.02	0.03	0.03	0.02	0.10	0.10	0.10	0.10	0.09	0.09	0.04	0.04	0.04	0.05	0.06	0.05	0.06	0.06	0.06	0.06	0.06	0.12	0.10	0.07	0.07	0.15
	GCU	0.52	0.52	0.53	0.53	0.55	0.54	0.53	0.54	0.31	0.31	0.31	0.32	0.32	0.32	0.39	0.39	0.39	0.39	0.36	0.36	0.36	0.37	0.36	0.35	0.35	0.31	0.29	0.35	0.36	0.32
C	UGC	0.18	0.21	0.20	0.18	0.20	0.19	0.19	0.17	0.67	0.68	0.68	0.68	0.60	0.60	0.52	0.52	0.52	0.53	0.56	0.55	0.57	0.57	0.57	0.63	0.64	0.59	0.72	0.65	0.59	0.57
	UGU	0.82	0.79	0.80	0.82	0.80	0.81	0.81	0.83	0.33	0.32	0.32	0.32	0.40	0.40	0.48	0.48	0.48	0.47	0.44	0.45	0.43	0.43	0.43	0.37	0.36	0.41	0.28	0.35	0.41	0.43
D	GAC	0.13	0.13	0.12	0.11	0.17	0.15	0.20	0.17	0.62	0.63	0.63	0.63	0.56	0.55	0.55	0.56	0.55	0.53	0.60	0.59	0.58	0.58	0.58	0.63	0.64	0.55	0.61	0.58	0.61	0.54
	GAU	0.87	0.87	0.88	0.89	0.83	0.85	0.80	0.83	0.38	0.37	0.37	0.37	0.44	0.45	0.45	0.44	0.45	0.47	0.40	0.41	0.42	0.42	0.42	0.37	0.36	0.45	0.39	0.42	0.39	0.46
E	GAA	0.80	0.86	0.93	0.93	0.79	0.85	0.83	0.82	0.39	0.36	0.37	0.37	0.36	0.35	0.39	0.39	0.39	0.40	0.37	0.37	0.38	0.39	0.38	0.37	0.36	0.35	0.30	0.37	0.31	0.37
	GAG	0.20	0.14	0.07	0.07	0.21	0.15	0.17	0.18	0.61	0.64	0.63	0.63	0.64	0.65	0.61	0.61	0.61	0.60	0.63	0.63	0.62	0.61	0.62	0.63	0.64	0.65	0.70	0.63	0.69	0.63
F	UUC	0.05	0.04	0.03	0.03	0.06	0.05	0.06	0.05	0.34	0.36	0.36	0.36	0.34	0.35	0.33	0.33	0.33	0.33	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.32	0.32	0.31
	UUU	0.95	0.96	0.97	0.97	0.94	0.95	0.94	0.95	0.66	0.64	0.64	0.64	0.66	0.65	0.67	0.67	0.67	0.67	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.68	0.68	0.69
G	GGA	0.32	0.33	0.38	0.38	0.21	0.23	0.20	0.21	0.22	0.20	0.20	0.20	0.20	0.21	0.21	0.22	0.21	0.21	0.20	0.20	0.20	0.20	0.20	0.21	0.21	0.20	0.21	0.22	0.19	0.20
	GGC	0.02	0.04	0.02	0.02	0.03	0.03	0.05	0.07	0.36	0.36	0.36	0.36	0.32	0.33	0.31	0.30	0.31	0.30	0.32	0.32	0.32	0.32	0.32	0.30	0.31	0.30	0.33	0.34	0.32	0.33
	GGG	0.02	0.02	0.01	0.00	0.03	0.02	0.05	0.04	0.20	0.27	0.27	0.27	0.26	0.25	0.20	0.19	0.20	0.20	0.21	0.21	0.21	0.21	0.23	0.23	0.23	0.29	0.24	0.20	0.27	0.25
	GGU	0.64	0.61	0.59	0.59	0.72	0.72	0.71	0.69	0.21	0.17	0.17	0.17	0.22	0.21	0.28	0.29	0.28	0.29	0.28	0.28	0.27	0.27	0.26	0.26	0.26	0.21	0.21	0.24	0.23	0.23
H	CAC	0.24	0.17	0.12	0.12	0.33	0.25	0.28	0.28	0.60	0.61	0.61	0.61	0.57	0.57	0.59	0.60	0.59	0.63	0.66	0.66	0.65	0.64	0.65	0.64	0.63	0.63	0.63	0.68	0.61	0.60
	CAU	0.76	0.83	0.88	0.88	0.67	0.75	0.72	0.72	0.40	0.39	0.39	0.39	0.43	0.43	0.41	0.40	0.41	0.37	0.34	0.34	0.35	0.36	0.35	0.36	0.37	0.37	0.38	0.32	0.39	0.40
I	AUA	0.39	0.39	0.40	0.39	0.41	0.42	0.43	0.43	0.25	0.25	0.25	0.24	0.26	0.25	0.24	0.24	0.24	0.24	0.23	0.23	0.24	0.24	0.24	0.25	0.26	0.23	0.23	0.26	0.29	0.25
	AUC	0.03	0.03	0.02	0.02	0.06	0.04	0.05	0.07	0.39	0.41	0.41	0.41	0.39	0.39	0.38	0.37	0.38	0.38	0.40	0.40	0.39	0.39	0.39	0.41	0.41	0.40	0.40	0.42	0.37	0.36
	AUU	0.58	0.59	0.58	0.59	0.52	0.54	0.52	0.50	0.36	0.34	0.34	0.35	0.35	0.35	0.38	0.39	0.39	0.38	0.37	0.37	0.37	0.37	0.37	0.34	0.34	0.38	0.37	0.33	0.34	0.39
K	AAA	0.90	0.92	0.96	0.96	0.88	0.91	0.88	0.89	0.47	0.45	0.45	0.45	0.43	0.43	0.49	0.49	0.48	0.50	0.47	0.48	0.48	0.48	0.48	0.44	0.45	0.45	0.43	0.42	0.40	0.43
	AAG	0.10	0.08	0.04	0.04	0.12	0.09	0.12	0.11	0.53	0.55	0.55	0.55	0.57	0.57	0.51	0.51	0.52	0.50	0.53	0.52	0.52	0.52	0.52	0.56	0.55	0.55	0.57	0.58	0.60	0.57
L	CUA	0.08	0.06	0.05	0.05	0.06	0.05	0.08	0.07	0.13	0.13	0.13	0.13	0.14	0.14	0.11	0.11	0.11	0.10	0.11	0.11	0.11	0.10	0.11	0.12	0.11	0.15	0.15	0.11	0.13	0.10
	CUC	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.26	0.27	0.27	0.27	0.25	0.25	0.24	0.23	0.24	0.24	0.26	0.26	0.26	0.26	0.26	0.27	0.28	0.26	0.27	0.25	0.26	0.24
	CUG	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.07	0.09	0.09	0.08	0.07	0.07	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.09	0.09	0.08	0.07	0.07	0.10
	CUU	0.14	0.14	0.13	0.13	0.13	0.12	0.12	0.13	0.21	0.21	0.21	0.22	0.22	0.22	0.27	0.28	0.25	0.25	0.25	0.25	0.25	0.23	0.23	0.20	0.20	0.26	0.23	0.26	0.23	0.25
	UUA	0.69	0.70	0.75	0.75	0.69	0.73	0.68	0.68	0.18	0.16	0.16	0.16	0.20	0.19	0.18	0.18	0.18	0.19	0.18	0.18	0.18	0.18	0.18	0.18	0.17	0.17	0.17	0.15	0.18	0.15
	UUG	0.07	0.08	0.05	0.05	0.09	0.08	0.09	0.10	0.14	0.14	0.14	0.14	0.13	0.12	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.15	0.14	0.13	0.16	0.13	0.16
M	AUG	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
N	AAC	0.20	0.21	0.14	0.14	0.25	0.20	0.23	0.24	0.56	0.63	0.63	0.63	0.53	0.54	0.57	0.57	0.57	0.57	0.57	0.55	0.55	0.56	0.63	0.63	0.65	0.55	0.54	0.67	0.59	0.62
	AAU	0.80	0.79	0.86	0.86	0.75	0.80	0.77	0.76	0.44	0.37	0.37	0.37	0.47	0.46	0.43	0.43	0.43	0.43	0.43	0.45	0.45	0.44	0.37	0.37	0.35	0.45	0.46	0.33	0.41	0.38
P	CCA	0.49	0.50	0.48	0.48	0.44	0.48	0.43	0.42	0.19	0.15	0.15	0.15	0.17	0.17	0.16	0.16	0.16	0.16	0.15	0.16	0.16	0.16	0.17	0.15	0.15	0.15	0.18	0.17	0.22	0.18
	CCC	0.01	0.01	0.01	0.01	0.03	0.02	0.05	0.05	0.45	0.45	0.45	0.45	0.42	0.42	0.43	0.41	0.43	0.41	0.45	0.44	0.44	0.43	0.43	0.40	0.40	0.43	0.50	0.45	0.43	0.36
	CCG	0.02	0.02	0.01	0.01	0.04	0.03	0.03	0.03	0.04	0.09	0.09	0.09	0.07	0.07	0.07	0.08	0.07	0.09	0.08	0.08	0.08	0.08	0.08	0.08	0.09	0.12	0.06	0.06	0.05	0.17
	CCU	0.48	0.48	0.50	0.50	0.50	0.48	0.48	0.50	0.32	0.30	0.31	0.31	0.33	0.33	0.34	0.35	0.34	0.34	0.32	0.33	0.32	0.33	0.32	0.37	0.36	0.30	0.26	0.32	0.29	0.29
Q	CAA	0.91	0.91	0.93	0.93	0.84	0.88	0.85	0.85	0.57	0.46	0.46	0.46	0.42	0.42	0.54	0.53	0.54	0.53	0.49	0.49	0.50	0.50	0.50	0.56	0.56	0.47	0.47	0.48	0.45	0.40
	CAG	0.09	0.09	0.07	0.07	0.16	0.12	0.16	0.15	0.43	0.54	0.54	0.54	0.58	0.58	0.46	0.47	0.46	0.47	0.51	0.51	0.50	0.50	0.50	0.44	0.44	0.53	0.53	0.52	0.55	0.60
R	AGA	0.88	0.86	0.87	0.87	0.73	0.79	0.73	0.76	0.19	0.18	0.18	0.18	0.22	0.22	0.24	0.24	0.24	0.21	0.20	0.20	0.21	0.21	0.21	0.22	0.22	0.20	0.18	0.22	0.20	0.14
	AGG	0.06	0.08	0.04	0.04	0.16	0.10	0.15	0.14	0.36	0.38	0.38	0.38	0.43	0.43	0.46	0.46	0.46	0.50	0.53	0.53	0.52	0.52	0.52	0.49	0.50	0.41	0.40	0.47	0.51	0.37
	CGA	0.02	0.02	0.03	0.04	0.02	0.03	0.03	0.02	0.13	0.13	0.13	0.14	0.08	0.08	0.09	0.10	0.10	0.09	0.09	0.09	0.09	0.09	0.09	0.08	0.07	0.12	0.12	0.09	0.08	0.09
	CGC	0.01	0.01	0.00	0.00	0.02	0.02	0.01	0.01	0.16	0.15	0.15	0.15	0.14	0.14	0.11	0.11	0.11	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.13	0.14	0.10	0.12	0.18
	CGG	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.08	0.07	0.07	0.07	0.05	0.05	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.04	0.04	0.07	0.08	0.03	0.04	0.12
	CGU	0.03	0.03	0.06	0.06	0.07	0.05	0.07	0.06	0.08	0.08	0.08	0.08	0.08	0.07	0.06	0.06	0.06	0.06	0.06	0.05	0.05	0.06	0.06	0.07	0.07	0.07	0.07	0.09	0.06	0.10
S	AGC	0.07	0.07	0.04	0.04	0.07	0.05	0.08	0.08	0.24	0.24	0.24	0.24	0.26	0.26	0.24	0.25	0.24	0.24	0.24	0.24	0.25	0.25	0.25	0.25	0.25	0.23	0.25	0.24	0.27	0.24
	AGU	0.25	0.28	0.24	0.24	0.24	0.27	0.21	0.21	0.08	0.08	0.08	0.09	0.08	0.08	0.10	0.09	0.10	0.09	0.10	0.09	0.10	0.10	0.10	0.08	0.08	0.09	0.07	0.09	0.09	0.09
	UCA	0.23	0.23	0.28	0.28	0.21	0.22	0.24	0.24	0.12	0.11	0.11	0.11	0.12	0.11	0.13	0.14	0.13	0.13	0.12	0.12	0.12	0.12	0.11	0.11	0.11	0.09	0.09	0.10	0.11	0.11
	UCC	0.01	0.01	0.01	0.01	0.02	0.01	0.03	0.03	0.18	0.19	0.19	0.19	0.18	0.18	0.16	0.16	0.16	0.17	0.17	0.17	0.17	0.17	0.19	0.19	0.19	0.21	0.21	0.18	0.18	0.18
	UCG	0.01	0.01	0.00	0.00	0.01	0.02	0.03	0.03	0.12	0.14	0.14	0.14	0.12	0.13	0.12	0.12	0.12	0.11	0.13	0.12	0.12	0.12	0.12	0.13	0.14	0.15	0.14	0.13	0.10	0.12
	UCU	0.42	0.41	0.42	0.42	0.44	0.43	0.42	0.42	0.25	0.24	0.24	0.24	0.25	0.25	0.24	0.24	0.25	0.25	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.23	0.23	0.26	0.25	0.25
T	ACA	0.51	0.49	0.48	0.49	0.50	0.46	0.46	0.47	0.23	0.20	0.20	0.19	0.22	0.21	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.19	0.19	0.19	0.19	0.19	0.22	0.20
	ACC	0.02	0.03	0.02	0.02	0.05	0.03	0.05	0.04	0.32	0.35	0.35	0.35	0.34	0.35	0.36	0.36	0.36	0.35	0.33	0.33	0.33	0.33	0.34	0.33	0.33	0.35	0.35	0.38	0.38	0.37
	ACG	0.02	0.02	0.02	0.02	0.01	0.01	0.04	0.03	0.23	0.23	0.23	0.23	0.20	0.20	0.17	0.18	0.17	0.18	0.20	0.20	0.20	0.19	0.19	0.19	0.23	0.23	0.24	0.21	0.17	0.20
	ACU	0.45	0.47	0.48	0.48	0.44	0.49	0.46	0.45	0.22	0.23	0.23	0.23	0.25	0.25	0.23	0.23	0.23	0.23	0.24	0.24	0.23	0.24	0.24	0.24	0.24	0.22	0.22	0.23	0.23	0.23
V	GUA	0.37	0.37	0.40	0.40	0.35	0.37	0.37	0.36	0.25	0.22	0.22	0.22	0.25	0.25	0.25	0.25	0.25	0.24	0.23	0.23	0.23	0.23	0.23	0.24	0.24	0.23	0.21	0.22	0.24	0.22
	GUC	0.03	0.01	0.02	0.02	0.02	0.02	0.03	0.03	0.24	0.26	0.27	0.27	0.22	0.22	0.25	0.25	0.26	0.25	0.27	0.27	0.27	0.27	0.27	0.26	0.27	0.26	0.26	0.26	0.25	0.23
	GUG	0.04	0.05	0.04	0.04	0.04	0.05	0.04	0.07	0.18	0.19	0.19	0.18	0.15	0.15	0.14	0.15	0.15	0.16	0.16	0.16	0.16	0.16	0.15	0.15	0.19	0.19	0.21	0.19	0.16	0.17
	GUU	0.57	0.57	0.54	0.55	0.58	0.57	0.53	0.54	0.32	0.33	0.33	0.33	0.37	0.37	0.36	0.36	0.36	0.36	0.34	0.34	0.34	0.34	0.35	0.34	0.34	0.32	0.32	0.33	0.35	0.38
W	UGA	0.96	0.95	0.98	0.98	0.95	0.98	0.96	0.97	0.57	0.49	0.49	0.49	0.58	0.58	0.52	0.51	0.53	0.55	0.51	0.50	0.51	0.51	0.51	0.52	0.51	0.52	0.54	0.47	0.45	0.43
	UGG	0.04	0.05	0.02	0.02	0.05	0.02	0.04	0.03	0.43	0.51	0.51	0.51	0.42	0.42	0.48	0.49	0.47	0.45	0.49	0.50	0.49	0.49	0.49	0.48	0.49	0.48	0.46	0.53	0.55	0.57
Y	UAC	0.19	0.18	0.12	0.12	0.22	0.15	0.20	0.21	0.58	0.59	0.59	0.59	0.57	0.57	0.55	0.54	0.55	0.56	0.56	0.56	0.56	0.56	0.56	0.55	0.55	0.61	0.63	0.52	0.60	0.59
	UAU	0.81	0.82	0.88	0.88	0.78	0.85	0.80	0.79	0.42	0.41	0.41	0.41	0.43	0.43	0.45	0.46	0.45	0.44	0.44	0.44	0.44	0.44	0.44	0.45	0.45	0.39	0.37	0.48	0.40	0.41

Supplementary Figure 14: Codon usage in *Paramecium* species. Shown is the frequency of each codon for each amino acid across the *Paramecium* species included in this study.

Supplementary Figure 15: Correlation between nucleotide diversity estimates at 0-fold degenerate sites relative to that at 4-fold degenerate between all SNPs and a conservative set of SNPs (excluding all potential NUMT contaminants).

Supplementary Figure 16: Distribution of SNP positions across mitochondrial genomes. *P. tetraurelia* (light blue), *P. sexaurelia* (dark blue), *P. caudatum* (orange) and *P. multimicronucleatum* (green). Top panel covers positions 1–10,000, second panel shows positions 10,000–20,000, third panel shows positions 20,000–30,000, and last panel shows positions 30,000-44,000.
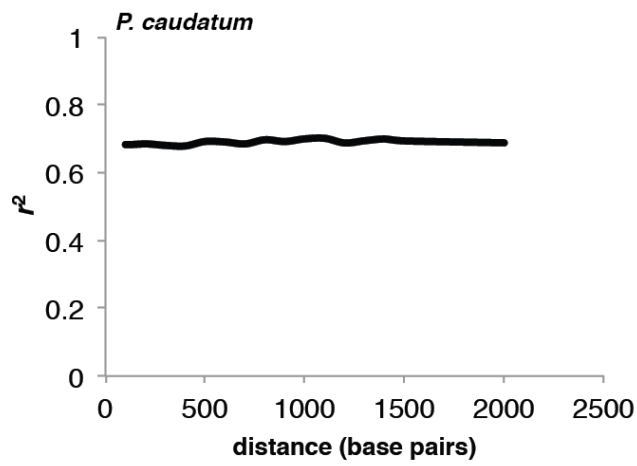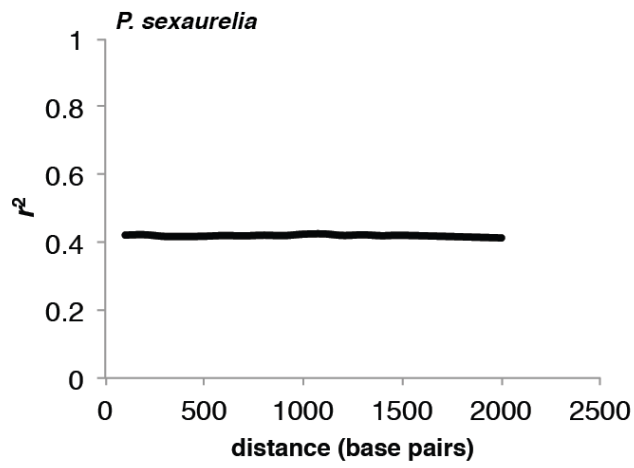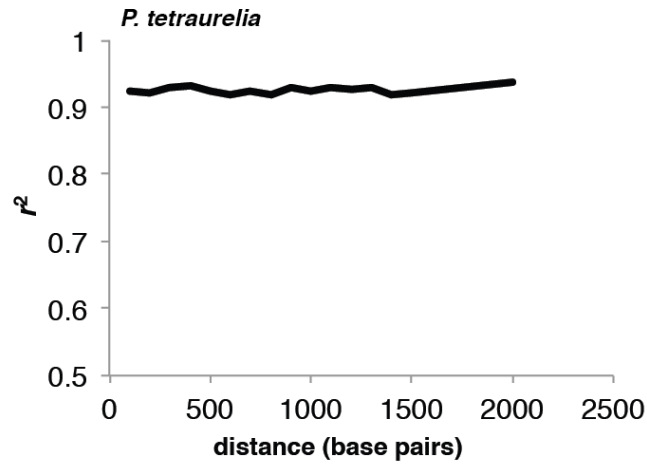
Supplementary Figure 17: Decay of linkage disequilibrium across the mitochondrial genomes of *P. tetraurelia*, *P. sexaurelia*, and *P. caudatum*.

Supplementary Tables

gene	dN/dS
NADH dehydrogenase subunit 10	0.0071
NADH dehydrogenase subunit 1 b	0.0159
NADH dehydrogenase subunit 7	0.0177
ribosomal protein L14	0.0220
apocytochrome b	0.0221
Ymf68	0.0234
NADH dehydrogenase subunit 3	0.0296
NADH dehydrogenase subunit 6	0.0305
cytochrome c oxidase subunit 1	0.0337
NADH dehydrogenase subunit 4	0.0338
Ymf57	0.0342
Ymf80	0.0347
NADH dehydrogenase subunit 1 a	0.0396
ribosomal protein S12	0.0406
cytochrome c oxidase subunit 2	0.0433
ribosomal protein L16	0.0482
ribosomal protein L12	0.0483
Ymf66	0.0491
ribosomal protein L6	0.0522
ribosomal protein S13	0.0544
NADH dehydrogenase subunit 4L	0.0559
NADH dehydrogenase subunit 5	0.0623
NADH dehydrogenase subunit 2	0.0661
Ymf79	0.0663
Ymf56	0.0703
Ymf78	0.0805
ribosomal protein S14	0.0818
Ymf65	0.0894
Ymf61	0.1018
Ymf67	0.1105
NADH dehydrogenase subunit 9	0.1158
Ymf84	0.1200
yejR	0.1241
Ymf59	0.1476
ribosomal protein S3	0.1574
Ymf63	0.1632
ATP synthase F0 subunit 9	0.1781
Ymf83	0.1803
Ymf76	0.1962
Ymf64	0.2154

Supplementary Table 2: List of genes under positive selection in *P. sexaurelia* and *P. caudatum*. All p values are corrected for multiple tests by Holm method.

P. sexaurelia				P. caudatum			
all sites included		Using well conserved sites		all sites included		Using well conserved sites	
gene	p	gene	p	gene	p	gene	p
Ymf64	1.69×10^{-13}	*Cox1*	5.01×10^{-3}	*Ymf63*	5.71×10^{-9}	*Ymf64*	3.48×10^{-7}
Ymf67	2.71×10^{-7}	*Ymf76*	9.49×10^{-3}	*Ymf64*	2.12×10^{-8}		
Ymf63	3.56×10^{-6}	*Ymf63*	2.99×10^{-2}	*rpS3*	2.78×10^{-4}		
Ymf84	1.84×10^{-5}			*Ymf59*	7.79×10^{-3}		
Cox1	2.28×10^{-4}			*Ymf61*	1.18×10^{-2}		
Nadh5	3.58×10^{-4}			*Ymf67*	2.65×10^{-2}		
rpL6	1.91×10^{-3}						
yejR	4.22×10^{-3}						
Ymf65	6.72×10^{-3}						
rpS13	8.23×10^{-3}						
cox2	1.05×10^{-2}						
Ymf76	2.72×10^{-2}						
Ymf61	2.79×10^{-2}						

Supplementary Table 3: Mitochondrial mutations identified in *P. biaurelia* and *P. sexaurelia* MA experiments

P. biaurelia			P. sexaurelia		
position	genome	variant	position	genome	variant
490	C	A	1577	A	G
606	C	A	1645	T	C
2646	T	A	3466	A	T
2887	T	G	3790	A	G
3155	A	G	4674	T	A
3155	A	T	4742	T	A
4779	C	A	4770	G	A
4794	G	A	4847	A	T
4811	T	G	5065	T	A
7442	A	T	5417	A	G
7498	A	G	6096	G	A
7529	T	A	6454	T	A
7728	T	A	6491	T	A
7753	T	A	6635	G	A
7979	T	A	6642	T	A
7998	A	G	6903	A	C
8071	T	A	7007	A	G
8076	T	A	7424	A	T
8077	A	T	7522	G	A
13086	T	A	7538	G	A
13140	A	C	7568	G	A
17709	T	A	7721	T	A
17709	T	C	7734	T	A
17761	T	A	7858	A	C
17787	G	A	8152	T	A
17790	C	A	8172	T	G
17841	T	A	8405	T	C
17841	T	G	8439	A	C
18090	A	T	8620	A	G
22843	T	A	8850	T	A
22843	T	G	9044	G	A
22955	A	T	9955	A	G
26509	T	A	10023	T	C

Continued on next page

P. biaurelia			P. sexaurelia		
position	genome	variant	position	genome	variant
26657	T	C	10382	A	G
26668	A	G	10444	A	G
26702	T	A	10919	T	A
26703	T	A	11283	T	A
26744	C	A	11900	T	A
26766	G	A	12902	A	G
26768	T	A	13649	T	C
28321	T	A	15256	T	A
28378	T	A	15771	A	T
28498	T	G	15786	T	C
32849	T	A	15799	A	G
32849	T	G	16239	A	G
33094	T	G	16394	T	C
33096	T	G	17103	A	T
33139	A	G	18194	A	G
35080	T	C	19729	T	G
35485	T	A	20044	T	G
35512	A	G	20398	A	G
35564	T	C	20529	T	C
35723	C	T	21060	A	G
37061	G	A	21204	A	T
37104	T	G	21423	A	T
			21615	A	T
			22111	T	C
			23241	A	G
			24268	C	T
			24806	A	G
			25196	T	C
			25199	A	C
			25227	A	G
			25290	T	A
			25347	T	A
			26548	T	A
			26672	T	G
			26934	T	A
			27174	T	C
			27714	T	G
			27924	A	C
			29490	A	T
			30667	T	C
			31217	C	T
			31870	A	G
			32870	T	G
			33090	T	A
			33794	A	G
			33800	T	G
			34300	C	T
			34334	G	A
			34348	G	A
			34736	T	A
			35045	T	A
			35700	C	T
			36667	T	A
			38460	T	A

Supplementary Table 4: Comparison of the total number and the overlap of SNP positions between the main SNP set and the stringent (excluding all possible NUMT contaminants) SNP set.

	P. tetraurelia		*P. sexaurelia*		*P. caudatum*		*P. multimicronucleatum*	
	All SNPs	Conservative SNPs	All SNPs	Conservative SNPs	All SNPs	Conservative SNPs	All SNPs	Conservative SNPs
Number of SNPs	510	565	5219	5255	2980	1393	534	350
Number of overlapping SNPs	508	508	5195	5195	1339	1339	259	259
Percentage overlap	99.6	89.9	99.5	98.9	44.9	96.1	48.5	74.0
Correlation of allele frequency (R^2)		0.93		0.84		0.39		0.63
Significance of correlation of allele frequency (p-value)		$\leq 2.2 \times 10^{-16}$		$\leq 2.2 \times 10^{-16}$		$\leq 2.2 \times 10^{-16}$		$\leq 2.2 \times 10^{-16}$
Average nucleotide diversity	0.0044	0.0060	0.0496	0.0487	0.0311	0.0157	0.0055	0.0060

Supplementary Table 5: Summary of results obtained from LDhat (pairwise) to detect the presence of recombination in mitochondrial genomes of *Paramecium* species. The p values (not corrected for multiple tests) indicate whether values of statistics calculated on observed data are significantly different from the same values obtained for permuted SNPs.

Model	θ	p (Composite Likelihood Test)	p (G4)	p (correlation between r^2 and distance)	p (correlation between D' and distance)	$4N_e r$ (Hudson's method with sites)	$4N_e r$ (estimated following Wakely, 1997)
				P. tetraurelia			
cross-over	0.001	0.333	0.000	0.194	0.000	0	0
	0.01	1.000	0.000	0.211	0.000	0	0
gene conversion	0.001	0.322	0.000	0.559	0.043	0	0
	0.01	1.000	0.000	0.549	0.043	0	0
				P. sexaurelia			
cross-over	0.01	1.000	0.965	0.980	1.000	0	0
	0.1	1.000	0.974	0.971	1.000	0	0
gene conversion	0.01	1.000	0.107	0.043	0.990	0	0
	0.1	1.000	0.099	0.050	0.998	0	0
				P. caudatum			
cross-over	0.01	0.273	1.000	0.995	0.998	0	0
	0.1	0.000	1.000	0.998	0.999	0	0
gene conversion	0.01	0.276	1.000	1.000	1.000	0	0
	0.1	0.000	1.000	1.000	1.000	0	0
				P. multimicronucleatum			
cross-over	0.01	0.236	0.811	0.782	0.466	0	0
gene conversion	0.01	0.225	0.879	0.806	0.502	0	0

Supplementary Table 6: Comparison of neutrality indices between the mitochondrial and nuclear genes that encode for proteins that are part of the oxidative phosphorylation pathway and those that form the structural components of the ribosome..

	Oxidative phosphorylation			Ribosomal proteins		
	π_n/π_s	$P_N/P_S/D_N/D_S$	$\pi_n/\pi_s/d_N/d_S$	π_n/π_s	$P_N/P_S/D_N/D_S$	$\pi_n/\pi_s/dN/dS$
			P. tetraurelia			
Number of nuclear genes	27	13	14	336	77	88
Mitochondrial	0.127	0.781	2.384	0.062	1.935	2.248
Nuclear	0.200	2.896	6.712	0.092	0.737	1.653
p	0.223	0.285	0.422	0.387	0.352	0.262
			P. sexaurelia			
Number of nuclear genes	22	18	16	429	119	138
Mitochondrial	0.124	0.328	5.180	0.035	0.089	0.754
Nuclear	0.146	1.243	7.579	0.158	1.047	3.759
p	0.535	0.444	0.145	0.093	0.124	0.308
			P. caudatum			
Number of nuclear genes	12	3	–	422	9	–
Mitochondrial	0.105	0.364	–	0.067	0.051	–
Nuclear	0.133	0.211	–	0.133	0.260	–
p	0.726	0.107	–	0.513	0.650	–

Supplementary Table 7: Neutrality indices (defined in the Methods section) in the mitochondria of *Paramecium* species (divergence estimated using all 13 taxa)

	P. tetraurelia		P. sexaurelia		P. caudatum	
	NI [SD]	NI_{TG}	NI [SD]	NI_{TG}	NI [SD]	NI_{TG}
All mitochondrial genes	1.521 [2.031]	0.765	0.281 [0.475]	0.213	0.500 [0.592]	0.314
Excluding *Ymf* genes	1.794 [2.515]	0.690	0.339 [0.601]	0.265	0.645 [0.706]	0.403
All mitochondrial genes with $dS < 1.0$	1.379 [1.454]	0.829	0.539 [0.843]	0.371	0.513 [0.618]	0.314
All mitochondrial genes with $dS < 1.0$, excluding *Ymf* genes	1.607 [1.890]	0.715	0.859 [1.211]	0.495	0.674 [0.894]	0.253

Supplementary Table 8: Number of nuclear DNA repair-associated genes in *Paramecium* species

Repair-related genes	Number of gene copies:			
	P. caudatum	P. tetraurelia	P. biaurelia	P. sexaurelia
ALKYLATED DNA REPAIR PROTEIN ALKB-RELATED	1	2	1	2
DNA CROSS-LINK REPAIR PROTEIN PSO2/SNM1	1	2	2	2
DNA-DAMAGE REPAIR PROTEIN DRT111		1	4	1
DNA MISMATCH REPAIR MUTS RELATED PROTEINS	2	2	2	2
DNA MISMATCH REPAIR PROTEIN MLH1	1	1	1	
DNA MISMATCH REPAIR PROTEIN PMS1	1	1	1	1
DNA REPAIR AND RECOMBINATION PROTEIN RAD54B		1	1	2
DNA REPAIR DEAD HELICASE RAD3/XP-D SUBFAMILY MEMBER		1		1
DNA REPAIR ENDONUCLEASE XPF	1	1	1	1
DNA REPAIR HELICASE RAD3/XP-D	1	3	2	
DNA REPAIR POLYMERASE UMUC / TRANSFERASE FAMILY MEMBER		1		
DNA REPAIR PROTEIN RAD51	1	2	2	2
DNA REPAIR PROTEIN XP-C / RAD4	1	2	2	1
DNA REPAIR/TRANSCRIPTION PROTEIN MET18/MMS19	1	1	1	1
DOUBLE-STRAND BREAK REPAIR PROTEIN MRE11A	1	2	2	2
EXCISION REPAIR CROSS-COMPLEMENTING 1 ERCC1		1		
RAD25/XP-B DNA REPAIR HELICASE		2		
UV EXCISION REPAIR PROTEIN RAD23	3	6	7	6
XP-G/RAD2 DNA REPAIR ENDONUCLEASE FAMILY		1	1	1
DNA-REPAIR PROTEIN XP-G	1			
RAD25/XP-B DNA REPAIR HELICASE	2		2	2
DNA REPAIR PROTEIN RECN			1	
NUCLEOTIDE EXCISION REPAIR COMPLEX SUBUNIT UVRC			1	