Supplemental Material for:

Lake Malawi cichlid pangenome graph reveals extensive structural variation driven by transposable elements

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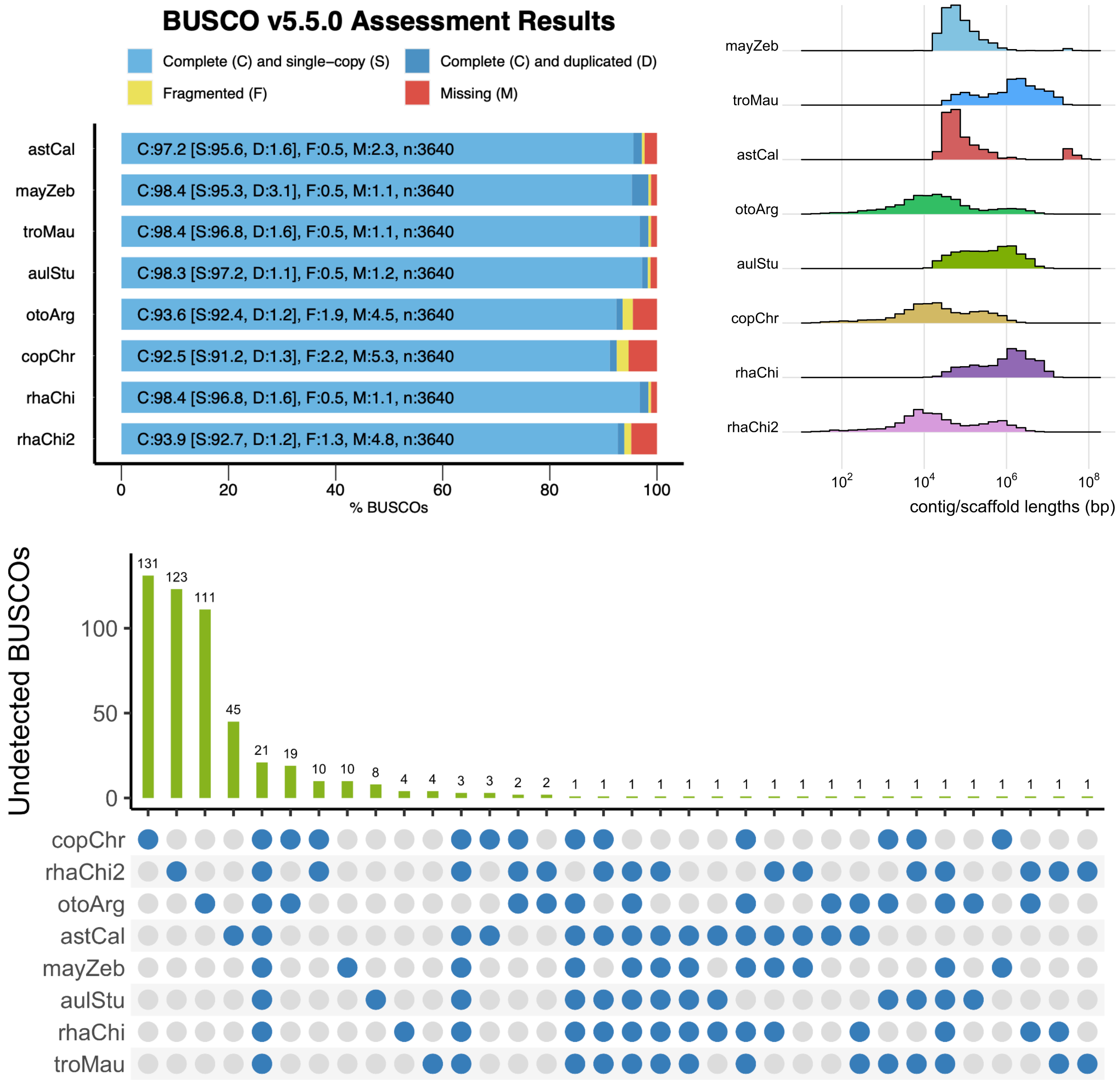
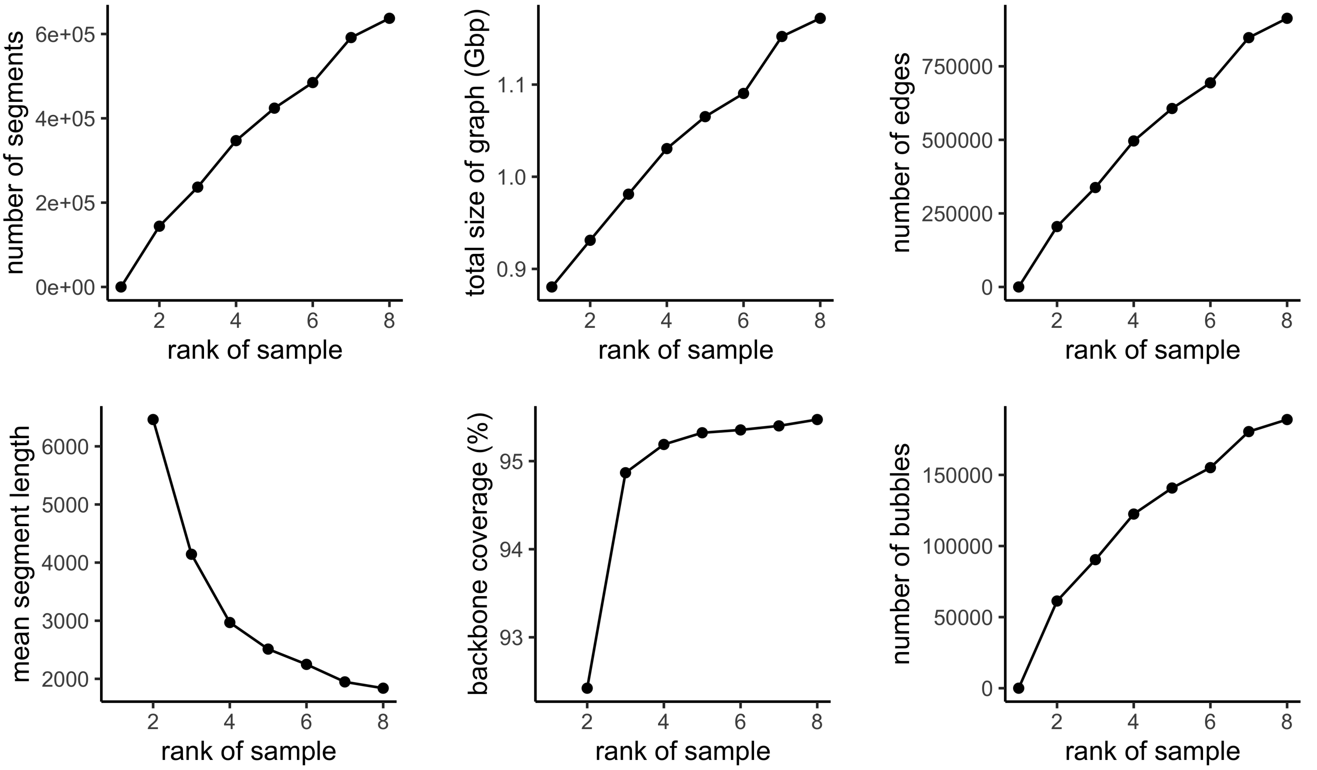
In Zenodo repository (https://doi.org/10.5281/zenodo.14029308):

- **FASTA files of six new assemblies for Lake Malawi cichlids**
- **Pangenome graph in minigraph GFA format**
- **Excel file of detected variants**
- **Excel files for gene lists used in Gene Ontology enrichment analysis**

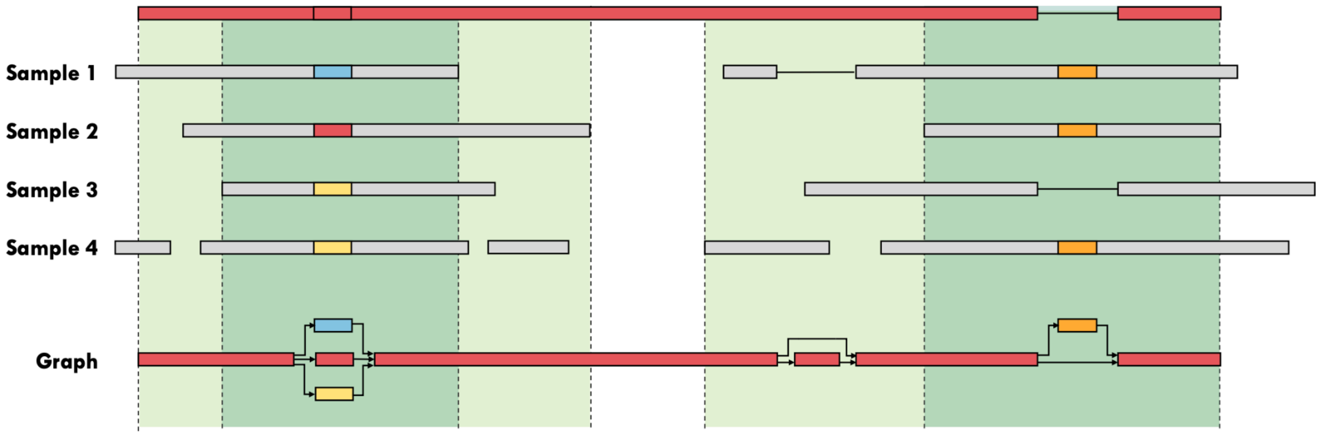
Supplemental Figures



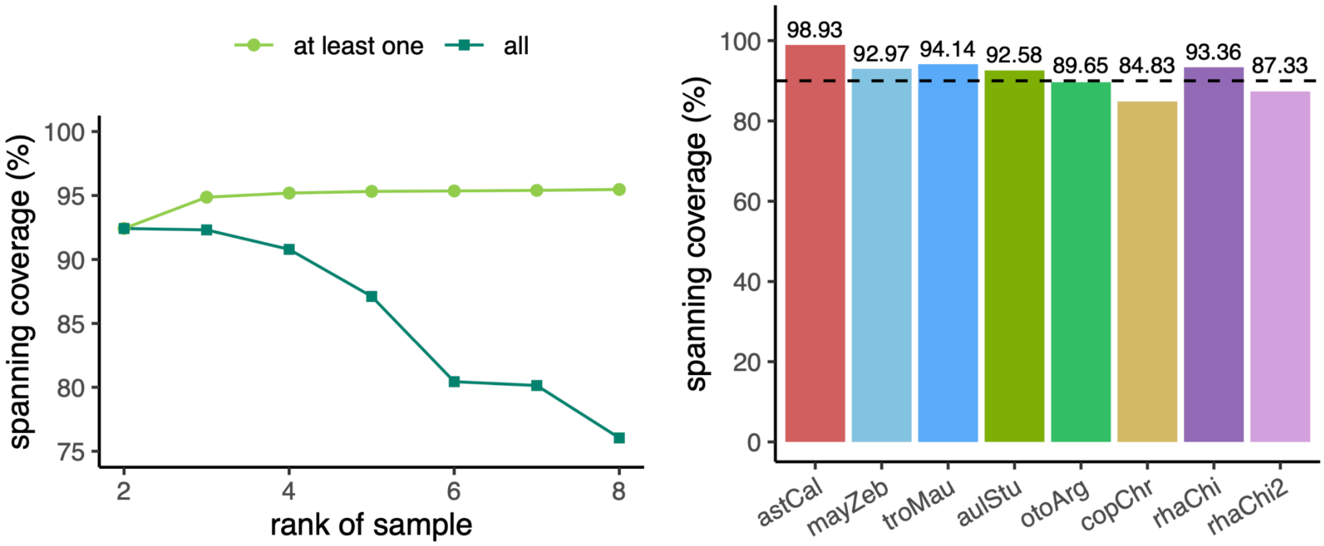
Supplemental Figure S1. (Top left) Benchmarking Universal Single-copy Orthologs (BUSCO) assessment of genome completeness. Gene completeness was evaluated based on the percentage of detectable genes out of 3,640 essential ray-finned fish genes in the "actinopterygii_odb10" dataset from OrthoDB. (Top right) Histogram showing distribution of contig or scaffold sizes for assemblies. (Bottom) Undetected BUSCOs. 30 (0.82%) BUSCOs are undetected by more than five assemblies, and only 21 (0.57%) are missed by all.



Supplemental Figure S2. Growth in the complexity and information content of Lake Malawi cichlid multiassembly graph with each incorporated assembly. Rank of sample (x-axis) denotes the number of assemblies incorporated into the graph, starting at 1 for the *A. calliptera* backbone alone, and rising to 8 as the other seven nonreference assemblies were added. As other assemblies are aligned onto the backbone, bubbles are formed to represent the SVs. This process "breaks" the backbone chromosomes into smaller segments that are joined by edges to other non-backbone segments. When only the *A. calliptera* backbone is present (rank = 1), the number of segments equals 248, corresponding to its 22 chromosomes and 226 unplaced scaffolds.

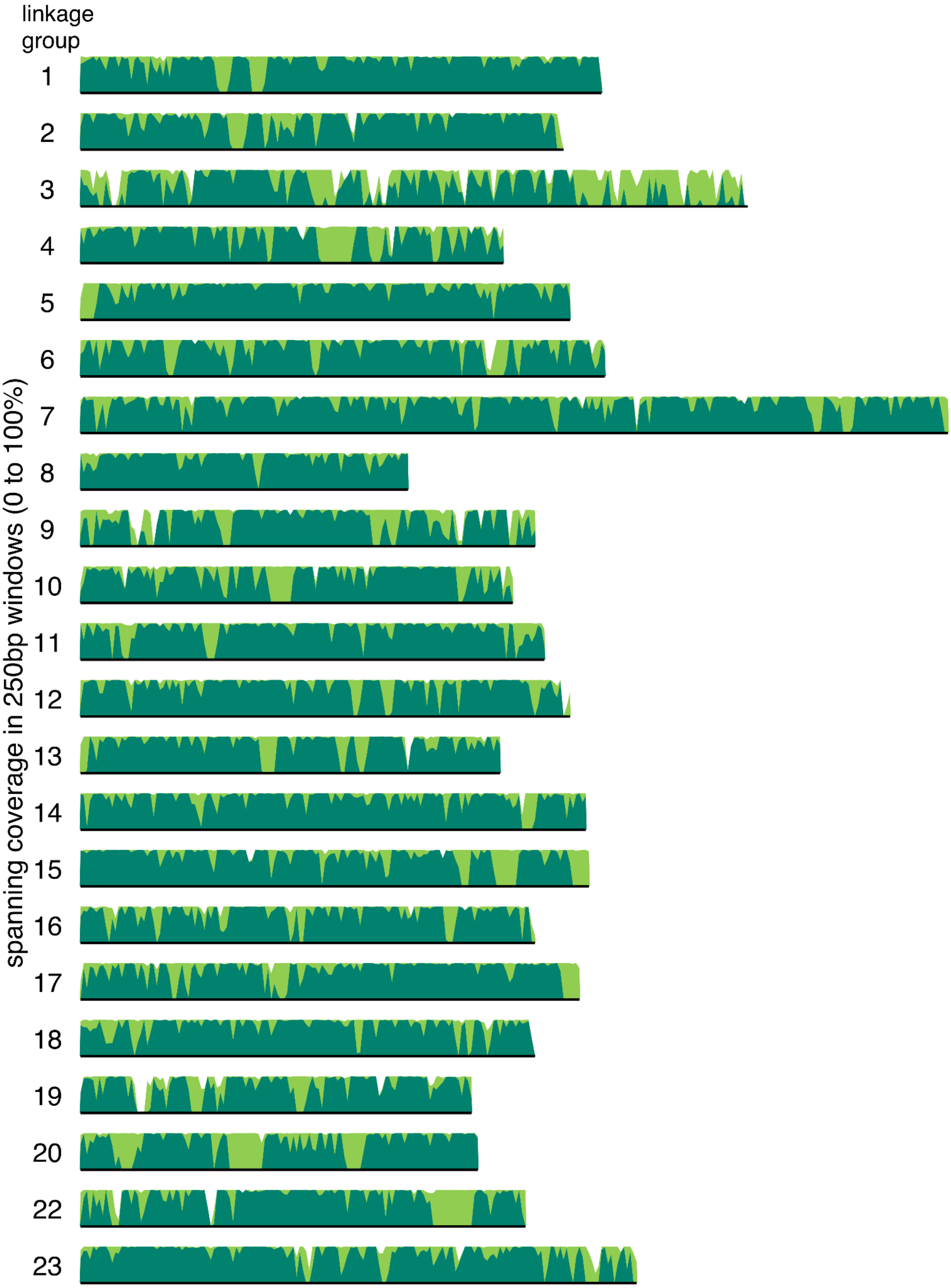


(a) Minigraph algorithm.

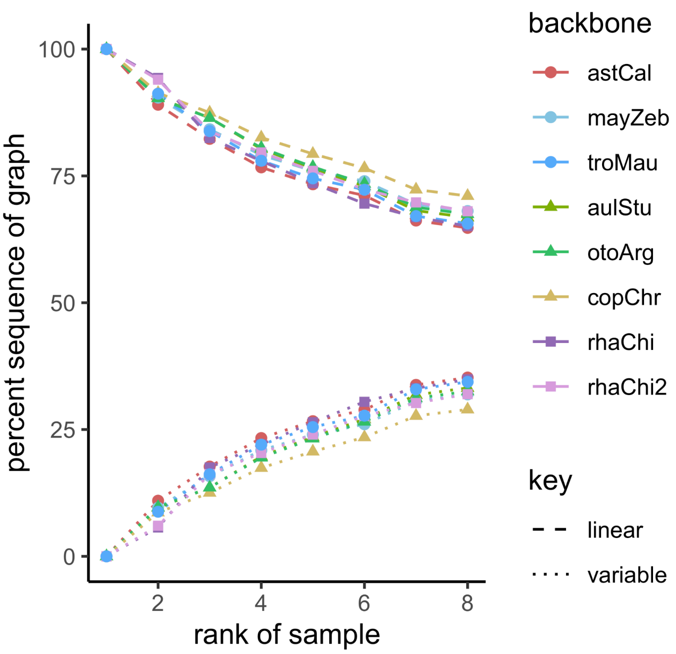


(b) Growth in overall backbone coverage. (c) Spanning coverage of individual assemblies.

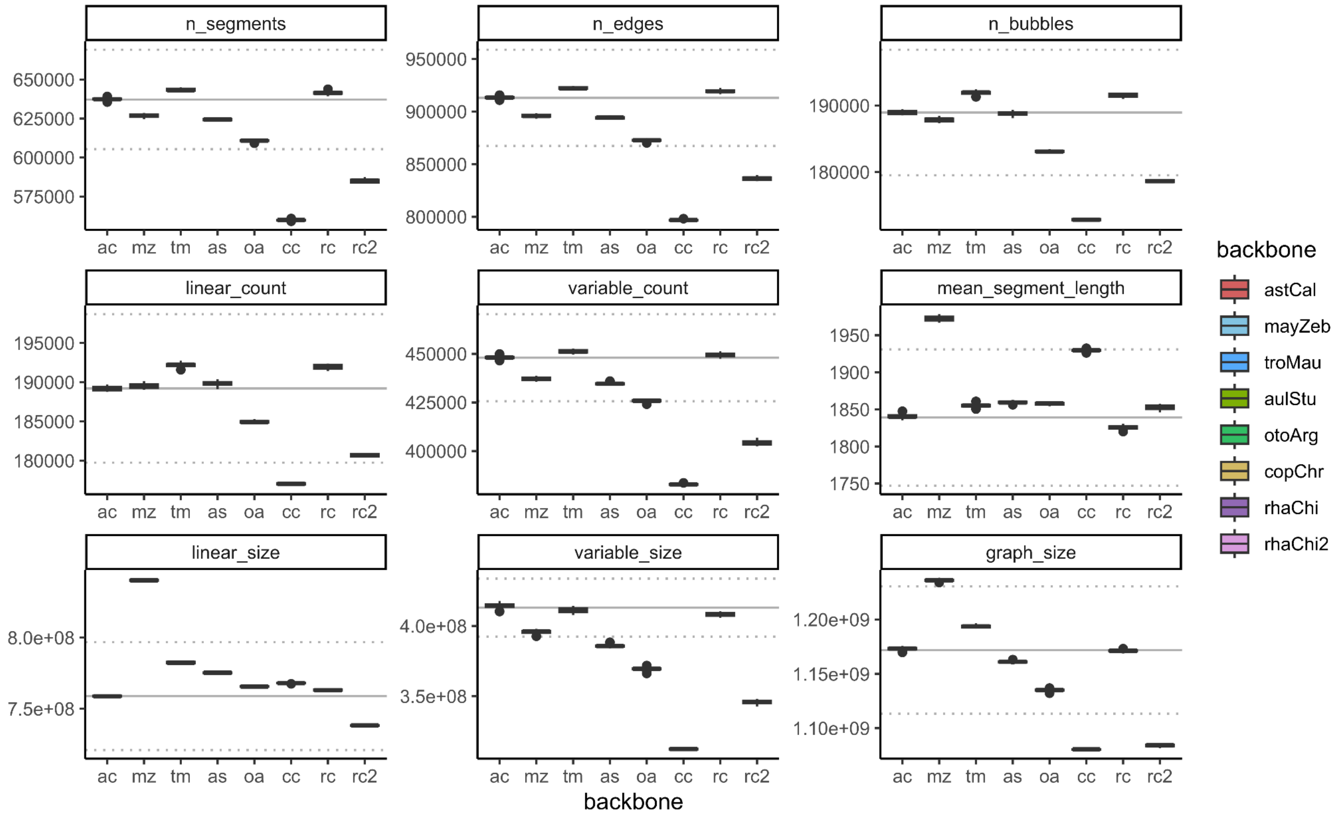
Supplemental Figure S3. Spanning coverage on the *A. calliptera* backbone during the multiassembly graph construction. (a) Illustration of how the minigraph algorithm works, depicting how bubbles are augmented onto the backbone (in red) by aligning the contigs from nonreference assemblies to produce the bottom multiassembly graph. Minigraph does not infer the presence of bubbles at gaps between nonreference contigs. Backbone regions with spanning coverage from at least one sample are colored light green, while those spanned by all are dark green. (b) Growth in the overall spanning coverage on *A. calliptera* fAstCal1.2 backbone as nonreference assemblies were incorporated into the graph. (c) Spanning coverage of individual assemblies when aligned separately on the backbone.



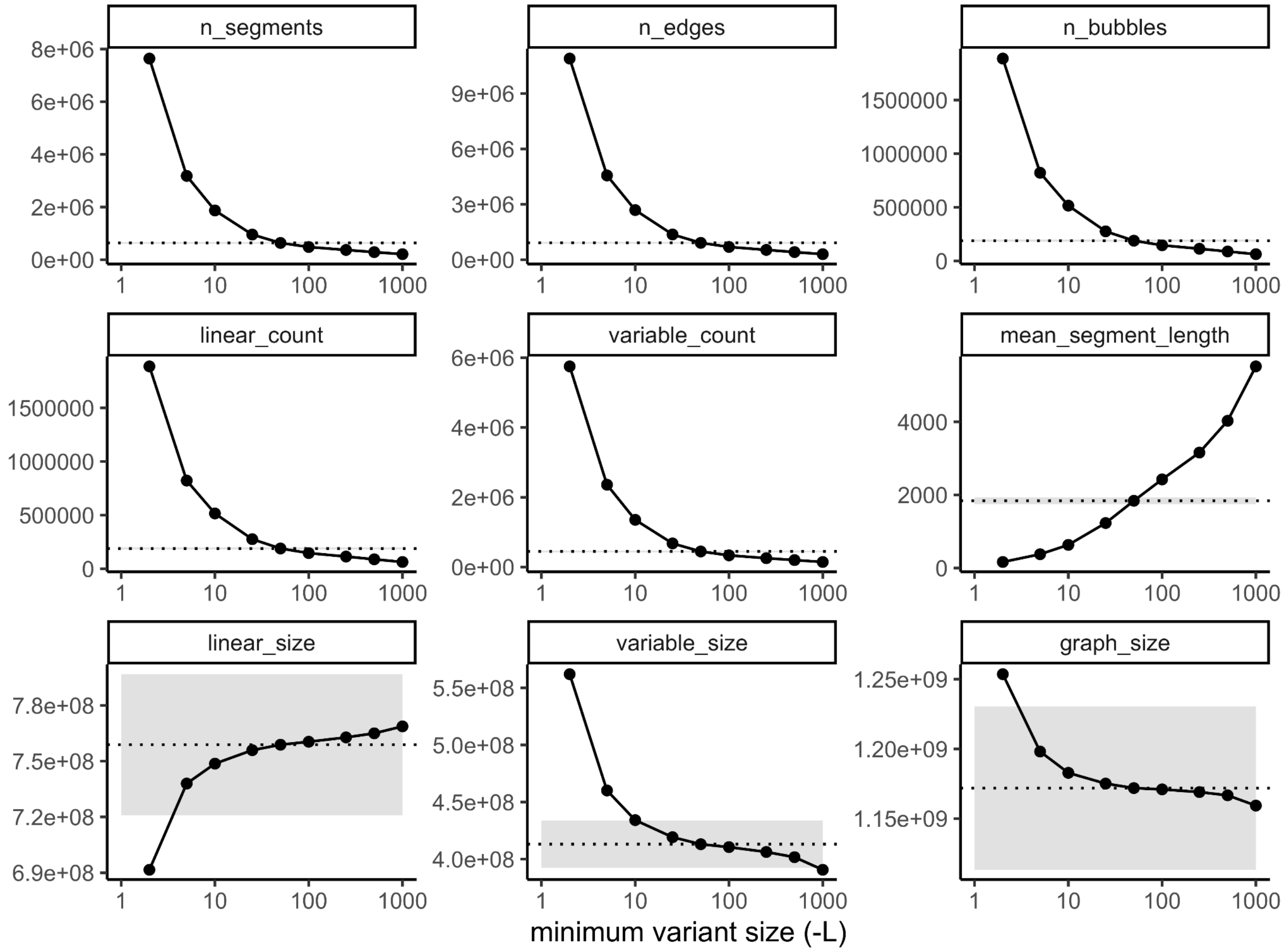
Supplemental Figure S4. Spanning coverage across the *Astatotilapia calliptera* fAstCal1.2 backbone assembly, showing 22 linkage groups representing chromosomes. The y-axis denotes sequencing coverage calculated with a window size of 250 bp. Light green denotes regions with coverage from at least one nonreference sample, while dark green denotes coverage from all.



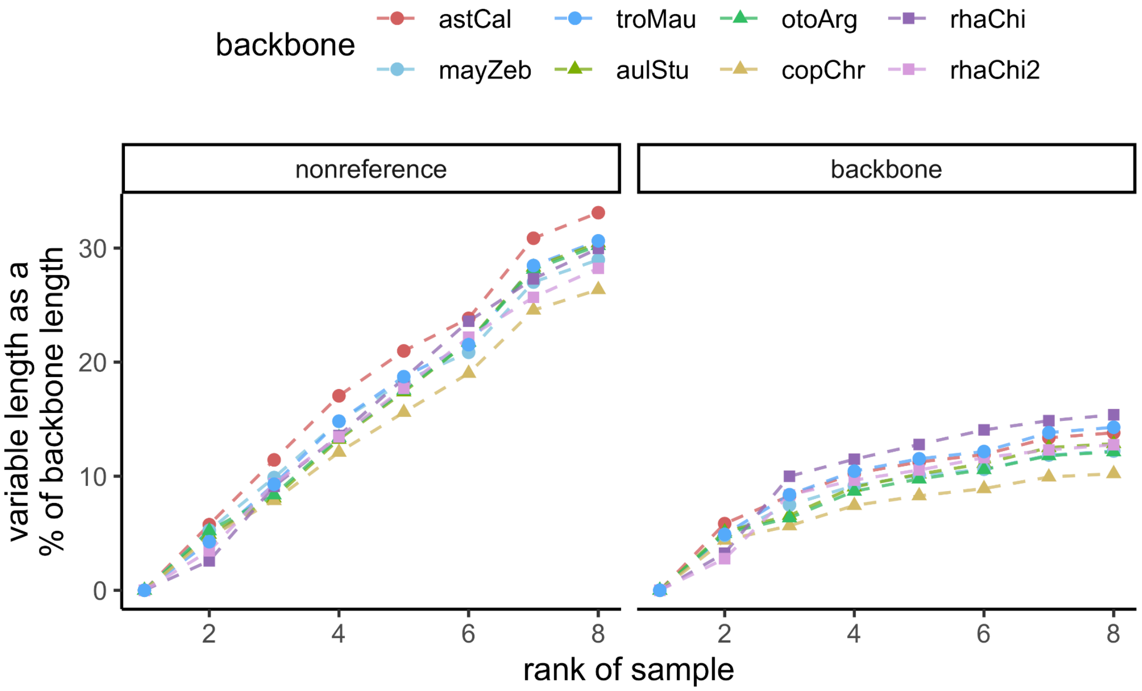
Supplemental Figure S5. Relative size of linear and variable components in Lake Malawi cichlid multiassembly graph across different backbone choices. The variable component represents the proportion of graph sequences that become encapsulated in bubbles as assemblies are added.



Supplemental Figure S6: Stability assessment of Lake Malawi cichlid multiassembly graph across backbone choice and species ordering. Boxplots denote the interquartile range of 30 permutations of incorporating subsequent assemblies for each given backbone in the x-axis. Gray solid lines denote the properties of the canonical graph, with ranges shown with dotted lines.

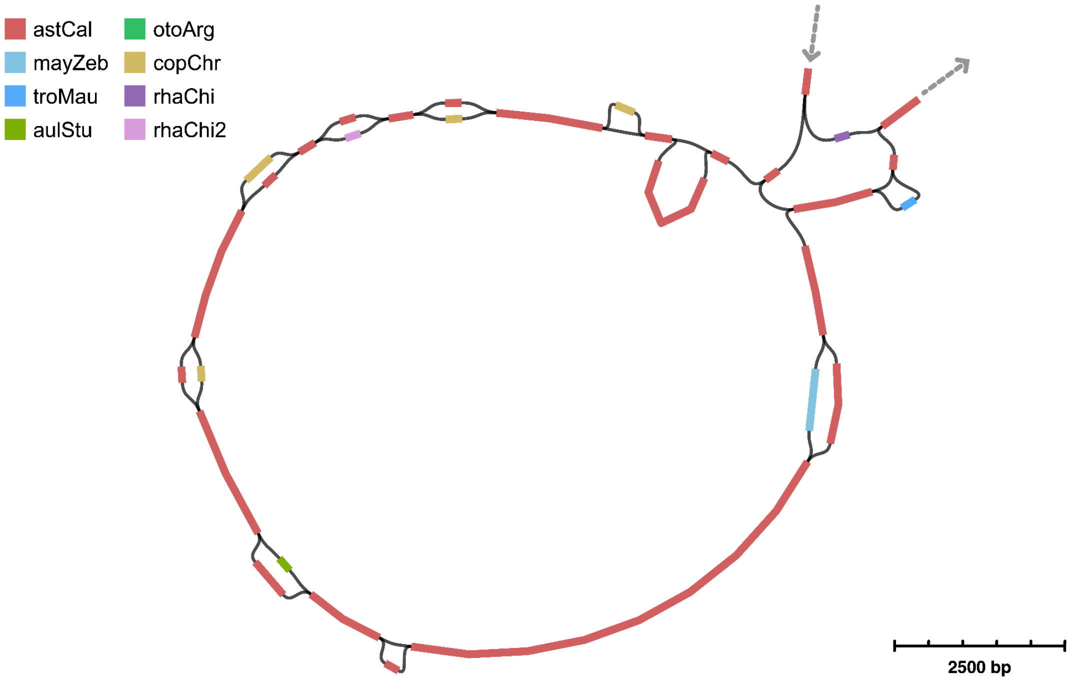


Supplemental Figure S7. Stability assessment of Lake Malawi cichlid multiassembly graph across minimum variant length L. Empirical properties were benchmarked across a parameter sweep, of which minigraph's default value L is 50. The canonical *Astatotilapia calliptera* fAstCal1.2 backbone and ordering of subsequent assemblies was used in all cases. Black dotted lines denote the properties of the default L = 50, with ±5% ranges shaded in gray. The results at L = 1 are omitted because it was an outlier and distorted the visualization of the current values, but are shown in Supplemental Table S2.

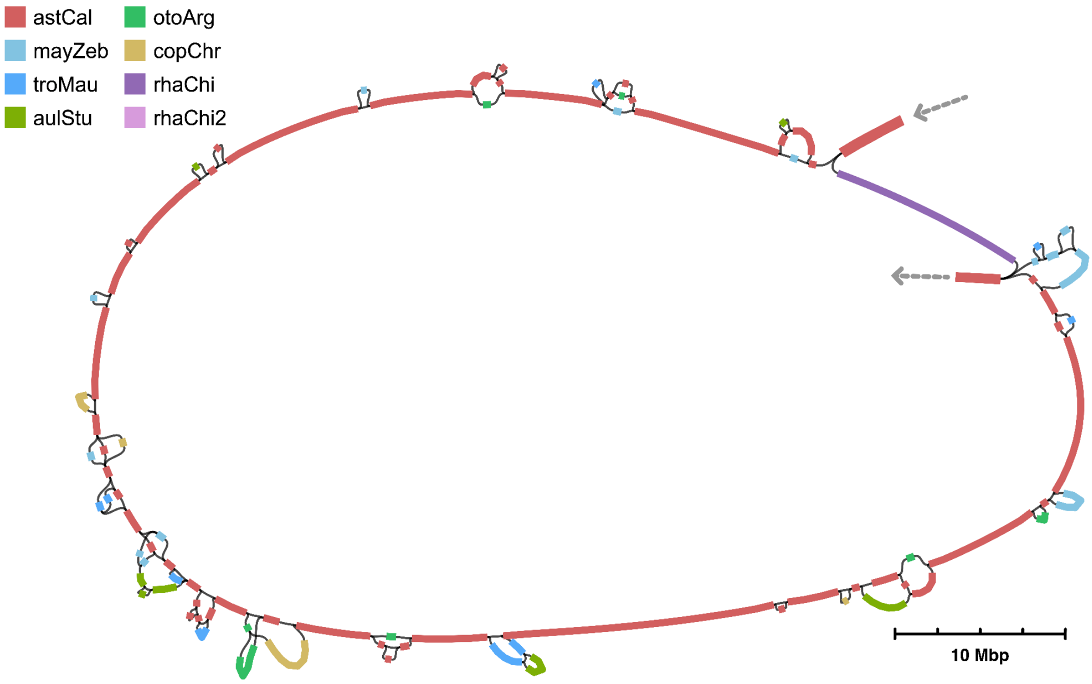


Supplemental Figure S8. Growth of the variable component in Lake Malawi multiassembly graph across different backbones. These plots show the amount of sequence that are included in bubbles, based on whether they originated from non-reference assemblies (left) or the backbone (right).

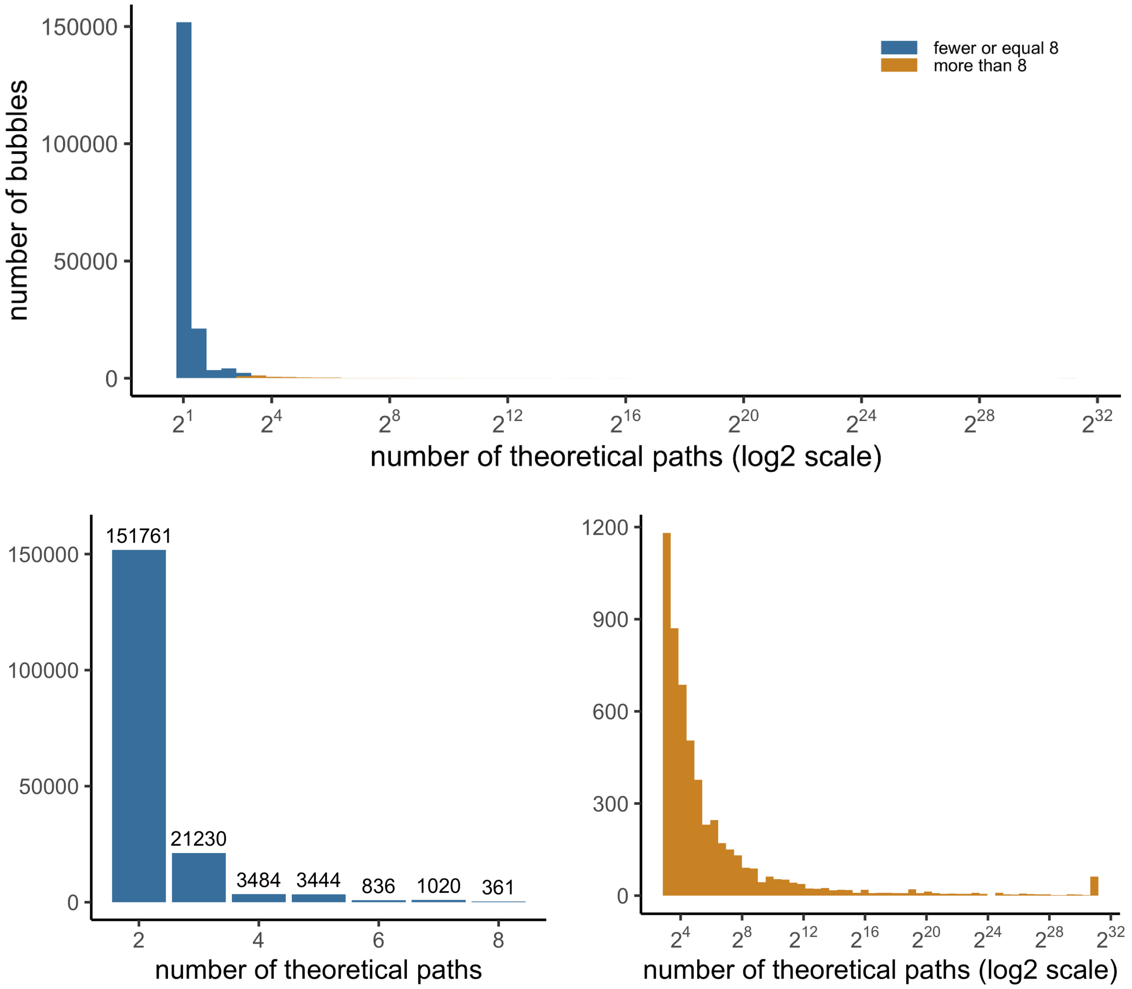
chr14:2790295-27923959 (segment 244073-244095), 32 segments, 1027 (210 + 3) paths



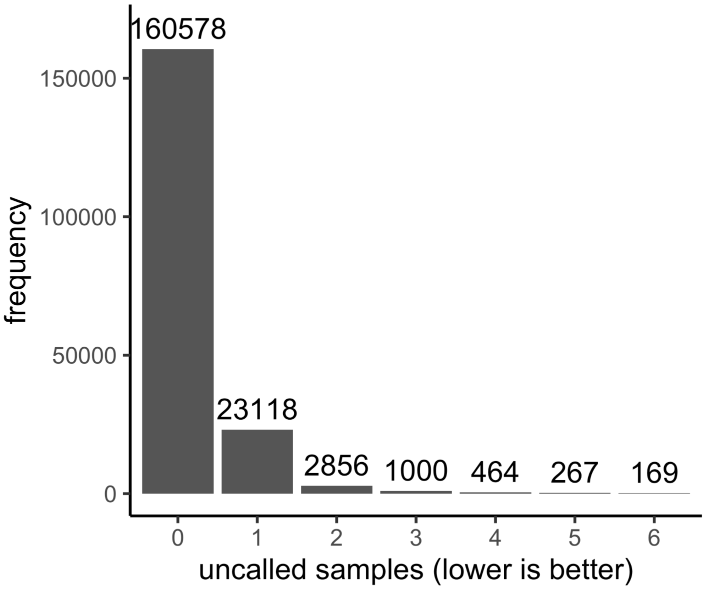
chr7:38163562-38266473 (segment 129577-129629), 94 segments, 2147483647 (231 − 1) paths



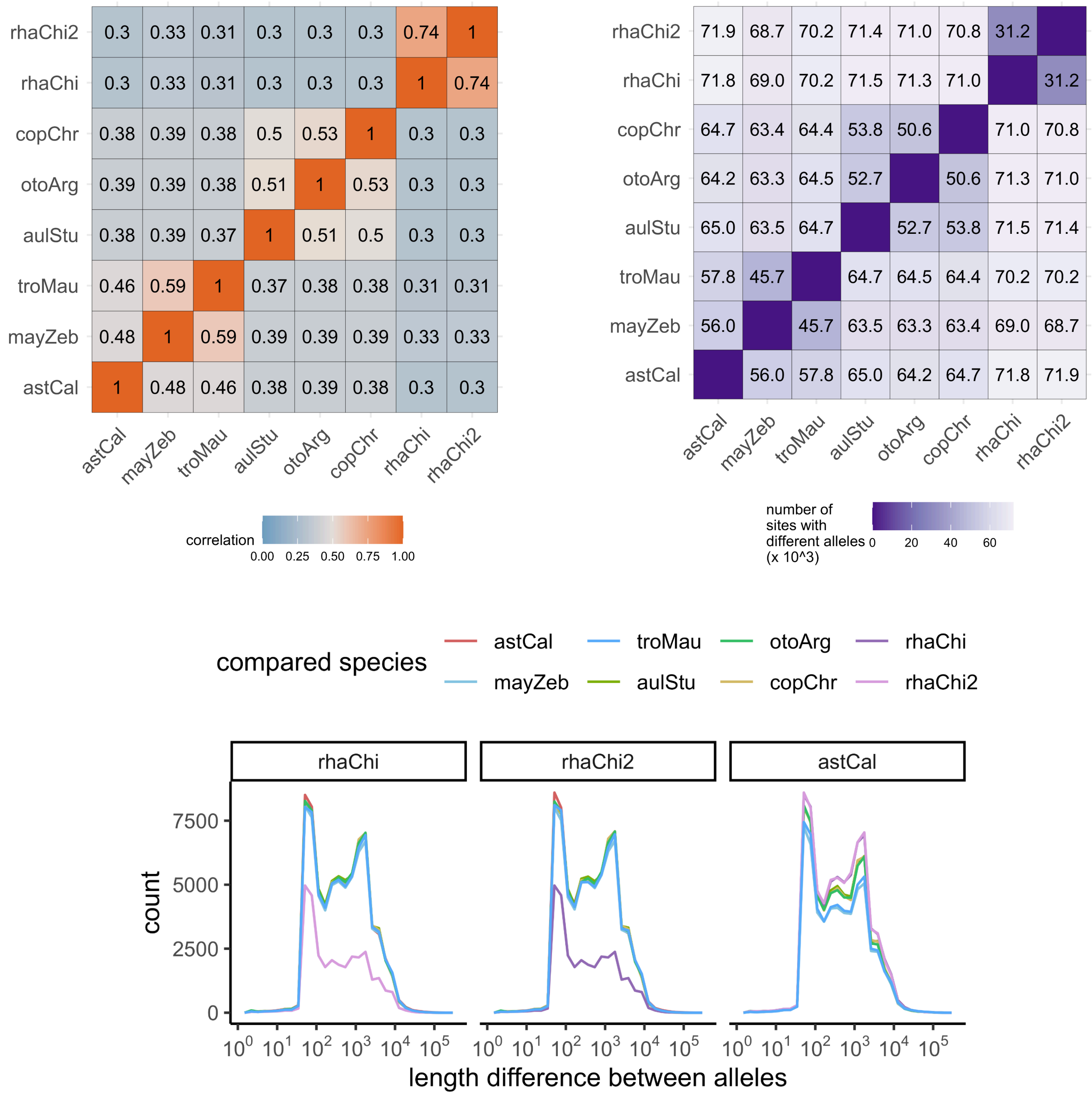
Supplemental Figure S9: Intuitive representation of complex structural variation in the multiassembly graph. Graph segments are coloured differently to reflect the original assemblies from which the sequences were derived, with backbone segments coloured red. Arrows denote entry and exit points based on the backbone coordinates.



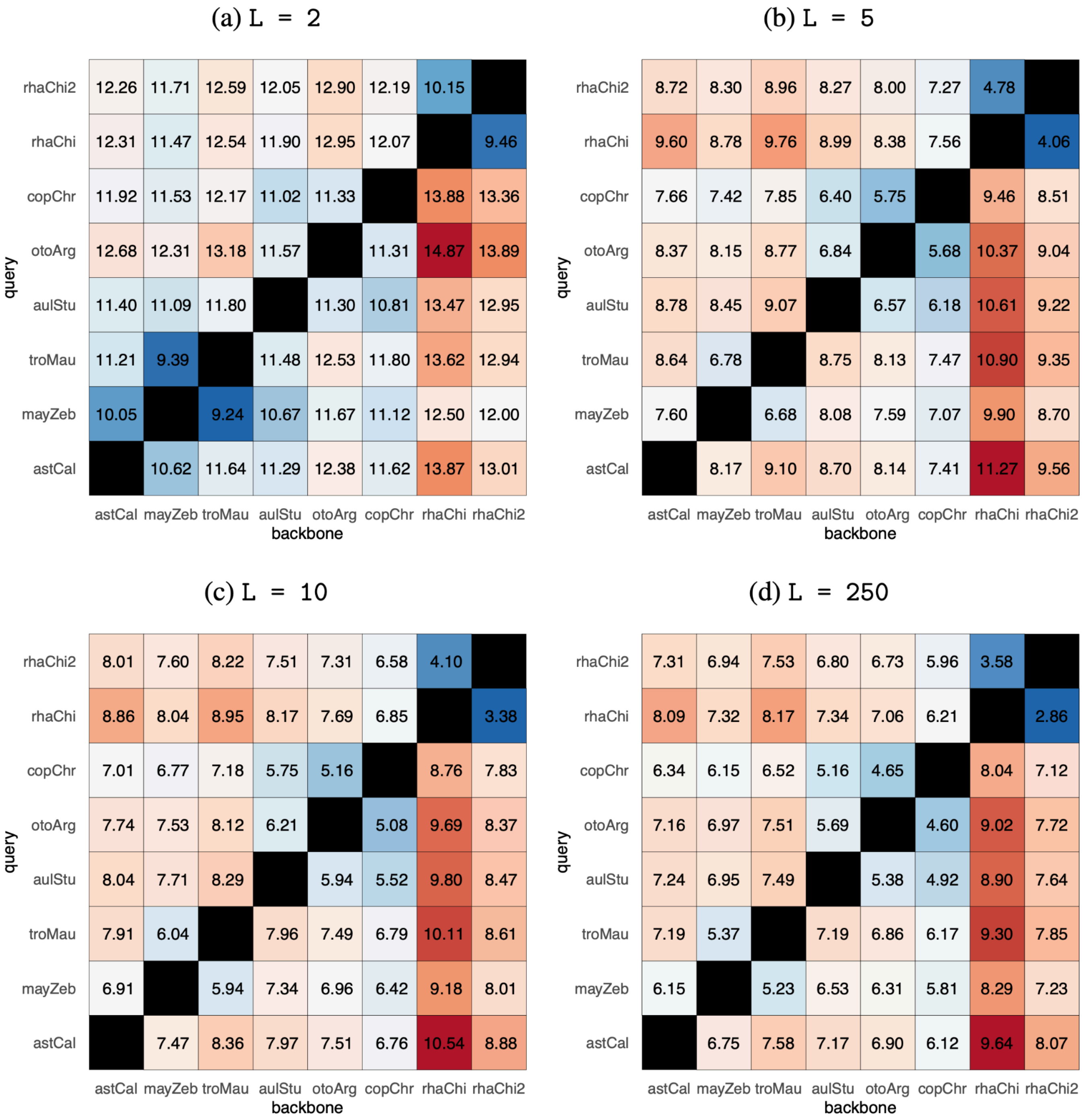
Supplemental Figure S10. Theoretical complexity of bubbles in the Lake Malawi cichlid graph. The measure of complexity (x-axis) was obtained by counting the number of theoretical paths for a sample to transverse the bubble.



Supplemental Figure S11. Success rate of allele calling across 188,452 graph bubbles.



Supplemental Fig. S12. Estimation of relationships between Lake Malawi cichlid assemblies. Analyses were performed for the 160,572 bubbles where there was complete allelic information for all the samples. The two *Rhamphochromis* individuals were closer in distance across all measures, which is as expected from SNP data in previous studies. (Top left) Pearson’s correlation matrix. (Top right) Number of sites where a pair of samples have different alleles. (Bottom right) Distribution of allele length differences between pairs of samples.



(a) L = 2

	astCal	mayZeb	troMau	aulStu	otoArg	copChr	rhaChi	rhaChi2
rhaChi2	12.26	11.71	12.59	12.05	12.90	12.19	10.15	
rhaChi	12.31	11.47	12.54	11.90	12.95	12.07		9.46
copChr	11.92	11.53	12.17	11.02	11.33		13.88	13.36
otoArg	12.68	12.31	13.18	11.57		11.31	14.87	13.89
aulStu	11.40	11.09	11.80		11.30	10.81	13.47	12.95
troMau	11.21	9.39		11.48	12.53	11.80	13.62	12.94
mayZeb	10.05		9.24	10.67	11.67	11.12	12.50	12.00
astCal		10.62	11.64	11.29	12.38	11.62	13.87	13.01

(b) L = 5

	astCal	mayZeb	troMau	aulStu	otoArg	copChr	rhaChi	rhaChi2
rhaChi2	8.72	8.30	8.96	8.27	8.00	7.27	4.78	
rhaChi	9.60	8.78	9.76	8.99	8.38	7.56		4.06
copChr	7.66	7.42	7.85	6.40	5.75		9.46	8.51
otoArg	8.37	8.15	8.77	6.84		5.68	10.37	9.04
aulStu	8.78	8.45	9.07		6.57	6.18	10.61	9.22
troMau	8.64	6.78		8.75	8.13	7.47	10.90	9.35
mayZeb	7.60		6.68	8.08	7.59	7.07	9.90	8.70
astCal		8.17	9.10	8.70	8.14	7.41	11.27	9.56

(c) L = 10

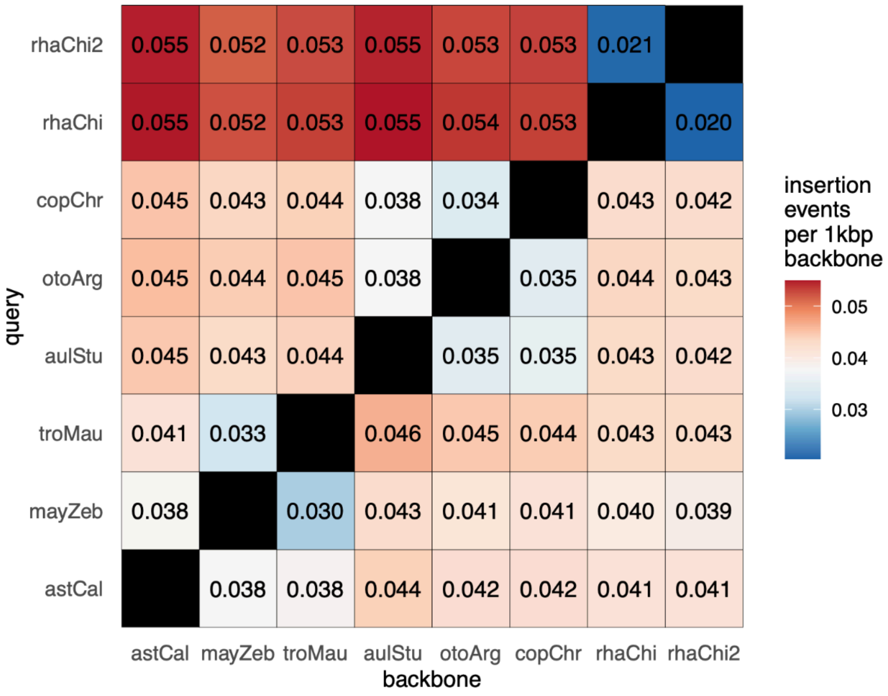
	astCal	mayZeb	troMau	aulStu	otoArg	copChr	rhaChi	rhaChi2
rhaChi2	8.01	7.60	8.22	7.51	7.31	6.58	4.10	
rhaChi	8.86	8.04	8.95	8.17	7.69	6.85		3.38
copChr	7.01	6.77	7.18	5.75	5.16		8.76	7.83
otoArg	7.74	7.53	8.12	6.21		5.08	9.69	8.37
aulStu	8.04	7.71	8.29		5.94	5.52	9.80	8.47
troMau	7.91	6.04		7.96	7.49	6.79	10.11	8.61
mayZeb	6.91		5.94	7.34	6.96	6.42	9.18	8.01
astCal		7.47	8.36	7.97	7.51	6.76	10.54	8.88

(d) L = 250

	astCal	mayZeb	troMau	aulStu	otoArg	copChr	rhaChi	rhaChi2
rhaChi2	7.31	6.94	7.53	6.80	6.73	5.96	3.58	
rhaChi	8.09	7.32	8.17	7.34	7.06	6.21		2.86
copChr	6.34	6.15	6.52	5.16	4.65		8.04	7.12
otoArg	7.16	6.97	7.51	5.69		4.60	9.02	7.72
aulStu	7.24	6.95	7.49		5.38	4.92	8.90	7.64
troMau	7.19	5.37		7.19	6.86	6.17	9.30	7.85
mayZeb	6.15		5.23	6.53	6.31	5.81	8.29	7.23
astCal		6.75	7.58	7.17	6.90	6.12	9.64	8.07

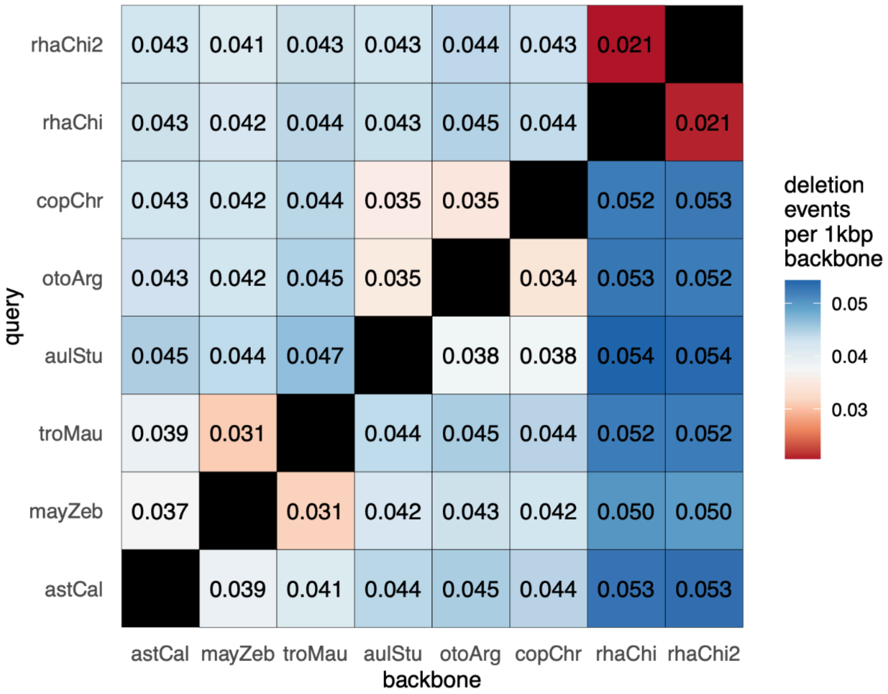
Supplemental Figure S13. Estimated percentage sequence of structural variants across parameter sweep of minimum variant size L. Values were calculated using bi-assembly graphs built from pairs of the Lake Malawi haplochromine cichlid assemblies. For each possible pair, the sequences of a query (y-axis) were aligned onto a backbone (x-axis), and the percentage of flexible sequence on the backbone was estimated based on how much of these backbone regions with spanning coverage was located inside bubbles. With higher L values, smaller variants were gradually excluded, causing the percentage scores to decrease and eventually stabilize. The genome graphs generated at higher L values should be more reliable as they exclude small sequencing errors due to potential sequencing errors in the PacBio CLR and ONT simplex reads used for assembly.

(a) Insertions



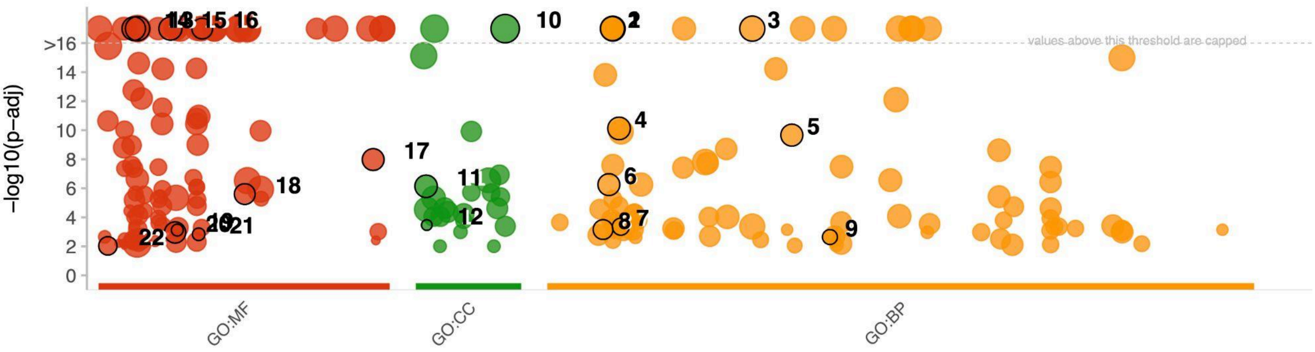
	astCal	mayZeb	troMau	aulStu	otoArg	copChr	rhaChi	rhaChi2
rhaChi2	0.055	0.052	0.053	0.055	0.053	0.053	0.021	
rhaChi	0.055	0.052	0.053	0.055	0.054	0.053		0.020
copChr	0.045	0.043	0.044	0.038	0.034		0.043	0.042
otoArg	0.045	0.044	0.045	0.038		0.035	0.044	0.043
aulStu	0.045	0.043	0.044		0.035	0.035	0.043	0.042
troMau	0.041	0.033		0.046	0.045	0.044	0.043	0.043
mayZeb	0.038		0.030	0.043	0.041	0.041	0.040	0.039
astCal		0.038	0.038	0.044	0.042	0.042	0.041	0.041

(b) Deletions



	astCal	mayZeb	troMau	aulStu	otoArg	copChr	rhaChi	rhaChi2
rhaChi2	0.043	0.041	0.043	0.043	0.044	0.043	0.021	
rhaChi	0.043	0.042	0.044	0.043	0.045	0.044		0.021
copChr	0.043	0.042	0.044	0.035	0.035		0.052	0.053
otoArg	0.043	0.042	0.045	0.035		0.034	0.053	0.052
aulStu	0.045	0.044	0.047		0.038	0.038	0.054	0.054
troMau	0.039	0.031		0.044	0.045	0.044	0.052	0.052
mayZeb	0.037		0.031	0.042	0.043	0.042	0.050	0.050
astCal		0.039	0.041	0.044	0.045	0.044	0.053	0.053

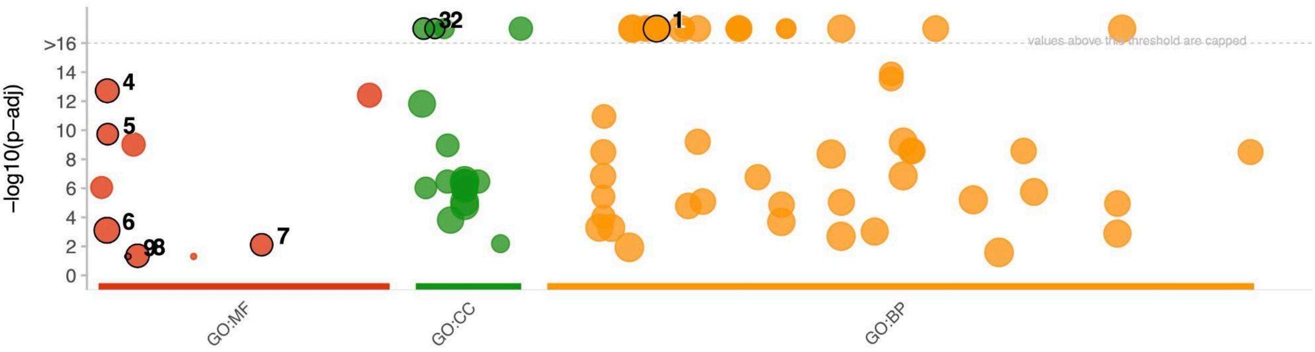
Supplemental Fig. S14. Insertion and deletion densities estimated from bi-assembly graphs between pairs of cichlid assemblies. For each possible pair, the sequences of a query (y-axis) were aligned onto a backbone (x-axis), and the density was estimated based on how many events are discovered per 1 kbp of backbone regions with spanning coverage. Minimum variant size L set to 50.



id	source	term_id	term_name	term_size	intersection_size		p_value
1	GO:BP	GO:0006810	transport	2177	1669	🟦	1.3e−38
2	GO:BP	GO:0006793	phosphorus metabolic process	1447	1120	🟦	3.6e−27
3	GO:BP	GO:0032502	developmental process	2513	1823	🟩	1.7e−19
4	GO:BP	GO:0007166	cell surface receptor signaling pathway	767	585	🟩	7.5e−11
5	GO:BP	GO:0035556	intracellular signal transduction	595	462	🟩	2.2e−10
6	GO:BP	GO:0006629	lipid metabolic process	560	425	🟩	5.6e−07
7	GO:BP	GO:0007268	chemical synaptic transmission	104	89	🟨	4.4e−04
8	GO:BP	GO:0006325	chromatin organization	250	195	🟨	7.0e−04
9	GO:BP	GO:0043087	regulation of GTPase activity	60	54	🟨	2.3e−03
10	GO:CC	GO:0110165	cellular anatomical entity	14687	9446	🟦	2.1e−31
11	GO:CC	GO:0005856	cytoskeleton	719	528	🟩	7.3e−07
12	GO:CC	GO:0005891	voltage−gated calcium channel complex	28	28	🟨	3.4e−04
13	GO:MF	GO:0005524	ATP binding	1636	1267	🟦	3.8e−38
14	GO:MF	GO:0005215	transporter activity	1163	933	🟦	4.4e−38
15	GO:MF	GO:0016301	kinase activity	923	731	🟦	4.3e−26
16	GO:MF	GO:0030695	GTPase regulator activity	355	300	🟩	1.3e−17
17	GO:MF	GO:0140657	ATP−dependent activity	559	422	🟩	1.1e−08
18	GO:MF	GO:0042578	phosphoric ester hydrolase activity	368	281	🟩	2.5e−06
19	GO:MF	GO:0016849	phosphorus−oxygen lyase activity	33	32	🟨	7.6e−04
20	GO:MF	GO:0016746	acyltransferase activity	436	317	🟨	1.1e−03
21	GO:MF	GO:0030215	semaphorin receptor binding	37	35	🟨	1.5e−03
22	GO:MF	GO:0003774	cytoskeletal motor activity	156	121	🟨	9.3e−03

g:Profiler (biit.cs.ut.ee/gprofiler)

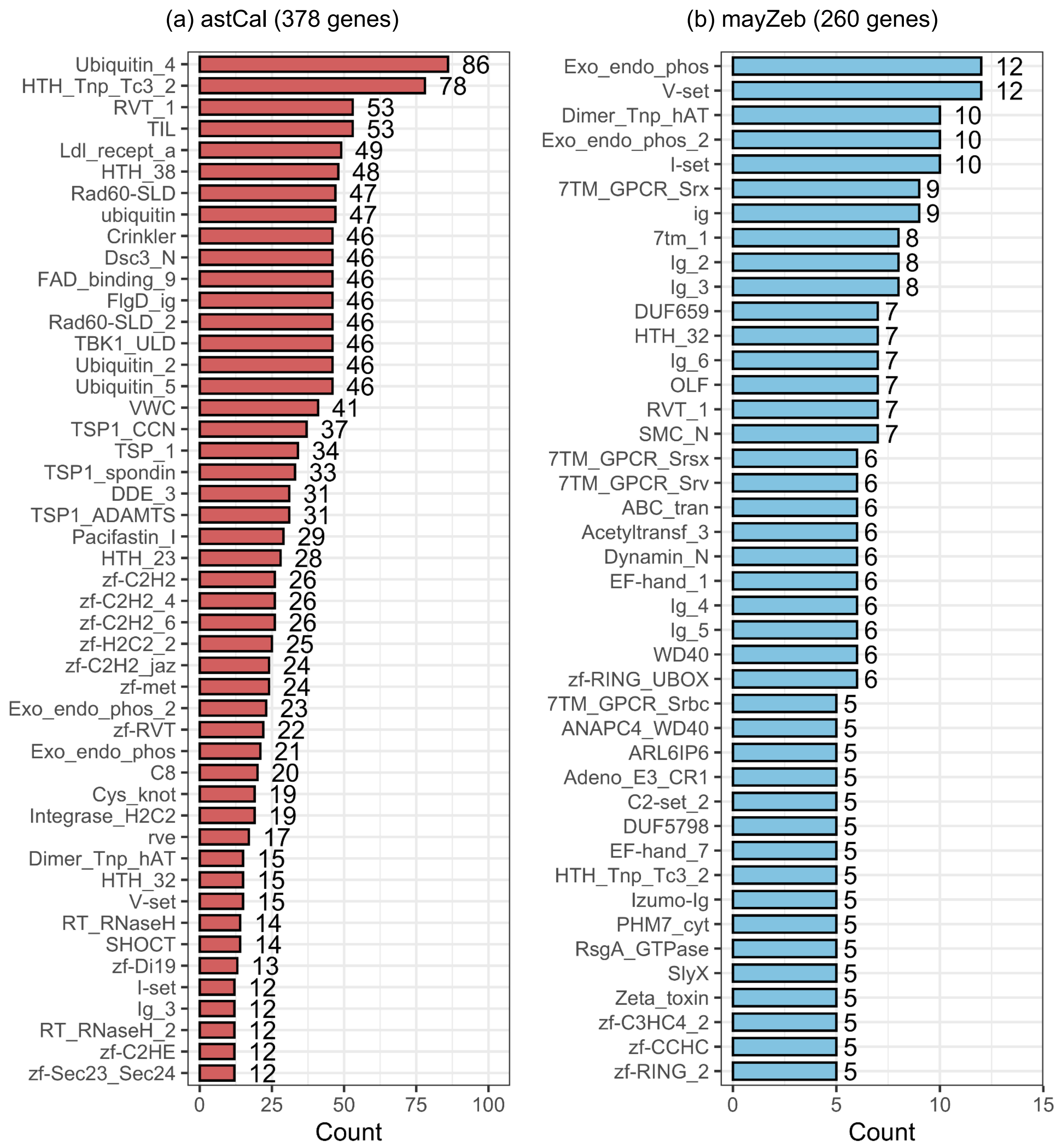
Supplemental Figure S15. Functional enrichment analysis of genes containing structural variants, with non-redundant driver terms as highlighted by g:Profiler. Full list of genes is provided as a separate Excel file in this Zenodo link https://doi.org/10.5281/zenodo.14029308.



id	source	term_id	term_name	term_size	intersection_size		p_value
1	GO:BP	GO:0010467	gene expression	2646	630	🟦	8.1e−40
2	GO:CC	GO:0016442	RISC complex	174	104	🟦	1.3e−37
3	GO:CC	GO:0005730	nucleolus	277	109	🟩	4.1e−19
4	GO:MF	GO:0003700	DNA−binding transcription factor activity	707	179	🟩	1.9e−13
5	GO:MF	GO:0003735	structural constituent of ribosome	290	88	🟩	1.9e−10
6	GO:MF	GO:0003677	DNA binding	1722	308	🟨	7.8e−04
7	GO:MF	GO:0046983	protein dimerization activity	398	86	🟨	7.8e−03
8	GO:MF	GO:0005525	GTP binding	602	117	🟨	4.5e−02
9	GO:MF	GO:0004860	protein kinase inhibitor activity	17	9	🟨	5.0e−02

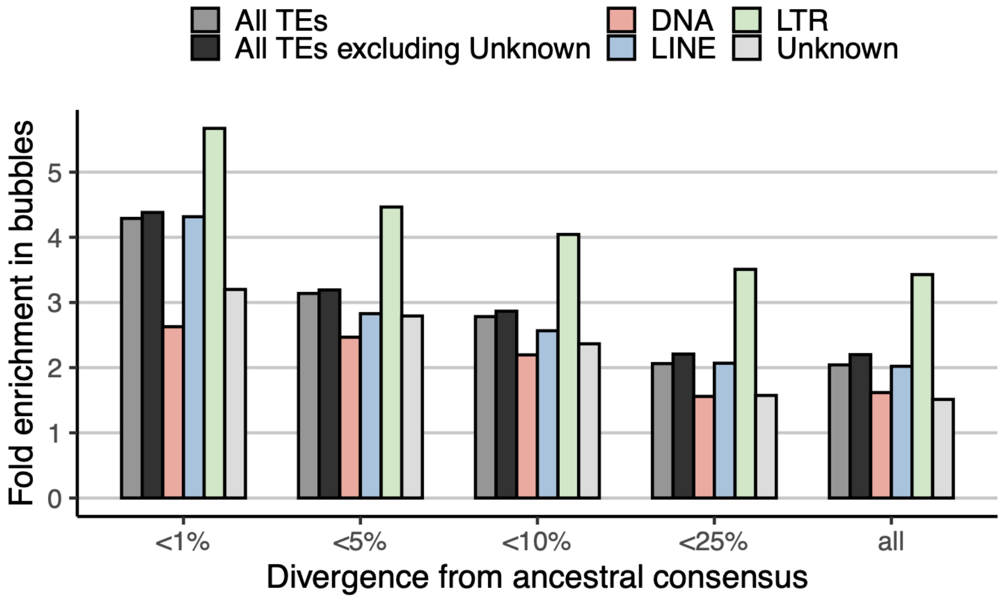
g:Profiler (biit.cs.ut.ee/gprofiler)

Supplemental Figure S16. Functional enrichment analysis of genes without structural variants in the gene body and 2000 bp upstream or downstream, with non-redundant driver terms as highlighted by g:Profiler. Full list of genes is provided as a separate Excel file in this Zenodo link https://doi.org/10.5281/zenodo.14029308.

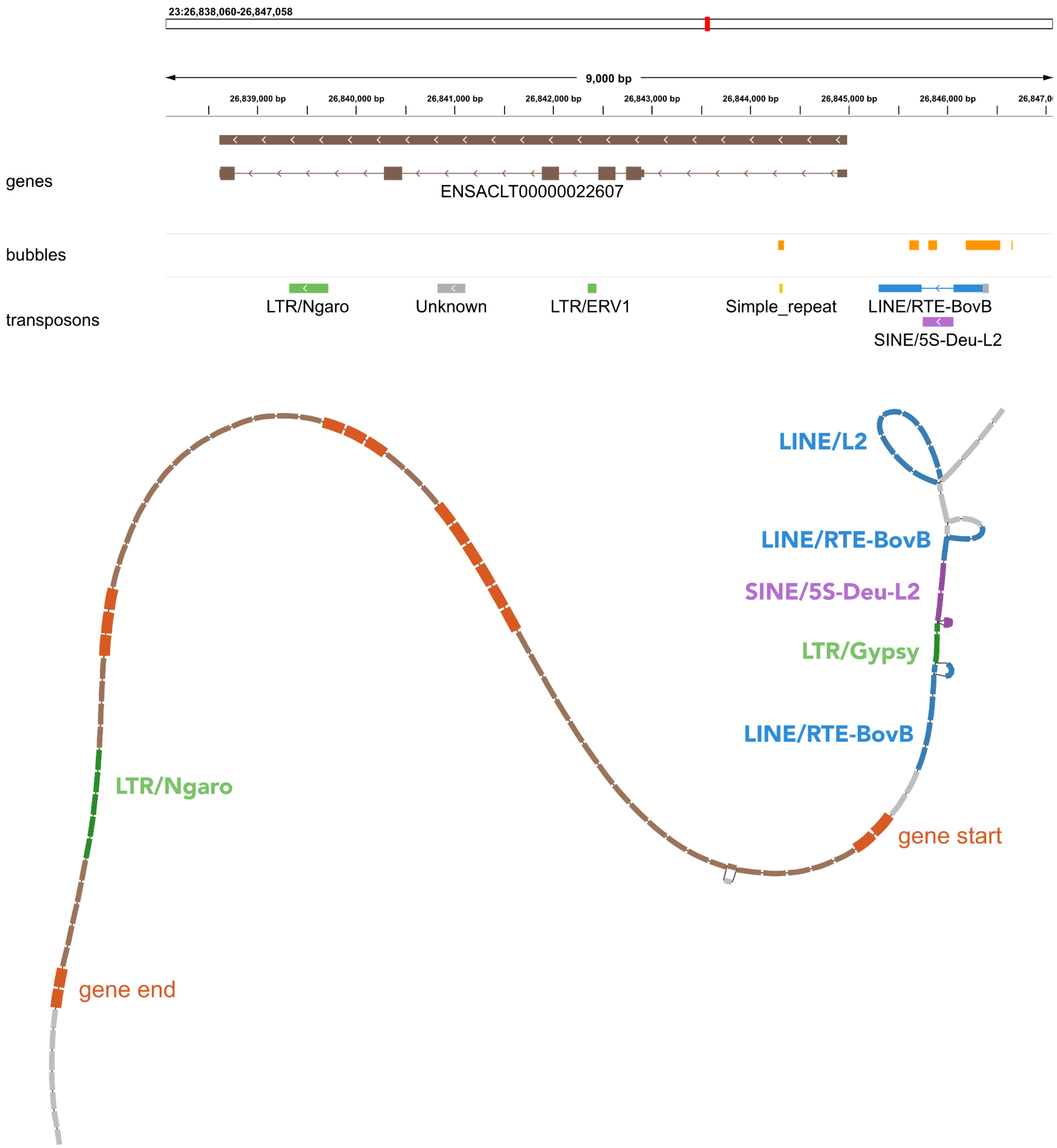


Supplemental Figure S17. Pfam domain annotation for "private" genes in *A. calliptera* and *M. zebra* reference genomes. These represent gene sequences that were not detectable in the other assemblies. Domain detection achieved by alignment of gene sequences against the Pfam database with HMMER 3.3.

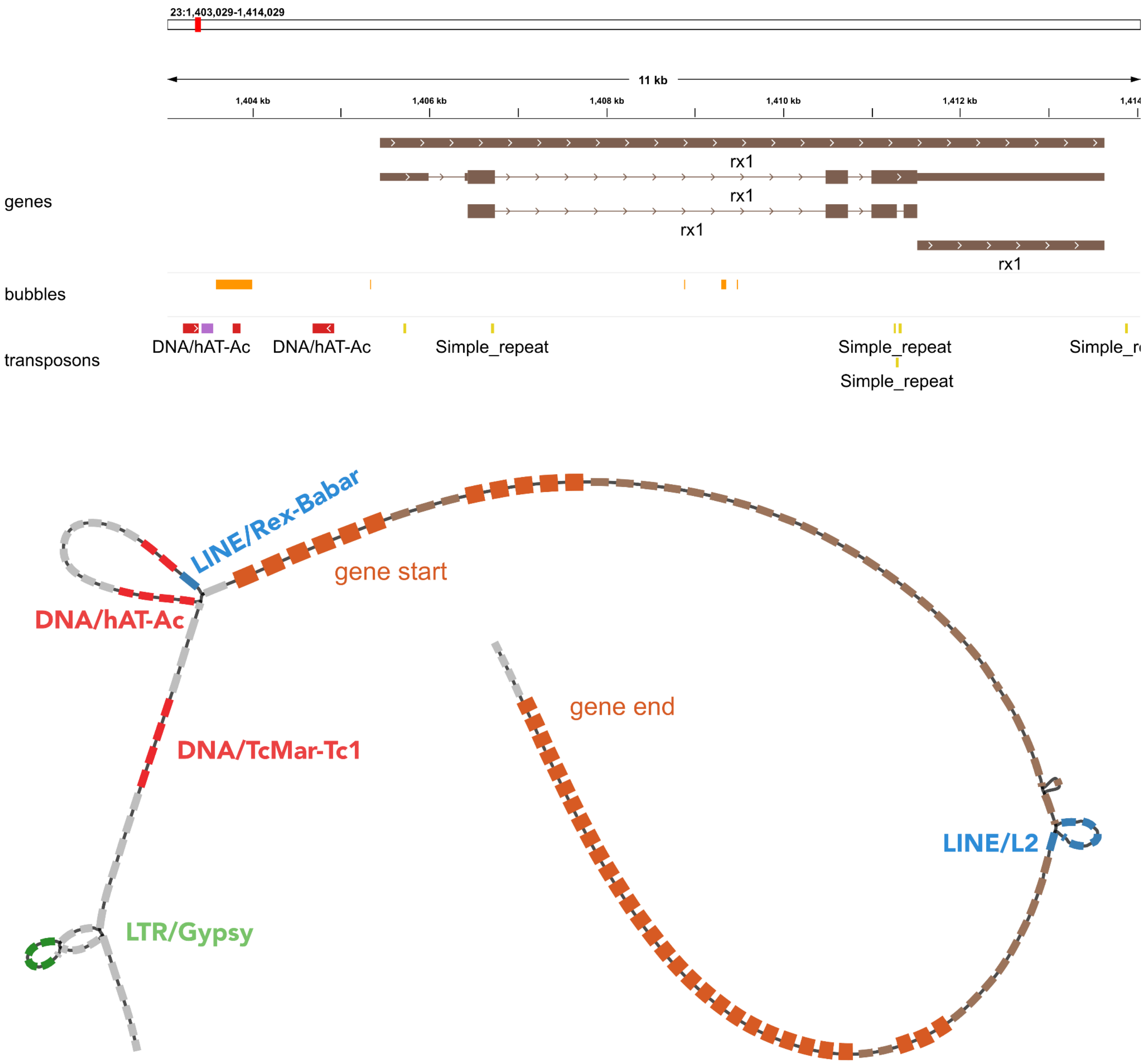
Supplemental Figure S18. Inaccurate structural variant detection at the hemoglobin MN locus caused by backbone assembly errors. Globin genes appear to be poorly assembled in the *A. calliptera* fAstCal1.2 genome (top), compared to in *Maylandia zebra*. The labeled globin alpha-B (red) and beta-A (blue) subunits in *A. calliptera* were determined by ODGI to be unique to the backbone, but actually had matching orthologs in *M. zebra* based on BioMart. Note that the orientation of the chromosomes are reversed.



Supplemental Figure S19. Enrichment of transposable elements in graph bubbles. Fold enrichment was measured for *A. calliptera* sequences in graph bubbles relative to its expected genome-wide composition. The x-axis represents RepeatMasker sequence divergence thresholds, where lower values are an approximate measure to filter for “younger” transposons. SINEs, Helitrons and Retroposons are not shown because their overly small values make the calculation unreliable.



Supplemental Figure S20. TE insertions around the *fhl2b* gene, showing the conserved SINE element in purple. Segments are coloured manually based on the presence of TEs. Exons and introns are coloured dark orange and brown respectively, with the former having a thicker width.



Supplemental Figure S21. E insertions around the *rx1* gene, showing the conserved SINE element in purple. Segments are coloured manually based on the presence of TEs. Exons and introns are coloured dark orange and brown respectively, with the former having a thicker width. The first bubble from the left is known from previous research, hosting three possible alleles: complete deletion, partial insertion of 413 bp and the full length of 831 bp, each of which contribute to different opsin palettes in cichlid vision.

Supplemental Tables

	astCal	mayZeb	troMau	aulStu	otoArg	copChr	rhaChi	rhaChi2
segment count	637,237	626,826	642,989	625,018	610,555	559,685	642,806	587,152
edge count	913,087	896,071	921,610	895,007	872,224	796,680	921,326	839,389
mean segment length	1,839.04	1,970.12	1,854.30	1,857.58	1,858.03	1,930.61	1,823.03	1,847.71
graph size, Gbp	1.172	1.235	1.192	1.161	1.134	1.081	1.172	1.085
linear percentage	64.75%	68.07%	65.61%	66.80%	67.47%	71.05%	65.10%	68.03%
variable percentage	35.25%	31.93%	34.39%	33.20%	32.53%	28.95%	34.90%	31.94%
backbone coverage	95.47%	89.26%	94.70%	95.09%	93.06%	89.68%	97.15%	94.94%
additional bases	33.11%	28.98%	30.64%	30.46%	30.22%	26.36%	29.98%	28.23%
flexible on backbone	13.81%	12.21%	14.29%	12.86%	12.14%	10.22%	15.39%	12.76%
bubble count	188,944	188,006	192,055	189,201	183,423	172,846	191,963	178,693
bubble density, per kbp	0.2146	0.1964	0.2104	0.2126	0.2106	0.2021	0.2129	0.2112
bubble density, per kbp (coverage corrected)	0.2248	0.2200	0.2222	0.2236	0.2263	0.2254	0.2192	0.2225

Supplemental Table S1. Empirical properties of Lake Malawi cichlid multiassembly graphs constructed with different backbones. Linear and variable percentages are with respect to the entire graph. Backbone coverage denotes percentage sequence with at least spanning coverage with one aligned assembly. Additional bases is the percentage of extra bases relative to the size of the backbone.

	1	2	5	10	25	50	100	250	500	1000
segment count	2,446	7,638,583	3,183,041	1,870,374	955,101	637,237	483,066	370,077	289,658	210,137
edge count	3,085	10,893,890	4,564,107	2,689,473	1,368,504	913,087	693,044	531,467	415,489	300,430
mean segment length	403618.03	164.11	376.40	632.40	1230.37	1839.04	2424.06	3158.94	4027.89	5517.15
graph size, Gbp	0.987	1.254	1.198	1.183	1.175	1.172	1.171	1.169	1.167	1.159
linear percentage	81.77%	55.17%	61.60%	63.30%	64.33%	64.75%	64.94%	65.25%	65.57%	66.31%
variable percentage	18.23%	44.83%	38.40%	36.70%	35.67%	35.25%	35.06%	34.75%	34.43%	33.69%
additional bases	12.13%	42.38%	36.08%	34.35%	33.47%	33.11%	33.00%	32.78%	32.52%	31.68%
flexible on backbone	8.31%	21.45%	16.18%	14.96%	14.14%	13.81%	13.63%	13.36%	13.11%	12.69%
bubble count	417	1,887,173	822,148	515,500	275,825	188,944	145,915	113,700	88,162	63,004
bubble density, per kbp	0.0005	2.1435	0.9338	0.5855	0.3133	0.2146	0.1657	0.1291	0.1001	0.0716

Supplemental Table S2. Stability assessment of Lake Malawi cichlid multiassembly graph across minimum variant length L. Empirical properties were benchmarked across a parameter sweep, of which minigraph's default value for L is 50. The canonical *A. calliptera* backbone and ordering of subsequent assemblies was used in all cases. Setting L = 1 resulted in an overly simple graph, which was likely an outlier behavior in minigraph.

(a) Bubbles with singleton SVs

Bubble	Nearest Gene	Ensembl ID	Gene Description	Score	Heterozygotes	Sanger Confirmation
3:9790027-9790027	rpgrip1	ENSACLG00000020186	RPGR interacting protein 1	5/5	-	Complete
20:15022145-15023430	klhl7	ENSACLG00000006581	kelch-like protein 7	5/5	-	Complete
23:12757935-12758244	tprg1	ENSACLG00000024576	tumor protein p63-regulated gene 1-like protein	5/5	-	Complete
7:42326247-42326247	anpepb	ENSACLG00000017196	aminopeptidase Ey-like	5/5	-	Complete
5:33820642-33820642	mfsd4aa	ENSACLG00000025203	major facilitator superfamily domain containing 4A	5/5	1/5 (T)	Complete
15:16298262-16298895	dtnba	ENSACLG00000027349	dystrobrevin, beta a	5/5	1/5 (O)	2/5 confirmed
5:30881965-30882447	mitfa	ENSACLG00000022427	melanocyte inducing transcription factor a	5/5	2/5 (O,C)	4/5 confirmed
16:5354683-5354683	lcp1	ENSACLG00000015018	lymphocyte cytosolic protein 1	4/5 (C)	-	Complete

(b) Bubbles with more common SVs

Bubble	Nearest Gene	Ensembl ID	Gene Description	Score	Heterozygotes	Sanger Confirmation
10:13464861-13465174	kcnab3	ENSACLG00000017346	voltage-gated potassium channel subunit beta-3-like	5/5	-	Complete
7:51031051-51031051	-	ENSACLG00000002767	coiled-coil-helix-coiled-coil-helix domain containing 3b	5/5	2/5 (O,R♀)	Complete
15:25833370-25833370	adam17b	ENSACLG00000014403	ADAM metallopeptidase domain 17	5/5	-	4/5 confirmed
10:2382458-2382689	dgat2	ENSACLG00000026652	diacylglycerol O-acyltransferase 2	5/5	-	3/5 confirmed
7:16423102-16423419	ptges	ENSACLG00000027791	prostaglandin E synthase	5/5	-	2/5 confirmed
3:41434172-41435346	-	ENSACLG00000021983	H-2 class II histocompatibility antigen, E-S beta chain-like	4/5 (O)	-	Complete
10:24736910-24737125	nsd1b	ENSACLG00000015768	histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific-like	4/5 (C)	-	Complete
9:28296874-28297388	gabrr3b	ENSACLG00000013807	gamma-aminobutyric acid receptor subunit rho-3-like	4/5 (C)	-	3/5 confirmed

Supplemental Table S3. Summary of PCR validation results on original samples. The score column indicates the number of samples with PCR bands that matched their predicted allele lengths from the graph. Individuals with a band not of the expected size are indicated in parentheses, as well as those that exhibited heterozygosity. Sanger sequencing has been attempted to check the amplified PCR products. O = *Otopharynx argyrosoma*, C = *Copadichromis chrysonotus*, R = *Rhamphochromis sp. "chilingali"*.

(a) *A. calliptera* backbone, universal reliable genes: 20,785

	Reliable genes for PAV analysis	**>95% conserved**		**>95% conserved, reliable for all samples**	
		gene body	**exon**	**gene body**	**exon**
astCal	25,639	25,615 (99.9%)	25,632 (100.0%)	20,765 (99.9%)	20,779 (100.0%)
mayZeb	24,461	20,687 (84.6%)	22,978 (94.0%)	17,786 (85.6%)	19,650 (94.5%)
troMau	24,568	20,779 (84.6%)	23,113 (94.1%)	17,794 (85.6%)	19,681 (94.7%)
aulStu	24,336	20,199 (83.0%)	22,781 (93.6%)	17,487 (84.1%)	19,573 (94.2%)
otoArg	23,695	19,693 (83.1%)	22,172 (93.6%)	17,480 (84.1%)	19,544 (94.0%)
copChr	22,919	19,144 (83.5%)	21,426 (93.5%)	17,455 (84.0%)	19,505 (93.8%)
rhaChi	24,421	20,060 (82.1%)	22,691 (92.9%)	17,356 (83.5%)	19,473 (93.7%)
rhaChi2	23,148	19,049 (82.3%)	21,516 (92.9%)	17,287 (83.2%)	19,434 (93.5%)

(b) *M. zebra* backbone, universal reliable genes: 20,266

	Reliable genes for PAV analysis	**>95% conserved**		**>95% conserved, reliable for all samples**	
		gene body	**exon**	**gene body**	**exon**
astCal	24,047	20,568 (85.5%)	22,917 (95.3%)	17,519 (86.4%)	19,436 (95.9%)
mayZeb	27,343	27,293 (99.8%)	27,324 (99.9%)	20,223 (99.8%)	20,248 (99.9%)
troMau	24,692	21,892 (88.7%)	23,719 (96.1%)	18,152 (89.6%)	19,583 (96.6%)
aulStu	24,316	20,500 (84.3%)	23,099 (95.0%)	17,295 (85.3%)	19,381 (95.6%)
otoArg	23,623	19,913 (84.3%)	22,439 (95.0%)	17,287 (85.3%)	19,365 (95.6%)
copChr	22,977	19,479 (84.8%)	21,798 (94.9%)	17,224 (85.0%)	19,279 (95.1%)
rhaChi	24,410	20,490 (83.9%)	23,044 (94.4%)	17,222 (85.0%)	19,263 (95.1%)
rhaChi2	23,075	19,409 (84.1%)	21,807 (94.5%)	17,181 (84.8%)	19,242 (94.9%)

Supplemental Table S4. Estimated gene sequence conservation by ODGI's presence-absence variation analysis (`odgi pav`) function. This analysis was restricted to gene sets meeting criteria for sufficient sequencing coverage, avoidance of complex graph regions and removal of falsely annotated TEs. A different set of reliable genes was used for each sample (first three columns), as well as a universally reliable set for all samples (last two columns). The table counts the number of genes showing 95% sequence conservation or above. Backbone assemblies are shaded in gray.

	Observed (O)	Genomewide (G)	Expected (E)	O/G ratio	O/E ratio
All Transposons (TEs)	74.65	36.55	42.46	2.04	1.76
All TEs w/o Unknown	62.25	28.30	34.57	2.20	1.80
DNA	19.55	12.09	12.08	1.62	1.62
LINE	16.82	8.32	10.05	2.02	1.67
LTR	25.31	7.38	12.18	3.43	2.08
SINE	0.35	0.40	0.30	0.88	1.17
Retroposon	0.50	0.24	0.33	2.08	1.52
Helitron	0.20	0.06	0.10	3.33	2.00
Unknown	12.92	8.54	8.50	1.51	1.52

Supplemental Table S5. Fold enrichment of TEs in SV regions of *Astatotilapia calliptera*. The percentage sequence of non-overlapping TE regions are shown for the structural variants (O) and whole genome (G). The E column are mean values estimated from 100 randomly shuffled sets of SV coordinates that still maintained proximity to the SV regions, in order to create more realistic baselines than the genomewide values.

(a) Genomewide

	astCal	mayZeb	troMau	aulStu	otoArg	copChr	rhaChi	rhaChi2
All Transposons (TEs)	36.55	38.29	37.54	37.13	37.09	36.32	37.49	36.96
All TEs w/o Unknown	28.30	29.36	28.71	28.20	28.39	28.03	29.02	28.65
DNA	12.09	12.23	12.08	12.12	12.03	11.81	12.22	12.12
LINE	8.32	8.77	8.50	8.64	8.46	8.47	8.60	8.46
LTR	7.38	7.65	7.48	7.53	7.32	7.21	7.57	7.45
SINE	0.40	0.40	0.40	0.40	0.39	0.39	0.40	0.39
Retroposon	0.24	0.35	0.26	0.24	0.29	0.27	0.28	0.28
Helitron	0.06	0.18	0.21	0.10	0.12	0.09	0.18	0.17
Unknown	8.54	9.22	9.11	8.61	8.98	8.57	8.76	8.61

(b) Structural variants

	astCal	mayZeb	troMau	aulStu	otoArg	copChr	rhaChi	rhaChi2
All Transposons (TEs)	74.65	73.29	72.26	73.85	74.25	75.74	74.82	76.60
All TEs w/o Unknown	62.25	61.74	59.99	62.78	62.79	65.10	62.79	65.32
DNA	19.55	19.32	18.36	19.36	19.11	19.59	19.76	20.39
LINE	16.82	17.85	16.91	18.21	18.40	19.34	17.75	18.36
LTR	25.31	24.09	23.82	24.88	24.92	25.81	24.77	26.19
SINE	0.35	0.33	0.32	0.31	0.31	0.32	0.29	0.29
Retroposon	0.50	0.47	0.41	0.41	0.41	0.44	0.43	0.43
Helitron	0.20	0.20	0.68	0.17	0.20	0.16	0.35	0.22
Unknown	12.92	12.02	12.74	11.56	11.95	11.15	12.59	11.84

Supplemental Table S6. Genomewide and structural variant TE composition across Lake Malawi cichlid assemblies.

Supplemental Methods

NCBI access information for raw reads

Sample	Species	Taxon	Library	BioProject	Biosample	Run ID(s)
troMau	Tropheops sp. 'mauve'	286600	PacBio - CLR	PRJEB80840	SAMEA11293786	ERR12954135
aulStu	Aulonocara stuartgranti	286572	PacBio - CLR	PRJEB72478	SAMEA115846654	ERR13382500
rhaChi	Rhamphochromis sp. 'chilingali'	454631	PacBio - CLR	PRJEB72870	SAMEA112818853	ERS14826389
otoArg	Otopharynx argyrosoma	323788	ONT - Simplex	PRJNA1144831	SAMN43044617	SRR30633342
copChr	Copadichromis chrysonotus	323762	ONT - Simplex	PRJNA1144838	SAMN43044710	SRR30633337, SRR30633338
rhaChi2	Rhamphochromis sp. 'chillingali'	454631	ONT - Simplex	PRJNA1144843	SAMN43044956	SRR30633436, SRR30633437, SRR30633438

Long read genome assemblies

The six new long read assemblies in this study were generated from DNA extracted from aquaria grown fish specimens. The *Tropheops* sp. “mauve” and two *Rhamphochromis* sp. "Chilingali" individuals (one male, one female) were reared by members of the Santos Lab at the local fish facility in the University of Cambridge. *Otopharynx argyrosoma* and *Copadichromis chrysonotus* were provided by Dr. Hannes Svardal’s lab at the University of Antwerp in Belgium, while *Aulonocara stuartgranti* were sourced from Professor George Turner at Bangor University, Wales. Pacific Biosciences (PacBio) sequencing was used for *Tropheops* sp. “mauve” (troMau), *Aulonocara stuartgranti* (aulStu) and the male *Rhamphochromis* sp. “Chilingali” (rhaChi), while Oxford Nanopore (ONT) was used for *Copadichromis chrysonotus* (copChr), *Otopharynx argyrosoma* (otoArg) and the female *Rhamphochromis* (rhaChi2). Depending on tissue availability, frozen tissue from either fin, muscle or gill tissue, with the variations in DNA extraction and library preparation protocols described below.

Pacific Biosciences

Genomic DNA extraction

Tissue samples were collected from muscle for *Tropheops* sp. “mauve” (troMau) and the male *Rhamphochromis* sp. “Chilingali” (rhaChi) sample, while fin and tail clips were used for *Aulonocara stuartgranti* (aulStu). High molecular weight DNA (HMW DNA) was extracted using the Bionano Genomics: IrysPrep® Animal Tissue DNA Isolation Soft Tissue protocol, which involved tissue disruption, cell lysis and DNA purification steps. The quantity of extracted genomic DNA was evaluated with the HS Qubit DNA kit and QC-assessed with the Femto Pulse instrument (Agilent).

Library preparation

Pacific Biosciences (PacBio) SMRT sequencing was performed with CLR (Continuous Long Reads) technology for the HWM DNA, following the official SMRTbell® Express Template Preparation Kit 2.0 protocol, with specific modifications to the DNA shearing step and library size selection using a Bluepippin instrument (Sage Science) depending on the sample.

- troMau: DNA shearing was not performed, because the sample was slightly degraded. Library size of 20 kb selected.

- aulStu: DNA sheared using a 26 gauge needle (5 passes through the needle). Library size of 7 kb selected
- rhaChi: DNA sheared using Megaruptor 3 at speed setting 5. Library size of 20 kb.

The prepared DNA libraries were sequenced on the Sequel II instrument using Sequencing kit v0.9 / Binding Kit v0.9. Contigs were generated from the reads using FALCON and FALCON-Unzip assembler software.

Oxford Nanopore

DNA extraction

Otopharynx argyrosoma, otoAtg: Fin tissue was pulverised in a mortar with liquid nitrogen, after which DNA was extracted using the QIAGEN Genomic Tip 100/G Kit (10243) with minor modifications to manufacturer instructions. After elution from the Genomic Tip column, the sample was divided into 4 x 1.25 mL aliquots in 2.0 mL DNA LoBind® tubes using wide-bore tips and the DNA precipitated with isopropanol, washed twice with 70% ethanol and each aliquot resuspended in 50uL elution buffer.

Copadichromis chrysonotus, copChr: Fin tissue was pulverised in a mortar with liquid nitrogen. Released cells were lysed in 5 mL Lysis Buffer (100 mM NaCl, 10 mM Tris pH, 25 mM EDTA pH, 0.5% SDS) with 2 uL RNaseA (100 mg/ml) at 37°C for 1 h with gentle inversion mixing every 20 minutes, followed by 50 uL Proteinase K (20 mg/ml) digestion at 55°C for 2 hours with 10 rpm in a rotating incubator. DNA was then extracted using Phenol-Chloroform-Isoamyl alcohol pH 8 in combination with MaXtract High Density 15 ml tubes (Qiagen) according to manufacturer instructions. The DNA was then precipitated with isopropanol in the presence of NaCl, spooled and transferred to DNA LoBind® tubes, washed twice with 70% ethanol, air dried for 10 minutes and resuspended in a 100 uL elution buffer. DNA was size selected on Bluepippin at >15kb followed by magnetic bead purification.

Rhamphochromis female, rhaChi2: Gill tissue was cut into smaller pieces and digested for 2.5h in ALT Buffer with 20 uL Proteinase K (20 mg/ml) at 56°C. Undigested cell debris removed by a very gentle centrifugation and supernatant transferred with wide-bore tip to DNA LoBind® tube. 4 uL RNaseA (100 mg/ml) was added and incubated for 10 minutes at room temperature. Tween 20 (to a 0.1% final concentration) was added with an equal volume of resuspended SPRIselect beads and incubated on a tube rotator at 10 rpm for 20 minutes. The beads were captured on a magnet and washed twice with fresh 70% ethanol for 30 seconds, briefly air dried, then removed from the magnet and resuspended with 55 uL elution buffer and mixed by gently tapping on the tube and incubated for a few minutes at room temperature. DNA was then separated from the beads on the magnet and supernatant transferred using wide bore tips to the new tube. Elution was repeated but collected into separate tubes. DNA was size selected on Bluepippin at >20kb followed by magnetic bead purification.

Library preparation and genome assembly

Prior to library preparation, DNA was QC assessed using Qubit (dsDNA BR Assay Kit), Nanodrop and Tapestation (Genomic DNA ScreenTape). Sequencing libraries were prepared from 1-5 ug of material using Ligation Sequencing Kit (SQL-LSK109) and sequenced on R9 MinION flow cells with minor modifications. End repair was performed at 20°C for 30 minutes, followed by 30 minutes at 65°C, after which adapter ligation was performed for 1 hour. Guppy v5.0.11 was used to call bases, and the resulting read sets were assembled into contigs with Shasta v0.7.0 (commit b64c4ad0755a6e0d5ba5ef9c1cffead5dc6fadc0) [182] under default settings, keeping contigs longer than 500bp in length.

PCR experiments

Fish maintenance and euthanasia

Astatotilapia calliptera, *Tropheops sp. "mauve"* and *Rhamphochromis sp. "chilingali"* animals were grown in 220 Litre tanks, with pH 8, at approximately 28°C, and with a 12h dark/light cycle. Males and females of each species were housed only with conspecifics. Feeding, housing, and handling were conducted in strict adherence to local regulations. Fish were fed twice a day with cichlid flakes and pellets (Vitalis). Tank environment was enriched with plastic plants, plastic hiding tubes, and sand substrate.

Aquaria grown animals were euthanized with 1 g/L MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Merck #E10521) and subsequent exsanguination by cutting the gill arches, in accordance with local regulations. Afterwards, required tissues were carefully dissected, swiftly snap frozen in dry ice and stored at approximately -80°C.

Lysis and DNA extraction

DNA was extracted from frozen fins or muscle tissue using the QIAamp DNA Mini kit (Qiagen, #51304), according to manufacturer's instructions. A small portion of fin or muscle tissue was lysed using the lysis buffer supplied in the kit, supplemented with Proteinase K. Lysis was performed at 37°C for 1-2 h. The quality and purity of the extracted DNA was checked using a Nanodrop 2000 (Thermo Fischer Scientific) and on an 1% agarose gel. 1-4 ng of genomic DNA was subsequently used as a template in each PCR reaction. DNA was extracted from two distinct sets of animals: 1) from the same fin tissue of the wild-caught animals whose DNA was sequenced to generate the genome assemblies (one male each of *Tropheops sp. "mauve"*, *Otopharynx argyrosoma*, and *Copadichromis chrysonotus*, and one male and one female of *Rhamphochromis sp. "chilingali"*, no fin tissue leftover for the other species); and 2) from the fins or muscle of aquaria grown individuals (five males and five females of *Astatotilapia calliptera salima*, nine *Tropheops sp. "mauve"* males, and five females and one male of *Rhamphochromis sp. "chilingali"*).

Experimental validation of SVs by PCR and Sanger sequencing

PCRs to validate SVs were performed using Taq DNA Polymerase (NEB #M0267) following manufacturer's instructions. 10 µl reactions were prepared and 1-4 ng of genomic DNA were used as template. Primers used to amplify distinct SVs in the vicinity of protein-coding genes are listed in below. Taq PCR reactions were performed as follows: 95°C for 1 minute; 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 68°C for varying periods of time; and a final cycle of 68°C for 5 minutes. PCR products were run on a 1% agarose gel stained with SYBR Safe (Thermo Fischer Scientific, #S33102) at 120V for 50 minutes.

For subsequent validation of amplicons by Sanger sequencing, PCRs were repeated using high-fidelity Q5 DNA Polymerase (NEB, #M0491), according to manufacturer's instructions. 50 µL reactions were prepared and 6-8 ng of genomic DNA was used as template. Primers used are the same used for Taq DNA Polymerase PCRs, and are listed below. When sequencing was inefficient, additional primers were designed, which include M13 primer sites for more efficient sequencing. 5 µl of the PCR product were run on a 1% agarose gel stained with SYBR Safe to confirm the presence of bands of expected size, and the remaining PCR product was sent to Azenta Life Sciences for Sanger sequencing with the primers listed or M13 universal primers. For animals heterozygous for a particular SV, as observed by the presence of two bands on an agarose gel, the entire PCR product was run on a 1% agarose gel and both bands were extracted using the QIAquick Gel Extraction kit (Qiagen, #28704), and each sent to Azenta Life Sciences for Sanger sequencing.

Table: PCR primers for selected bubbles

Oligo ID	Fw/Rev	Sequence (5' to 3')	SV ID (proximal protein-coding gene)	PCR Extension Time	Expected PCR Product Size (in base pairs)*						Notes
					astCal	troMau	otoArg	copChr	rhaChi	rhaChi2	
MVA380	Fw	CAGTGGAGGAGGATCTCAGC	s180017 (*ENSACLG00000015768* aka *nsd1b*)	1 min 30 sec	944	729	944	944	729	729	
MVA381	Rev	TTTTGCTTTTCTGCCTCGAT									
MVA391	Fw	TGCAGAAGACGCTCTGATCT	s438346/s54791 (ENSACLG00000021983)	1 min 30 sec	1306	169	169	1306	1306	1306	
MVA392	Rev	TGCAGCAACATTTCAAAACAA									
MVA395	Fw	GAGACTCTCAGCCGTTTTACG	s461001 (*ENSACLG00000002767* aka *chchd3b*)	1 min	281	588	588	588	281	281	
MVA396	Rev	TCCACACACTGCATGTAGGC									
MVA399	Fw	AAATGGGTCATTTGGAGCTG	s175418 (*ENSACLG00000017346* aka *kcnab3*)	40 sec	446	133	446	446	133	133	
MVA400	Rev	CCCAATAGTGTCCACTGACATC									
MVA401	Fw	CTGAACCCGTTTCTCACACA	s120363 (*ENSACLG00000027791* aka *ptges*)	40 sec	460	143	143	143	460	460	
MVA402	Rev	CCAGTGCTGGGTTTTGAGAT									
MVA409	Fw	TCAGGTAGAGGGCAGGTGTT	s165551 (*ENSACLG00000013807* aka *gabrr3b*)	1 min	852	338	338	338	852	852	
MVA410	Rev	CTGTCACGTCATTGGCTGAT									
MVA413	Fw	TGAGGAGGAAGAGGATTTGG	s473871 (*ENSACLG00000014403* aka *adam17b*)	40 sec	207	413	207	207	413	413	
MVA414	Rev	CTGCAGTCAGCTGGGTTTTT									
MVA415	Fw	CATGCTTTCTGCATGCATCT	s171149 (*ENSACLG00000026652* aka *dgat2*)	40 sec	558	327	558	558	327	327	
MVA416	Rev	ACGTGCTTGGCTTTCAAGAG									
MVA421	Fw	GGAGGGAAAACAGCCAAAAT	s88741 (*ENSACLG00000022427* aka *mitfa*)	1 min 35 sec	1037	555	1037	1037	1037	1037	
MVA422	Rev	CACCGACGTAATGGGAAACT									
MVA431	Fw	AGGTTCCTGCTGAAGGTCAA	s483914 (*ENSACLG00000025203* aka *mfsd4aa*)	1 min	230	592	230	230	230	230	
MVA432	Rev	GGTCGACGGAATTCTCATGT									
MVA435	Fw	GGATTTGGCACTACCTTCCA	s336440 (*ENSACLG00000006581* aka *klhl7*)	1 min 35 sec	234	1519	234	234	234	234	
MVA436	Rev	ATGCCCCAACAACTTGAAAA									
MVA439	Fw	GCCACCACATGTCTCTCAAA	s457179 (*ENSACLG00000020186* aka *rpgrip1*)	1 min 35 sec	215	1234	215	215	215	215	
MVA440	Rev	CCACAGACTGCTTGACCTGA									
MVA445	Fw	ATGTCCATCTTGAGGGCTGT	s473698 (*ENSACLG00000017196* aka *anpepb*)	1 min 10 sec	218	863	218	218	218	218	
MVA446	Rev	GCATCAAGACGTACCGTCAA									
MVA449	Fw	GCGCATCTGGACTCATTTGT	s256152 (*ENSACLG00000027349* aka *dtnba*)	1 min 10 sec	826	193	826	193	193	193	
MVA450	Rev	TGTTTTTCAGCCAACTGTGG									
MVA451	Fw	ACCCAAAGGAGACGAAGAGG	s423054 (*ENSACLG00000015018* aka *lcp1*)	1 min 10 sec	640	1062	640	1062	1062	1062	
MVA452	Rev	CTCGCTTCAGGTCGTCTTTC									
MVA457	Fw	GTTCTCAGCGTTTGGCTGAT	s364746 (*ENSACLG00000024576* aka *tprg1*)	1 min 10 sec	598	289	598	598	598	598	
MVA458	Rev	ACAACAGCCCCAGCTTCTC									
MVA459	Fw	**GTAAAACGACGGCCAG**GGCGTGTACTCTGAACCCGTTTCTCACACA	s120363 (*ENSACLG00000027791* aka *ptges*)	1 min	518	201	201	201	518	518	With M13 Fw (Bold)
MVA460	Rev	**CAGGAAACAGCTATGAC**AGGTCTCTGATAACACCAGTGCTGGGTTTTGAGAT									With M13 Rev (Bold)
MVA461	Fw	**GTAAAACGACGGCCAG**GAGACTCTCAGCCGTTTTACG	s461001 (*ENSACLG00000002767* aka *chchd3b*)	1 min	314	621	621	621	314	314	With M13 Fw (Bold)
MVA462	Rev	**CAGGAAACAGCTATGAC**TCCACACACTGCATGTAGGC									With M13 Rev (Bold)
MVA463	Fw	**GTAAAACGACGGCCAG**GCGCATCTGGACTCATTTGT	s256152 (*ENSACLG00000027349* aka *dtnba*)	1 min	879	246	879	246	246	246	With M13 Fw (Bold)
MVA464	Rev	**CAGGAAACAGCTATGAC**AATTAAATATTTTTGCTCAATGTTTTTCAGCCAACTGTGG									With M13 Rev (Bold)
MVA465	Fw	**GTAAAACGACGGCCAG**AGGTTCCTGCTGAAGGTCAA	s483914 (*ENSACLG00000025203* aka *mfsd4aa*)	1 min	263	625	263	263	263	263	With M13 Fw (Bold)
MVA466	Rev	**CAGGAAACAGCTATGAC**GGTCGACGGAATTCTCATGT									With M13 Rev (Bold)

*PCR product size indicated for each of the species used for PCR. Species notation used is the same as in the main text.