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1 **Table S1 Summary of sites in ATP α 1 implicated in cardenolide-sensitivity by site-directed**
 2 **mutagenesis and structural studies.**

Amino Acid Position	Location	Ancestral State	Derived States	Fold Change	References
104	H1	C	F, A, Y	6.3-500	[1,2]
108	H1	Y	A, D	9.1	[2]
111	H1-H2	Q	R, H, L, V, T	3-12.5	[3-5]
112	H1-H2	A	T, V, K	-	[6]
114	H1-H2	T	M, S	-	[6,7]
115	H1-H2	S	D	-	[6,8]
116	H1-H2	E	D, L	-	[6,8]
117	H1-H2	E	*	-	[8]
118	H1-H2	P	K, F	4.4	[2]
119	H1-H2	A	S	-	[7]
120	H1-H2	D	R	-	[9]
121	H1-H2	D	E, N, A, S	25-60	[10,11]
122	H1-H2	N	H, D	8-25	[3-5,12]
308	H3-H4	Y	C, F	1.5-2.4	[13]
312	H3-H4	D	R	-	[14]
314	H4	V	M	-	[14]
315	H4	I	V	-	[14]
316	H4	F	*	-	[8]
319	H4	G	A, S	-	[14,15]
322	H4	V	*	-	[8]
323	H4	A	*	-	[8]
324	H4	N	Y	-	[14]
330	H4	L	Q	7.9	[16]
331	H4	A	G	3.1	[16]
338	H4	T	N, A	3.2-3.3	[16]
367	H4-H5	C	*	-	[17]
656	H4-H5	A	*	-	[17]
778	H5	P	A	-	[15,18]
783	H5	F	Y	-	[14]
786	H5	F	N, I	11-19	[16,19]
793	H5-H6	L	P, N, K	2.8-118	[12, 20]
797	H5-H6	T	A, V, N, C, S	40-67	[13,19,21,22]
802	H6	C	F	-	[16,20]
804	H6	D	E	-	[22]
863	H7	F	L	5.7	[12]
874	H7-H8	K	S	-	[7]
880	H7-H8	R	P	7.9	[23]
886	H7-H8	K	*	-	[8]
898	H7-H8	E	E	-	[7]
972	H9-H10	R	*	-	[8]
982	H10	F	S	6.3	[17]

* Revealed by structural prediction.

1 **Table S2. List of species and collection information**

Species	Sex	Life Stage	Tissue	Location	Collected by
<i>Aularches miliaris</i>	F	Adult	Head, muscle, gut, 3 biological replicates	Honey Valley, Karnataka, India	N. Achari, R. Deshmukh
<i>Taphronota calliparea</i>	F	Adult	Gut, antennae, mouthparts	Sofala, Cheringoma Plateu, Mozambique	R. Mariño-Pérez
<i>Dictyophorus griseus</i>	F	Adult	Gut, antennae, mouthparts	Sofala, Cheringoma Plateu, Mozambique	R. Mariño-Pérez
<i>Chrotogonus hemipterus</i>	F	Adult	Head	Sofala, Gorongosa, Mozambique	R. Mariño-Pérez, B. Foquet
<i>Atractomorpha acutipennis</i>	F	Adult	Head	Sofala, Gorongosa, Mozambique	R. Mariño-Pérez, B. Foquet
<i>Zonocerus elegans</i>	M	Adult	Head	KwaZulu-Natal, Mpophomeni Hill, South Africa	H. Song, G. Cowper, R. Mariño-Pérez, A. Gomez
<i>Poecilocerus pictus</i>	M + F	Adult	Head, muscle, gut, 6 biological replicates	Chitoor, Andhra Pradesh, India	N. Achari, R. Deshmukh
<i>Phymateus leprosus</i>	unknown	Nymph	Head, muscle, gut	KwaZulu-Natal, Ntunjambili Forest, South Africa	H. Song, G. Cowper, R. Mariño-Pérez, A. Armstrong, A. Gomez
<i>Sphenarium purpurascens</i>	F	Adult	Head	Oaxaca, Near La Colorada, Mexico	R. Mariño-Pérez, B. Foquet, S. Sannabria-Urban, M.E. Pocco
<i>Ochrophlebia cafra</i>	F	Adult	Head	KwaZulu-Natal, Hlabisa, South Africa	H. Song, G. Cowper, R. Mariño-Pérez, A. Armstrong, A. Gomez

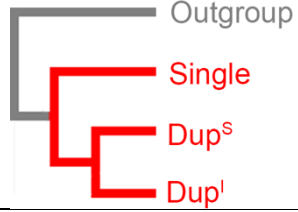
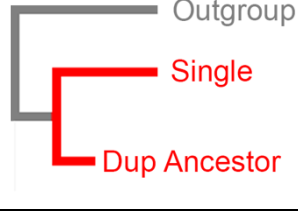
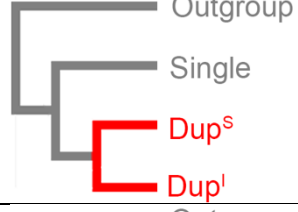
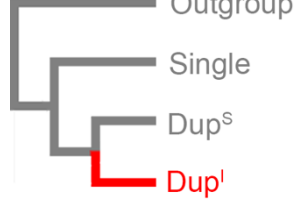
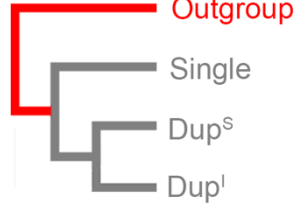
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2**Table S3. Raw RNA-seq read counts of ATP α 1A/B of *Poekilocerus pictus***

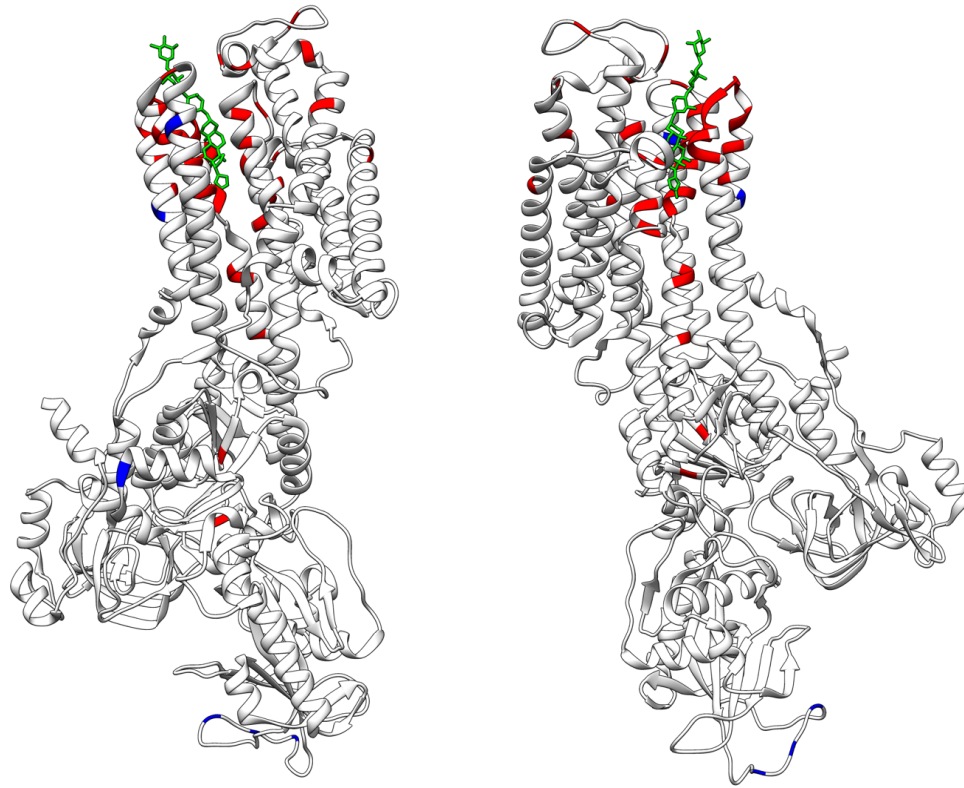
	Individual	#1	#2	#3	#4	#5	#6
Head	ATPα1A	1870	2145	1573	455	227	1365
	ATPα1B	52	1397	1283	14	43	95
	# mapped	7933248	11522005	15682417	18295987	7587412	14911717
Foregut	ATPα1A	888	2943	457	975	3142	1022
	ATPα1B	21	70	17	19	46	18
	# mapped reads	17148883	12282284	5196066	5702546	11186498	6746261
Thorax	ATPα1A	1641	1973	706	588	714	252
	ATPα1B	46	344	177	298	22	40
	# mapped reads	11435872	12950380	11050566	8775458	24748961	20277512

3

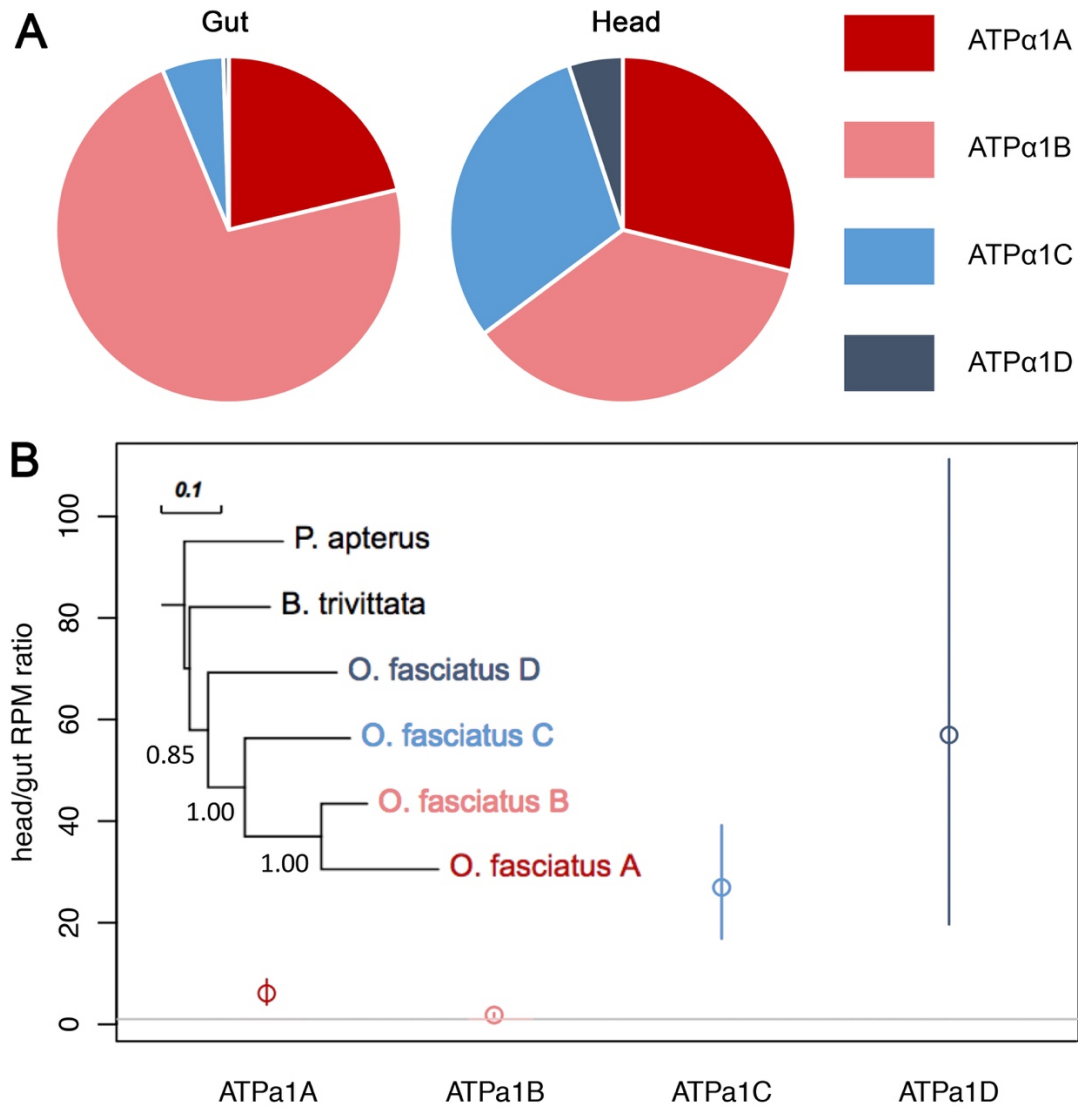
1 **Table S4. Probability of positively selected sites under different models**

Model No.	Model description	Schematic of model	Positively selected sites	BEB Prob.
1	Selection in all Apocynaceae-specialist lineages;		111 Q	1.000*
2	Selection on all Apocynaceae-specialist lineages with single copies of ATP α 1 (including ancestral lineages prior to duplication);		111 Q	1.000*
3	Selection on all Apocynaceae-specialist lineages with duplicate copies of ATP α 1;		301V 566 C	0.966* 0.952*
4	Selection on all putatively less-insensitive ATP α 1 duplicate lineages		102 I 106 I 115 V 118 P 122 N 163 T 177 D 274 E 315 I 428 P 431 D 460 V 560 V 563 K 566 C 667 N 787 I 790 D	0.953* 0.664 0.956* 0.998* 0.993* 0.997 0.787 0.568 0.852 0.553 0.530 0.690 0.980* 0.991* 0.996* 0.981* 0.659 0.544
5	Selection on all outgroup lineages.		None	None

2 NOTE – Asterisks denote BEB posterior probability >0.95 Sites implicated in cardenolide-
3 sensitivity are highlighted in bold. See Methods for more detail on models.



1
2 **Figure S1.** Amino acid sites implicated in cardenolide-sensitivity shown on the ATP1A1 crystal
3 structure. 41 amino acid sites (red) implicated in cardenolide-sensitivity are scattered throughout
4 ATP1A1 (*Sus scrofa*, PDB 4RET). Sites in blue are additional sites that have been identified as
5 positively-selected under one of the selection models evaluated in this study (**Figure 7** and **Table**
6 **S5**). The bound cardenolide digoxin is shown in green.



1

2 **Figure S3.** Relative expression of four ATPα1 copies in the head and gut of large milkweed bugs

3 (*Oncopeltus fasciatus*). **A**) The average percentages of ATPα1A-D of the total ATPα1 in both

4 gut and head. **B**) The normalised counts (reads per million, or RPM) ratio of head to gut. The

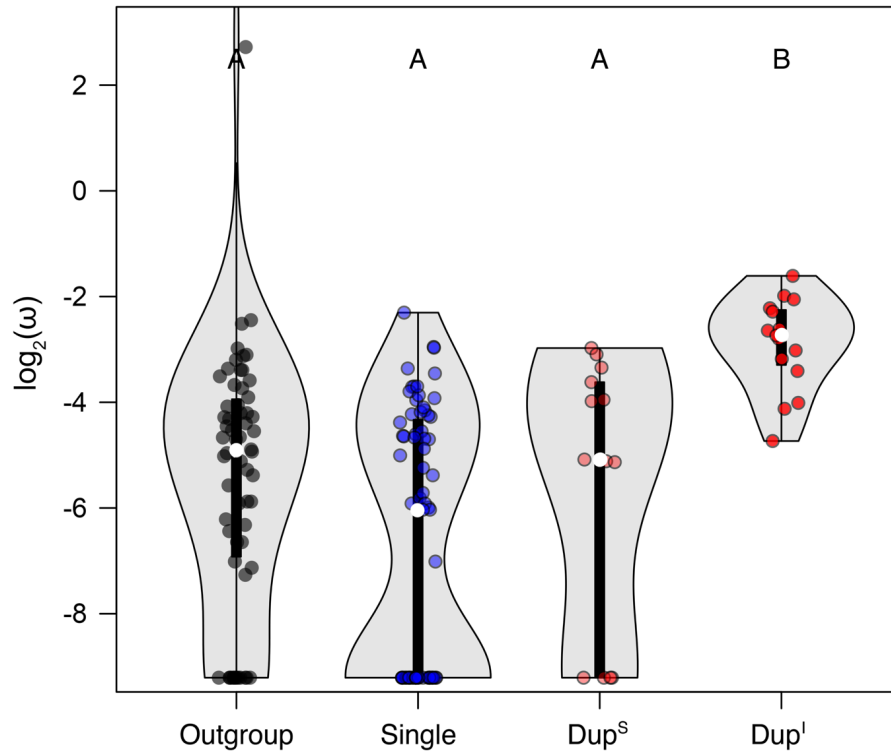
5 grey horizontal line represents the head/gut ratio of 1. Open circles and whiskers represent means

6 and approximate 95% confidence interval determined by bootstrap resampling the count data by

7 individual. The phylogeny was constructed using a maximum likelihood method implemented in

8 SeaView based on nucleotide-coding nucleotide sequences of ATPα1 with bootstrap values

9 shown.



1
2 **Figure S4.** Distributions of lineage-specific omega (dN/dS) estimates for ATP α 1 excluding the
3 41 implicated in cardenolide-sensitivity. The distributions of omega estimates are shown with
4 median and 95% confidence intervals. Letters A and B indicate significantly different categories.
5 There is a significant difference between the omega ratios of Dup^I and those of the three other
6 groups (Dup^I vs Outgroup P=6e-5, Dup^I vs Single P=4e-8, Dup^I vs Dup^S P=3e-3). P-values were
7 estimated using Dunn's test of multiple comparisons using rank sums as implement in R
8 (dunn.test) and corrected using the Benjamini-Hochberg method.

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