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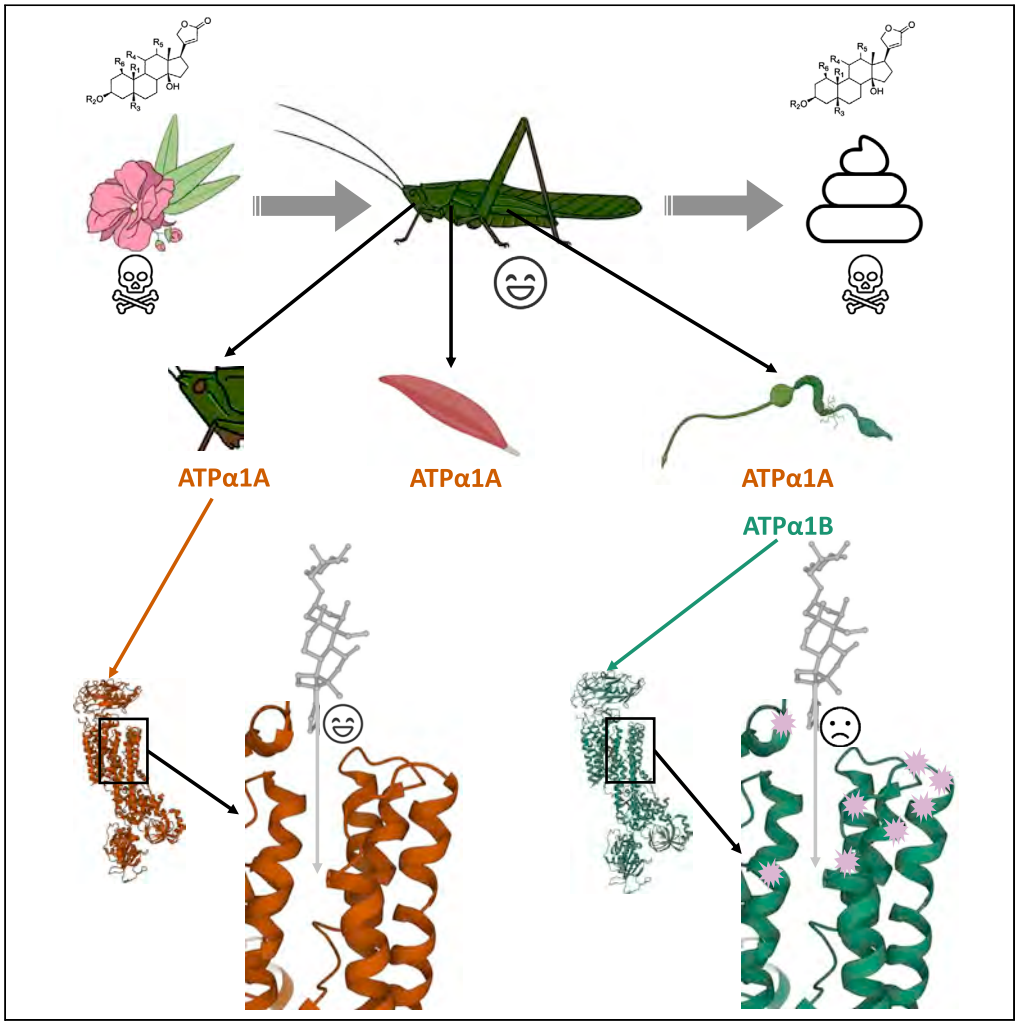

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Highlights

First case of cardenolide resistance in an omnivorous katydid

Molecular convergence in ATPα with cardenolide-resistant specialist herbivores

A gut-specific resistant copy of ATPα in leaf katydids (Phaneropterinae)

Evolution of resistance before currently known cardenolide-producing plants

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Gut-specific cardenolide-resistant sodium pump primed an omnivore to feed on toxic oleander

Tianyu Wang,[1,2,3,4] Lina Shi,[2,3,4] and Ying Zhen[2,3,4,5,*]

SUMMARY

Apocynaceae plants produce toxic cardenolides to defend against their herbivores. Cardenolides could inhibit the α subunit of Na^+/K^+-ATPase (ATPα), which plays critical roles in biological processes such as muscle contraction, neural function, and osmoregulation. Numerous herbivores that specialized on cardenolides-producing milkweeds carry parallel molecular changes in ATPα that confer resistance. We found that Pacific Ducetia (*Ducetia japonica*), an omnivorous katydid, could feed on cardenolides-rich oleander leaves. ATPα in *D. japonica* has undergone parallel molecular changes just like milkweed specialists. These changes evolved at the common ancestors of the leaf katydids before the diversification of Apocynaceae and may prime species in this lineage to feed on cardenolides-rich food. In summary, we reported the first case of cardenolide resistance in katydid, with convergent molecular evolution in ATPα, also an unusual case of cardenolides resistance in nonspecialist species that evolved earlier than the currently known cardenolide-producing plants.

INTRODUCTION

Several lineages of plants, including many plant species in the dogbane family (Apocynaceae), produce cardiac glycosides named cardenolides to defend against their herbivores.[1] Cardenolides are toxic to most animals because they could bind with and inhibit the activity of the α subunit of Na^+/K^+-ATPase (ATPα), a multi-subunits Na^+ and K^+ transporter that plays critical roles in many important biological processes such as muscle contraction, neural function, and osmoregulation.[2] However, numerous insects have been found to convergently evolve the ability to feed on cardenolides-containing plants, including the most well-known monarch butterflies (*Danaus plexippus*) that specialized on milkweeds.[3,4] In the 1990s, researchers discovered that monarch butterflies carry amino acid substitution (N122H) in ATPα1 that confers resistance to cardenolides.[5] Several studies identified molecular changes in ATPα1 underlying cardenolides resistance in a number of milkweed specialists from six insect orders, including Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, and Orthoptera.[6–10] Target site insensitivity in ATPα1 in these cardenolides-resistant milkweed specialists was found to evolve at a highly predictable manner. This includes repeated amino acid substitutions observed at a limited number of sites in ATPα1 protein, repeated duplication of ATPα1 gene, and repeated tissue-specific expression pattern of duplicated ATPα1 copies.[8–10] Negative pleiotropy and epistasis are found to constrain the adaptive evolution of cardenolides insensitivity in these specialized herbivores.[4,9–11]

Previous studies mostly examined phytophagous milkweed-specialized herbivores.[8–10] Oleander (*Nerium oleander*) in Apocynum also contains high amounts of cardenolides and is commonly cultivated as an ornamental plant. Here, we report for the first time that an omnivore and generalist, the Pacific Ducetia (*Ducetia japonica*), also occasionally feeds on toxic oleander leaves; it is also the first species in katydids family (Tettigoniidae) to feed on cardenolides-rich food. The Pacific Ducetia (*D. japonica*) is an old-world species and one of the most widespread katydids.[12] It is unclear whether *D. japonica* evolved similar molecular changes that confer constitutive cardenolides insensitivity similar as specialists or utilizes different mechanisms to deal with occasional cardenolides-rich food.

To address this question, we collected Pacific Ducetia (*D. japonica*) from the wild and reared them in the laboratory. We first confirmed that *D. japonica* could use cardenolides-rich oleander leaves as sole food source. We further investigated whether *D. japonica* sequesters cardenolides and whether it evolved target site insensitivity at molecular level as an omnivore and generalist that only occasionally consumes cardenolides-rich food.

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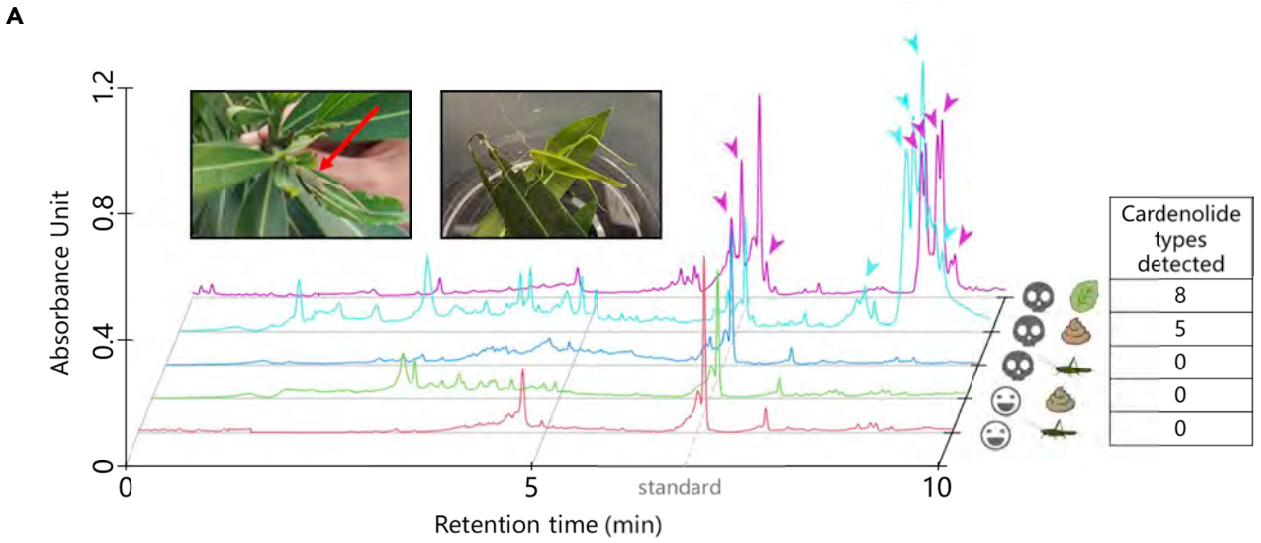
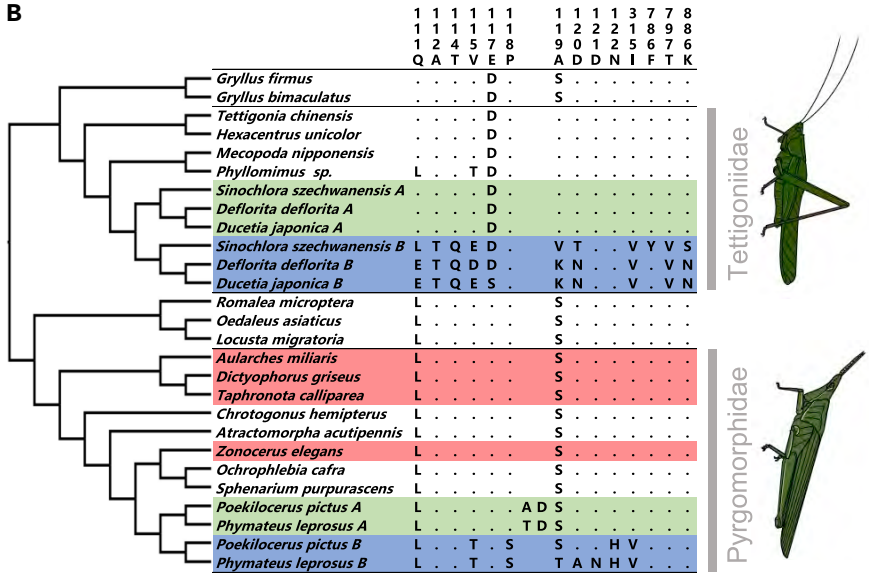
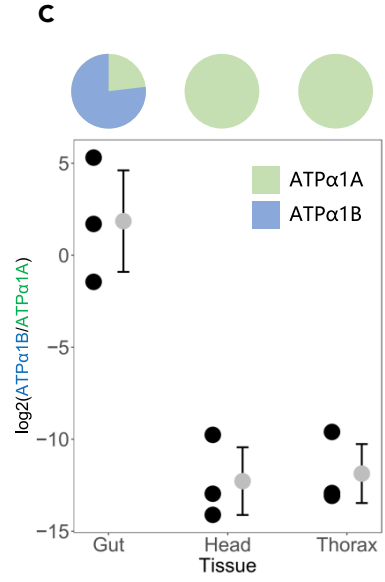
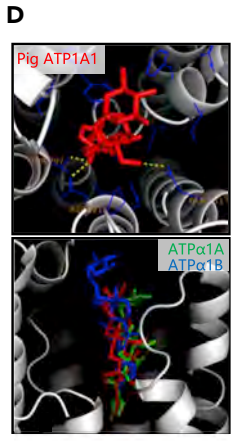

A
Absorbance Unit
1.2
0.8
0.4
0
0
5
10
standard
Retention time (min)
Cardenolide types detected
8
5
0
0
0


B
Gryllus firmus
Gryllus bimaculatus
Tettigonia chinensis
Hexacentrus unicolor
Mecopoda nipponensis
Phyllomimus sp.
Sinochlora szechwanensis A
Deflorita deflorita A
Ducetia japonica A
Sinochlora szechwanensis B
Deflorita deflorita B
Ducetia japonica B
Romalea microptera
Oedaleus asiaticus
Locusta migratoria
Aularches miliaris
Dictyophorus griseus
Taphronota calliparea
Chrotogonus hemipterus
Atractomorpha acutipennis
Zonocerus elegans
Ochrophlebia cafra
Sphenarium purpurascens
Poekilocerus pictus A
Phymateus leprosus A
Poekilocerus pictus B
Phymateus leprosus B
Tettigoniidae
Pyrgomorphidae


C
ATPα1A
ATPα1B
log2(ATPα1B/ATPα1A)
5
0
−5
−10
−15
Gut
Head
Thorax
Tissue


D
Pig ATP1A1
ATPα1A
ATPα1B

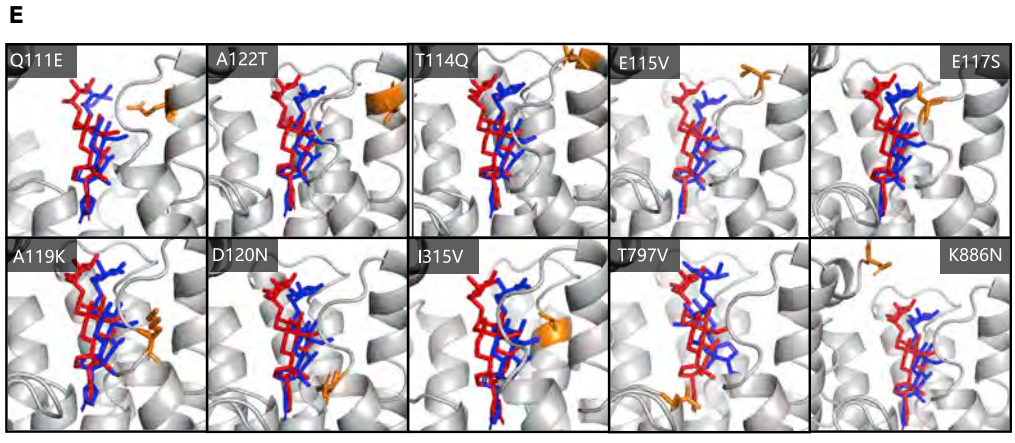

E
Q111E
A122T
T114Q
E115V
E117S
A119K
D120N
I315V
T797V
K886N

Figure 1. ATPα1 duplication gave rise to a gut-specific resistant ATPα1B and a sensitive ATPα1A in the Pacific Ducetia (*Ducetia japonica*)

(A) *Ducetia japonica* could feed on oleander leaves but does not sequester cardenolides. Photos show nymph of *Ducetia japonica* found on oleander tree in the wild (left) and adult raised on fresh oleander leaves under laboratory condition (right). Traces show absorbance spectra of cardenolides at 218 nm from HPLC. From top to bottom, traces represent extracts from samples of oleander leaves (purple), frass of *Ducetia japonica* feeding on oleander leaves (cyan), *Ducetia japonica* body without the gut after feeding on oleander leaves (blue), frass of *Ducetia japonica* feeding on soybean leaves (green), and *Ducetia japonica* body without the gut after feeding on soybean leaves (red). Toxic symbols at the right indicate three traces related to toxic oleander. Arrows with corresponding colors highlight detected cardenolides peaks. Peaks at about 7 min are internal digitoxin standards added to each sample (gray dashed line).

(B) Amino acid substitutions of Orthopterans at sites implicated in cardenolides sensitivity for ATPα1. Only sites with substitution are shown. Numbered columns are based on sheep ATP1A1 (*Ovis aries*) (GenBank: NC019458.2). Colored rows correspond to putatively cardenolides-adapted species and red, green, and blue denote single copy ATPα1, ATPα1A, and ATPα1B, respectively. Dots indicate identity with the reference.

(C) Tissue-specific gene expression of ATPα1 duplicates in *Ducetia japonica*. qPCR was based on three biological replicates. Green: ATPα1A; blue: ATPα1B. Each gray point represents the mean ± SEM of three biological replicates.

(D) (top)Structure of the ouabain binding pocket of *Sus scrofa* ATP1A1 bound to ouabain (in red).[13] Yellow lines represent possible hydrogen bonds between ATP1A1 and ouabain. (bottom) Best docking position of ouabain onto native ATPα1A (in green) and ATPα1B (in blue) of *Ducetia japonica*.

(E) Molecular docking simulations for each candidate substitution. Blue denotes the best docking and red denotes ouabain in the co-crystal structure. The substituted amino acid is highlight in gold.

See also Tables S1–S5.

Interestingly, we found *D. japonica* has ATPα1 duplication that results in one conserved cardenolides-sensitive ATPα1A and one gut-specific cardenolides-resistant ATPα1B that also evolved at a faster rate. By examining transcriptomes of sister species, we found that the duplication of ATPα1 happened in the common ancestor of the leaf katydids (Phaneropterinae) that may predate the evolution of cardenolides-producing plant species.

RESULTS AND DISCUSSION

D. japonica feeds on toxic oleander while does not sequester cardenolides

Oleander plants (*N. oleander*) produce high amounts of cardenolides and have few specialized herbivores. During field trips, we occasionally observed Pacific Ducetia (*D. japonica*) on oleander plants (Figure 1A). *D. japonica* belongs to katydids (Tettigoniidae) in Orthoptera, is a widespread katydid, and is well known to eat diverse food including leaves of leguminous plants and small insects.[14] Nymph of *D. japonica* has recently been observed to hunt fireflies at the Shanghai Botanical Garden (Video S1). Some firefly species contain toxic lucibufagins with similar structure to cardenolides that also inhibit ATPα1,[15–17] although whether the specific firefly species in the video contains lucibufagin toxins has not been reported. Such generalist and omnivorous insects have so far not been reported to have specialized molecular adaptation to cardenolides, a group of relatively uncommon toxins.

To confirm Pacific Ducetia (*D. japonica*) indeed could feed on cardenolides-rich oleander, we collected their nymphs and adults from the wild and reared them in the laboratory. *D. japonica* adults reared in the laboratory have been observed to feed on both fresh oleander leaves and soybean leaves and could use either one as the only food source for at least two weeks without any apparent problem. *D. japonica* nymphs could use oleander leaves as sole food sources and grow into adults without apparent adverse effects. ATPα1 is a highly conserved and essential animal pump; thus, almost all previously reported herbivores that could feed on cardenolides-containing milkweeds are specialized to use milkweeds to some extent. To the best of our knowledge, Pacific Ducetia (*D. japonica*) is the first katydid (Tettigoniidae) and the first omnivore and generalist with diverse natural diets known to naturally feed on cardenolides-rich plants.

Several milkweed specialists are known to sequester cardenolides.[4] Pacific Ducetia (*D. japonica*) may only occasionally feed on oleander under natural conditions and has cryptic green color that blends well with the environment, thus probably does not sequester cardenolides. We next examined whether *D. japonica* could sequester cardenolides from oleander leaves using high-performance liquid chromatography (HPLC). We found that *D. japonica* did not have any detectable level of cardenolides in its body tissue (without gut) after feeding solely on oleander leaves for two weeks, same as the control individuals that feed on cardenolides-free soybean leaves. Similar cardenolides profiles were found in oleander leaves and frass of *D. japonica* that feed on these oleander leaves, suggesting *D. japonica* does not actively sequester cardenolides (Figure 1A).

Duplication and substitutions of ATPα1 in *D. japonica*

Has Pacific Ducetia (*D. japonica*) evolved similar molecular changes as previously reported specialized herbivores that confer constitutive cardenolides target site insensitivity or does it deal with occasional cardenolides-rich food through different mechanism? We collected RNA sequencing (RNA-seq) data and *de novo*

Table 1. Molecular docking simulations of ouabain onto pig ATP1A1 carrying specific amino acid substitution from *Ducetia japonica* ATPα1B, and onto native *Ducetia japonica* ATPα1A and ATPα1B

	RMSD from co-crystal in 3N23 (Å)	RMSD from best[a] WT docking position (Å)	Affinity
Q111E	2.351	0.998	−7.6
A112T	2.387	0.768	−8.1
T114Q	2.516	0.844	−8.2
E115V	2.479	0.023	−8.2
E117S	2.474	0.042	−8.2
A119K	2.348	0.632	−8.2
D120N	2.472	0.051	−8.2
I315V	2.341	0.779	−8.3
T797V	**3.856**	**4.725**	**−7.5**
K886N	2.487	0.062	−8.2
ATPα1B	**5.215**	**4.928**	**−8.2**
ATPα1A	2.475	0.021	−8.2
WT	2.479		−8.2

WT denotes pig ATP1A1 in the reference co-crystal structure (PDB 3N23A). The best docking is defined as closest to the co-ordinates of ouabain in the reference co-crystal structure. Large effects with RMSD exceeds 4.6 Å (the resolution of 3N23A) are in bold.

[a]The 'best' WT docking was defined as the structure in the top 10 highest affinity dockings that was closest to the co-crystal coordinates of ouabain.

assembled transcriptomes from three tissues of *D. japonica*, including gut, head, and thorax muscle. Surprisingly, from *D. japonica* gut transcriptome, we identified two copies of ATPα1, *i.e.*, ATPα1A and ATPα1B. Patterns of amino acid substitutions suggest that ATPα1A is a highly conserved cardenolides-sensitive copy and ATPα1B may be cardenolides resistant. Substitutions at 41 sites in ATPα1 have been previously implicated in cardenolides sensitivity.[4,10,11] In ATPα1A, only one substitution E117D was observed among the 41 sites, and this exact substitution is shared with ATPα1B. In ATPα1B, we found 10 amino acid substitutions (Q111E, A112T, T114Q, V115E, E117S, A119K, D120N, I315V, T797V, and K886N) in the 41 sites (Figure 1B). Of these, T797V could increase ouabain resistance 70 times in *in vitro* mutagenesis experiments.[13] A122T and substitutions at position 114 and 120 have been found in cardenolides-resistant toads.[18] V115E has been found in cardenolides-sequestering large and small milkweed bugs. Compared with two previously reported milkweed-specialized grasshoppers that also have two copies of ATPα1 in the sister clade spear-headed grasshoppers (Pyrgomorphidae), Pacific Ducetia (*D. japonica*) ATPα1A and ATPα1B have distinct pattern of substitutions, suggesting these two lineages have undergone independent duplication events Figure 1B).[9]

We used molecular docking simulations to examine the effect of individual substitution based on the known crystal structure of the pig ortholog ATP1A1 bound to cardenolide ouabain (PDB: 3N23A)[19] (Figure 1D). We compared the ouabain docking positions onto ATP1A1 carrying each of the 10 amino acid substitutions found in Pacific Ducetia (*D. japonica*) ATPα1B with wild-type ATP1A1 docking position. We found T797V most drastically influences the binding between ouabain and ATP1A1 by blocking ouabain from entering the binding pocket, resulting in weaker affinity and larger root-mean-square deviation (RMSD) (Figure 1E and Table 1). Q111E may also affect the binding affinity of ouabain and ATP1A1. Other eight substitutions each has relatively minor effect on ouabain binding based on affinity and RMSD (Figure 1E and Table 1). We further compared the ouabain docking positions onto native Pacific Ducetia (*D. japonica*) ATPα1A and ATPα1B by homology modeling (Figure 1D). We found the RMSD between the best docking position onto ATPα1B and pig ATP1A1 is 5.215 Å, greater than resolution of reference structure 3N23A, suggesting ATPα1B is cardenolides resistant (Figure 1D and Table 1).

Gut-exclusive expression of ATPα1B in *D. japonica*

ATPα1A transcripts were found in transcriptomes of all three tissue types, including gut, head, and thorax muscle. However, ATPα1B was only assembled from gut transcriptome, but not from the head or thorax

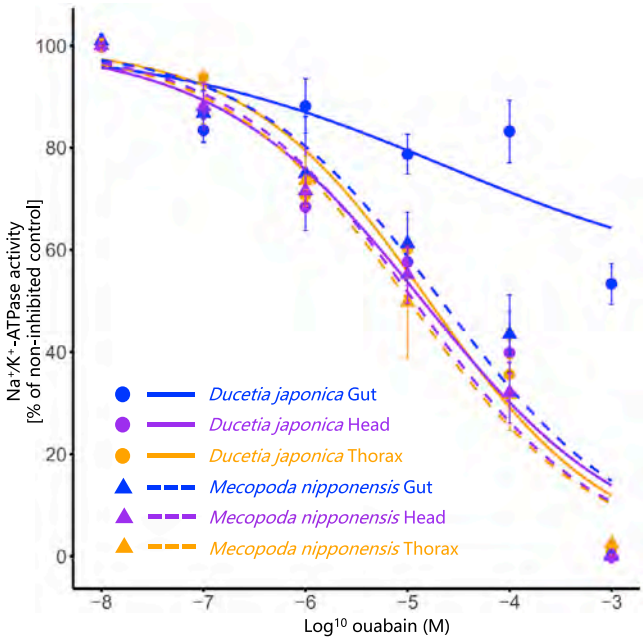


Figure 2. Na^+/K^+-ATPase from the gut of *Ducetia japonica* is more resistant to cardenolide ouabain

Relative Na^+/K^+-ATPase activity from different tissues was plotted as a function of increasing concentrations of ouabain. Head, gut, and muscle tissues of both Pacific Ducetia (*Ducetia japonica*) and cardenolide-sensitive outgroup species *Mecopoda nipponensis* were assayed. Each data point represents the mean ± SEM of three biological replicates. See also Table S3.

transcriptomes. We further examined whether there is a similar tissue-specific expression pattern of the two copies of ATPα1 in Pacific Ducetia (*D. japonica*) using qPCR and copy-specific primers. We confirmed that expression of the cardenolides-resistant copy ATPα1B is approximately 3.61-fold higher than that of the cardenolides-sensitive ATPα1A in the gut, while ATPα1B expression is extremely low in the head and thorax (Figure 1C).

Previous studies have reported six milkweed specialists with multiple copies of ATPα1 [4]. In these milkweed specialists, the relative expression of resistant copy to sensitive copy is higher in the gut compared to the head and muscle, probably because the gut is a primary location to process large amounts of cardenolides and nerve tissues are protected by barriers such as glial sheath.[9,10,20] Our finding in the generalist Pacific Ducetia (*D. japonica*) is similar to the reported milkweed specialists, with more restricted and higher expression of the cardenolides-resistant ATPα1B in the gut compared to the head and thorax.

Na^+/K^+-ATPase from *D. japonica* gut is resistant to cardenolides

To confirm that ATPα1B is resistant to cardenolides inhibition, we carried out Na^+/K^+-ATPase activity assays on three tissues, *i.e.* gut, head and muscle, from both Pacific Ducetia (*D. japonica*) and *Mecopoda nipponensis*, an outgroup species that is not resistant to cardenolides. We found that activity of Na^+/K^+-ATPase from head and muscle tissues of *D. japonica*, as well as Na^+/K^+-ATPase from all three tissues from *M. nipponensis*, showed similar responses to cardenolide (ouabain) treatments at different concentrations (Figure 2). However, Na^+/K^+-ATPase from the gut of *D. japonica* exhibits significantly higher resistance to ouabain than all the other tissues from both *D. japonica* and cardenolide-sensitive *M. nipponensis* (Figure 2). This is consistent with the fact that cardenolides-resistant ATPα1B specifically expresses in the gut of *D. japonica*, which enables it to tolerate cardenolides and feed on cardenolides-containing oleander plants.

Dating the ATPα1 duplication to the common ancestor of the leaf katydids (Phaneropterinae)

To determine when the ATPα1 duplication happened, we collected fresh samples and sequenced the gut, head, and thorax muscle transcriptomes of five additional species in katydids (Tettigoniidae), *i.e., Sinochlora szechwanensis, Hexacentrus unicolor, Deflorita deflorita, M. nipponensis,* and *Phyllomimus sp.* From *de novo* assembled transcriptomes of each species, we found that *Deflorita deflorita* and *S. szechwanensis*, two species

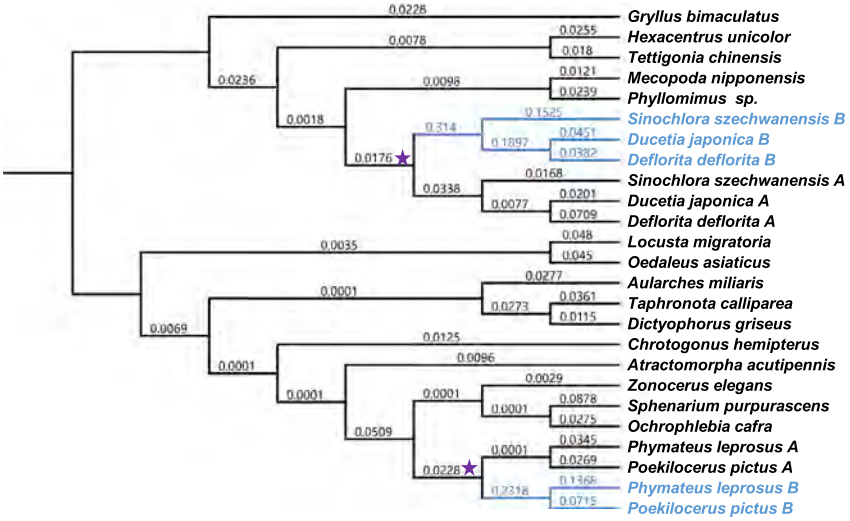


Figure 3. d_N/d_S of ATPα1 in Orthoptera calculated using PAML codeml

Purple stars correspond to ATPα1 duplications and branches of ATPα1B are highlighted in blue.
See also Tables S2–S4.

that are more closely related to Pacific Ducetia (*D. japonica*) and belong to the same subfamily Phaneropterinae, the leaf katydids, also have two copies of ATPα1 found almost exclusively from their gut transcriptomes. In contrast, three more distantly related katydids in the Tettigoniidae family, *i.e., H. unicolor*, *Phyllomimus sp,*. and *M. nipponensis,* have only a single conserved and sensitive copy of ATPα1 *de novo* assembled from transcriptomes of all three tissues (Figure 1B). Using similar pipeline, we also only found one single copy of ATPα1 from publicly available transcriptome data from one additional katydid (Tettigoniidae) *Tettigonia chinensis* and one outgroup Gryllidea species *Gryllus bimaculatus*. Previous study also reported one single copy of ATPα1 in *Gryllus firmus*.[9] As summary, we found two copies of ATPα1 in all three leaf katydids (Phaneropterinae), including Pacific Ducetia (*D. japonica*), *Deflorita deflorita,* and *S. szechwanensis*, and only one copy in all the other katydids (Tettigoniidae) and outgroup species.

For the three species in the leaf katydids subfamily (Phaneropterinae) with two copies of ATPα1, DNA sequence similarity between paralogous ATPα1A and ATPα1B of same species is 77.4%–81.1%, while pairwise DNA sequence similarity is 88.5%–93.5% between ATPα1A orthologs and 86.7%–92.1% between ATPα1B orthologs (Table S1). Higher sequence similarity between ATPα1 orthologs than between ATPα1 paralogs suggests that the ATPα1 duplication event probably happened in the common ancestor of the leaf katydids (Phaneropterinae).

ATPα1B evolved at a faster rate in Phaneropterinae

ATPα1B in Pacific Ducetia (*D. japonica*), *Deflorita deflorita,* and *S. szechwanensis* has accumulated more amino acid substitutions than ATPα1A (Figure 1). d_N/d_S of ATPα1B in the three species of the leaf katydids subfamily (Phaneropterinae), as well as in the two species of spear-headed grasshoppers (Pyrgomorphidae), are higher than that of ATPα1A, which is similar as single copy ATPα1 in other lineages (Figure 3). Using two-ratio model in Phylogenetic Analysis by Maximum Likelihood (PAML),[21] d_N/d_S are estimated to be 0.150 for ATPα1B in the leaf katydids (Phaneropterinae) and 0.144 for ATPα1B in *Poekilocerus pictus* and *Phymateus leprosus*, more than 7-fold higher than the background d_N/d_S of 0.019 across the rest of the ATPα1 phylogeny. The two-ratio model fits significantly better to observed data than one-ratio model (p value < 0.001), suggesting ATPα1B in three species of the leaf katydids (Phaneropterinae) and two species of spear-headed grasshoppers (Pyrgomorphidae) both evolved at a faster rate.

The duplication of ATPα1 in the common ancestor of phaneropterinae may predate the evolution of known cardenolides-producing plants

Cardenolides have convergently evolved in multiple lineages of plants as defensive secondary metabolites. Plant species from 12 families have been reported to produce cardenolides, with relatively sporadic phylogenetic distribution and a higher prevalence in phylogenetically younger angiosperm orders.[1] The

dogbane family (Apocynaceae) is the most well-known plant family dominated by a large number of cardenolides-producing plant species. The dogbane family (Apocynaceae) was estimated to evolve approximately 58.96 mya (95% highest probablity density (HPD): 58–60.81 mya).[22] The divergence times of most other cardenolides-producing plants with their respective cardenolides-free sister group were estimated to be even more recent (Table S2). The time of ATPα1 duplication in two species of aposematic milkweed-specialized grasshoppers in spear-headed grasshoppers (Pyrgomorphidae) were estimated to be ~36 mya,[9] after the emergence of cardenolides-producing plants, consistent with driving role of host plant adaptation in the evolution of ATPα1 in these two specialist grasshopper species.

Intriguingly, we found that the ATPα1 duplication and the functional diversification of the two duplicated copies occurred at the common ancestor of the leaf katydids (Phaneropterinae), before the evolution of extant plant families with known cardenolides-producing plant species (Table S2). Several previous studies have estimated the divergence times of orthoptera clade including the leaf katydids (Phaneropterinae). Based on different fossil calibrations, genetic datasets, and species sampling, the most recent common ancestor (MRCA) of the leaf katydids (Phaneropterinae) is estimated to be 125.11 mya (95% HPD: 98.25–152.83 mya),[23] 106.74 mya (95% HPD: 97.21–117.73 mya),[24] and 100.92 mya (95% HPD: 73.56–131.62 mya)[25] from three studies with relatively consistent results. This suggests that duplication of ATPα1 and the evolution of the resistant ATPα1B at the MRCA of the leaf katydids (Phaneropterinae) might have happened before the origin of currently known cardenolides-producing plants and not due to adaptation to cardenolides-producing host plants. Instead, acquisition of a gut-specific cardenolides-resistant ATPα1B might prime this group of omnivores to feed on cardenolides-producing plants. Alternatively, it is also possible that some other unknown or extinct ancient cardenolides-producing plant lineages might drive the evolution of cardenolides resistance in the common ancestors of the leaf katydids (Phaneropterinae). Further evidence is needed to distinguish these two hypotheses.

In summary, we report that the omnivore Pacific Ducetia (*D. japonica*) could feed on cardenolides-rich oleander plants; it is also the first species in katydids (Tettigoniidae) reported to be cardenolides resistant. We found Pacific Ducetia (*D. japonica*) does not sequester cardenolides. Although Pacific Ducetia (*D. japonica*) is well known as a generalist and omnivore, it has parallel molecular changes in ATPα1 same as previously reported milkweed specialists, including ATPα1 duplication that gave rise to a cardenolides-sensitive ATPα1A and a cardenolides-insensitive ATPα1B, as well as tissue-specific expression of ATPα1B in the gut where large amounts of cardenolides from foods are processed. Among ten candidate amino acid substitutions in ATPα1B that might influence the target site sensitivity of ATPα1B, we highlight T797V and Q111E that may contribute the most to cardenolides resistance through docking simulations. Interestingly, we found duplication of ATPα1 at the common ancestor of the leaf katydids (Phaneropterinae), implying that duplication of ATPα1 and evolution of the cardenolides-resistant ATPα1B might have happened before the diversification of cardenolides-producing plants and primed insects in the leaf katydids (Phaneropterinae) the ability to explore broader selection of food, including cardenolides-rich oleanders.

Limitations of the study

We are currently not able to breed Pacific Ducetia (*D. japonica*) in the lab, so all samples were collected from the wild. Owing to limitation of samples, we could not determine whether *D. japonica* could survive solely on toxic oleander from early developmental stages. Also, due to sample limitation, we could not quantitatively measure the preference of *D. japonica* on different types of food choice, although in preliminary assays we found that *D. japonica* did not show apparent preference between oleander leaves and gardenia leaves. In addition, the survey of cardenolides-containing plant species may be incomplete and the understanding of time of cardenolides biosynthesis evolution across phylogeny may be incomplete; thus, we could not rule out the possibility that an unknown but more ancient cardenolides-containing plant lineage and/or an extinct cardenolides-containing plant lineage might have driven the evolution of resistance in the common ancestors of the leaf katydids (Phaneropterinae).

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105616.

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AUTHOR CONTRIBUTIONS

Y.Z. designed and supervised research; T.W. collected samples, performed research, and analyzed data; L.S. and T.W. performed the enzyme activity assay; Y.Z. and T.W. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Ducetia japonica	See Table S2	N/A
Sinochlora szechwanensis	See Table S2	N/A
Hexacentrus unicolor	See Table S2	N/A
Deflorita deflorita	See Table S2	N/A
Mecopoda nipponensis	See Table S2	N/A
Phyllomimus sp.	See Table S2	N/A
Critical commercial assays		
Ea-step Super Total RNA Extraction Kit	Promega	cat#LS1040
NEB Next Ultra RNA Library Prep Kit	Illumina	cat#E7530
Ea-step RT Master Mix Kit	Promega	cat#LS2050
Takara PrimeSTAR DNA Polymerase	Takara	cat#R045A
AxyPrep DNA Gel Extraction Kit	Corning	cat#AP-GX-250
E. coli Top10 competent cell	KangTi Life Technology	cat#11635018
Plasmid miniprep kit	EASY-DO	cat#DR0201050
Go Taq qPCR Master Mix	Promega	cat#A6001
Oligonucleotides		
ATPα1A primers	See Table S4	N/A
ATPα1B primers	See Table S4	N/A
ATPα1A qPCR primers	See Table S4	N/A
ATPα1B qPCR primers	See Table S4	N/A
Deposited data		
Raw sequencing reads	This paper	BioProject: PRJNA892789
ATPα mRNA sequences	This paper	GenBank: OP712493-OP712501
Software and algorithms		
FastQC	Andrews,[26] 2010	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Trimmomatic-0.38	Bolger et al.,[27] 2014	http://www.usadellab.org/cms/?page=trimmomatic
Trinity-v2.8.4	Haas et al.,[28] 2013	https://github.com/trinityrnaseq/trinityrnaseq/releases
Blast	Camacho et al,[29] 2009	https://ncbiinsights.ncbi.nlm.nih.gov/2020/11/12/blast-2-11-0/
Geneious 2021.2.2	Kearse et al.,[30] 2012	http://www.geneious.com
PAML	Yang,[21] 2007	http://abacus.gene.ucl.ac.uk/software/paml.html
PyMOL-2.3.4_0	Delano,[31] 2002	http://www.pymol.org
MODELLER	Fiser and Sali,[32] 2003	https://salilab.org/modeller/
UCSF Chimera	Pettersen et al,[33] 2004	https://www.cgl.ucsf.edu/chimera/
AutoDock Tools	Morris et al,[34] 2009	https://autodock.scripps.edu/
AutoDock Vina	Trott and Olson,[35] 2010	https://vina.scripps.edu/
R	R core team, 2021	https://www.R-project.org/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ying Zhen (zhenying@westlake.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Raw sequencing reads are deposited in NCBI SRA database (BioProject: PRJNA892789). mRNA sequences of ATPα are deposited to NCBI nucleotide database (GeneBank: OP712493-OP712501).
- This paper does not report original codes.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

This study does not include experiments model or subjects.

METHOD DETAILS

Sampling, sequencing and *de novo* transcriptome assembly

Samples of Pacific Ducetia (*Ducetia japonica*) were collected from Hangzhou, Zhejiang province and Tongling Grand Canyon, Guangxi province in China. Live samples were brought back to the laboratory. To confirm *Ducetia japonica* could survive using oleander leaves as the sole food source, nymph and adult samples were reared on fresh oleander leaves, unless specified otherwise. Five additional katydids (Tettigoniidae), *i.e., Sinochlora szechwanensis, Hexacentrus unicolor, Deflorita deflorita, Mecopoda nipponensis* and *Phyllomimus sp.*, were collected from MT. Tianmu, Zhejiang province (Table S3). Samples were identified to species using morphological characteristics and COI barcoding sequences.

For each species, head, gut and thorax tissues were dissected from a fresh sample in phosphate-buffered saline solution. RNA extractions were proceeded immediately using Eastep Super Total RNA Extraction Kit following the manufacturer's protocol (Promega, Shanghai, China). RNAseq library for each tissue was prepared with NEB Next Ultra RNA Library Prep Kit (Illumina) and sequenced with paired-end 150 bp reads on an Illumina Novaseq 6000 at Novogene (Hangzhou, China).

The overall quality of raw reads was checked using FastQC.[26] Reads were trimmed for adapters and by quality using Trimmomatic-0.38 using default parameters.[27] Transcriptome for each tissue was *de novo* assembled with Trinity-v2.8.4 using default parameters.[28] ATPα1 orthologs were identified from the assembled transcriptomes using tblastn[29] and ATPα1 protein sequence from *Drosophila melanogaster* (AF044974.1) as query. Raw reads were mapped back to the identified orthologs and manually examined to confirm correct assembly.

RNAseq data of one additional katydid (Tettigoniidae) species *Tettigonia chinensis* and one outgroup Gryllidea species *Gryllus bimaculatus* were downloaded from NCBI (Table S4), and transcriptome of each species was *de novo* assembled and ATPα1 orthologs were identified using the same methods as described above.

Confirmation of ATPα1 duplicates

The duplicated copies of ATPα1 in Pacific Ducetia (*Ducetia japonica*) were verified by cloning and sequencing. Total RNA from gut tissue of a *Ducetia japonica* sample was extracted as described above, and reverse transcribed to cDNA using Ea-step RT Master Mix Kit (Promega, Shanghai, China). Based on the *de novo* assembled sequences of ATPα1A and ATPα1B, we designed three pairs of overlapping and copy-specific PCR primers to amplify the full length of each ATPα1 copy (Primer3web-version 4.1.0) (Table S5). PCR reactions were set up using Takara PrimeSTAR DNA Polymerase (Takara, Dalian, Liaoning Province, China). The PCR products were examined on 1% agarose gel and then purified using AxyPrep DNA Gel Extraction Kit (Corning, Wujiang, Jiangsu Province, China). Purified PCR products were cloned into TA vector (pMD19-T) following the manufacturer's instructions (Takara, Dalian, Liaoning Province, China). The recombinant plasmids were transformed into *E. coli* Top10 competent cell (KangTi Life Technology, Shenzhen, China). Three single colonies were picked per transfection and plasmids from cultured bacteria were extracted using Plasmid miniprep kit (EASY-DO, Zhejiang, China). The purified plasmids were sequenced with corresponding amplification primers from both ends by 3730xl DNA analyzer (Sunya Biology).

Tissue specific expression of ATPα1 copies using qPCR

We dissected head, thorax and gut tissues from three additional Pacific Ducetia (*D.ucetia japonica*) individuals and extracted total RNA from each tissue. 1ug total RNA per sample was reverse transcribed to cDNA as above. Copy specific qPCR primers were designed using Primer3web-version 4.1.0 (Table S5). Amplification efficiency and specificity of each primer pair was confirmed using plasmids carrying ATPα1A and ATPα1B sequences as templates. qPCR was performed using Go Taq qPCR Master Mix (Promega, Shanghai, China) on a CFX Maestro (Bio-Rad). For each qPCR, three technical replicates were performed, and the mean Ct values were used for expression analysis. Relative expression of ATPα1B and ATPα1A was compared across three different tissues.

Na^+/K^+-ATPase enzyme assay

Enzyme inhibition assay was performed on three types of tissues from both Pacific Ducetia (*Ducetia japonica*) and *Mecopoda nipponensis*. Samples were prepared and the activities of Na^+/K^+-ATPase were measured following the methods described in[4,36] with modifications. For both species, samples were collected from the field and kept alive in the lab until dissection. Gut, head and thorax muscle tissues were dissected from 1 to 2 alive insects and processes immediately. Dissected tissues were washed with PBS and placed into a 1.5 mL EP tube on ice, and 300-400 μL of pre-cooled deionized water was added. Samples were homogenized with a pestle, and centrifuged at 5,000×g at 4°C for 8 min. The supernatant was taken and split into technical replicates. Samples were incubated in a series of six different concentrations of ouabain solutions from 0.01 μM to 1 mM (with 100 mM NaCl, 20 mM KCl, 4 mM MgCl2, 50 mM imidazol) at 37°C for 10 min. Then 2.5 mM ATP was added at 37°C for 20 min. A non-inhibited positive control for full activity was without addition of ouabain, and the negative control for background activity was without KCl. Absorbance was measured after inorganic phosphate staining at 700 nm on an Imaging Multi-Mode Microplate Reader (Agilent BioTek, USA). Each biological replicate was averaged over three technical replicates, and three biological replicates were assayed per sample per treatment concentration.

Relative activities of Na^+/K^+-ATPase (abs[full activity]-abs[inhibited activity])/(abs[full activity]-abs[background activity])) were plotted with $\log_{10}$(ouabain concentration). Curve fitting was performed with the nls function in R using the function: $Y = \min + (\max - \min)/(1 + 10^{((\log10[IC50] - X) \times hs)})$ where Y is the percentage of non-inhibited control; IC50 is the concentration of ouabain at which 50% of sodium pump activity is inhibited; X is the concentration of ouabain; and hs is a coefficient; min and max were set to be 0 and 100 (100%) for most of the samples, because the relative activities were seen to go from 100 to 0. The min was set to 53 (53%) for Pacific Ducetia (*Ducetia japonica*) gut since that was the lowest relative activity it got to at the highest ouabain concentration (1 mM).

Molecular evolution of ATPα1 duplicates

ATPα1 coding sequences were aligned using MUSCLE implemented in Geneious 2021.2.2 (http://www.geneious.com).[30] We used branch-model in codeml of PAML[21] to calculate d_N/d_S in each branch and to examine molecular evolution of ATPα1 copies in Orthoptera with free-ratio model M1. In the two-ratio model M2, ATPα1B of three species in the leaf katydids (Phaneropterinae) and the two species in spear-headed grasshoppers (Pyrgomorphidae) was set as foreground branches. The one ratio model M0 was set as null hypothesis. Model comparison was performed between M2 and M0. The p values were estimated assuming a null-distribution that is a 50:50 mixture of a $\chi 2$ distribution and a point mass at zero.

Molecular docking simulations

We used molecular docking simulations to examine the effects of amino acid substitutions on binding affinity between ATPα1 and ouabain (cardenolide). We downloaded the co-crystal structure of pig ATP1A1 (*Sus scrofa* ortholog of ATPα1) bound to ouabain in the E2P form (PDB Accession Number 3N23; [19]) and used PyMOL-2.3.4_0 [31] to separate the ligand ouabain and ATP1A1. Then we added individual candidate amino substitution observed at sites implicated in cardenolides binding to ATP1A1. We also performed protein homology modeling of native Pacific Ducetia (*D. japonica*) ATPα1B using MODELLER.[32] ATP1A1 carrying one specific substitution, as well as native *D. japonica* ATPα1B, were used as receptors in docking simulation with ouabain. Specifically, we added polar hydrogen, computed Gasteiger charges and converted to pdbqt files using UCSF Chimera[33] and AutoDock Tools (ADT)[35] implemented in MGLTools 1.5.6 (https://ccsb.scripps.edu/mgltools/1-5-6/). The co-crystal structures of ouabain and mutated and

native ATP1A1 were created with a grid box of 20 × 20 × 30 Å for the docking searching space and an exhaustiveness of 10 in AutoDock Vina.[35] The results were visualized in PyMOL-2.3.4_0.

High-performance liquid chromatography (HPLC)

Wild collected Pacific Ducetia (*D. japonica*) adults were divided into two groups, feeding solely on soybean leaves or oleander leaves, respectively. After two weeks, *D. japonica* frass and body (without gut) in both groups were collected. These collected samples, as well as the same batches of leave samples they were feed on, were frozen at −80°C and freeze-dried at −40°C (ALPHA 2-4 LD PLUS Freeze-dryer) for HPLC. Each sample was ground to fine powder. To extract cardenolides, 1.6 mL of MeOH were added to 50 mg aliquots of each of the powdered samples. Then samples were agitated twice for 45 s at a speed of 6.5 m/s on a FastPrep-24 homogenizer and centrifuged at 17,000 g for 15 min 1 mL of supernatant and 20 μg digitoxin were added to each tube as internal standard. After drying with LNG-T100 vacuum concentrator, 200 μL of MeOH were added to each sample. The re-suspended samples were analyzed using HPLC (Waters/Acquity UPLC H-class plus BIO/QDa) based on the method established in.[37] Cardenolides were eluted with a constant flow rate of 0.7 mL/min of 0.25% phosphoric acid in water and acetonitrile gradient as follows: 0–2 min 16% acetonitrile, 2-12 min 70% acetonitrile, 12-13 min 70% acetonitrile, 13-14 min 95% acetonitrile, 14–16 min 95% acetonitrile.

Absorbance spectra at 218 nm (internal standard digitoxin absorbance spectra) were recorded. We considered peaks with symmetrical absorption maxima between 216 and 222 nm to be cardenolides following previous practices.[38]

ADDITIONAL RESOURCES

This study does not include additional resources.