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

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Highlights
First case of cardenolide resistance in an omnivorous katydid
Molecular convergence in ATPa with cardenolide-resistant specialist herbivores
A gut-specific resistant copy of ATPa in leaf katydids (Phaneropterinae)
Evolution of resistance before currently known cardenolide-producing plants
Gut-specific cardenolide-resistant sodium pump primed an omnivore to feed on toxic oleander

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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 ATPα that confers resistance to cardenolides.5 Several studies identified molecular changes in ATPα 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 ATPα 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 ATPα protein, repeated duplication of ATPα gene, and repeated tissue-specific expression pattern of duplicated ATPα copies.6–10 Negative pleiotropy and epistasis are found to constrain the adaptive evolution of cardenolides insensitivity in these specialized herbivores.5,9–11

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.
and essential animal pump; thus, almost all previously reported herbivores that could feed on cardenolides-rich leaves as sole food sources and grow into adults without apparent adverse effects. ATP1A and ouabain. (bottom) Best docking position of ouabain onto native ATP1A1. The substituted amino acid is illustrated in gold.

Interestingly, we found D. japonica has ATP1A duplication that results in one conserved cardenolides-sensitive ATP1A1 and one gut-specific cardenolides-resistant ATP1B that also evolved at a faster rate. By examining transcriptomes of sister species, we found that the duplication of ATP1A 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. Nymph of D. japonica has recently been observed to hunt fireflies at the Shanghai Botanical Garden (Video S1). Some firefly species occasionally feed on oleander under natural conditions and have 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 ATP1 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
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 ATP$_\alpha$1, i.e., ATP$_\alpha$1A and ATP$_\alpha$1B. Patterns of amino acid substitutions suggest that ATP$_\alpha$1A is a highly conserved cardenolides-sensitive copy and ATP$_\alpha$1B may be cardenolides resistant. Substitutions at 41 sites in ATP$_\alpha$1 have been previously implicated in cardenolides sensitivity. In *D. japonica* ATP$_\alpha$1A, only one substitution E117D was observed among the 41 sites, and this exact substitution is shared with ATP$_\alpha$1B. In ATP$_\alpha$1B, we found 10 amino acid substitutions (Q111E, A112T, T114Q, E115V, E117S, A119K, D120N, I315V, T797V, and K886N) in the 41 sites (Figure 1B). Of these, T797V could increase ouabain resistance 70 times in *in vitro* mutagenesis experiments. A122T and substitutions at position 114 and 120 have been found in cardenolides-resistant toads. V115E has been found in cardenolides-sequestering large and small milkweed bugs. Compared with two previously reported milkweed-specialized grasshoppers that also have two copies of ATP$_\alpha$1 in the sister clade spear-headed grasshoppers (*Pyrgomorphidae*), Pacific Ducetia (*D. japonica*) ATP$_\alpha$1A and ATP$_\alpha$1B have distinct pattern of substitutions, suggesting these two lineages have undergone independent duplication events.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*) ATP$_\alpha$1B with wild-type ATP1A1 docking position. We found T797V most drastically influences the binding between ouabain and ATP1A1 by blocking ouabain from entering the binding pocket, resulting in weaker affinity and larger root-mean-square deviation (RMSD) (Figure 1E and Table 1). Q111E may also affect the binding affinity of ouabain and ATP1A1. Other eight substitutions each has relatively minor effect on ouabain binding based on affinity and RMSD (Figure 1E and Table 1). We further compared the ouabain docking positions onto native Pacific Ducetia (*D. japonica*) ATP$_\alpha$1A and ATP$_\alpha$1B by homology modeling (Figure 1D). We found the RMSD between the best docking position onto ATP$_\alpha$1B and pig ATP1A1 is 5.215 Å, greater than resolution of reference structure 3N23A, suggesting ATP$_\alpha$1B is cardenolides resistant (Figure 1D and Table 1).

### Table 1. Molecular docking simulations of ouabain onto pig ATP1A1 carrying specific amino acid substitution from *Ducetia japonica* ATP$_\alpha$1B, and onto native *Ducetia japonica* ATP$_\alpha$1A and ATP$_\alpha$1B

| Substitution | RMSD from co-crystal in 3N23 (Å) | RMSD from best 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     |
| ATP$_\alpha$1B | 5.215                        | 4.928                                 | −8.2     |
| ATP$_\alpha$1A | 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 coordinates of ouabain in the reference co-crystal structure. Large effects with RMSD exceeds 4 Å (the resolution of 3N23A) are in bold.

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.

*Gut-exclusive expression of ATP$_\alpha$1B in *D. japonica*

ATP$_\alpha$1A transcripts were found in transcriptomes of all three tissue types, including gut, head, and thorax muscle. However, ATP$_\alpha$1B was only assembled from gut transcriptome, but not from the head or thorax
transcriptomes. We further examined whether there is a similar tissue-specific expression pattern of the two copies of ATP\(a1\) in Pacific Ducetia (D. japonica) using qPCR and copy-specific primers. We confirmed that expression of the cardenolides-resistant copy ATP\(a1B\) is approximately 3.61-fold higher than that of the cardenolides-sensitive ATP\(a1A\) in the gut, while ATP\(a1B\) expression is extremely low in the head and thorax (Figure 1C).

Previous studies have reported six milkweed specialists with multiple copies of ATP\(a1\) \(^4\). 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 ATP\(a1B\) in the gut compared to the head and thorax.

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

To confirm that ATP\(a1B\) 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 ATP\(a1B\) specifically expresses in the gut of D. japonica, which enables it to tolerate cardenolides and feed on cardenolides-containing oleander plants.

**Dating the ATP\(a1\) duplication to the common ancestor of the leaf katydids (Phaneropterinae)**

To determine when the ATP\(a1\) 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...
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 ATP\_A 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 ATP\_A de novo assembled from transcriptomes of all three tissues (Figure 1B). Using similar pipeline, we also only found one single copy of ATP\_A 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 ATP\_A in Gryllus firmus. As summary, we found two copies of ATP\_A 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 ATP\_A, DNA sequence similarity between paralogous ATP\_A and ATP\_B of same species is 77.4%–81.1%, while pairwise DNA sequence similarity is 88.5%–93.5% between ATP\_A orthologs and 86.7%–92.1% between ATP\_B orthologs (Table S1). Higher sequence similarity between ATP\_A orthologs than between ATP\_B orthologs suggests that the ATP\_A duplication event probably happened in the common ancestor of the leaf katydids (Phaneropterinae).

ATP\_B evolved at a faster rate in Phaneropterinae

ATP\_B in Pacific Ducetia (D. japonica), Deflorita deflorita, and S. szechwanensis has accumulated more amino acid substitutions than ATP\_A (Figure 1). \( d_{ij}/d_{ij} \) of ATP\_B 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 ATP\_A, which is similar as single copy ATP\_A in other lineages (Figure 3). Using two-ratio model in Phylogenetic Analysis by Maximum Likelihood (PAML), \( d_{ij}/d_{ij} \) is estimated to be 0.150 for ATP\_B in the leaf katydids (Phaneropterinae) and 0.144 for ATP\_B in Poekilocerus pictus and Phymateus leprosus. More than 7-fold higher than the background \( d_{ij}/d_{ij} \) of 0.019 across the rest of the ATP\_A phylogeny. The two-ratio model fits significantly better to observed data than one-ratio model (p value < 0.001), suggesting ATP\_B 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 ATP\_A 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.
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 probability density (HPD): 58–60.81 mya).\(^2\) 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 ATP\(\alpha\)1 duplication in two species of aposematic milkweed-specialized grasshoppers in spear-headed grasshoppers (Pyrgomorphidae) were estimated to be \(\sim\)36 mya,\(^9\) after the emergence of cardenolides-producing plants, consistent with driving role of host plant adaptation in the evolution of ATP\(\alpha\)1 in these two specialist grasshopper species.

Intriguingly, we found that the ATP\(\alpha\)1 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 ATP\(\alpha\)1 and the evolution of the resistant ATP\(\alpha\)1B 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 ATP\(\alpha\)1B 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 ATP\(\alpha\)1 same as previously reported milkweed-specialists, including ATP\(\alpha\)1 duplication that gave rise to a cardenolides-sensitive ATP\(\alpha\)1A and a cardenolides-insensitive ATP\(\alpha\)1B, as well as tissue-specific expression of ATP\(\alpha\)1B in the gut where large amounts of cardenolides from foods are processed. Among ten candidate amino acid substitutions in ATP\(\alpha\)1B that might influence the target site sensitivity of ATP\(\alpha\)1B, we highlight T797V and Q111E that may contribute the most to cardenolides resistance through docking simulations. Interestingly, we found duplication of ATP\(\alpha\)1 at the common ancestor of the leaf katydids (Phaneropterinae), implying that duplication of ATP\(\alpha\)1 and evolution of the cardenolides-resistant ATP\(\alpha\)1B 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:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
Materials availability
Data and code availability

EXPERIMENTAL MODEL AND SUBJECT DETAILS

METHOD DETAILS
- Sampling, sequencing and de novo transcriptome assembly
- Confirmation of ATPα1 duplicates
- Tissue specific expression of ATPα1 copies using qPCR
- Na ’K’-ATPase enzyme assay
- Molecular evolution of ATPα1 duplicates
- Molecular docking simulations
- High-performance liquid chromatography (HPLC)

ADDITIONAL RESOURCES

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        |
| Sinociliana 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** |        |            |
| ATP1A primers | See Table S4 | N/A        |
| ATP1B primers | See Table S4 | N/A        |
| ATP1A qPCR primers | See Table S4 | N/A        |
| ATP1B qPCR primers | See Table S4 | N/A        |
| **Deposited data** |        |            |
| Raw sequencing reads | This paper | BioProject: PRJNA892789 |
| ATPa 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.usadelab.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 | Kease et al., 30 2012 | http://www.geneious.com |
| PAML | Yang, 31 2007 | http://abacus.gene.ucl.ac.uk/software/paml.html |
| PyMOL-2.3.4_0 | Delano, 32 2002 | http://www.pymol.org |
| MODELLER | Fiser and Sali, 33 2003 | https://salilab.org/modeller/ |
| UCSF Chimera | Pettersen et al., 34 2004 | https://www.cgl.ucsf.edu/chimera/ |
| AutoDock Tools | Morris et al., 35 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 ATPα orthologs were identified from the assembled transcriptomes using tblastn 29 and ATPα 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 ATPα orthologs were identified using the same methods as described above.

Confirmation of ATPα duplicates
The duplicated copies of ATPα 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 ATPαA and ATPαB, we designed three pairs of overlapping and copy-specific PCR primers to amplify the full length of each ATPα 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 to perform transfection and plasmids from cultured bacteria were extracted using Plasmid miniprep kit (EASY-DQ, 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. 1 ug 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 in5,36 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 log10(ouabain concentration). Curve fitting was performed with the nls function in R using the function: Y = min + (max – min)/(1 + 10((log10[IC50] – X) × 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 PAML21 to calculate dS/dd to 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 (Pygromorphaeae) 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 χ² 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 ATP1A1) 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 Chimera33 and AutoDock Tools (ADT)35 implemented in MGLTools 1.5.6 (https://cbsb.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. 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 leaf 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. A 1 mL 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. 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.

**ADDITIONAL RESOURCES**

This study does not include additional resources.