Museum Genomics Confirms that the Lord Howe Island Stick Insect Survived Extinction

Graphical Abstract

Highlights

- Mitogenomic data confirm that the Lord Howe Island stick insect escaped extinction

- *D. australis* has a massive, most likely polyploid, genome over 4 Gb in size

- The recently discovered population should be suitable for reintroduction

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In Brief

The largest insect driven to extinction in recent history was the flightless Lord Howe Island stick insect. In 2001, a small population of similar insects was discovered nearby, and preparations are already underway for reintroduction. Using DNA from museum collections, Mikheyev et al. show that the rediscovered population is indeed the same species.
Museum Genomics Confirms that the Lord Howe Island Stick Insect Survived Extinction

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SUMMARY

The Lord Howe Island stick insect, Dryococelus australis, was once common on the island but was driven to extinction after the arrival of ship rats in the early 20th century [1, 2]. It was thought to be extinct for decades, until a tiny population of similar-looking stick insects was discovered 20 km away, on the islet of Ball’s Pyramid, in 2001 [2]. Individuals from this population are currently being reared in Australia and elsewhere in the world, with the eventual goal of recolonizing Lord Howe Island [3]. Recent surveys of the wild population on Ball’s Pyramid suggest that it is among the world’s rarest species. However, there are significant morphological differences between Ball’s Pyramid and museum specimens, and there has never been a genetic confirmation of the rediscovered population’s species identity. Because Dryococelus is monotypic, there are also no known extant relatives for comparison. Using shotgun genomic data from the Ball’s Pyramid population, we assembled a draft genome and the complete mitochondrial genome. We found that the genome is massive, over 4 Gb in size, and is most likely hexaploid. We re-sequenced mitochondrial genomes from historic museum specimens collected on Lord Howe Island before the extinction event. Sequence divergence between the two populations is less than 1% and is within the range of intraspecific differences between the museum specimens, suggesting that they are conspecific and that D. australis has successfully evaded extinction so far. This work highlights the importance of museum collections for taxonomic validation in the context of ongoing conservation efforts.

RESULTS AND DISCUSSION

Worldwide, only around 60 insect species have been recorded as recently extinct [4], but hundreds have disappeared, and populations of many more are in steep decline [5]. Reintroduction efforts aim to restore species to previously occupied habitat or to reinforce numbers in existing populations, and these have most commonly been applied to vertebrates and plants [6]. In these cases, part of the risk management strategy is to determine whether the introduced populations or species are appropriate for translocation. However, such efforts are frequently hindered by uncertain taxonomy, and novel methods relying on museum specimens can be necessary to establish species identity [7].

Although the discovery of giant stick insects on Ball’s Pyramid made it probable that D. australis has indeed been rediscovered [2], there are several compelling reasons why the species identification needs to be verified genetically. First, in the paper announcing the rediscovery, Priddel et al. [2] acknowledged that Ball’s Pyramid specimens were morphologically distinct from those found on Lord Howe Island (see Figure 1). A later morphometric analysis of captive-reared and museum insects likewise found significant differences, though it remained unclear whether they were due to genetics or environment [8]. Second, surprisingly, the giant “tree lobster” phenotype, characterized by flightlessness and a stocky dorsally flattened body with square-edged thoracic segments, has evolved repeatedly, raising the possibility of morphological convergence [9]. Finally, the Ball’s Pyramid population may have had a common origin with that on Lord Howe but isolated for an extended period of time. All of these scenarios could potentially complicate conservation efforts planned on Lord Howe Island.

Genome Sequencing and Resequencing

Unfortunately, no genetic resources existed for this species, and species verification was technically challenging, given the massive genome size of related stick insects [10] and the absence of closely related genomic reference material [9]. To remedy this situation, we used fresh material from the captive-bred population at the Melbourne Zoo to assemble nuclear and mitochondrial genomes for D. australis. The bioinformatic nuclear genome size estimate was indeed massive, at 4.2 Gb, and the assembly contained 3.4 Gb, with a contig N50 of 17,265 bp. The mitochondrial genome assembly was 16,604 bp long and was not substantially different from other phasmid genomes in length or composition (Figure 2) [11]. We
also found that *D. australis* is most likely hexaploid, though this finding should be confirmed through future cytogenetic work (Figure 3). Polyploidy is not uncommon in stick insects, particularly in parthenogenetic lineages [12], and parthenogenesis may occur in *D. australis* as well [3]. Polyploidy does pose challenges for population genetic analysis, since many methods are specifically developed for diploid data. Furthermore, any investigations of nuclear genetic diversity should rely on methods capable of detecting allelic ratios, which will require high sequencing depths.

To mitigate difficulties posed by high ploidy and large nuclear genome size, we leveraged low-coverage resequencing of museum specimens to recover complete mitochondrial genomes in order to compare between- and within-island genetic diversity between Lord Howe Island and Ball’s Pyramid. Mitochondrial genome coverage was high (at least 50×) for all specimens, allowing for accurate genotype calls and no missing data (Table 1). High coverage also eliminates errors introduced by DNA degradation [13], which can complicate population genetic analyses [14]. None of the four pinned zoo-bred specimens had detectable differences from the mitochondrial genome reference, which is consistent with their being descendants of the original female collected on Ball’s Pyramid. By contrast, both museum specimens from Lord Howe Island had a number of genetic differences from the reference, though within typical range of variation expected within a species (Figure 2). These within- and between-island differences were of the same order, and less than 1% overall, suggesting that the two populations most likely diverged after the origin of this species and not long enough ago for speciation to have taken place.

**Conservation Implications**

Both Lord Howe Island and Ball’s Pyramid were considerably larger during the Last Glacial Maximum, though apparently not in contact with each other [15], and could have harbored allopatric stick insect populations for an extended period of time. Estimating the actual age of separation would require more museum specimens from Lord Howe Island, and possibly more of those from Ball’s Pyramid. In addition, accurate calibration of the mitochondrial clock would also be necessary. Current studies investigating phasmid phylogenies have confidence intervals at the tips that are far too wide to provide informative insights into population-level splits [16]. Alternatively, more sophisticated approaches using methods such as approximate Bayesian computation could be employed, but they would...
Potential Role of Polyploidy for D. australis Evolution and Conservation

Polyploidy is relatively rare in animals [17], though it has been detected in a variety of stick insects, particularly in parthenogenetic lineages (reviewed by Scali [18]). It is difficult to state with any degree of certainty what role, if any, polyploidy may play in D. australis. However, extensive work on plants allows us to propose two effects: a resistance to inbreeding and an explanation for the large body size in this species.

Extensive research in plants has shown that polyploids are overrepresented among invasive species [19, 20], and polyploidy may help invasions by masking recessive mutations [21] or slowing progression toward full homozygosity in inbred populations [22]. Thus, polyploidy may be a reason for the successful survival of D. australis on the marginal habitat of Ball’s Pyramid. It may also slow the onset of inbreeding depression in captive populations, but not eliminate it. Consequently, we recommend that genetic diversity in these captive populations be assessed and actively maintained or enhanced through appropriate breeding strategies, including the collection and integration of new founders from Ball’s Pyramid.

Another common outcome of polyploidy is an increase in body size [23]. Though the mechanism underlying this relationship is unclear and not always manifest, hexaploids are often the largest size class in an autoploid plant series (reviewed by [24]). Thus, polyploidy could be a mechanism underlying the repeated evolution of the giant “tree lobster” form in stick insects [9] and, even more generally, larger body sizes. If this is the case, we make the easily testable prediction that other tree lobster genera should have higher ploidy numbers.

Conclusions

Previous morphological assessment of the stick insects discovered on Ball’s Pyramid suggested that they were a relic population of D. australis [2]. This current study provides genetic support for this conclusion and may help facilitate efforts toward the recovery of this species. Pending final approvals, a rodent eradication program is planned to commence on Lord Howe Island in mid-2018 [25]. Although ship rat eradication is challenging, and the resident human population on Lord Howe Island will make it even more so, historically >80% of tropical island eradication programs have succeeded [26, 27]. If successful, this will provide the first opportunity for the reintroduction of this species to its former range. However, had our findings demonstrated that the Ball’s Pyramid population was a distinct species, the release of these insects on Lord Howe Island would be regarded as an introduction (the release of a species outside its indigenous range [28]). Introductions can be justified on the basis that a population or species faces a high risk of extinction in its current range or that it would most likely restore an important ecological function that has been lost by the extinction of another species. Nevertheless, introductions generally require a greater level of scrutiny and sometimes face legislative barriers because they can result in negative ecological and/or economic impacts that may often be difficult to foresee [28]. Consequently, the greater certainty provided by this study that the insects from Ball’s Pyramid are indeed surviving representatives of D. australis can only strengthen the argument for reintroduction to Lord Howe Island should the opportunity arise.

Table 1. Summary Statistics from Sequencing and Re-mapping Reads to the Mitochondrial Reference Assembly

| Sample ID Type | Total Yield (Gb) | Read Length ± SD | Mapped Reads | Coverage ± SD |
|----------------|-----------------|-----------------|--------------|--------------|
| museum 15_000002 | 6.3 ± 0.38 | 6,842,938 | 412 ± 0.301 |
| museum 15_000003 | 13 ± 0.42 | 2,801,149 | 167 ± 0.117 |
| zoo 15_000004 | 12.5 ± 0.44 | 9,073,150 | 548 ± 0.421 |
| zoo 15_000005 | 10.8 ± 0.40 | 3,216,964 | 194 ± 0.143 |
| zoo 15_000006 | 1.5 ± 0.40 | 935,265 | 56 ± 0.28 |
| zoo 15_000007 | 9.7 ± 0.43 | 3,711,997 | 223 ± 0.94 |

Museum samples were collected on Lord Howe Island, and zoo samples were related to the individual used to make the reference sequence, having descended from the pair captured for captive breeding [3]. Although the total yield was high, mapping rates to the genome reference were relatively low, resulting in low overall coverage. By contrast, mitochondrial coverage was high enough to overcome stochastic errors introduced by DNA degradation due to age [13] and to produce reliable genotypes.
STAR METHODS

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

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  - Sequencing and reference nuclear and mitochondrial genome assemblies
  - Re-mapping museum specimens
  - Assessment of "index hopping"
  - Ploidy estimation
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

AUTHOR CONTRIBUTIONS

Conceptualization, A.S.M., D.Y., and A.Z.; Investigation, A.S.M., M.L.G., and L.Q.; Resources, M.J.L.M.; Data Curation, M.L.G.; Writing – Original Draft, A.S.M., M.J.L.M., and D.Y.; Visualization, Y.N.S. and A.S.M.; Funding Acquisition, A.S.M. and D.Y.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological Samples** | | |
| *D. australis* specimens | This study | ANIC Database # 15-000002 to 15-000007 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| SiMAG/FK-Silanol (100 mg/mL, Ø 1.0 μm) | Chemicell | Cat#1101 |
| Dynabeads MyOneTM Carboxylic Acid | ThermoFisher Scientific | Cat#65012 |
| Buffer PE | QIAGEN | Cat#19065 |
| Buffer EB | QIAGEN | Cat#19086 |
| UltraPure guanidine isothiocyanate | Invitrogen | Cat#15535016; CAS: 593-84-0 |
| Trizma hydrochloride | Sigma-Aldrich | Cat#T5941; CAS: 1185-53-1 |
| Ethylenediaminetetraacetic Acid | Nacalai Tesque | Cat#15105-35; CAS: 60-00-4 |
| N-Lauroylsarcosine sodium salt | Sigma-Aldrich | Cat#L9150; CAS: 137-16-6 |
| 2-mercaptoethanol | Sigma-Aldrich | Cat#M6250; CAS: 60-24-2 |
| Ethanol | Nacalai Tesque | Cat#14712-05; CAS: 64-17-5 |
| Agarose -LE, Classic Type | Nacalai Tesque | Cat#01157-95; CAS: 9012-36-6 |
| Polyethylene Glyco #6000 | Nacalai Tesque | Cat#28254-85; CAS: 25322-68-3 |
| 5mol/l-Sodium Chloride Solution | Nacalai Tesque | Cat#31334-51; CAS: 7647-14-5 |
| FastAP Alkaline phosphatase | ThermoFisher Scientific | Cat#EF0651 |
| NEBuffer 4 | New England Biolabs | Cat#B7004S |
| 2.5mM CoCl2 | New England Biolabs | Cat#B0252S |
| Terminal Transferase | New England Biolabs | Cat#M0315L |
| 100 mM GTP | Takara | Cat#4042 |
| klenow fragment (3’→5’ exo-) | New England Biolabs | Cat#M0212M |
| 10 mM dNTP mix | Promega | Cat#U151A |
| BSA | New England Biolabs | Cat#B9000S |
| T4 DNA polymerase | New England Biolabs | Cat#M0203S |
| Adenosine 5’-Triphosphate (ATP) | New England Biolabs | Cat#P0756S |
| Calf Intestinal Alkaline Phosphatase | ThermoFisher Scientific | Cat# 18009-019 |
| 2x Quick Ligase Reaction Buffer | New England Biolabs | Cat#B2200S |
| T4 DNA ligase | New England Biolabs | Cat#M0202L, M0202M |
| 5x Phusion HF Buffer | ThermoFisher Scientific | Cat#F-518 |
| Phusion DNA polymerase | ThermoFisher Scientific | Cat#F-530L |
| 2-Log DNA Ladder | New England Biolabs | Cat#N3200L |
| EZ-Vision one | AMRESCO | Cat#N472-KIT |
| **Critical Commercial Assays** | | |
| Truseq DNA LT sample Prep KIT | Illumina | Cat#FC-121-2002 |
| MinElute Reaction Cleanup Kit | QIAGEN | Cat#28206 |
| Quant-IT PicoGreen dsDNA Assay Kit | ThermoFisher Scientific | Cat#P7589 |
| High Sensitivity DNA Kit | Agilent | Cat#5067-4626 |
| KAPA SYBR FAST Universal qPCR kit | KAPA Biosystems | Cat#KK4601 |
| Illumina DNA Standards and Primer Premix Kit | KAPA Biosystems | Cat#KK4808 |
| Sequence assembly and raw data | DDBJ/ENA/GenBank | GenBank: PRJNA387351 |
| Oligonucleotides | | |
| CCCCC_TS: GTGACTGGAGTTCAAGCTGCTCCTCCC | This study | N/A |
| GATCCCCC | | |
| Truseq PCR1: AATGATACGGCGACAGATCTACA | This study | N/A |

(Continued on next page)
### CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests may be directed to and will be fulfilled by the Lead Contact, Alexander S. Mikheyev (sasha@homologo.us).

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| I7_D701 primer_long: 5'-CAAGCAGAAGACGGCATACGAGATGCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT | This study | N/A |
| I7_D702 primer_long: 5'-CAAGCAGAAGACGGCATACGAGATGCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT | This study | N/A |
| I7_D705 primer_long: 5'-CAAGCAGAAGACGGCATACGAGATGCTCAGAAGTGCTGGAGTTCAGACGTGTGCTCTTCCGAT | This study | N/A |
| I7 truseq upper: 5'-p-ATCGGAAGAGCACACGTCTGAACTCCAGT*/ddC/ (* = phosphorothioate linkage) | [29] | N/A |
| I7-D701 FL truseq lower: 5'-CAAGCAGAAGACGGCATACGAGATGCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*C*CCC (* = phosphorothioate linkage) | [29] | N/A |
| I7-D703 FL truseq lower: 5'-CAAGCAGAAGACGGCATACGAGATGCTCAGAAGTGCTGGAGTTCAGACGTGTGCTCTTCCGAT*C*C*CCC (* = phosphorothioate linkage) | [29] | N/A |
| I5 truseq upper: CCCTACACGACGCTCTTCCGATCT/ddC/ | [29] | N/A |
| I5-D501 FL truseq lower: 5'-p-GAGATCGGAAGAGCGTCGTGTAAGGGAAAGAGTGAGCTAGATCTCGGTGGTCCGGCGATATCATT | [29] | N/A |
| I5-D502 FL truseq lower: 5'-p-GAGATCGGAAGAGCGTCGTGTAAGGGAAAGAGTGAGCTAGATCTCGGTGGTCCGGCGATATCATT | [29] | N/A |
| I5-D503 FL truseq lower: 5'-p-GAGATCGGAAGAGCGTCGTGTAAGGGAAAGAGTGAGCTAGATCTCGGTGGTCCGGCGATATCATT | [29] | N/A |
| I5-D504 FL truseq lower: 5'-p-GAGATCGGAAGAGCGTCGTGTAAGGGAAAGAGTGAGCTAGATCTCGGTGGTCCGGCGATATCATT | [29] | N/A |
| I5-D506 FL truseq lower: 5'-p-GAGATCGGAAGAGCGTCGTGTAAGGGAAAGAGTGAGCTAGATCTCGGTGGTCCGGCGATATCATT | [29] | N/A |

### Software and Algorithms

| Software | URL | DOI |
|----------|-----|-----|
| Bowtie   | http://bowtie-bio.sourceforge.net/index.shtml | [30] |
| FreeBayes| https://github.com/ekg/freebayes | [31] |
| Geneious (R8.1) | https://www.geneious.com/ | [32] |
| MITObim  | https://github.com/chrishah/MITObim | [33] |
| Newbler  | http://www.roche.com/ | [34] |
| NextGenMap| http://cibiv.github.io/NextGenMap/ | [35] |
| ParDRe   | https://sourceforge.net/projects/pardre/ | [36] |
| PEAR     | https://sco.h-its.org/exelixis/software/pear/ | [37] |
| ploidyNGS | https://github.com/diriano/ploidyNGS | [38] |
| Samtools | http://samtools.sourceforge.net/ | [39] |
| VCFTools | http://vcftools.sourceforge.net/ | [40] |
EXPERIMENTAL MODEL AND SUBJECT DETAILS

A male (ANIC Database # 15-000002) and a female specimen (# 15-000003) of *D. australis* in the Australian National Insect Collection (ANIC) were sampled. These two specimens originated from Lord Howe Island proper and are part of the W. W. Froggatt collection; the collecting dates are unknown. In addition to historical museum specimens, we included four more recent control individuals from the Ball’s Pyramid, which were captive-reared specimens from the Melbourne Zoo deposited in the ANIC (# 15-000004 to 15-000007).

METHOD DETAILS

**Library preparation**

A library from an ethanol-preserved zoo-bred specimen from Ball’s Pyramid was constructed using a Truseq DNA LT Sample Prep Kit. The library was size-selected so that many of the forward and reverse reads would overlap to facilitate assembly.

Most libraries for the museum specimens were prepared using a PCR-free approach as described previously, while libraries for samples 15-000002 and 15-000004 were amplified with a limited number of PCR cycles (less than eight cycles) [29, 41]. Libraries were purified with 17% PEG-6000 [42], analyzed using a Bioanalyzer High Sensitivity Kit and then pooled together in equal amounts.

**Sequencing and reference nuclear and mitochondrial genome assemblies**

Reads for the genomic reference made from the ethanol-preserved Ball’s Pyramid specimen were sequenced on an Illumina HiSeq2500 in PE250 mode, producing 97 million read pairs. PCR duplicates were removed using ParDRe [36], and were then stitched together using PEAR with parameters–min-overlap 10–memory 48G–threads 10 -n 200 -m 600 -p 0.0001 [37]. This resulted in 18.9 Gb of data with an average read length of 428 ± 51 (SD). The resulting super-reads were assembled using Newbler with parameters-large -m -cpu 10 -mi 95 -siom 450 -l 1000 -a 500 -urt -novs -a 1000 -minlen 45 -het [34]. The genome size estimate was reported by Newbler. Mitochondrial DNA was assembled separately using MITObim [33] after aligning reads to the *Extatosoma tiaratum* mitochondrial genome (GenBank: NC_017748.1) [11] using NextGenMap [35]. MITObim had difficulties assembling the repetitive mitochondrial control region, resulting in a gap that was filled in manually by identifying super-reads that mapped to both sides of the gap. The *D. australis* mitochondrial assembly was annotated using the *E. tiaratum* sequence as a template in Geneious (r8.1), which was also used for interactive exploration and visualization of the data [32].

**Re-mapping museum specimens**

Libraries were sequenced using a HiSeq 2000 in SE50 mode. Reads were trimmed to remove 3’ adaptor sequences and mapped to the *D. australis* mitochondrial genome assembly using bowtie [30]. Variants were then called using freebayes in diploid mode [31]. Diploid mode was used because a small number of sites in the alignment had apparent mixtures of genotypes, possibly from nuclear copies or repetitive regions, making them difficult to resolve using short-read data from museum specimens. Therefore, 30 sites with heterozygous genotype calls were filtered from the variant call file, along with sites having quality scores less than 40. The final analysis retained 161 total variant sites, all of them single nucleotide polymorphisms. Because divergence from the reference sequence can cause poor mapping and variants to be missed, the mitochondrial read alignments were visually inspected for uniform coverage and other abnormalities.

**Assessment of “index hopping”**

Each sample was barcoded for sequencing using a unique combination Illumina adaptors, and sequenced in the same lanes. We quantified the extent of molecular recombination between the indexes by dividing the total number of invalid index combinations by the total number of valid index combinations (omitting indexes with any ambiguous calls). There was a low rate of index hopping (0.361%). It was a substantially smaller value than the average percentage of mapped reads assigned to mitochondrial DNA (2.1% ± 1.4% (SD)), and should not have influenced the result.

**Ploidy estimation**

Analysis of several candidate microsatellite loci developed from the reference genome suggested the presence of more than two alleles (L.Q. and A.S.M., unpublished data). However, since genetic variability in the captive population is limited, and microsatellites can give additional peaks as a result of PCR artifacts, these data were inconclusive, and we decided to further investigate ploidy of *D. australis* across multiple loci. We used ploidyNGS, a model-free approach for estimating ploidy [38], to calculate the frequencies of alleles in the ten longest scaffolds (2,617,109 bp) of the nuclear genome assembly, using super-reads remapped to the reference. This approach counts the relative frequencies of encountered alleles, which occur in predictable ratios that correspond to organismal ploidy. For example, diploid individuals will have biallelic sites predominantly at frequencies of 0, ½ and 1, whereas triploids will have 0, $\frac{1}{3}$, $\frac{2}{3}$ and 1, etc.
QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were conducted in R (version 3.4) [https://www.R-project.org/], using built-in functions (mean and sd) to compute coverage means and standard deviations, respectively. Statistical differences in coverage were tested using the t.test function (Figure 3).

DATA AND SOFTWARE AVAILABILITY

The accession number for the sequences reported in this paper is GenBank: PRJNA387351.