Can Long-Range PCR Be Used to Amplify Genetically Divergent Mitochondrial Genomes for Comparative Phylogenetics? A Case Study within Spiders (Arthropoda: Araneae)

Andrew G. Briscoe
*Bangor University*

Sara Goodacre
*University of Nottingham*

Susan E. Masta
*Portland State University*

Martin I. Taylor
*Bangor University*

Miquel A. Arnedo
*Universitat de Barcelona*

Follow this and additional works at: [https://pdxscholar.library.pdx.edu/bio_fac](https://pdxscholar.library.pdx.edu/bio_fac)

Let us know how access to this document benefits you.

**Citation Details**
Briscoe AG, Goodacre S, Masta SE, Taylor MI, Arnedo MA, et al. (2013) Can Long-Range PCR Be Used to Amplify Genetically Divergent Mitochondrial Genomes for Comparative Phylogenetics? A Case Study within Spiders (Arthropoda: Araneae). PLoS ONE 8(5): e62404.

This Article is brought to you for free and open access. It has been accepted for inclusion in Biology Faculty Publications and Presentations by an authorized administrator of PDXScholar. Please contact us if we can make this document more accessible: pdxscholar@pdx.edu.
Authors
Andrew G. Briscoe, Sara Goodacre, Susan E. Masta, Martin I. Taylor, Miquel A. Amedo, David Penny, John Kenny, and Simon Creer

This article is available at PDXScholar: https://pdxscholar.library.pdx.edu/bio_fac/1
Can Long-Range PCR Be Used to Amplify Genetically Divergent Mitochondrial Genomes for Comparative Phylogenetics? A Case Study within Spiders (Arthropoda: Araneae)

Andrew G. Briscoe1, Sara Goodacre2, Susan E. Masta3, Martin I. Taylor1, Miquel A. Arnedo4, David Penney5, John Kenny6, Simon Creer1*

1 Environment Centre Wales Building, Molecular Ecology and Fisheries Genetics Laboratory, School of Biological Sciences, College of Natural Sciences, Bangor University, Bangor, United Kingdom, 2 Institute of Genetics, Queens Medical Centre, University of Nottingham, Nottingham, United Kingdom, 3 Department of Biology, Portland State University, Portland, Oregon, United States of America, 4 Departament Biologia Animal, Universitat de Barcelona, Barcelona, Spain, 5 Faculty of Life Sciences, The University of Manchester, Manchester, United Kingdom, 6 Centre for Genomic Research, School of Biological Sciences, University of Liverpool, Liverpool, United Kingdom

Abstract

The development of second generation sequencing technology has resulted in the rapid production of large volumes of sequence data for relatively little cost, thereby substantially increasing the quantity of data available for phylogenetic studies. Despite these technological advances, assembling longer sequences, such as that of entire mitochondrial genomes, has not been straightforward. Existing studies have been limited to using only incomplete or nominally intra-specific datasets resulting in a bottleneck between mitogenome amplification and downstream high-throughput sequencing. Here we assess the effectiveness of a wide range of targeted long-range PCR strategies, encapsulating single and dual fragment primer design approaches to provide full mitogenomic coverage within the Araneae (Spiders). Despite extensive rounds of optimisation, full mitochondrial genome PCR amplifications were stochastic in most taxa, although 454 Roche sequencing confirmed the successful amplification of 10 mitochondrial genomes out of the 33 trialled species. The low success rates of amplification using long-Range PCR highlights the difficulties in consistently obtaining genomic amplifications using currently available DNA polymerases optimised for large genomic amplifications and suggests that there may be opportunities for the use of alternative amplification methods.

Introduction

Mitochondrial DNA markers are the cornerstones of contemporary molecular systematics and contribute greatly to our understanding of organellar evolution [1,2]. However, robust phylogenetic reconstruction is often impeded by low sequence volume and number of comparative loci. Prior to the turn of the century, single gene partitions were commonly used to infer phylogenetic histories, but poor nodal support and discordance between phylogenies derived from separate markers clearly revealed that additional data are needed for robust phylogenetic reconstruction [3].

As more sequence data becomes available for elucidating the tree of life, large-scale sequencing efforts and interrogation of expressed sequence tag (EST) libraries or sequenced transcriptomes [4,5] have begun to yield large numbers of nuclear markers that can be used for phylogenetic reconstruction [6]. However, true phylogenomic analyses are still not practical for the de novo construction of phylogenetic hypotheses for taxa without sequenced transcriptomes [7,8]. A compromise between traditional phylogenetic methods and phylogenomics lies in the phylogenetic analysis of whole mitochondrial genomes [9–11]. However, the routine amplification of complete mitochondrial genomes from divergent taxa remains a significant hurdle to the widespread adoption of mitogenomic approaches.

The number of informative phylogenetic characters within the mitochondrial genome has been appreciated for some time [1] but most studies have limited themselves to only exploring parts of this information to resolve relationships at multiple levels [12]. The increase in whole mitochondrial genome datasets has provided new characters potentially allowing more robust phylogenies to be constructed. These markers, known as rare genomic changes (RGCs) have become increasingly popular for resolving complex phylogenetic relationships where traditional methods have produced ambiguous, unresolved results [12]. RGCs, defined as large-scale mutational changes occur much less frequently than base substitutions and have long been used in phylogenetics as supporting data embedded in DNA sequences. Examples of RGCs include changes in organelle gene order, gene duplications and genetic code variants [13–16].
Whilst the potential benefits of analysing whole mitochondrial genomes (including both coding regions and structural characters) in furthering our understanding of the mechanisms behind the evolution of organellar DNA are clear, all of the data produced to date have been of comparatively low volume. The lack of empirical data means that we still cannot accurately assess the utility of full mitogenomic sequences as a tool for resolving complex phylogenies in many taxa [17]. Advances in sequencing technology (e.g 454 Roche GSFLX series and Solexa/Illumina) are likely to resolve the sequencing throughput issue [18]. However, there are still clear limitations associated with the large-scale PCR amplification of divergent, interspecific, whole mitochondrial genomes. In spite of the recent revolution in sequencing technologies, current mitogenomic studies have been characterised by either analysing data from large, but incomplete mitochondrial genome fragments [10], low numbers of species [19] or by focusing on nominally intra-specific datasets [9]. In order to investigate the resolution potential, and associated problems with the amplification of large, interspecific mitogenomic data sets we focus here on spiders (Arthropoda: Araneae).

The Araneae are among the oldest and most diverse group of terrestrial organisms [20,21], with a current diversity of more than 43,240 described species, placed in 111 families [22]. Spiders are an unequivocally ecologically important guild, being the dominant predators of insects in natural and managed ecosystems [23]. However, they have been relatively understudied from a higher-level molecular systematic perspective, and very little is known about inter-family relationships [24]. More recent attempts to resolve the phylogeny of the Araneae, have revealed significant topological incongruence between morphological and multiple loci phylogenies [25]. This makes the Araneae an ideal order to test the application of using mitochondrial phylogenomics to resolve complex relationships and better understand the evolutionary mechanisms underlying speciation and diversification. Furthermore, recent studies have shown repeated RNA gene translocations [26,27] in combination with an extensive fossil record can be utilised in resolving high-level relationships, via calibrated gene trees in the Araneae [28–31]. Here, we investigate the utility of direct long-range PCR amplification of whole mitochondrial spider genomes, using a large range of currently available long-range Taq polymerases. The PCR approach was chosen as it circumvents the need for (often unavailable) large starting biomass associated with the direct pelleting of mitochondria. Whilst mitochondrial genomes can be readily amplified in large numbers of small fragments, this is both costly and labour intensive. To this end, we adopted both single and dual fragment amplification approaches across the phylogenetic breadth of the order to assess the feasibility of both conserved and directed approaches for expedient whole mitochondrial DNA amplification.

Materials and Methods

Sample Collection and DNA Extraction

Spiders were obtained from across the United Kingdom and Gambia by the authors and members of the British Arachnological Society (BAS) and either ethanol preserved (70%–100%, stored at 4°C) or freshly frozen (stored at −80°C) directly from living individuals (Table 1). Samples of Selenops annulatus, Dendops sp. and Citharion praedonius had been stored in 70% ethanol and were protected and the field studies did not involve protected or endangered species. All four legs were removed from the left side of the thorax prior to DNA extraction. The rest of the body was stored in 100% ethanol for vouchering and subsequent identification purposes. Whole genomic DNA was extracted from a single femur of each species using the Qiagen DNeasy Blood & Tissue Kits (Qiagen).

Primer Anchoring Strategy

In order to identify an efficient approach for the amplification of whole mitochondrial genomes we adopted two long-range PCR strategies to amplify the complete mitogenome in one or two large fragments [32]. DNA from two anchoring regions, a ca. 650 b.p. region of the Cytochrome Oxidase I (COI) and a 450 b.p. region spanning the large ribosomal subunit (16 s rRNA), were amplified for 33 taxa. Both amplifications were performed in 25 μl reactions using the primer combinations of CHELF1 (5'- TACTCTAC- TAACTCATAAAGACATTGG) and CHELR2 (5'- GGAATGCC-GAAAAATTCAAAATAATG) (COI) [33] and primers LR-N-13398 (5'- CGGCCGTGTATAAAACAAATAG) and LR-J-12887 (5'- CCGGTTCTGAACCTAGATCGT) (16 s) [25]. PCR reactions comprised 1× PCR buffer, 1.5 mM MgCl2, 0.4 μM of each primer, 0.625 units ThermoPrime Taq DNA polymerase (Thermo Scientific) and 1 μl template DNA and thermocycling was performed using a DNA engine (Tetrad 2) Peltier Thermal Cycler (BIORAD). Cycling conditions were 60 seconds at 94°C, 5 cycles of 60 seconds at 94°C, 90 seconds at 45°C and 90 seconds at 72°C followed by 35 cycles of 60 seconds at 94°C, 90 seconds at 50°C and 90 seconds at 72°C [33]. Amplification success was checked using a 1% agarose gel stained with Ethidium Bromide (EtBr). Successful amplifications were cleaned with 1 U shrimp alkaline phosphatase (Promega) to dephosphorylate residual deoxyribonucleotides and 0.5 U Exonuclease I (Promega) to degrade excess primers [34] and subsequently sequenced bidirectionally (Macrogen Inc, Seoul, Korea) using the same primers as for amplification.

Long-Range Primer Design and PCR

Following the Sanger sequencing of the COI and 16s anchoring regions, chromatograms were checked and sequences were manually edited where necessary, using CodonCode Aligner (v. 2.0.6, Codon Code Corporation), prior to alignment using Clustal W [35]. The initial aim of this process was to amplify regions of sufficient length, on opposite sides of the mitochondrial genome, from which ‘universal’ primers could be designed [26,36,37]. However, no conserved regions were found that would facilitate the design of long-range primers that could be used to amplify homologous loci from multiple families. Long-range primers were subsequently designed for individual taxa in order to amplify the entire mitochondrial genome in one or two large fragments that overlapped with the conserved COI and/or 16s ribosomal subunit using the Primer 3 software [38]. Default values were used with the exception of length (22–30 bp), primer Tm (57.0–70.0°C) and GC content (40–60%) which followed consensus recommendations from the Taq manufacturers (Primer sequences available on DRYAD entry doi:10.5061/dryad.8ld3n).

For the single fragment protocol, primers were designed within the COI sequences, with the light strand primer situated downstream of the heavy strand primer, thus taking advantage of the circular nature of the genome. For the dual fragment protocols, two sets of primers were designed for each taxon, to bridge the gaps between the COI and 16 s regions (Figure 1).

The PCRs were performed initially in 50 μl volumes using multiple polymerases recommended for long-range PCR amplification, including: Clontech Advantage 2, Clontech Advantage.
Table 1. Taxonomic information, storage conditions and GenBank accession numbers for specimens.

| Family         | Genus            | Specific epithet | Sample ID | Locality       | COI 16 s | Sample storage |
|----------------|------------------|------------------|-----------|----------------|----------|----------------|
| Agelenidae     | Malthonica       | silvestris       | 464_SC_AB | Kent, UK       | JQ412460 | Frozen         |
| Amaraubidiidae | Amaurobius       | similis          | 483_SC_AB | Lancashire, UK | JQ406635 | Frozen         |
| Anyphaenidae   | Anyphaena        | accentuata       | 522_SC_AB | Kent, UK       | JQ412439 | JQ406633 Frozen |
| Araneidae      | Agalenatea       | redii            | 507_SC_AB | Kent, UK       | JQ406637 | Frozen         |
| Araneidae      | Araneus          | diadematus       | 571_SC_AB | Dorset, UK     | JQ412440 | JQ406621 Frozen |
| Cithaeronidae  | Cithaeron        | praedonius       | 455_SC_AB | Kotu, Gambia   | JQ412441 | Ethanol        |
| Clubionidae    | Clubiona         | terrestris       | 518_SC_AB | Kent, UK       | JQ412442 | Frozen         |
| Corinnidae     | Messapus         | martini          | 453_SC_AB | Kerr Serign, Gambia | JQ406616 | Ethanol        |
| Corinnidae     | Phnarlolithus    | festivus         | 503_SC_AB | Kent, UK       | JQ406632 | Ethanol        |
| Ctenidae       | Anaita           | sp.              | 460_SC_AB | Kotu, Gambia   | JQ406614 | Ethanol        |
| Cybaeidae      | Argyroneta       | aquatica         | 581_SC_AB | Dorset, UK     | JQ406617 | Frozen         |
| Deinopidae     | Deinopis         | sp.              | 451_SC_AB | Gunjir, Gambia | JQ412443 | Ethanol        |
| Dictynidae     | Dictyna          | laten            | 499_SC_AB | Kent, UK       | JQ412444 | JQ406629 Ethanol |
| Dyserideridae  | Dysera           | erythrina        | 479_SC_AB | Kent, UK       | JQ412445 | JQ406627 Ethanol |
| Eresidae       | Stegodaphus      | sp.              | 454_SC_AB | Bijilo, Gambia | JQ412459 | Ethanol        |
| Gnaphosidae    | Zelotes           | apricorum        | 462_SC_AB | Kent, UK       | JQ412463 | Frozen         |
| Idiopidae      | Gorgyrella       | sp.              | 448_SC_AB | UK Pet Trade   | JQ412447 | Frozen         |
| Linyphidae     | Unknown          | Unknown          | 559_SC_AB | Gwynedd, UK    | JQ412448 | JQ406636 Frozen |
| Lycosidae      | Pardosa           | nigriceps        | 477_SC_AB | Kent, UK       | JQ412452 | JQ406631 Ethanol |
| Philodromidae  | Philodromus      | dispar           | 517_SC_AB | Kent, UK       | JQ412453 | JQ406634 Frozen |
| Pisauridae     | Pisaura          | mirabilis        | 502_SC_AB | Kent, UK       | JQ412454 | JQ406630 Ethanol |
| Pholidae       | Pholius           | philangioloides  | 484_SC_AB | Lancashire, UK | JQ406625 | Frozen         |
| Salticidae     | Salticus         | scenicus         | 423_SC_AB | Gwynedd, UK    | JQ412456 | JQ406628 Ethanol |
| Segestridae    | Segestria        | senoculata       | 583_SC_AB | Gwynedd, UK    | JQ412457 | JQ406615 Ethanol |
| Selenopidae    | Selenops         | annulatus        | 448_SC_AB | Kotu, Gambia   | JQ412458 | Ethanol        |
| Sparassidae    | Micrommata       | virescens        | 461_SC_AB | Kent, UK       | JQ412451 | JQ406618 Frozen |
| Tetragnathidae | Meta             | menardi          | 481_SC_AB | Gwynedd, UK    | JQ412449 | JQ406620 Frozen |
| Theraphosidae  | Eupalaeustrus     | campestratus     | 446_SC_AB | UK Pet Trade   | JQ412446 | JQ406626 Frozen |
| Theraphosidae  | Grammostola      | rosea            | 445_SC_AB | UK Pet Trade   | JQ412446 | JQ406624 Frozen |
| Theraphosidae  | Psalmopeus       | cambridgei       | 447_SC_AB | UK Pet Trade   | JQ412455 | JW406623 Frozen |
| Theridiosomatidae | Theridiosoma   | gemmosum         | 489_SC_AB | Glamorgan, UK  | JQ412461 | Frozen         |
| Thomisidae     | Xysticus          | audax            | 521_SC_AB | Kent, UK       | JQ412462 | JQ406622 Frozen |
| Uloboridae     | Miagrommopes     | sp.              | 452_SC_AB | Bijilo, Gambia | JQ412450 | JQ406619 Ethanol |

Sample storage indicates the methods in which the specimens were preserved on collection; either freshly frozen at – 80°C (frozen) or stored in 70–100% ethanol at 4°C.

doi:10.1371/journal.pone.0062404.t001

**Amplicon Shearing, Library Construction and 454 Roche Sequencing**

Amplions from eleven purified samples (Meta menardi, Xysticus audax, Psalmopeus cambridgei, a Gorgyrella sp., Eupalaeustrus campestratus, a Linyphidae sp., Zelotes apricorum, Malthonica silvestris, Araneus diadematus, Pisaura mirabilis & Dysdera erythrina) were fragmented using a Covaris DNA shearer (Covaris) at 10% duty cycle, intensity: 4 with 200 cycles per burst for 65 seconds. Following quantification using a Qubit 2.0 fluorometer (Invitrogen), MID barcoded adapted sequencing libraries were constructed using the NEBNext DNA sample prep master mix set in accordance with manufacturer’s instructions (New England Biolabs), pooled in equimolar concentrations and sequenced at low putative coverage on 1/16th of a 454 Roche GSFLX Titanium platform (Centre for Genomic Research, University of Liverpool).
Please note that the sequencing step here was intended as a quality control measure and not intended to sequence to a depth required for full mitochondrial genome assembly (sequence quality data available on DRYAD entry doi:10.5061/dryad.8dd3n). The 454 Roche sequences were assembled using GS De Novo Assembler software (Roche). Following trials with Roche’s GS De Novo software, MIRA (B. Chevreux) and CLC Genomics Workbench (CLC bio, Aarhus, Denmark), no substantial differences in number of contigs or length were present using any of the approaches. Therefore, in accordance with the proven functionality of the 454 Roche software, all sequences were assembled using the GS De Novo program. Following assembly, the resulting contigs where compared to existing Araneae mitochondrial DNA sequences within GenBank via BLAST [40] in order to investigate sequence homology (Genbank accession numbers: AY309258, AY309259, AY452691, NC_005942, NC_010777, NC010780).

Results

Anchoring Regions

Of the 33 species used, 27 and 24 taxa were successfully amplified for the COI and the 16 s regions respectively, yielding anchoring points for a total of 26 families (Table 1) (GenBank accession numbers: COI - JQ412439 - JQ412463 and 16 s - JQ406614 - JQ406637).

Mitochondrial Genome PCR Amplifications

Initially, five specimens, covering a broad taxon range, were used to test the performance of the Taq polymerases for single fragment mitochondrial genome amplification (Table 2). Following repetition of the Taq testing when opting for a dual fragment approach, it was found that NEB LongAmp was equally as effective as Clontech Advantage 2 Taq polymerase. Subsequently, complete mitochondrial genomes were successfully amplified in c. 7–9 kb or c. 15 kb fragments using Clontech Advantage 2 (single
Discussion

We were able to amplify most of the target genes (COI and 16s) across our taxon range, but were unable to identify or develop degenerate primers for any other potential anchoring region within the spider mitochondrial genome following sliding window analyses of all regions most commonly used in spider phylogenetics. Sliding window analysis was performed on all 13 protein coding genes and both ribosomal RNAs (rRNAs) of the six previously published spider mitochondrial genomes using the Drosophila Polymorphism database, SNP Graphics (http://dpdb.uab.es/dpdb/diversity.asp) (sliding window plots available on DRYAD entry doi:10.5061/dryad.8dd3n). This most likely highlights the high level of mitochondrial genetic diversity that can be found within the spiders along with the absence of universal primers available for no more than a handful of protein coding genes, resulting in few reference sequences available for comparison for large portions of the Araneae mitogenome [41].

Predictably, the 454 Roche low coverage sequencing step resulted in a wide variation of read coverage per amplicon pool, most likely due to differences in MID primer tag design and read recovery. Nevertheless, even highly covered mitochondrial genomes did not result in a full assembly, suggesting further optimisation may be required in either the shearing or bioinformatic steps of mitogenome assembly [42]. The BLAST search of the assembled contigs showed that spider mitochondrial genomes had indeed been amplified and that we had not inadvertently amplified nuclear DNA or DNA from a contaminating source had indeed been amplified and that we had not inadvertently amplified nuclear DNA or DNA from a contaminating source such as the Wolbachia bacterium [43]. The need to use searches focused on Araneae accessions, in order to get positive matches, most likely highlights the genetic divergence between the trialled taxa and the complete mitogenomic data currently available for 6 out of the 112 described families of spiders.

Nevertheless, even following an extensive campaign of PCR strategies and optimization, using a variety of long range Taqs, we could not consistently amplify approximately two thirds of the species, a substantial proportion of our sample taxa. We have no reason to attribute PCR failure to degraded DNA resulting from sample storage conditions, since all specimens were preserved directly from living organisms using tried and tested preservation media. Moreover, of the three samples stored in 70% EtOH, although sample Selensops annulus did not amplify, samples Deinopis sp. and Cithaeron praedonius could be amplified, albeit inconsistently, suggesting that 70% EtOH preservation over ca. three years may be sufficient to preserve mitogenome integrity for PCR amplification. However, we could not rule out the possibility that suboptimal storage conditions can adversely affect the availability of

| Sample reference | Clontech | NEB LongAmp | Expand Long Range |
|------------------|----------|-------------|-------------------|
|                  | Advantage2 | AdvantageLA |                   |
| 448_SC_AB        | Stochastic | No amplification | No amplification |
| 462_SC_AB        | Good      | No amplification | Weak              |
| 464_SC_AB        | Good      | No amplification | No amplification  |
| 446_SC_AB        | No amplification* | No amplification* | No amplification* |
| 451_SC_AB        | Stochastic | No amplification | Stochastic        |

Clontech Advantage Titanium and Roche GeneAmp XL are not shown but failed to amplify any samples.

*Indicates good amplification from a dual fragment PCR approach. The term Good indicates robust and consistent amplification. The term Stochastic indicates a non-consistently reproducible amplification.

doi:10.1371/journal.pone.0062404.t002
suitable templates for long-range PCR. It is also unlikely that the primers affected long-range PCR success rate, since comparative analysis between the primer sets revealed no discernable statistical differences in physical or chemical properties (i.e. GC content, length, Tm). The arachnid mitochondrial genome, as for most animal mitochondrial genomes, is a small extra-chromosomal genome comprising 37 genes including 22 coding for transfer RNAs (tRNAs), 13 coding for proteins and 2 coding for ribosomal RNAs (rRNAs) [1]. As well as the 37 coding genes, mitochondrial genomes also contain a small, approximately 1–2 kb, non-coding control region, named due to its perceived role in controlling the transcription and replication of the mtDNA molecule [44]. However, the protein coding gene arrangement of spiders is highly conserved and shared amongst many other Chelicerates and so is not likely to cause differences in amplification success. Previously published Araneae mitochondrial genomes are considered to be very A/T rich (64–76%), reflecting the low G/C content of the DNA, no definitive reason is forthcoming for the failure to obtain more consistent long PCR amplifications from our target mitochondrial genomes. Whilst the larger region (COI-16 s) of the dual fragment approach amplified successfully more often than the shorter 16 s-COI primer sets, it is possible to avoid the amplification of non-specific nature of the amplification process. By using random hexamers, as opposed to synthesising bespoke taxon-specific oligonucleotides, it is possible to isolate only the mtDNA locus are needed to optimise organellar EST libraries is possible [54,55], but expedient mechanisms to isolate only the mtDNA lcoi is another key avenue of exploration in contemporary phylogenetics [51]. Although we present data on a single order (Araneae – Spiders) we believe that the results highlight the limitations to the feasibility of generating diverse, interspecific complete mitochondrial genome data sets from long-range PCR amplifications, in terms of both cost and efficiency. Whilst many studies have successfully used a multitude of PCR amplifications in order to generate mitochondrial genomes [48–50], this is both labour and time intensive. This highlights the potential need to utilise alternative, non-PCR based methods that are able to amplify complete mitochondrial genomes both quickly and cost effectively. Direct recovery of organellar genomes and mitochondrial genome partitions from whole shotgun genome sequencing and EST libraries is possible [54,55], but expedient mechanisms to isolate only the mtDNA locus are needed to optimise organellar coverage for large numbers of taxa. While methods such as Rolling Circle Amplification (RCA) [56] have been trialled successfully on a limited number of species, further investigations across a range of taxa will be desirable to investigate their full potential to create divergent, multi-taxon datasets for comparative mitogenomics. Such methods hold advantages over PCR-based strategies due to the non-specific nature of the amplification process. By using random hexamers, as opposed to synthesising bespoke taxon-specific oligonucleotides, it is possible to avoid the amplification failure shown by this study whilst allowing for the creation of large, diverse mitochondrial genome datasets from low amounts of starting material.

### Table 3. Sequencing information following 454 GS FLX run and subsequent GS De Novo Assembler contig assembly.

| Species Name       | Sample Reference | Number of Reads | Number of Contigs | Average Contig Length | Roche MID Identifier | Longest Contig Length |
|--------------------|------------------|-----------------|-------------------|-----------------------|----------------------|-----------------------|
| Araneus diadematus | 571_SC_AB        | 1,313           | 20                | 340                   | 1                    | 1,058                 |
| Psalmopoeus cambridgei | 447_SC_AB    | 1,689           | 17                | 944                   | 2                    | 4,483                 |
| Eupalaestra campestratus | 446_SC_AB | 380             | 5                 | 849                   | 3                    | 1,916                 |
| Gorgyrella sp.     | 448_SC_SB       | 6,203           | 49                | 665                   | 4                    | 2,849                 |
| Xysticus audax     | 521_SC_AB       | 4,887           | 35                | 423                   | 5                    | 2,257                 |
| Pisaura mirabilis  | 502_SC_AB       | 1,192           | 14                | 505                   | 6                    | 872                   |
| Dysdera erythrina | 479_SC_AB       | 1,554           | 16                | 733                   | 7                    | 1,523                 |
| Lynxiphiidae sp.  | 559_SC_AB       | 503             | 12                | 540                   | 8                    | 1,310                 |
| Zelotes apricorum  | 462_SC_AB       | 9,081           | 26                | 619                   | 9                    | 6,434                 |
| Meta menardi       | 481_SC_AB       | 1,090           | 18                | 532                   | 11                   | 1,452                 |

Number of reads per sample represents only those from which the indicator MID sequence was recovered. Number of contigs represents only those formed with a length greater than 100 bases. Average contig length refers only to contigs over 100 bases in length. Longest contig length shows the largest single contig assembled. 

**Araneae Mitogenome Amplification Challenges**

doi:10.1371/journal.pone.0062404.t003
Acknowledgments

The authors would like to thank the members of the British Arachnological Society, especially Tony Russell-Smith, Jennifer Newton, John Harper, Rob Cummings & Richard Gallon for the provision of samples and specimen identification. We also thank Wendy Grail (Molecular Ecology & Fisheries Genetics Laboratory), Christine Hertz-Fowler, Christian Bourne and the staff at the Centre for Genomic Research, University of Liverpool, for sequencing support.

Author Contributions

Critical review of the manuscript: SG SM MIT MAA DP JK. Conceived and designed the experiments: AGB SC. Performed the experiments: AGB. Analyzed the data: AGB SC. Contributed reagents/materials/analysis tools: AGB SC JK. Wrote the paper: AGB SC.

References

1. Roore JL (1999) Animal mitochondrial genomes. Nucleic Acids Research 27: 1767–1780.
2. Moritz C, Dowling TE, Brown WM (1987) Evolution of animal mitochondrial-DNA - relevance for population biology and systematics. Annual Review of Ecology and Systematics 18: 269–292.
3. Deluc F, Brinkmann H, Philippe H (2005) Phylogenetic trees and the reconstruction of the tree of life. Nature Reviews Genetics 6: 361–375.
4. Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, et al. (2002) Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature 418: 563–573.
5. Murchison EP, Towar C, Hua A, Bender HS, Khareddour P, et al. (2010) The Tasmanian Devil Transcripome Reveals Swannell Cell Origins of a Clonally Transmissible Cancer. Science 327: 827–832.
6. Dellaporta SL, Ax A, Sagasser S, Jakob W, Moreno MA, et al. (2006) Mitochondrial genome of Tischbeinidha advenarum supports Placeozoa as the basal lower metazoaan phylum. Proceedings of the National Academy of Sciences of the United States of America 103: 8751–8756.
7. Dortk J, Fisse F, Porschke G, Struck TH (2010) Phylogenetic position of Spunica from multi-gene and phylogenomic data and its implication for the evolution of segmentation. Journal of Zoological Systematics and Evolutionary Research 48: 197–207.
8. Struck TH, Paul C, Hill N, Hartmann S, Hoedl C, et al. (2011) Phylogenomic analyses unravel ameloid evolution. Nature 471: 95–U113.
9. Morin PA, Archer FL, Foote AD, Västrup J, Allen EE, et al. (2010) Complete mitochondrial genome phylogenetic analysis of killer whales (Orcinus Orca) indicates multiple species. Genome Research 20: 988–916.
10. Timmermans MJTN, Dodsworth S, Culverwell CL, Bocak L, Ahrens D, et al. (2010) Complete mitochondrial genome sequence of the predatory mite Triops cancriformis: phylogenetic analysis and dating evolutionary divergence within canidae. Molecular Biology Reports 36: 197–207.
11. Zhang H, Chen L (2011) The complete mitochondrial genome of rhode Caus albicum: phylogenetic analysis and dating evolutionary divergence within canidae. Molecular Biology Reports 38: 1651–1660.
12. Rokas A, Holland PWH (2000) Rare genomic changes as a tool for phylogenetics. Trends in Ecology & Evolution 15: 445–459.
13. Boore JL, Brown WM (1998) Big trees from little genomes: mitochondrial gene sequences reveal the higher-level phylogenetic relationships of animals. Journal of Molecular Evolution 47: 689–674.
14. Venkatesh B, Ning Y, Brenner S (1999) Late changes in spliceosomal introns define clades in vertebrate evolution. Proceedings of the National Academy of Sciences of the United States of America 96: 10267–10271.
15. Masta SE, McCall A, Longhorn SJ (2010) Rare genomic changes and mitochondrial sequences provide independent support for congruent relationships among the sea spiders (Arthropoda, Pycnogonida). Molecular Phylogenetics and Evolution 57: 59–70.
16. Derman W, Vanhoorne B, Tiiry L, Van Leuvenen T (2010) Mitochondrial genome analysis of the predatory mite Phytoseius persimilis and a review of the Metaseiidae occidentalis mitochondrial genome. Genome 53: 301–311.
17. Williams EE, Gilbert MT, Binladen J, Ho SYW, Campos PF, et al. (2009) Complete mitochondrial genomes from extinct and extant rhinoceroses reveals lack of phylogenetic resolution. BMC Evolutionary Biology 9: doi:10.1186/1471-2148-9-95.
18. Corp. I (Released 2011) IBM SPSS Statistics for Windows, Version 20.0. Araneae Mitogenome Amplification Challenges
19. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007) Clustal W and clustal X version 2.0. Bioinformatics 23: 2947–2948.
20. Dimitrov D, Austrin JJ, Huber BA (2012) Pholcid spider molecular systematics revisited, with new insights into the biogeography and the evolution of the group. Linniates 29: 132–146.
21. Dimitrov D, Lepardo L, Giribet G, Arnone MA, Alvarez-Padilla F, et al. (2012) Tangled in a sparse spider web: single origin of orb weavers and their spinning work unravelled by denser taxonomic sampling. Proceedings of the Royal Society B-Biological Sciences 279: 1341–1346.
22. Boder MR, Madding WP (2012) The biogeography and age of salticid spider radiations (Araneae: Salticidae). Molecular Phylogenetics and Evolution 65: 213–240.
23. Barnes WM (1994) PCR amplification of up to 35-KB DNA with high-fidelity and high-yield from lambda-bacteriophage templates. Proceedings of the National Academy of Sciences of the United States of America 91: 2216–2220.
24. Barrett RDH, Hebert PDN (2005) Identifying spiders through DNA barcodes. Canadian Journal of Zoology 83: 481–491.
25. Jansen T, Schneider C, Remmer M, Volker M, Fieni W (1994) Convenient single-step, one tube purification of PCR products for direct sequencing. Nucleic Acids Research 22: 4354–4355.
26. Larkin MA, Blackshields G, Brown NP,_chenna R, McGettigan PA, et al. (2007) Clustal W and clustal X version 2.0. Bioinformatics 23: 2947–2948.
27. Hwang UW, Park CJ, Yong TS, Kim W (2001) One-step PCR amplification of complete arthropod mitochondrial genomes. Molecular Phylogenetics and Evolution 19: 345–352.
28. Gai Y, Song D, Sun H, Yang Q, Zhou K (2008) The complete mitochondrial genome of Symphyplema sp (Myriapoda: Symphyplea): Extensive gene order rearrangement and evidence in favor of Progoneata. Molecular Phylogenetics and Evolution 49: 574–503.
29. Rozen S, Skalatsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Totowa: Humana Press. 365–380.
30. Corp. I (Released 2011) IBM SPSS Statistics for Windows, Version 20.0. Araneae Mitogenome Amplification Challenges
31. Altschul SF, Gish W, Miller WA, Myers EW, Lipman DJ (1990) Basic Local Alignment Search Tool. Journal of Molecular Biology 215: 403–410.
32. Carvalho DC, de Almeida RN, Beheregaray LB (2012) The molecular phylogeny of crab spiders (Araneae: Thomisidae). Cladistics 28: 701–722.
33. vẫn ND, Pham HT, Nguyen VT (2007) Mitochondrial genome of the wolf spider Araneae: Lycosidae: A hierarchical analysis of nucleotide sequence variation. Journal of Molecular Evolution 63: 38–45.
45. Beard CB, Mills Hamm D, Collins FH (1993) The mitochondrial genome of the mosquito Anopheles gambiae. DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects. Insect Molecular Biology 2: 103–124.

46. Hu M, Jex AR, Campbell BE, Gasser RB (2007) Long PCR amplification of the entire mitochondrial genome from individual helminths for direct sequencing. Nature Protocols 2: 2339–2344.

47. Zhang DX, Hewitt GM (1997) Insect mitochondrial control region: A review of its structure, evolution and usefulness in evolutionary studies. Biochemical Systematics and Ecology 25: 99–120.

48. Ernsting BR, Edwards DD, Aldred KJ, Fites JS, Neff CR (2009) Mitochondrial genome sequence of Unionicola foili (Acari: Unionicolidae): a unique gene order with implications for phylogenetic inference. Experimental and Applied Acarology 49: 305–316.

49. Quinn TW, Wilson AC (1993) Sequence evolution in and around the mitochondrial control region in birds. Journal of Molecular Evolution 37: 417–425.

50. Wenink PW, Baker AJ, Tilmans MGJ (1994) Mitochondrial control-region sequences in 2 shorebird species, the Turnstone and the Dunlin, and their utility in population genetic-studies. Molecular Biology and Evolution 11: 22–31.

51. Cummings MP, Otto SP, Wakeley J (1995) Sampling properties of DNA-sequence data in phylogenetic analysis. Molecular Biology and Evolution 12: 814–822.

52. Sorenson MD, Ast JC, Dimcheff DE, Yuri T, Mindell DP (1999) Primers for a PCR-based approach to mitochondrial genome sequencing in birds and other vertebrates. Molecular Phylogenetics and Evolution 12: 105–114.

53. Jiang S-T, Hong G-Y, Yu M, Li N, Yang Y, et al. (2009) Characterization of the complete mitochondrial genome of the giant silkworm moth, Eriogyna pyretorum (Lepidoptera: Saturniidae). International Journal of Biological Sciences 5: 351–365.

54. Kane N, Sveinsson S, Dempewolf H, Yang JY, Zhang D, et al. (2012) Ultra-barcoding in cacao (Theobroma spp.; Malvaceae) using whole chloroplast genomes and nuclear ribosomal DNA. American Journal of Botany 99: 320–329.

55. Groenenberg DSJ, Pierro W, Gittenberger E, Schilthuizen M (2012) The complete mitogenome of Cylindrus oblaus (Helicidae, Ariantinae) using Illumina next generation sequencing. BMC Genomics 13: 4 doi:10.1186/1471-2164-13-114.

56. Tang S, Hyman BC (2005) Rolling circle amplification of complete nematode mitochondrial genomes. Journal of Nematology 37: 236–241.