Title: Cost-efficient high throughput capture of museum arthropod specimen DNA using PCR-generated baits

Running title: Capture of museum specimens using PCR baits

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Abstract:

1. Gathering genetic data for rare species is one of the biggest remaining obstacles in modern phylogenetics, particularly for megadiverse groups such as arthropods. Next generation sequencing techniques allow for sequencing of short DNA fragments contained in preserved specimens >20 years old, but approaches such as whole genome sequencing are often too expensive for projects including many taxa. Several methods of reduced representation sequencing have been proposed that lower the cost of sequencing per specimen, but many remain costly because they involve synthesizing nucleotide probes and target hundreds of loci. These datasets are also frequently unique for each project and thus generally incompatible with other similar datasets.
2. Here, we explore utilization of in-house generated DNA baits to capture commonly utilized mitochondrial and ribosomal DNA loci from insect museum specimens of various age and preservation types without the a priori need to know the sequence of the target loci. Both within species and cross-species capture are explored, on preserved specimens ranging in age from one to 54 years old.
3. We found most samples produced sufficient amounts of data to assemble the nuclear ribosomal rRNA genes and near complete mitochondrial genomes and produce well-resolved phylogenies in line with expected results. The dataset obtained can be straightforwardly combined with the large cache of existing Sanger-sequencing-generated data built up over the past 30 years and targeted loci can be easily modified to those commonly used in different taxa. Furthermore, the protocol we describe allows for inexpensive data generation (as low as ~$35/sample), of at least 20 kilobases per

specimen, for specimens at least as old as ~1965, and can be easily conducted in most laboratories.

4. If widely applied, this technique will accelerate the accurate resolution of the Tree of Life especially on non-model organisms with limited existing genomic resources.

Keywords: [4-6]

Insects, phylogeny, host plant, Miridae

Introduction:

Natural history museums host troves of biological material and sometimes the only known representatives of extinct or rare species (Coddington, Agnarsson, Miller, Kuntner, & Hormiga, 2009; Lim, Balke, & Meier, 2011). In these cases, museum specimens represent the only accessible sources of genetic data for a given species and gathering data from such specimens in a cost-effective way is one of the primary obstacles yet to be overcome in modern phylogenetics. Specimens in museums may also allow for the inclusion of a temporal variable into analyses by comparing DNA sequence of individuals across different sampling dates and can even be used for the analysis of short-term evolutionary trends (Hartley et al., 2006; DiEuliis, Johnson, Morse, & Schindel, 2016).

Preservation conditions of museum material can dramatically impact the viability of obtaining DNA sequence data. Traditional approaches used amplification of target regions of DNA followed by Sanger sequencing. This method is highly dependent on residual DNA fragment size and the proportion of endogenous DNA in the extract. While targeting shorter gene regions can mitigate the issue of DNA fragmentation, a low endogenous content is harder to overcome

(Burrell, Disotell, & Bergey, 2015), and even innovative new PCR techniques are only capable of somewhat reliably amplifying fragments of less than 600 bp (Mitchell, 2015). The development of next generation sequencing (NGS) has expanded the array of methods for DNA sequencing from museum specimens. For whole genome sequencing, an NGS library is prepared from the original DNA extract and this library is then combined with other samples for multiplex sequencing and allocated a certain proportion of reads on a sequencer, depending on desired sequencing depth and the total budget (Cridland, Ramirez, Dean, Sciligo, & Tsutsui, 2018; Kanda, Pflug, Sproul, Dasenko, & Maddison, 2015; Maddison & Cooper, 2014). However, even low-coverage whole genome sequencing is currently still prohibitively expensive for all but very well-funded projects or studies focusing on relatively few samples.

As a way to decrease the cost per sample while still generating sufficient amounts of data for accurate phylogenetic placement, several methods of reduced representation sequencing have been proposed. Typically, these methods include selective hybrid capture of target loci, where the type and number of loci being captured depends on the scope and context of the study. During the past few years, utilization of commercially synthesized probes or microarray kits for the capture of conserved DNA regions has become popular for phylogenetic studies and can be applied to historical museum specimens (Bi et al., 2013; Blaimer, Lloyd, Guillory, & Brady, 2016; McCormack, Tsai, & Faircloth, 2015). These kits are designed based on existing reference genomes or transcriptomes and typically enrich many loci (~500-5000), thus a large amount of data is generated for each sample, but the cost per sample is relatively high. Other kits are designed to enrich mitochondrial genomes, including a kit specifically designed for mitochondrial DNA across insects (Liu et al., 2016). However, all methods relying on

78 commercially synthesized kits are relatively expensive and might not be feasible for low-budget
79 projects. These kits are also limited by the original design and probe composition cannot be
80 adjusted after synthesis.

81 These limitations led us to explore an approach that uses in-house generated DNA baits for
82 hybrid enrichment (Maricic, Whitten, & Pääbo, 2010). These baits can be produced from
83 amplicons generated by PCR of short gene regions (Peñalba et al., 2014), or by long-range PCR
84 of complete mitochondrial (Li et al., 2015; Maricic et al., 2010) or chloroplast genomes (Mariac
85 et al., 2014), or even from ddRAD library fragments (Suchan et al., 2016). PCR-generated baits
86 have so far only been applied to vertebrates and plants, and only in a few cases tested on archival
87 specimens (Li et al., 2015). Appealing features of this approach include affordable synthesis of
88 baits, independence from the need of a good quality reference, and flexibility of the synthesis
89 workflow for low-cost modifications of the bait set (e.g., pooling different combinations of bait
90 amplicons, using same primers to obtain bait amplicons from different taxa, or generating
91 additional baits with new sets of primers).

92 The diversity of arthropods is staggering, with estimates of about 80% of species still
93 undescribed (Stork, 2018) and an increasing number of species going extinct every day
94 (Hallmann et al., 2017). While modern phylogenetic studies of vertebrates sometimes approach
95 complete sampling of extant diversity, complete extant sampling of any large clade of arthropods
96 is almost impossible due to the abundance of rare species, limited material, and the huge diversity
97 of arthropods (Coddington et al., 2009; Lim et al., 2011). However, near-complete sampling is
98 useful for many downstream analyses, including unbiased estimation of lineage diversification
99 rates (Cusimano, & Renner, 2010; Cusimano, Stadler, & Renner, 2012; Höhna, Stadler, Ronquist,

& Britton, 2011). Scientists have just started to utilize the enormous resources of arthropod specimens deposited in natural history collections for gathering large DNA datasets (Stork, McBroom, Gely, & Hamilton, 2015; Stork, 2018). We argue that insects in particular are an apt test case for the application of new NGS approaches to illuminating the dark areas in the Tree of Life, because most material in entomological collections is stored as dried and pinned or point-mounted specimens, which are often suitable for the retrieval of fragmented DNA. Previous applications of this approach on vertebrate and plant samples employed destructive extraction protocols to generate adequate amounts of DNA for capture. But DNA extraction can be performed without destroying external or genitalic morphological features and from individual and small specimens as in many insects. For a complete taxonomic sampling of large clades, already existing data should be compatible with character-rich new datasets generated at low costs.

Here, we test the efficiency of PCR-generated DNA baits (targeting the mitochondrial genome, nuclear ribosomal operon, and one nuclear protein-coding gene) to capture DNA sequences from museum-deposited insect specimens with different collection dates, preservation methods, and evolutionary relatedness, using phyline plant bugs (Insecta: Hemiptera: Miridae: Phylinae) as our test case. These loci were selected for optimal integration with existing, Sanger-based sequence data and to allow adequate coverage when multiplexing hundreds of libraries. Plant bugs are a group of > 11,000 described species that include serious plant pests and beneficial insects (Cassis & Schuh, 2012). Phylogenetic hypotheses for the entire group are in their infancy (Jung & Lee, 2011), but studies targeting selected subfamilies including the Phylinae now provide testable

hypotheses (Konstantinov & Knyshov, 2015; Menard, Schuh, & Woolley, 2014; Namyatova, Konstantinov, & Cassis, 2015; Tatarnic & Cassis, 2012). The taxonomic diversity of plant bugs in the Western U.S. is fairly well understood (Cassis & Schuh, 2012; Weirauch et al., 2016), but few species have been incorporated into phylogenetic analyses, and some are only known from the type specimen(s). As the first test case, we selected a putatively monophyletic group of native oak-associated plant bugs, the so called "Orange Oak Bugs" (OOB) (Weirauch, 2006a, 2006b), where some species may be monophagous on specific species of oaks, while at least two widespread and polymorphic species (*Phallospinophylus setosus* Weirauch and *Pygovepres vaccinicola* (Knight)) feed on a variety of host plants (including Fagaceae, Rhamnaceae, and Rosaceae). We sampled specimens of these two species from a range of localities and host plants, together with several additional species of OOB that had not yet been included in phylogenetic analyses (Menard et al., 2014) to test efficacy of capture across closely related samples and to investigate potential cryptic host plant races. As second test case, we selected the genus *Tuxedo* Schuh with seven described species associated with host plants in several families (Schuh, 2004); phylogenetic relationships within this genus are unknown. We aimed to sample several individuals from each of the seven species, including paratype specimens, to investigate capture efficiency at deeper phylogenetic levels and to explore host plant shifts within the genus. Both datasets were analyzed together with a Sanger-derived phylogenetic dataset of Phylinae (Menard et al., 2014), demonstrating the feasibility of combining existing and newly generated NGS data.

Material and methods:

Taxon Sampling and Vouchering

Specimens for this study were loaned from the American Museum of Natural History (AMNH), the Entomology Research Museum (UCRC), and the Zoological Institute, Russian Academy of Sciences (ZISP). Tentative voucher identification was done based on habitus and host association data using Weirauch (2006a, 2006b) and Schuh (2004). Age of specimens at the moment of DNA extraction varied from one to 54 years. Specimens of *Tuxedo*, *Leucophoroptera* Poppius, *Ausejanus* Menard and Schuh, and *Pseudophylus* Yasunaga were imaged using a Leica DFC 450 C imaging system. Image vouchers and specimen information are available through the Heteroptera Species Pages (http://research.amnh.org/pbi/heteropteraspeciespage/). After clearing soft abdominal tissues during the DNA extraction process, we examined male genitalic characters to confirm our tentative identifications. In cases where different diagnostic characters were in conflict (e.g., in some *Tuxedo* spp., see results and discussion), we based our identification on genitalic characters.

DNA Extraction

In most cases, only the abdomen (1-1.5 mm in length) was used for non-destructive DNA extraction, which was performed using a Qiagen DNeasy® Blood & Tissue kit (for relatively fresh ethanol specimens) or a combination of the previous kit with a Qiagen QIAquick® PCR purification kit (for dry specimens, see supplemental text S1), since the latter is commonly used for DNA extraction from degraded samples (Lee et al., 2010; Yang, Eng, Waye, Dudar, & Saunders, 1998). Abdomens were soaked in the extraction buffer, such that cuticular structures remain undamaged, and mirrored standard dissection procedures for plant bug specimens. This approach allows for subsequent remounting of the abdominal cuticle and genitalia with the rest of the specimen, or in a genitalic vial.

Bait Synthesis

Freshly collected specimens of *Phallospinophylus setosus* and *Tuxedo drakei* Schuh were selected as bait donors for the OOB and *Tuxedo* subprojects, respectively. Primers for obtaining long range PCR products are listed in Table S1. Details on the primer design are available in supplemental text S1. Target regions included mitochondrion, nuclear ribosomal operon, and a fragment of the cytoplasmic dynein heavy chain gene.

To prepare baits, six long-range (LR) PCRs per specimen were performed. For this and all subsequent PCR described in this paper, we used Takara PrimeSTAR® GXL polymerase, a hot-start high-fidelity enzyme that is able to amplify long products. The PCR mix contained 10 µl PrimeSTAR® GXL buffer, 4 µl 2.5M dNTPs, 1 µl PrimeSTAR® GXL polymerase, 32 µl water, 1.5 µl of each primer (10 µM), and 1 µl of DNA template. The thermocycler program included initial denaturation at 98° for 3 min, 35 cycles of denaturation for 10 sec at 98°, followed by annealing at variable temperatures for 15 sec, followed by elongation at 68° for a variable amount time, and with the final incubation at 68° for 15 min. Additional details on long-range PCR conditions are available in Table S1.

After clean up with custom Solid Phase Reversible Immobilization (SPRI) beads (Glenn et al., 2016; Rohland, & Reich, 2012), mitochondrial, nuclear ribosomal, and nuclear protein-coding products were mixed in molar ratios of 1:1:5, following recommendations of Peñalba et al. (2014) regarding capture of low copy nuclear genes. Mixtures were diluted to the volume of 100 µl and sonicated on a Diagenode Bioruptor® UCD-200 with 30/30 cycles for 6 runs of 5 minutes. Sheared PCR products were subjected to a bait library preparation generally following the protocol of Li et al. (2015) with the exception that regular dNTPs instead of a dUTP-containing

mixture were used, since NaOH melting was used to subsequently elute captured libraries instead of off-bead amplification. Three pools of ready-to-use bait were produced by amplifying M13-adaptor-ligated bait libraries with 5' biotinylated primers using PCR conditions outlined above with the following modifications: 6 µl of template was used, and annealing temperature set to 55°.

Preparation of Illumina-compatible Libraries

Since DNA sequence of bait donors was also of interest in this project, we also sequenced amplicons used for bait production. These LR PCR products were mixed in equimolar ratios and sonicated as described above. Following sonication, Illumina®-compatible libraries were prepared using the protocol from Li, Hofreiter, Straube, Corrigan, and Naylor (2013), with the following modifications: end prep mix contained 50% 2X Takara EmeraldAmp® GT PCR mix and after incubation at 25° for 15 min and 12° for 5 min was incubated at 72° for 20 min in order to obtain a-tailed fragments. We utilized with-bead SPRI method as originally described in Fisher et al. (2011), carrying same SPRI beads through the library preparation steps. T-tailed loop adaptors from NEBNext® Multiplex Oligos for Illumina® kit E7600s were ligated to the DNA and a PCR with indexing primers from the same kit was conducted using PCR conditions outlined above with the following modifications: 6 µl of template was used, annealing temperature set to 60°, number of cycles set to 16.

To prepare target libraries, DNA extracts were run on a gel with Biotium GelRed® premixed loading buffer in ratios 1:2 to check average fragment size and determine if sonication was needed (i.e., for younger samples). These DNA extracts were quantified using Qubit™ fluorometer, and for more consistent sonication results approximately 70 ng of DNA (where

209 possible, also see Table 1) were used for sonication. Library preparation followed the protocol
210 outlined above with the exception that after adaptor ligation, libraries were amplified with short
211 IS7/IS8 primers following Li et al. (2013). The same PCR conditions as above were used,
212 however number of cycles were varied from 16 to 21 depending on the amount of starting
213 material.

214 *First Sequencing Run – Target Capture, Pooling and Sequencing*

215 In our first sequencing run, target captures generally followed the protocol of Li et al. (2015).
216 Every sample was captured individually as in Li et al. (2015), 10 µl of Invitrogen Dynabeads®
217 M-270 and 10 ng of bait library was used for most samples, whereas all remaining bait library
218 was used for the last few captured samples (for details on bait amount used, see Table 1). DNA
219 concentration of input target library was not quantified, and we used 6 µl of target library in each
220 capture reaction. Elution was conducted with NaOH melting as in Maricic et al. (2010), and
221 double capture was performed following suggestions of Peñalba et al. (2014). After the second
222 round of capture, the supernatant was cleaned, and eluted in 50 µl of 10 mM Tris-HCl. Post-
223 capture PCR followed the same PCR procedure as outlined above, however indexing primers and
224 20 µl of template were used, and variable number of cycles was performed (16-24).

225 After indexing PCR, products were cleaned and normalized with Just-a-Plate™ 96 PCR
226 Purification and Normalization Kit. Since using Bioanalyzer on all 60 samples was prohibitively
227 expensive, libraries were first run on a gel with GelRed® to check average fragment size, pooled
228 together into nine groups according to their size, which were then analyzed on a single
229 Bioanalyzer chip to obtain more accurate fragment size distribution. Then libraries were pooled
230 equimolarly with the exception of sheared amplicon libraries (samples ph32 and ph47), which

were pooled at twice higher concentrations. The pool of 60 indexed libraries then was mixed in molar proportion of 50:50 with unrelated samples from other projects and sequenced on a single run of Illumina® MiSeq® V3 2x300bp at the UCR IIGB Core Facility.

Second Sequencing Run – Library Preparation, Target Capture and Sequencing

In the second sequencing run, we followed the protocol of Maricic et al. (2010) with modifications. DNA extracts from the same specimen of *Tuxedo drakei* as above was used as a source for bait preparation. The procedure differed from described above in that only nuclear rRNA operon and mitochondrial PCR products were used. We extracted one more specimen of *Pseudophylus* and prepared a library as outlined above. Five libraries (samples ph45, ph54, ph57, ph59, and a new *Pseudophylus* library) were carried through indexing PCR, quantified using Qubit™, checked on an agarose gel, and pooled equimolarly to obtain about 450 ng of DNA. Because indexed libraries were used, we added additional blocking oligos as in Maricic et al. (2010) to block longer adaptor fragments. Approximately 500 ng of bait and 5 µl of Dynabeads® as in Maricic et al. (2010) were used for each round of capture (two rounds total as in the first sequencing run). Post-capture amplification was done using IS5 and IS6 primers and was carried over in two aliquots. After PCR, the products were combined and purified, they were then sequenced on 5% of another Illumina® MiSeq® V3 2x300bp run at the UCR IIGB Core Facility.

Post-Sequencing Data Processing

Raw sequences were demultiplexed and adaptors were removed using bcl2fastq software (Illumina®) at the UCR IIGB Core Facility. Trimmomatic v0.36 (Bolger, Lohse, & Usadel, 2014) was used to trim off low quality ends of the sequences as well as perform more thorough

adaptor trimming. Reads were assembled into contigs with SPAdes (Bankevich et al., 2012). In cases where assembly did not yield complete target regions, we obtained them by mapping shorter contigs onto full length assemblies of other related samples. Assembled contigs were checked for misassembled regions and manually curated in Geneious v.10 (https://www.geneious.com, Kearse et al., 2012). We mapped reads on these contigs using BWA (Li & Durbin, 2009) to assess the coverage depth (see Table 1), prior to average coverage calculations, reads were deduplicated using PRINSEQ (Schmieder & Edwards, 2011).

We aligned all resulting 18S, 28S, and mitochondrial contigs using MAFFT v.7 (Katoh & Standley, 2013). Manual inspection of alignments and trimming was performed. Since accurate assembly of the mitochondrial control region with short reads without a close reference was problematic due to presence of repeats, we excluded it from the analysis. The remainder of the mitochondrion was annotated by aligning it with mitochondrial genome of another plant bug available on GenBank (NC_024641.1).

Phylogenetic Analysis

For phylogenetic analysis, the dataset was concatenated and divided into 18 partitions with protein coding genes split further into codon positions. Substitution models and partitioning scheme were optimized using PartitionFinder 2.1.1 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016) or ModelFinder (Kalyaanamoorthy, Minh, Wong, von Haeseler, & Jermiin, 2017), which is an IQ-TREE built-in model and partition test. Phylogeny estimation was performed in RAxML v8.2.11 (Stamatakis, 2014) and IQ-TREE v1.5.4 (Nguyen, Schmidt, von Haeseler, & Minh, 2014). Branch support was calculated using Rapid Bootstrap (Stamatakis, Hoover, & Rougemont, 2008) which is shown on Fig. 2, Ultrafast Bootstrap (Minh, Nguyen, & von

Haeseler, 2013), and SH-aLRT (Anisimova & Gascuel, 2006), which are shown on Figs S2 and S3.

To test how well our data can be combined with previously generated data, we combined our data with the dataset of Menard et al. (2014) which is the most comprehensive set of genetic data for related species. After downloading the sequences from GenBank, we extracted only 18S, 28S, 16S and COX1 sequences from our data, performed alignment and manual trimming. Alignments were then concatenated, optimized for model and partitioning scheme, and phylogenetically analyzed as above.

Illustrations for Figs 2, 3, and S1 were drafted using R v3.4.3 and packages APE (Paradis, Claude, & Strimmer, 2004), phytools (Revell, 2012), ggtree (Yu, Smith, Zhu, Guan, & Lam, 2016) and ggbio (Yin, Cook, & Lawrence, 2012). Relief image for Fig. 3 was taken from the SimpleMappr website (http://www.simplemappr.net/).

Results and Discussion

Expenses

Total expenses after bait and target library preparations, target capture, and sequencing and including all reagents and supplies came to about $54 per specimen or about $2.8 per 1 Kb of data in the first sequencing run, and about $39 per specimen or about $2.1 per 1 Kb of data in the second sequencing run (Table S2). Our estimates suggest that pooled capture together with using a higher throughput sequencer (e.g., a HiSeq® lane or a NextSeq® run) can generate the same amount of data for about half the price (up to $25 per specimen), however a greater number of samples (at least 360) need to be pooled together to efficiently utilize the sequencer.

295 *DNA extraction*

296 The amount of DNA extracted greatly varied across samples (see Table 1). The minimum amount
297 of DNA that was used for library preparation was 2.75 ng (sample 42). The average fragment size
298 for ethanol preserved material was large: we always detected a bright band larger than 10 Kb in
299 size, with many extracts also with a smear of fragments spanning down to 300 bp. For dry point-
300 mounted material we observed two types of fragmentation: extracts that had fragments of 500-
301 700 bp on average in addition to long (~8Kb) fragments (dry specimens collected within past ten
302 years), and extracts with only fragments shorter than 1000 bp (dry specimens collected more than
303 ten years ago).

304 *Sequencing and assembly*

305 A total of 45% of an Illumina® MiSeq® V3 lane was used for the samples in the first sequencing
306 run. The amount of reads obtained per sample is listed in Table 1 (average of 152670, σ =
307 39070). For bait samples, we obtained full bait contigs for the nuclear ribosomal operon, the
308 dynein fragment, and entire mitochondrial genome, although unambiguous assembly of the
309 control region was problematic due to lack of a close reference and long read data. For other
310 samples, we obtained full or partial mitochondrial contigs and nuclear ribosomal gene contigs for
311 the majority of samples (see Table 1). Mitochondrial completeness is indicated on Fig. 2B and
312 excludes the control region, and mitochondrial average coverage depth is indicated in Fig. 2C.
313 We were able to obtain reliable ribosomal data for 48 taxa, but some sequences exhibited cross
314 contamination of about 1% of reads by the bait taxon as detailed in supplemental text S1. In the
315 second sequencing run, we observed a higher percent of ribosomal operon reads on target (on
316 average 7.33% in the second run compared to 2.1% in the first run for the same samples) and

317 both for recaptured libraries, as well as for the library prepared after the first sequencing run was
318 complete (ph61), we have not detected contaminating reads.

319 *Capture efficiency*

320 Percent of reads on target varied from 0.61% to 33.95% and was on average 8.19% in the OOB
321 subproject and 4.02% in *Tuxedo* subproject. The percent of reads on target was slightly larger for
322 samples that are close to bait specimens (Figs 2A, 2E, Fig. S4). We also observed a significant
323 variation of percentage on-target across samples of close phylogenetic relatedness, which may be
324 attributed to variation in total amount of target DNA submitted to capture reactions (equal
325 volumes of target libraries were used in all reactions). Capture in the *Tuxedo* subproject
326 performed worse, which could be attributed to the higher sequence divergence from the bait (Fig.
327 2D).

328 Baits for a nuclear protein-coding gene (dynein) performed unsatisfactorily, even though they
329 were five times more concentrated. Although we do not have a clear explanation as to why this
330 bait performed suboptimally (see supplemental text S1), the large middle intron may have been
331 detrimental for bait efficacy.

332 On the contrary, mitochondrial baits were only 14.3% of total bait pool, yet were able to
333 considerably enrich for mitochondrial DNA. Typical sequencing of non-enriched DNA libraries
334 from insect museum specimens yields from 0.002% to 0.08% of total reads mapping to the
335 mitochondrial genome (Staats et al., 2013), however, we recovered on average of 2.13% (σ =
336 2.59%, range 0.24%-15.64%) representing an enrichment of at least 25x on average for our first
337 sequencing run. Given the amount of reads we allocated for our samples, an unenriched library

would produce only about 120 mitochondrial reads, where we achieved on average ~3,500 reads (an enrichment of 29x), sufficient for assembling the whole mitochondrial genome.

Suboptimal capture performance in our first sequencing run could be also attributed to the amount of bait. Overall, we observed an increase in the amount of reads on target in capture reactions where more bait was used (Table 1, samples ph29-ph31, ph33-ph35, and ph55-ph60). Thus, we repeated sequencing of five selected samples captured with a modified protocol (see Materials & Methods) where more bait was used. We also explored a pooled capture approach, which is significantly cheaper than the individual sample captures. In the result of the second sequencing run, we observed on average 8.65% on target reads as opposed to 3.42% for the same samples in the first sequencing run (see Table 1). We also noticed a larger variation of total amount of reads received for a given sample in the pool. This might be due to unequal divergence of samples in the pool with the respect to the bait or difference in library quality due to the age of the specimens. Because of this, we recommend balancing sample pools prior to capture and performing individual captures for sensitive samples.

Our results show no difference in capture efficiency as related to the age of the specimen (Fig. 2, specimens older than 20 years denoted with red asterisks). We thus expect that even older specimens can be used (Blaimer et al., 2016), but for this pilot study the youngest available specimens were chosen. Further adjustments of hybridization temperature and duration may further improve capture success, however need to be modified on an individual basis.

Phylogenetic analyses

Using the obtained data, we reconstructed a well resolved phylogeny, contributing new insights. Our phylogenetic analysis supports the monophyly of *Tuxedo* + *Pseudophylus*, the OOB clade, *Phallospinophylus setosus* and *Pygovepres vaccinicola* with the highest branch support (Fig. 2A, Fig. S2). As part of the *Tuxedo* subproject, we sampled two specimens of *Pseudophylus stundjuki* (Kulik) since this species from Far East Asia rendered the Western Nearctic *Tuxedo* paraphyletic in a previous analysis (Menard et al., 2014). "*Tuxedo*" is here confirmed to be paraphyletic with respect to *Pseudophylus*, after thorough examination of our sequence data and comparison with data from Menard et al. (2014) and Jung and Lee (2011). All primarily Fagaceae-feeding species of "*Tuxedo*" form a well-supported monophyletic group. Species other than *Tuxedo flavicollis* (Knight) and *Tuxedo susansolomonae* Schuh were recovered as monophyletic and conform with genitalic-based identifications. Phylogenetic analysis recovered two highly supported monophyletic groups within the *T. flavicollis/susansolomonae* species group, however composition of each group is not congruent with either genitalic structure or coloration. One specimen (ph57) initially identified as *T. susansolomonae* is distantly related from other members of *T. flavicollis/susansolomonae* clade and is recovered as sister taxon to *T. nicholi* (Knight), and likely represents an undescribed species. Species within the OOB clade represented by multiple specimens are monophyletic with high support. Our analysis did not find support for our hypothesis on the presence of host plant races within each of the widespread and polyphagous OOB species (Fig. 3). In contrast, the phylogenetic structure in *Phallospinophylus setosus* is more likely explained by geographic proximity between sampled localities.

Combined with existing data of Menard et al. (2014), phylogenetic hypotheses inferred from our dataset are congruent with those presented in prior studies (Fig. 4, Fig. S3). Deep level

relationships within Oncotylina as well as the monophyly of the subtribe itself remain poorly supported based on this data set. As in Menard et al. (2014), "*Tuxedo"* + *Pseudophylus* are recovered as the sister group to Leucophoropterini, although with low support. Sampled species of Leucophoropterini were recovered in expected phylogenetic positions.

Conclusions

In conclusion, we were able to cost-efficiently ($2.8/sample/Kb) sequence long-range PCR products as well as perform hybrid enrichment using in-house generated baits and obtain DNA sequences (~20 Kb) from archival specimens (up to 54 years old) using a minimal amount of DNA. This approach offers a much lower cost of bait production than other approaches, however, especially if LR PCR is chosen for amplicon generation, a high-quality sample of a related species is needed. While it is hard to scale up this method to produce baits for 500 targets, it is well suited to generate commonly used high-copy gene sequences for both archival and recently collected samples. It fits within a narrow 'Goldilocks' zone in terms of adequate data for accurately reconstructing phylogenies and relative cost effectiveness with the ability to multiplex at least ~120 individuals per MiSeq® run given the number of loci captured. While the amount of reads on target in our project was not high, we were able to assemble genes of interest for most captured samples.

Data obtained showed no evidence for host plant races in OOB. For *Pygovepres*, we could not detect any phylogenetic structure within the species, whereas the structure within *Phallospinophylus* could be explained by distribution. We also reconstructed the phylogeny of the genus *Tuxedo* and sampled all described species, some of which were rarely collected species that are based on specimens from type series.

Finally, it is straightforward to combine such data with previously generated data using conventional Sanger sequencing. Commonly used primers for different genes for use in phylogenetic analysis of other groups are easy to add to our protocol. When applied to museum specimens, this approach is optimal for generating complete phylogenetic sampling for clades of interest and relatively cheaply contributing confidently resolved twigs to the Tree of Life.

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Data Accessibility:

- DNA sequences: GenBank accessions [annotated mitochondrial genomes, ribosomal genes, and dynein fragments for baits will be uploaded to GenBank, and accession numbers will be indicated in Table S3]; NCBI SRA: SRP136090, accession numbers for individual samples are indicated in the Table S3.

603 - Final DNA sequence alignments and partitioning schemes: will be uploaded to Dryad
604 repository.

605 - Voucher specimen information including photographs: available through the Plant Bug
606 Planetary Biodiversity Inventory Project website
607 (http://research.amnh.org/pbi/heteropteraspeciespage/), linked to the unique specimen identifier
608 (See Table 1, the USI column) [photographs are in the process of being uploaded].

609

610 **Author contributions**

611 AK, ERLG & CW designed the research, AK and CW performed the research, AK analyzed the
612 data and AK, ERLG and CW wrote the paper.

613

Tables and Figures

Table 1. List of samples used in the project, voucher specimen information, and sequencing information.

ph#	USI	Species	Type status	Geographic region	Host plant	Preservation method	Collecting year	[dsDNA], ng/ul	DNA mass used for library prep (ng)	Amount of bait used, ng	PF Clusters	Filtered and merged reads	Filtered and merged reads (dedup)	Duplication rate	Mito: mapped reads	Mito: percent mapped	Mito: coverage depth	18S: mapped reads	18S: percent mapped	18S: coverage depth	28S: mapped reads	28S: percent mapped	28S: coverage depth	Dynein: mapped reads	Dynein: percent mapped	Total: reads mapped	COX1 distance between a bait and a sample
First sequencing run																											
ph1	UCR_ENT 00127382	Roburocoris exiguus	none	Cleveland NF	Quercus	EtOH	2010	0.276	11.04	10	130078	128027	126880	0.90%	1041	0.81%	13.8616	30	0.02%	3.09044	43	0.03%	4.33417	2	0.00%	1116	22.03%
ph2	UCR_ENT 00127369	Quercophylus gonoporospinus	none	Tehachapi	Quercus	dry	2009	6.52	78.24	10	153428	153550	152743	0.53%	1394	0.91%	19.0812	NA†	NA†	NA†	NA†	NA†	NA†	232	0.15%	1626	11.51%
ph3	UCR_ENT 00127383	Rubeospineus bicorniger	none	Los Angeles NF	Quercus	EtOH	2009	1.71	68.4	10	177774	177483	176589	0.50%	703	0.40%	9.27753	NA†	NA†	NA†	248	0.14%	21.7522	1438	0.81%	2389	10.98%
ph4	UCR_ENT 00127370	Rubeospineus bicorniger	none	Baja	Quercus	dry	2009	0.616	9.24	10	204208	199266	195784	1.75%	2735	1.37%	32.9303	NA†	NA†	NA†	301	0.15%	20.4866	1459	0.73%	4495	10.91%
ph5	UCR_ENT 00127371	Quernocoris caliginosus	none	Cleveland NF	Quercus	dry	2010	6.38	76.56	10	146948	145344	144916	0.29%	1372	0.94%	17.9123	NA†	NA†	NA†	227	0.16%	17.8322	1015	0.70%	2614	11.92%
ph6	UCR_ENT 00127372	Insulaphylus sp.	none	Baja	Quercus	dry	2009	0.308	9.24	10	163679	157591	152074	3.50%	13537	8.59%	110.284	646	0.41%	44.917	738	0.47%	50.1585	3209	2.04%	18130	14.82%
ph7	UCR_ENT 00127384	Vesperocoris paddocki	none	Los Padres	Quercus	EtOH	2007	2.52	75.6	10	142576	142125	141404	0.51%	1623	1.14%	20.869	NA†	NA†	NA†	918	0.65%	76.1237	8204	5.77%	10745	10.87%
ph8	UCR_ENT 00127385	Pygovepres vaccinicola	none	Cleveland NF	Quercus	EtOH	2010	2.86	74.36	10	79353	80147	74500	7.05%	2470	3.08%	33.1782	61	0.08%	6.18787	98	0.12%	7.86628	2111	2.63%	4740	9.65%
ph9	UCR_ENT 00127373	Pygovepres vaccinicola	none	Cleveland NF	Ceanothus	dry	2010	7.06	70.6	10	125133	123562	120335	2.61%	1889	1.53%	23.9774	987	0.80%	75.7632	1754	1.42%	114.08	6721	5.44%	11351	9.66%
ph10	UCR_ENT 00127386	Pygovepres vaccinicola	none	Cleveland NF	Prunus	EtOH	2010	6.06	74.538	10	235524	236912	232620	1.81%	977	0.41%	13.0746	NA†	NA†	NA†	NA†	NA†	NA†	957	0.40%	1934	9.66%
ph11	AMNH_PBI 00082512	Pygovepres vaccinicola	none	Cleveland NF	Marah	dry	1978	2.2	33	10	234450	227057	223678	1.49%	998	0.44%	10.1069	1107	0.49%	63.5918	1699	0.75%	92.6817	7713	3.40%	11517	9.13%
ph12	UCR_ENT 00127387	Pygovepres vaccinicola	none	San Bernardino NF	Quercus	EtOH	2016	4.2	72.66	10	168633	167206	163864	2.00%	3200	1.91%	34.7008	30	0.02%	2.98168	48	0.03%	3.7232	1775	1.06%	5053	9.65%
ph13	UCR_ENT 00127388	Pygovepres vaccinicola	none	Los Angeles NF	Ceanothus	EtOH	2016	0.76	38	10	159836	159278	158346	0.59%	4398	2.76%	53.8989	157	0.10%	14.6003	174	0.11%	14.7588	3153	1.98%	7882	9.58%
ph14	UCR_ENT 00127389	Pygovepres vaccinicola	none	Los Angeles NF	Prunus	EtOH	2016	2.64	75.24	10	156298	154959	154104	0.55%	1611	1.04%	21.5415	155	0.10%	13.105	183	0.12%	14.1757	1903	1.23%	3852	9.66%
ph15	UCR_ENT 00127390	Pygovepres vaccinicola	none	San Jacinto NF	Quercus	EtOH	2009	3.82	74.49	10	176319	178192	168409	5.49%	1459	0.82%	18.3962	93	0.05%	8.8446	117	0.07%	10.4295	3477	1.95%	5146	9.66%
ph16	UCR_ENT 00127374	Pygovepres vaccinicola	none	Baja	Quercus	dry	2009	0.226	9.04	10	261616	254113	228454	10.10%	10191	4.01%	98.982	218	0.09%	13.355	265	0.10%	15.6146	8531	3.36%	19205	9.58%
ph17	UCR_ENT 00127413	Pygovepres vaccinicola	none	Tehachapi	Quercus	EtOH	2009	4.32	75.6	10	161744	160556	158629	1.20%	786	0.49%	9.87092	116	0.07%	9.11229	132	0.08%	10.8353	2229	1.39%	3263	9.74%
ph18	UCR_ENT 00127414	Pygovepres vaccinicola	none	Tehachapi	Prunus	EtOh	2009	3.18	74.73	10	140057	137063	136356	0.52%	716	0.52%	7.96659	221	0.16%	16.5306	224	0.16%	16.3917	3254	2.37%	4415	9.82%
ph19	AMNH_PBI 00082518	Pygovepres vaccinicola	none	Shasta Co.	Quercus	dry	1984	7.72	77.2	10	195042	191489	167488	12.53%	1688	0.88%	15.8911	1151	0.60%	69.0346	2184	1.14%	110.787	10182	5.32%	15205	9.74%
ph20	UCR_ENT 00127415	Phallospinophylus setosus	none	Cleveland NF	Quercus	EtOH	2010	3.56	71.2	10	248114	245729	214508	12.71%	7434	3.03%	72.8828	380	0.15%	26.3917	527	0.21%	38.7424	10354	4.21%	18695	0.13%
ph21	UCR_ENT 00127375	Phallospinophylus setosus	none	Cleveland NF	Ceanothus	dry	2010	5.54	83.1	10	159940	157529	132835	15.68%	4094	2.60%	37.6226	359	0.23%	25.6961	508	0.32%	35.7781	8974	5.70%	13935	0.07%
ph22	UCR_ENT 00127416	Phallospinophylus setosus	none	Cleveland NF	Prunus	EtOH	2010	1.87	74.8	10	182847	179982	173458	3.62%	3377	1.88%	34.178	301	0.17%	22.728	385	0.21%	27.5876	9703	5.39%	13766	0.13%
ph23	UCR_ENT 00127395	Phallospinophylus setosus	none	San Bernardino NF	Quercus	EtOH	2016	3.5	70	10	175372	174075	161160	7.42%	4609	2.65%	45.4893	295	0.17%	23.5045	563	0.32%	40.8127	6932	3.98%	12399	0.00%
ph24	UCR_ENT 00127396	Phallospinophylus setosus	none	Los Angeles NF	Prunus	EtOH	2009	2.92	73	10	158979	156929	149370	4.82%	3508	2.24%	32.5592	484	0.31%	39.8366	830	0.53%	66.1537	5936	3.78%	10758	0.00%
ph25	UCR_ENT 00127376	Phallospinophylus setosus	none	San Jacinto NF	Prunus?	dry	2010	4.16	74.88	10	187491	183024	92752	49.32%	4265	2.33%	25.8512	389	0.21%	16.2267	565	0.31%	19.4696	7891	4.31%	13110	0.00%
ph26	UCR_ENT 00127397	Phallospinophylus setosus	none	San Jacinto NF	Quercus	EtOH	2009	4.44	75.48	10	201740	198281	133357	32.74%	4106	2.07%	32.4041	306	0.15%	15.2422	461	0.23%	23.259	10519	5.31%	15392	0.00%
ph27	UCR_ENT 00127398	Phallospinophylus setosus	none	Baja	Quercus	EtOH	2009	6.06	72.72	10	203169	199019	99699	49.90%	4010	2.01%	24.0353	682	0.34%	28.8785	1128	0.57%	35.8972	13222	6.64%	19042	1.12%
ph28	UCR_ENT 00127399	Phallospinophylus setosus	none	Tehachapi	Quercus	EtOH	2009	4.26	72.42	10	243084	236322	65515	72.28%	NA†	NA†	NA†	NA†	NA†	NA†	NA†	NA†	NA†	NA†	NA†	NA†	NA†
ph29	AMNH_PBI 00082453	Phallospinophylus setosus	none	Tulare Co.	Quercus	dry	1978	3.44	51.6	25	174628	164972	155883	5.51%	9744	5.91%	77.2913	1059	0.64%	57.5104	1626	0.99%	83.4698	25175	15.26%	37604	1.79%
ph30	AMNH_PBI 00082567	Phallospinophylus setosus	none	Tulare Co.	Ceanothus	dry	1978	2.52	37.8	25	231106	215919	194434	9.95%	4413	2.04%	36.0177	3048	1.41%	124.559	3756	1.74%	140.556	39847	18.45%	51064	1.66%
ph31	AMNH_PBI 00082575	Phallospinophylus setosus	none	San Bernardino NF	Ceanothus	dry	1977	0.674	10.11	25	137771	129184	115784	10.37%	11023	8.53%	89.2148	1973	1.53%	97.604	3688	2.85%	150.804	27180	21.04%	43864	0.00%
ph32‡	UCR_ENT 00127400	Phallospinophylus setosus	none	Los Angeles NF	Prunus	EtOH	2016	2.26			477874	488748	188234	61.49%													
ph33	UCR_ENT 00061245	Phallospinophylus setosus	none	Los Angeles NF	Quercus	dry	2009	4.3	64.5	25	117627	115080	108265	5.92%	8019	6.97%	69.6609	1096	0.95%	72.8249	1913	1.66%	107.528	24564	21.35%	35592	0.00%
ph34	UCR_ENT 00127401	Insulaphylus sp.	none	Los Angeles NF	Quercus	EtOH	2016	4.38	74.46	25	158420	157638	155235	1.52%	2556	1.62%	34.8036	NA†	NA†	NA†	3038	1.93%	221.822	3229	2.05%	8823	14.64%
ph35	UCR_ENT 00127402	Rubellomiris bispinosus	none	San Jacinto NF	Quercus	EtOH	2009	1.83	73.2	25	113379	115573	112310	2.82%	3755	3.25%	60.1884	NA†	NA†	NA†	2166	1.87%	141.893	4834	4.18%	10755	13.55%
ph36	UCR_ENT 00127403	Tuxedo flavicollis	none	San Gabriels	Ceanothus	EtOH	2016	0.902	36.08	10	92094	91688	90313	1.50%	221	0.24%	4.17547	461	0.50%	35.7073	533	0.58%	41.2995	31	0.03%	1246	20.38%
ph37	UCR_ENT 00127404	Tuxedo cruralis	none	Baja	Ceanothus	EtOH	2009	2.2	74.8	10	152070	150714	147489	2.14%	1022	0.68%	12.9058	289	0.19%	23.1464	240	0.16%	18.2607	12	0.01%	1563	18.62%
ph38	UCRC ENT 277960	Tuxedo cruralis	none	Santa Catalina	Cercocarpus	dry	2008	1.55	62	10	189047	187115	184503	1.40%	996	0.53%	13.0652	550	0.29%	42.5184	609	0.33%	42.9142	22	0.01%	2177	19.51%
ph39	UCR_ENT 00061761	Tuxedo cruralis	none	Los Padres	Ceanothus	dry	1977	1.4	21	10	121381	117839	115773	1.75%	660	0.56%	7.66924	558	0.47%	37.8137	673	0.57%	42.8548	20	0.02%	1911	19.39%
ph40	UCR_ENT 00127377	Tuxedo cruralis	none	Cleveland	Cercocarpus	dry	2010	1.12	44.8	10	166127	156472	154217	1.44%	380	0.24%	4.38708	240	0.15%	17.3591	321	0.21%	19.2929	15	0.01%	956	19.08%
ph41	UCR_ENT 00127378	Tuxedo cruralis	none	Los Padres	Quercus	dry	2007	0.106	4.24	10	199572	193698	176738	8.76%	30296	15.64%	258.987	231	0.12%	16.5828	249	0.13%	17.012	8	0.00%	30784	18.97%
ph42	UCR_ENT 00124208	Tuxedo elongatus	paratype	Siskiyou	Chrysolepis	dry	1985	0.11	2.75	10	154837	149029	136729	8.25%	3401	2.28%	32.7475	295	0.20%	23.6585	305	0.20%	28.5825	5	0.00%	4006	20.65%
ph43	UCR_ENT 00124209	Tuxedo nicholi	none	Arizona	Quercus	dry	1983	0.566	14.15	10	128082	124762	122707	1.65%	988	0.79%	10.5853	580	0.46%	37.5604	607	0.49%	38.2207	50	0.04%	2225	20.46%
ph44	AMNH_PBI 00271922	Ausejanus albisignatus	none	Australia		dry	1999	0.142	5.68	10	122419	118609	117116	1.26%	933	0.79%	8.41977	213	0.18%	15.193	210	0.18%	14.3961	1	0.00%	1357	15.31%
ph45	AMNH_PBI 00234761	Pseudophylus stundjuki	none	Russia	Pyrus domestica	dry	1963	0.618	15.45	10	128411	124971	122725	1.80%	1764	1.41%	17.2437	174	0.14%	11.8394	382	0.31%	12.1518	10	0.01%	2330	17.60%
ph46	AMNH_PBI 00322474	Tuxedo drakei	none	Kern Co.	Fremontodendron	dry	2004	3.16	72.68	10	217404	212868	200164	5.97%	8941	4.20%	76.7723	689	0.32%	50.1347	828	0.39%	55.9677	324	0.15%	10782	0.66%
ph47‡	UCR_ENT 00127405	Tuxedo drakei	none	San Gabriels	Fremontodendron	EtOH	2016	3.1			437390	415926	175841	57.72%													
ph48	UCR_ENT 00127379	Tuxedo bicinctus	none	San Gabriels	Cercocarpus	dry	2009	0.234	9.36	10	218974	211310	206211	2.41%	1966	0.93%	21.359	1226	0.58%	74.2586	1482	0.70%	79.4659	101	0.05%	4775	17.31%
ph49	UCR_ENT 00127406	Tuxedo bicinctus	none	San Bernardino	Cercocarpus	EtOH	2016	1.21	48.4	10	167268	165486	161883	2.18%	1146	0.69%	14.2143	1065	0.64%	78.7786	1187	0.72%	84.441	88	0.05%	3486	17.37%
ph50	UCR_ENT 00127380	Tuxedo bicinctus	none	~Cleveland	Ceanothus	dry	1979	1.53	22.95	10	247749	242705	235526	2.96%	1048	0.43%	10.2613	1753	0.72%	103.295	1879	0.77%	103.383	64	0.03%	4744	17.65%
ph51	AMNH_PBI 00303164	Tuxedo bicinctus	none	Kern Co.	Cercocarpus	dry	2004	1.51	60.4	10	199541	197218	194672	1.29%	1937	0.98%	20.821	308	0.16%	23.9846	363	0.18%	26.5047	52	0.03%	2660	18.35%
ph52	UCR_ENT 00127407	Tuxedo flavicollis	none	San Gabriels	Quercus	EtOH	2011	0.936	37.44	10	151620	151613	150329	0.85%	1751	1.15%	20.5295	606	0.40%	51.3449	285	0.19%	21.7794	10	0.01%	2652	20.78%
ph53	UCR_ENT 00127417	Tuxedo flavicollis	none	Baja	Quercus	EtOH	2009	1.09	43.6	10	100264	99989	98713	1.28%	1231	1.23%	15.3749	842	0.84%	72.2523	872	0.87%	70.0371	14	0.01%	2959	20.35%
ph54	UCR_ENT 00124210	Tuxedo flavicollis	none	San Luis Obispo	Quercus	dry	1985	4.52	67.8	10	169211	164906	163348	0.94%	1463	0.89%	13.5354	1039	0.63%	64.6738	943	0.57%	57.8907	22	0.01%	3467	20.40%
ph55	UCR_ENT 00127418	Tuxedo flavicollis	none	Tehachapi	Quercus	EtOH	2009	0.846	33.84	21	150306	149362	129184	13.51%	996	0.67%	12.804	10655	7.13%	562.762	13241	8.87%	609.559	67	0.04%	24959	20.60%
ph56	UCR_ENT 00127419	Tuxedo flavicollis	none	Los Padres	Quercus	EtOH	2007	1.21	48.4	21	119391	120440	116373	3.38%	2946	2.45%	45.1772	2757	2.29%	233.799	3444	2.86%	251.834	77	0.06%	9224	20.42%
ph57	UCR_ENT 00127420	Tuxedo nr susansolomonae	none	Baja	Quercus	EtOH	2009	0.914	36.56	21	180677	182114	175173	3.81%	4351	2.39%	56.4749	2828	1.55%	222.789	3531	1.94%	250.415	69	0.04%	10779	20.30%
ph58	UCR_ENT 00127412	Tuxedo susansolomonae	none	San Gabriels	Quercus	EtOH	2016	0.696	27.84	21	147887	147486	137036	7.09%	1576	1.07%	22.7188	8088	5.48%	513.552	6378	4.32%	367.853	69	0.05%	16111	20.35%
ph59	UCR_ENT 00127381	Tuxedo susansolomonae	paratype	San Jacinto	Quercus	dry	1976	0.772	19.3	21	183084	176924	173139	2.14%	951	0.54%	10.0994	2635	1.49%	159.152	3126	1.77%	166.138	26	0.01%	6738	20.26%
ph60	AMNH_PBI 00272096	Leucophoroptera quadrimaculata	none	Australia		dry	2004	2.96	74	21	225540	218222	203023	6.96%	3206	1.47%	33.3436	3008	1.38%	159.906	NA†	NA†	NA†	17	0.01%	6231	19.16%
Second sequencing run																											
ph61	AMNH_PBI 00234872	Pseudophylus stundjuki	none	Russia		dry	1985	8.03	40.15		120233	118580	57411	51.58%	809	0.68%	4.23413	3442	2.90%	96.6703	5987	5.05%	87.6388	NA	NA	10238	17.83%
ph45	AMNH_PBI 00234761	Pseudophylus stundjuki	none	Russia	Pyrus domestica	dry	1963	0.618	15.45		229282	226703	112806	50.24%	5330	2.35%	23.4212	2543	1.12%	70.8435	5503	2.43%	76.2825	NA	NA	13376	17.60%
ph59	UCR_ENT 00127381	Tuxedo susansolomonae	paratype	San Jacinto	Quercus	dry	1976	0.772	19.3	500	111690	110430	52175	52.75%	500	0.45%	3.17897	6064	5.49%	163.543	6205	5.62%	157.164	NA	NA	12769	20.26%
ph54	UCR_ENT 00124210	Tuxedo flavicollis	none	San Luis Obispo	Quercus	dry	1985	4.52	67.8		150714	149727	64773	56.74%	1320	0.88%	5.81821	5143	3.43%	148.684	5103	3.41%	139.368	NA	NA	11566	20.40%
ph57	UCR_ENT 00127420	Tuxedo nr susansolomonae	none	Baja	Quercus	EtOH	2009	0.914	36.56		248967	255061	103903	59.26%	5654	2.22%	36.255	9152	3.59%	434.985	9206	3.61%	407.608	NA	NA	24012	20.30%

† Excluded from calculations and analyses due to sample cross contamination

‡ For samples ph32 and ph47 amplicons used for bait synthesis were sequenced

620 Figure 1. Procedure flowchart.

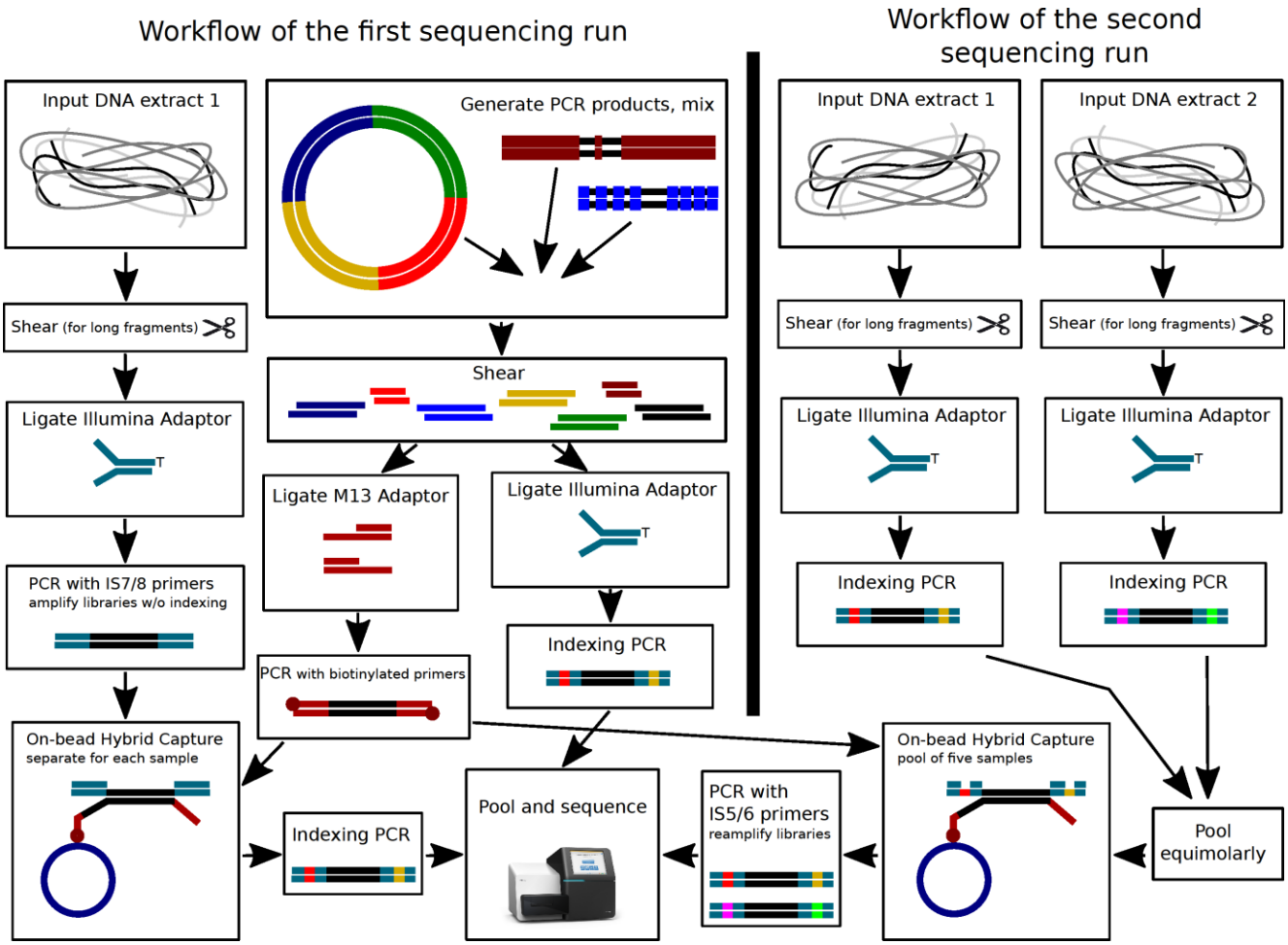

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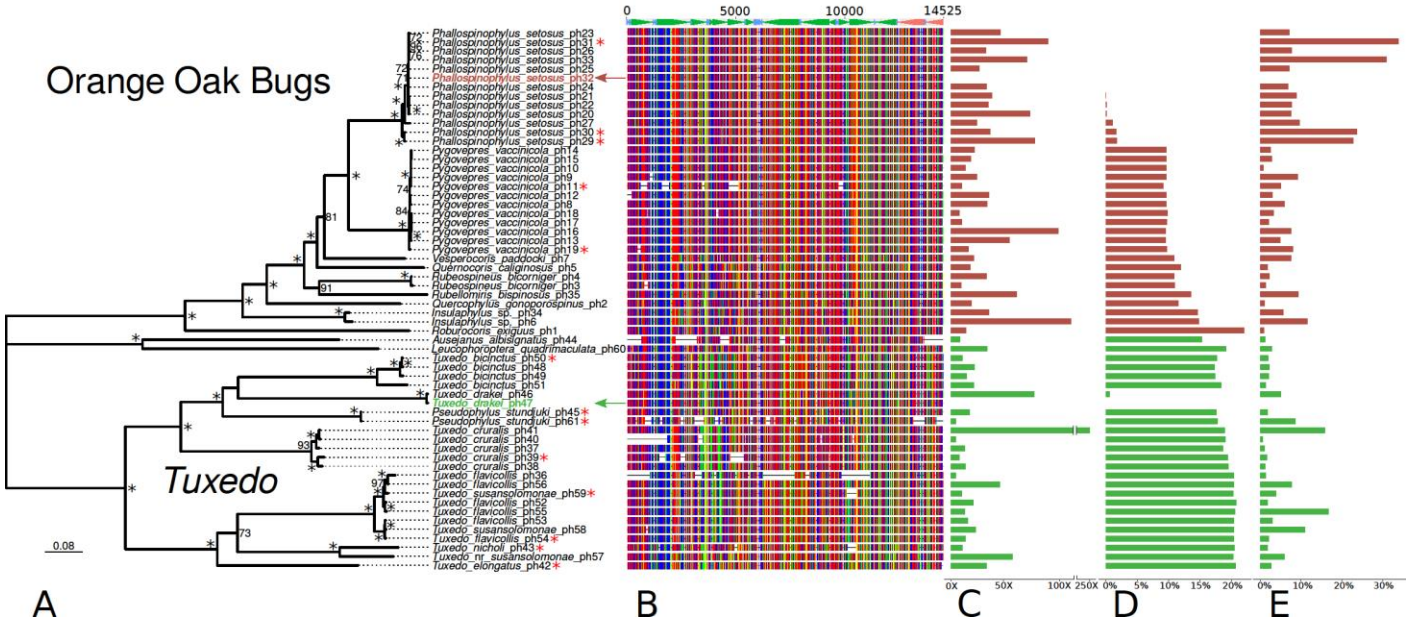
Figure 2. A. Combined phylogeny of the OOB and *Tuxedo* subprojects, generated in RAxML, values at nodes represent Rapid Bootstrap Support, values below 70 are not shown, asterisks indicate full support, arrows denote bait samples for the OOB (red) and *Tuxedo* (green) subprojects, red asterisks denote samples older than 20 years. B. Mitochondrial alignment completeness, control region excluded. C. Average coverage of mitochondrial contig(s), control region excluded. D. Pairwise COX1 distances between a bait and a captured sample. E. Total percent of reads mapping to target including mitochondrial genome, 18S, 28S, and dynein.



633 Figure 3. Host and distribution data for the Orange Oak Bug subproject, aligned with phylogeny
634 (branches not to scale) and with host plant of specimens mapped using representative leaf shapes
635 of plant genus.

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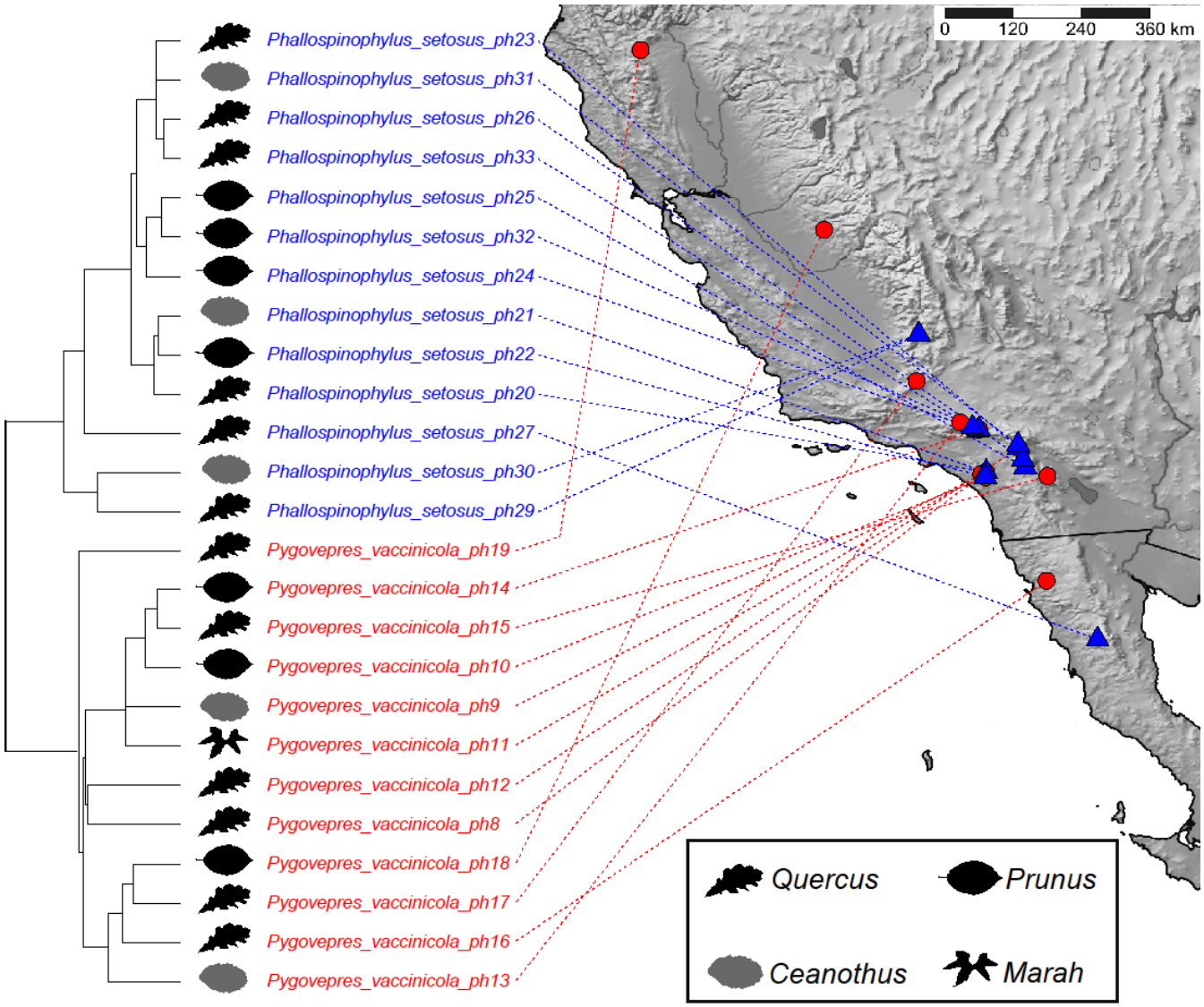

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Figure 4. Phylogeny of Phylinae, generated in RAxML, with specimens for which new data was gathered in bold font, values at nodes represent Rapid Bootstrap Support, values below 70 not shown.