Angiosperm to Gymnosperm host-plant switch entails shifts in microbiota of the *Welwitschia* bug, *Probergrothius angolensis* (Distant, 1902)

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Abstract
The adaptation of herbivorous insects to new host plants is key to their evolutionary success in diverse environments. Many insects are associated with mutualistic gut bacteria that contribute to the host’s nutrition and can thereby facilitate dietary switching in polyphagous insects. However, how gut microbial communities differ between populations of the same species that feed on different host plants remains poorly understood. Most species of Pyrrhocoridae (Hemiptera: Heteroptera) are specialist seed-feeders on plants in the family Malvaceae, although populations of one species, *Probergrothius angolensis*, have switched to the very distantly related *Welwitschia mirabilis* plant in the Namib Desert. We first compared the development and survival of laboratory populations of *Pr. angolensis* with two other pyrrhocorids on seeds of *Welwitschia* and *Malvaceae* host plants, respectively, to assess their bacterial and fungal community profiles using high-throughput amplicon sequencing. Comparison with long-term laboratory-reared insects indicated stable associations of *Pr. angolensis* with core bacteria (*Commensalibacter*, *Enterococcus*, *Bartonella* and *Klebsiella*), but not with fungi or yeasts. Phylogenetic analyses of core bacteria revealed relationships to other insect-associated bacteria, but also found new taxa indicating potential host-specialized nutritional roles. Importantly, the microbial community profiles of bugs feeding on *Welwitschia* versus *Malvaceae* revealed stark and consistent differences in the relative abundance of core bacterial taxa that correlate with the host-plant switch; we were able to reproduce this result through feeding experiments. Thus, a dynamic gut microbiota may provide a means for insect adaptation to new host plants in new environments when food plants are extremely divergent.

Keywords
adaptation, bacteria, development and evolution, insects, life history evolution, microbial biology
1 | INTRODUCTION

Microbial symbionts are known to facilitate insect adaptation to novel foods and environments (Sudakaran, Kost, & Kaltenpoth, 2017) and can be especially important for supplementing limited nutrients to insects that feed on imbalanced food sources (Douglas, 2009; Feldhaa, 2011; Frago, Dicke, & Godfray, 2012; Hansen & Moran, 2014; Oliver & Martinez, 2014; White, Giorgini, Strand, & Pennacchio, 2013). Microbial symbionts, for example, allow insects to break down complex plant polymers (Brune, 2014; Cragg et al., 2015; Salem et al., 2017), supplement vitamins and amino acids (Douglas, 2009), protect against abiotic stressors such as temperature and desiccation (Brumin, Kontsedalov, & Ghanim, 2011; Engl et al., 2017; Montllor, Maxmen, & Purcell, 2002; Russell & Moran, 2006), detoxify plant secondary metabolites and other substances (Berasetegui et al., 2017; Kikuchi et al., 2012; Shen & Dowd, 1991), and influence the spectrum of host plants that can be fed upon (Tsuda, Koga, & Fukatsu, 2004; Wagner et al., 2015). Similarly, shifts in host plant utilization between insect populations or species can be mediated by bacterial symbionts (Chu, Spencer, Curzi, Zavala, & Seufferheld, 2013; Henry et al., 2013; Hosokawa, Kikuchi, Shimada, & Fukatsu, 2007), and selection on individual bacteria can influence the invasive potential of their host (Brown, Huynh, Bolender, Nelson, & McCutcheon, 2014). Gut microbial communities can be especially diverse in composition and function (Douglas, 2016; Engel & Moran, 2013) and, as a particularly dynamic part of the insect, they may be able to aid in even more rapid adaptation. A better understanding of the shifts in microbial taxa that influence their hosts’ ability to adapt to new foods and environments is relevant for understanding insect ecology and evolution and elucidating the success of particular taxa in changing environments or upon invasion of new habitats.

The heteropteran family Pyrrhocoridae, commonly known as “firebugs,” “red bugs” and “cotton strainers,” consists of over 300 currently described, mostly Old World, species that primarily feed on seeds from malvaceous plants such as Gossypium (cotton), Tilia (linden), Hibiscus, Adansonia (baobab), Ceiba (kapok) and Sterculia (tropical chestnut) (Panizzi & Grazia, 2015; Schaefer & Ahmad, 2000). These seeds are chemically defended and toxic to many animals (Nixon, Eisele, Wales, & Sinnhuber, 1974; Rani & Rajasekharreddy, 2009, 2010; Schmid & Patterson, 1988; Schneider, Sheehan, Vavich, & Kemmerer, 1968), yet pyrrhocorids have evolved to utilize them as their primary nutrition source. In the process, the gut microbial community of Pyrrhocoridae has diverged considerably from that of their sister families (Gordon, McFrederick, & Weirauch, 2016; Kikuchi, Hosokawa, & Fukatsu, 2011; Sudakaran, Retz, Kikuchi, Kost, & Kaltenpoth, 2015). Many species of pyrrhocorids from multiple genera maintain a characteristic, nutritionally important, gut microbiota (Salem et al., 2014; Salem, Kreutzer, Sudakaran, & Kaltenpoth, 2013; Sudakaran et al., 2015). However, the microbial communities of a more ancestral genus Probergothius were notably different from the rest of the Pyrrhocoridae, mostly lacking the Coriobacteriaceae symbionts that are involved in B-vitamin provisioning (Salem et al., 2014; Sudakaran et al., 2015).

Probergothius angolensis (Distant, 1902), like most other pyrrhocorids, feeds on the seeds of plants in the family Malvaceae, although isolated populations within its native range of southwest Africa are found on Welwitschia mirabilis (Hooker, 1863) plants in and around the Namib Desert (Figure 1). These insects are the only members of the hemipteran suborder Heteroptera known to subsist exclusively on the seeds of Welwitschia. Welwitschia mirabilis is the only extant species of its family, Welwitschiaceae, and represents one of only three extant genera in the gymnosperm phylum Gnetophyta, separated from Malvaceae plants by at least 300 million years of evolution (Clarke, Warnock, & Donoghue, 2011). In both cases, the insects feed on the seeds of the plants, so there are probably some similarities in nutritional content and even known similarities in rare toxic fatty acids (Aitzetmüller & Vosmann, 1998). However, given such a large evolutionary distance between host plants, Pr. angolensis probably has to contend with differences in nutritional composition and plant defences (Chen, 2008; Fürstenberg-Hägg, Zagrobelny, & Bak, 2013; War et al., 2012), which may be especially strong in desert plants that need to protect their limited resources (Coley, Bryant, & Chapin, 1985).

In Namibia, Pr. angolensis is best known for feeding on Welwitschia plants, but populations have also been reported to live outside the range of Welwitschia on Sterculia and Adansonia trees. It remains unclear, however, whether these bugs require special adaptations to utilize such different host plants as nutritional resources, so we first compare their developmental success on Welwitschia and three Malvales plants, respectively, with two other pyrrhocorid species: the African cotton stainer (Dysdercus fasciatus) that is known to feed on both Sterculia and Adansonia, and the European firebug (Pyrrhocoris apterus) that feeds on a variety of European Malvaceae, especially Tilia. Then, because nutrient-provisioning symbiotic microbes are extremely common within the insect order Hemiptera, we used a combination of experimental assays, field collections, and high-throughput bacterial and fungal amplicon sequencing, to determine whether microbial communities differ between populations of Pr. angolensis feeding on Malvaceae plants versus on Welwitschia and may be involved in this divergent host plant adaptation.

2 | MATERIALS AND METHODS

2.1 | Study organisms and lab rearing

Our laboratory colony of Pr. angolensis was originally collected from Welwitschia plants in Namibia in 2011 (Table 1) and has since been maintained in large colonies that are provided with sand substrate, autoclaved water and an artificial diet (Table S1) that is occasionally supplemented with seeds from Tilia spp., Adansonia spp., Sterculia spp. and Welwitschia. All cages and experiments are kept at a constant 28°C and 60% relative humidity with 16-hr light/8-hr dark cycles in environmental incubators (Fitotron).

Two other species of Pyrrhocoridae are also maintained in large colonies under the same conditions as Pr. angolensis, except
that both are fed a continuous supply of seeds from *Tilia* trees. European firebugs, *Py. apterus* (Linnaeus, 1758), were collected from *Tilia* spp. in Mainz, Germany (continuously collected), and African cotton stainers, *D. fasciatus* (Signoret, 1861), were collected from the Ivory Coast in 2001. Cotton stainers, including *D. fasciatus*, are widespread throughout sub-Saharan Africa and are known to generalize on several plants in the family Malvaceae, including *Gossypium*, *Hibiscus*, *Adansonia* and *Sterculia*. The European firebug, *Py. apterus*, is common throughout central Europe and is known to be closely associated with *Tilia* trees, but can also be found on other minor Malvaceae hosts such as *Althaea, Lavatera, Malva, Hibiscus* and *Urocarpidium*, among other species in this plant family (Schaefer & Ahmad, 2000).

In recent decades, there has been some confusion over the species identity of the *Welwitschia* bug. Multiple species of *Probergrothius* (formerly *Odontopus*) are widely distributed throughout sub-Saharan Africa (Robertson, 2004) and many of the characteristics used to define each species, such as size and colour patterns, are variable, so a modern revision of this genus may be warranted. The *Welwitschia* bug, *Pr. angolensis*, was first described in 1902 from Angola, southwest Africa (Distant, 1902) and the morphological characteristics and geographical range of specimens collected and reared for our study align with this description. Due to physical similarities, it has also been referred to as *Probergrothius sexpunctatus* (e.g., Cooper-Driver, Wagner, & Kolberg, 2000; Costa, 1995, 2012; Wetschnig & Depisch, 1999) but this is probably a misnomer because *Pr. sexpunctatus* was
first described in 1832 from Senegal in northwest Africa (Laporte de Castelnaud, 1833), outside of the range of the *W. mirabilis* plant, found only in Namibia and Angola in southwest Africa, and *Pr. sexpunctatus* has never been reported from Namibia.

### Table 1  Probergrothius angolensis collection locations in Namibia

| Location ID | GPS               | Location description                        | Host plant information          |
|-------------|-------------------|--------------------------------------------|---------------------------------|
| 1           | 23°36′58.2″S 15°10′19.7″E | Namib-Naukluft Park, Welwitschia Wash, Gobabeb Research Station | On *Welwitschia mirabilis*       |
| 2           | 22°46′52.0″S 14°55′08.0″E | South of Welwitschia Plain | On *W. mirabilis*                |
| 3           | 22°39′02.0″S 15°01′30.0″E | Welwitschia Plain | On *W. mirabilis*                |
| 4           | 21°31′57.0″S 15°45′42.0″E | Erongo Plateau, SW of Omaru | On *Sterculia* sp.               |
| 5           | 18°02′48.0″S 13°49′59.0″E | Near Opuwo | On *Sterculia* sp.               |
| 6           | 18°20′43.0″S 13°46′34.0″E | On C43 S. of Opuwo, ‘Baobab Bend’ | On *Colophospermum* bark, scavenging seeds from several plants (*Welwitschia* nearby but heavily grazed by livestock) |
| 7           | 20°26′22.0″S 14°36′21.0″E | Torra Conservancy, Petrified Forest nr. C39 | On *Adansonia digitata*          |
| 8           | 20°14′01.0″S 13°52′31.0″E | Torra Conservancy, 26 km E. of Springbokwasser on C39 | On *W. mirabilis*                |
| 9           | 20°15′26.0″S 13°45′08.0″E | Torra Conservancy, 12 km E. of Springbokwasser on C39 | On *W. mirabilis*                |
| 10          | 20°21′53.0″S 13°26′01.0″E | Torra Conservancy, Skeleton Coast Park on C39 | On *W. mirabilis*                |
| 11          | 23°34′11.0″S 15°17′05.0″E | Namib-Naukluft Park, Gobabeb near Hope Mine Ruins | On *W. mirabilis*                |
| 12          | 23°24′39.0″S 16°03′33.0″E | Rooiklip Guest Farm, Near Gamsberg Nature Reserve | On *Sterculia* sp.               |
| 13          | 23°24′22.1″S 16°03′38.4″E | Rooiklip Guest Farm, Near Gamsberg Nature Reserve | On *Vachellia/Senegalia* bark, near *Sterculia* |
| Laboratory colony | 22°38′S 15°10′45″E | Welwitschia Plain | On *W. mirabilis*                |

To determine whether the capacity of feeding on *Welwitschia* seeds is unique to *Pr. angolensis*, we measured the survival and fresh weight of three pyrrhocorid species, *Pr. angolensis*, *Py. apterus* and *D. fasciatus*, fed on seeds from Malvaceae plants, *Adansonia*, *Sterculia* and *Tilia*, and also seeds from *Welwitschia*. For use in all laboratory experiments, *Adansonia* and *Sterculia* seeds were purchased from a commercial supplier (www.rarepalmsseeds.com), *Tilia* seeds were collected locally (Mainz, Germany) and *Welwitschia* seeds were collected near the Gobabeb Research Station (Table 1, Location 11). For each seed-diet, five replicates of 10–12 third-instar nymphs were placed in small cages with sterile sand substrate and were provided with a constant supply of seeds and water for each respective treatment. All seed shells were cracked open prior to feeding. Life stage, fresh weight and survival were recorded after 25 days for *Py. apterus* and *D. fasciatus*, but were recorded at 40 days for *Pr. angolensis* due to its longer development time due to its larger size. Results were analysed using Kruskal–Wallis and Steel–Dwass tests for pairwise multiple comparisons (JMP-Statistical-Software, 2016).

### 2.3 Collection of *Pr. angolensis* on different host plants

Specimens of *Pr. angolensis* were collected on *W. mirabilis*, *Adansonia digitata* and *Sterculia quinqueloba* in Namibia in April 2017 (see Table 1 for detailed collection information). All specimens were collected live and immediately preserved in 75% ethanol for transport, then stored at −20°C until DNA extraction. Note that four individuals, collected near *Welwitschia* plants (Table 1: Location 7), were categorized as Malvaceae-feeding because the *Welwitschia* plants in this area had been heavily grazed by livestock, removing all of the seed cones and most leaves; these insects were not found on or near *Welwitschia* and were foraging on seeds from other host plants. This location (Table 1: Location 7) was the most heterogeneously vegetated area that also contained *Welwitschia* plants that we encountered, providing more secondary hosts for the insects.

### 2.4 Taxonomic comparisons of *Pr. angolensis* populations

To determine whether *Pr. angolensis* populations that feed on *Welwitschia* are taxonomically distinct from those found on other food sources, we polymerase chain reaction (PCR)-amplified DNA from a single individual from each collection location: eight from *Welwitschia*, four from *Sterculia* and one from *Adansonia*. Nine variable gene regions that are commonly used for taxonomic identification
of insects were amplified and sequenced: cytochrome oxidase 1, cytochrome oxidase 2, cytochrome B, mitochondrial 16S, homeobox (scr, abd-A, dfd-2), and internal transcribed spacers (ITS) 1 and 2 of the rRNA operon. Heterozygous portions of ITS1 and 2 were removed prior to identity calculations and phylogenetic inferences. Primers and their references, including reaction conditions, are presented in Table S2. Sequences were aligned using MUSCLE and trimmed to the same length before calculating the percentage identity between sequences, then concatenated (4,163-bp total alignment) for phylogenetic reconstruction (GENEIOUS 9.17). Substitution models for each gene were selected using jmodeltest (Darriba, Taboada, Doallo, & Posada, 2012) and phylogenies were inferred using MrBayes (Huelsenbeck & Ronquist, 2001) with subsampling every 100 generations; substitution models on concatenated gene sequences were partitioned on individual genes.

### 2.5 Bacterial and fungal community profiling

Prior to DNA extraction, legs and wings were removed from each preserved individual, and the body was dipped in absolute ethanol and briefly placed in a flame to eliminate any surface contaminants. DNA from whole insects was extracted using the EZNA Insect DNA Kit (Omega Bio-Tek) and stored at −20°C. Our methodology cannot exclude the possibility that some of the bacteria detected are present outside the gut in other insect tissues, although we discuss them as gut microbiota based on their detection in gut dissections and faeces using quantitative PCR (qPCR) as well as culture-based methodologies.

For bacterial community analysis of Namibia-collected specimens, the ~410-bp V4–V5 region of the 16S rRNA gene from a maximum of two males, two females and two fifth-instar nymphs (individually) from each of 13 different Pr. angolensis populations (62 individual samples in total, plus a negative control), was sequenced on an Illumina Miseq platform (StarSEQ), using v3 chemistry and universal bacterial 16S primers 515F and 909R (Table S2). Amplicons were generated using a high-fidelity polymerase (AccuStart II PCR ToughMix; Quantabio). The amplicons were then normalized to equimolar concentrations using a high-fidelity polymerase (AccuStart II PCR ToughMix; Quantabio). Libraries were mixed with Illumina-generated PhiX control libraries and denatured using fresh NaOH. 25% PhiX was used to balance the runs and 600-bp V3 chemistry was used for sequencing. We used MISEQ CONTROL SOFTWARE version 2.6, RTA version 1.18.54, MISEQ REPORTER version 2.6, BASESPACE BROKER version 2.5.3 and MISEQ RECIPES version 3.1.0.

Results were analysed using qiime2 version 2017.9 (Caporaso et al., 2010), and the full analysis pipeline can be accessed online (see Data Availability Statement). Sequencing yielded a total of 3,494,684 reads after quality filtering, with an average of 57,254 sequences per sample. Briefly, paired-end reads were paired, denoised and chimera-filtered using DADA2 (Callahan et al., 2016), unique sequences only present in a single sample were removed, taxonomy was assigned using the Silva release_128 (28 September 2016) 99% consensus-clustered database (Quast et al., 2012; Yilmaz et al., 2013), and sequences identified as chloroplast, mitochondria or host DNA were removed. Finally, after all other quality filtering steps, sequences representing <0.01% of total reads were removed. For principal coordinate analysis (PCoA) of microbial communities, the samples were first normalized by rarefaction to 15,000 sequences per sample, which excluded a single sample. Sequences were then aligned with MAFFT (Katoh & Standley, 2013) and a phylogenetic tree was generated using FASTTREE (Price, Dehal, & Arkin, 2010). Results were analysed with Jaccard, Bray–Curtis and Weighted UniFrac beta diversity metrics and visualized using EMPEROR (Vázquez-Baeza et al., 2017). Generalized linear models (GzLMs) with binomial distribution and logit link functions were used to analyse the proportions of selected bacterial taxa using host plant (Welwitschia vs. Malvaceae) and life stage (fifth-instar nymph vs. adults) as variables. GzLM data were overdispersed, so final results are reported with a quasibinomial adjustment (Computer software, 2016). Finally, infection frequencies of five amplicon sequence variants (ASVs) of bacteria identified as Commensalibacter were compared between Welwitschia (n = 29) and Malvaceae (n = 33) populations using Fisher’s exact test.

Microbiota profiling by 16S rRNA analyses of our laboratory colony was done separately from wild-caught specimens. DNA from eight individuals was pooled for each life stage: first to fifth instars, respectively, adult males and adult females. Illumina sequencing resulted in a total of 658,586 sequences after quality filtering, which were then analysed as above, without subsequent PCoA diversity analyses.

Fungal internal transcribed spacer (ITS) regions of field-collected and laboratory-reared specimens were also sequenced using Illumina Miseq version 3 chemistry and universal fungal primers ITS1F and ITS2 (Table S2). The same Pr. angolensis samples as above were used, but were pooled into 10 samples based on collection location (Table 1); however, due to their relative proximity and same host plant, locations 1/11, 8/9 and 12/13 were combined prior to analyses. Sequencing yielded a total of 44,533 sequences after quality filtering. Due to the variable length of this region, only forward sequences were analysed using qiime2 version 2018.2 (Caporaso et al., 2010). Forward reads were trimmed of any reverse primer and subsequent sequence using cutadapt (Martin, 2011) and de-noised as well as chimera-checked with DADA2 as above. Taxonomy was assigned using the UNITE 01.12.2017 (dynamic) fungal ITS database (requiring at least 90% identity and 75% coverage), and host reads were removed (UNITE-Community, 2017).

### 2.6 Phylogeny of Bartonella, Klebsiella, Commensalibacter and Enterococcus symbionts

Almost complete 16S rRNA sequences were obtained through a combination of amplicon cloning/sequencing and culture-based methods. For the latter, the whole midgut region of Pr. angolensis was dissected out and homogenized in lysogeny broth (LB) medium, serially diluted, plated on LB and Columbia agar (CA) + 5% defibrinated sheep’s blood, and maintained at 37°C under both aerobic and microaerophilic conditions. The dominant ASV of Klebsiella was
isolated on LB, with small beige colonies visible after 24 hr under both aerobic and microaerophilic conditions. The dominant ASV of Bartonella was originally isolated on a 1:1 mixture of Grace’s medium, TC100 and SF900 plates, and small white colonies were visible after 3–4 days under aerobic conditions; we subsequently found that this strain of Bartonella would also grow on CA blood plates or LB plates under aerobic conditions. The dominant ASV of Enterococcus was isolated on CA plates, with small beige colonies were visible after 24 hr under aerobic conditions. The 16S rRNA sequences from both Klebsiella and Bartonella were amplified using primers 27f and 1525r, and Enterococcus was amplified using 27f and 909R (Table S2) and sequenced in both directions on an Applied Biosystems 3130XL Bioanalyzer. Identification of dominant strains was done by comparison of isolate 16S sequences to the most abundant 16S ASV 3130XL Bioanalyser. Identification of dominant strains was done by comparison of isolate 16S sequences to the most abundant 16S ASV

3.1 | Only Pr. angolensis can subsist on Welwitschia seeds

The seeds of Welwitschia and three Malvaceae plants (Adansonia, Sterculia and Tilia) were fed to three species of Pyrrhocoridae (Py. apterus, D. fasciatus and Pr. angolensis), and their survival, developmental stage and fresh weight were measured to determine performance. When fed the Malvaceae seeds, we found variation in survival and fresh weight depending on the seed type (Figure 2), but all three pyrrhocorid species were capable of developing to adulthood even when fed on seeds not found in their native region—Tilia seeds are non-native for Pr. angolensis and D. fasciatus, and Sterculia/Adansonia seeds are non-native for Py. apterus. When fed Welwitschia seeds, we found that Pr. angolensis had the highest survival rate and the majority of individuals reached adulthood (Figure 2a), whereas D. fasciatus (Figure 2b) and Py. apterus (Figure 2c) suffered their lowest survival rates and none of the individuals reached adulthood, indicating that these species are incapable of completing their development on Welwitschia seeds.

2.7 | Recapitulating effects of diet on microbiota composition

We then reared Pr. angolensis on the seeds of different host plants to determine whether diet influences the differential abundance of bacteria observed in field populations. We reared same-day hatchlings of Pr. angolensis together with adults on artificial diet (Table S1) and water until they reached the third instar (10 days), thereby ensuring a similar initial gut inoculum. These nymphs were then split into three treatments that differed in being fed dried Welwitschia, Sterculia or Adansonia seeds, respectively. They were reared on each seed diet for 40 days (when most had reached adulthood). DNA was then extracted from whole insects and qPCR was performed with symbiont-specific primers (Table S2). All DNA extractions for this experiment were performed identically, at the same time, and with the same final elution volume (300 µl). Copies per insect (copies/µl x 300 µl) measured here with qPCR represents the estimated absolute abundance from these DNA extractions. Absolute quantification standard curves (10^3–10^5 copies/µl) were created for the primer sets of each measured bacterium for comparison in qPCR. qPCR conditions were as follows (on Qiagen Rotorgene Q): [one cycle] 95°C (5 min); [six cycles] 95°C (15 s), 70–65°C Touchdown (−1°C per cycle, 20 s), 72°C (15 s); [34 cycles] 95°C (15 s), 64°C (20 s), 72°C (15 s); 65–99°C melting curve (increasing 0.5°C every 5 s).

To make sure that the seeds did not contain the bacteria being measured, 20 of each seed type were pooled, ground in liquid nitrogen and homogenized in sterile water. A 1-ml aliquot from each was taken and centrifuged in 1.5 -ml microcentrifuge tubes at 7,000 g for 1 min. DNA was extracted from the water portion of the supernatant using the same kit used previously, EZNA Insect DNA Kit (Omega Bio-Tek), and amplified using the same qPCR primers and procedures as above (Figure S4).

3 | RESULTS

3.2 | Life history of Pr. angolensis

In western Namibia, we observed all life stages of Pr. angolensis gregariously feeding on seeds of W. mirabilis, Sterculia quinqueloba, Sterculia africana and Adansonia digitata (Figure 1). As previously reported for Pr. angolensis and other Pyrrhocoridae (Bornman, 1970; Socha & Zemek, 2000; Wetschnig & Depisch, 1999), both male and female Pr. angolensis appeared to be flightless, even when provoked, despite the presence of fully developed wings. As a result, any migration probably only takes place from plant to plant on foot, limiting the dispersal ability of isolated populations in the desert. The Adansonia spp. and Sterculia spp. host plants, as well as other Malvaceae plants in general, are widely distributed throughout sub-Saharan Africa, including Namibia (Figure 3). However, W. mirabilis is only known from a narrow desert corridor along the Namibian–Angolan coast (Figure 3), with large arid and sparsely vegetated expanses between populations of these plants. As a result, we expect that most of the populations of Welwitschia-feeding Pr. angolensis are isolated from each other and from the Adansonia- and Sterculia-feeding populations.
There is no published information on the reproductive biology of *P. angolensis*, but in our laboratory colony the males and females pair up and mate continuously upon sexual maturity. Females lay ~20–60 eggs at a time and bury them in sand beneath heavy objects such as water tubes and glass Petri dishes that are provided for this purpose; females will often, but not always, bury seeds with their egg clutches. We have not observed egg-laying in nature, but females probably bury their eggs under leaf litter, sand or rocks at the base of the host plants. Eggs begin to hatch after about 7 days, hatchlings undergo five nymphal instars before reaching adulthood after 30–45 days, and total lifespan is several months. These observations are consistent with other Pyrrhocoridae (Schaefer & Ahmad, 2000).

### 3.3 | Probergrothius populations feeding on Welwitschia and Malvaceae belong to the same species

To determine whether the different *P. angolensis* populations might in fact represent distinct species, we sequenced nine different gene regions from a single individual from each of the 13 collection locations (Table 1). Alignment length, genes and percentage similarity across all individuals include: 632-bp cytochrome oxidase 1 (CO1) >99.6%, 664-bp cytochrome oxidase 2 (CO2) >99.6%, 424-bp cytochrome B (CytB) >98.8%, 485-bp mitochondrial 16S rDNA >99.4%, 452-bp homeobox Abd-A >98.2%, 282-bp homeobox Dfd-2 >98.9%, and 527-bp homeobox Scr >98.8%. The most variable regions were from the rRNA internal transcribed spacers (ITS1 and ITS2), although these sequences were highly heterozygous, resulting in ambiguous base calls, so alignment columns containing ambiguities were stripped prior to calculating percentage identity. Percentage identity was: 297-bp ITS1 >92.2% and 400-bp ITS2 >94.5%. Phylogenetic reconstruction using individual genes and concatenation of all sequences (4,163 bp total alignment) revealed that most well-supported clades were formed based on the proximity of the populations to each other, although this was not always true (Figure S2). For example, population #4-*Sterculia* clustered with the distant #5-*Sterculia* and #6-*Adansonia* populations despite being closer to several *Welwitschia* populations; additionally, populations #1/11-*Welwitschia* did not cluster with the relatively close
#12/13- Sterculia populations, instead appearing more closely related to #2/9- Welwitschia populations that are a similar distance (#2) or even further away (#9) (Figure S2). This provides some evidence suggesting genetic differentiation based on host plant use and the potential presence of host-specialized races (Dres & Mallet, 2002), although the >99.6% similarity of the CO1 gene across all individuals measured is well within the 3% and 5% interspecies thresholds proposed previously (Hebert, Cywinska, & Ball, 2003; Kaur & Sharma, 2017; Li et al., 2014), indicating that all are members of the same species.

FIGURE 3 (a) Collection and host plant information of Probergothius angolensis in Namibia. Principal coordinate analyses comparing bacterial communities of individual Pr. angolensis feeding on Welwitschia versus on Malvaceae; purple = Sterculia, green = Adansonia, black = Welwitschia, and blue = other (b–d). (b) Jaccard = presence/absence, (c) Bray-Curtis = presence/absence and abundance, (d) Weighted-UniFrac = presence/absence, abundance, phylogeny [Colour figure can be viewed at wileyonlinelibrary.com]
3.4 | Bacterial communities of Welwitschia-feeding Pr. angolensis differ from those that feed on Malvaceae plants

Sixty-two individual Pr. angolensis, collected from 13 locations in Namibia, were submitted for high-throughput bacterial community sequencing. PCoA, using three distance metrics (Jaccard, Bray-Curtis, and Weighted UniFrac), showed significant differences in bacterial communities between Welwitschia-feeding bugs and those feeding on Malvaceae plants (Figure 3). A comparison of bacterial diversities between Welwitschia-feeding populations versus Malvaceae populations revealed that the Welwitschia populations were significantly less diverse than the Malvaceae populations (Faith’s phylogenetic diversity: $df = 1, H = 15.0, p = .0001$), (Shannon index: $df = 1, H = 25.0, p < .0001$).

The bacterial communities of all individuals were dominated by Proteobacteria and Firmicutes, but some individuals contained high proportions of Bacteriodetes and Actinobacteria (Figure S1). The most common and abundant bacterial genera found in the majority of individuals were Commensalibacter (56/62), Enterococcus (60/62), Klebsiella (56/62) and Bartonella (46/62), but their relative abundance differed based on host plant and life stage (Figure 4a). Less abundant but prevalent bacteria included Lactococcus (43/62) and Serratia (27/62). An initial GzLM, comparing males to females from each host plant type, showed no significant differences in core bacterial relative abundance related to sex ($p = .16$). A subsequent GzLM revealed significant differences in relative abundances of core bacterial taxa between host plants (Welwitschia vs. Malvaceae) and life stages (adult vs. fifth-instar nymphs); there was also a significant interaction between host plant and life stage, indicating that adults and nymphs should be analysed independently (Table 2). After performing separate GzLM analyses for adults and nymphs, we found that Commensalibacter was present in significantly higher relative abundance in Welwitschia- than Malvaceae-feeding populations, in both nymphs and adults (Figure 4b,c). We found five ASVs of Commensalibacter in our sampling, two of which (ASV-1 and ASV-2) were present significantly more often and in higher abundance in Welwitschia populations than Malvaceae populations and remained the most abundant ASVs in our laboratory colony (Figure S3). Enterococcus was significantly lower in Welwitschia-feeding adults (Figure 4b), but higher in nymphs (Figure 4c). Klebsiella was significantly higher in the nymphs of Malvaceae-feeding populations. Further differences between populations were observed for other bacteria; for example, an unidentified genus with closest relatives in the family Holosporaceae was mostly found in Welwitschia-feeding populations and very few Malvaceae-feeders; two other abundant bacterial taxa in the genera Clostridiales and Proteus were only found in Malvaceae-feeding populations (Figure 4a).

3.5 | Core bacteria are stably inherited and present in all life stages

Our laboratory colony of Pr. angolensis was originally collected from Welwitschia plants in 2011 and has since been maintained in the laboratory. We used high-throughput bacterial 16S sequencing on all nymphal stages and adults to determine the stability of bacterial associations after long-term rearing in the laboratory as well as to understand how the bacterial community changes.
throughout development. We found that all of the most abundant core bacterial genera from wild populations, including *Commensalibacter*, *Enterococcus*, *Klebsiella* and *Bartonella*, were still present in all developmental stages of our laboratory colonies (Figure 5). We also found that some of the less common bacteria, such as *Lactococcus*, *Serratia*, *Holosporeae*, *Lactobacillus* and *Stenotrophomonas*, remained present in the laboratory colonies. However, bacteria not detected in any wild populations were also found in our laboratory colonies (Figure 5; grey shading), but these were present in low relative abundance and may represent transient infections.

The bacterial community was most diverse in first instars before the successful establishment of the core bacteria that is evident in subsequent instars (Figure 5), which is consistent with earlier observations from another pyrrhocorid species, *Py. apterus* (Sudakaran, Salem, Kost, & Kaltenpoth, 2012). *Enterococcus*, which was one of the most abundant bacteria in wild collections, was present at much lower proportions in the laboratory colony than in wild-caught specimens.

### 3.6 | Phylogenetic placement of *Commensalibacter, Bartonella, Klebsiella and Enterococcus* symbionts

Phylogenetic affiliations of the four major symbiotic bacteria were inferred using 16S rRNA sequences from bacterial clones or isolates from our laboratory colonies of *Pr. angolensis*. Before phylogenetic reconstruction, sequences were first confirmed to be 100% similar to the corresponding ~370-bp most-abundant operational taxonomic unit (second most abundant for Klebsiella) attained from high-throughput Illumina sequencing. We constructed phylogenies to show their placement relative to other closely related bacteria, highlighting bacteria with symbiotic relationships with insects (Figure S5). Notably, we found that the *Bartonella* symbiont forms a well-supported clade with other uncultured Hemiptera-associated Bartonellaceae. These are distant and separate from all other described *Bartonella*, making them undescribed members of this genus (Figure S5c).

### 3.7 | Fungi and yeast mycobiome

To uncover the mycobiota of *Pr. angolensis* associated with Malvaceae- and Welwitschia-feeding populations, we submitted 10 regionally pooled samples (minimum of six *Pr. angolensis* per sample) for high-throughput Illumina MiSeq amplicon sequencing with fungus-specific ITS primers. We also submitted a pooled sample from our 6-year-old laboratory colony to determine whether any of the fungi formed long-term relationships with the host.

From the wild-caught specimens, the most abundant fungal genera, also found in the majority of samples, were *Eremothecium* (syn. Ashbya) in 7/10 samples, *Cladosporium* in 9/10, *Aspergillus* in 10/10 and *Fusarium* in 6/10 samples; all of these are known plant pathogens, but symbiotic roles with *Pr. angolensis* cannot be ruled out. Of these four, the laboratory colony only maintained association with *Aspergillus*, although this fungus is common in many environments. The laboratory colony also contained *Penicillium*, which was also found in some wild populations, and most surprisingly maintained two yeasts in high relative abundance, *Meyerozyma* and *Debaromyces*, which were not found in any of the wild populations, probably making them laboratory acquisitions (Figure 6).
Wilcoxon Pairwise, arthropods (Schaefer & Ahmad, 2000). Even these species, however, also opportunistically forage on other plants and scavenge on dead less patterns of core bacterial abundance to insects collected in the field (Schaefer & Panizzi, 2000). Here we report on an insect species, are usually restricted to plants within the same order or family (Deguine, Martin, & Leclant, 1999; Jaenike, 1990; Thorsteinson, 1960), but, especially within Hemiptera, the insects rous insect groups (Deguine, Martin, & Leclant, 1999; Jaenike, 1990; Thorsteinson, 1960), but, especially within Hemiptera, the insects 4 | Discussion

Host plant switching and polyphagy are common in many herbivo rous insect groups (Deguine, Martin, & Leclant, 1999; Jaenike, 1990; Thorsteinson, 1960), but, especially within Hemiptera, the insects are usually restricted to plants within the same order or family (Schaefer & Panizzi, 2000). Here we report on an insect species, , in which separate populations feed and reproduce on Malvaceae and Welwitschia, respectively, which differ above the phylum level (Angiosperm vs. Gymnosperm) and are separated by at least 300 million years of evolution (Clarke et al., 2011). Many other Pyrrhocoridae are known to generalize on different Malvaceae plants, and several members such as Py. apterus and D. fasciatus will also opportunistically forage on other plants and scavenge on dead arthropods (Schaefer & Ahmad, 2000). Even these species, however, showed high mortality and were incapable of completing development when fed seeds of Welwitschia plants (Figure 2), indicating that this is an ability unique to , which is also the only pyrrhocorid reported to feed on the plant despite the presence of other pyrrhocorids in this region (Robertson, 2004). While we cannot rule out genetic components to polyphagy within , a previous study showed pronounced differences in the microbiota between and other Pyrrhocoridae (Sudakaran et al., 2015) that potentially predisposes to broader host-plant use. Using this information, we investigated whether microbes may have facilitated the host plant switch. We first confirmed that the Welwitschia-feeding and Malvaceae-feeding popula tions of were indeed the same species with low levels of genetic differentiation (Figure S2), although they may represent host-specialized races (Dres & Mallet, 2002). We then revealed large differences between populations in both the qualitative and the quantitative composition of their associated bacterial communities (Figure 4). Additionally, we found evidence of long-term symbiont associations (Figure 5), indicating that the bacteria stably colonize the gut and are probably functionally important to these insects.

Because of their often specialized diets, herbivorous Hemiptera generally need to overcome nutrient imbalance (e.g., limited essential amino acids and B-vitamins) of their diets that are commonly supplemented by their symbiotic microbes (Douglas, 2009). However, many seeds are a relatively rich nutrition source, containing high proportions of fats, proteins and carbohydrates. For example, Gossypium (cotton) seeds, the major food source for several pest Pyrrhocoridae, contain all essential amino acids and most B-vitamins except B12-cobalamin (U.S. Department of Agriculture, Agricultural Research Service, 2019), which is an essential cofactor for cellular metabolism and is only known to be produced by bacteria.
Probergrothius angolensis, which lacks the Coriobacteriaceae bacteria common in other Pyrrhocoridae (Sudakaran et al., 2015), may derive B-vitamins (at least cobalamin) from other bacteria. In addition to nutritional limitations, pyrrhocorids may also need to overcome plant defences, and both Malvaceae and Welwitschia seeds are known to contain high proportions of cyclopropenoic fatty acids (CPFAs) that are toxic to many insects and vertebrates (Aitzetmüller & Vosmann, 1998; Greenberg & Harris, 1982; Rani & Rajasekharreddy, 2009, 2010). While B-vitamin limitation and the presence of CPFAs, with some variation in amounts, are characteristics that Malvaceae and Welwitschia seeds share, clearly other differences exist that make Welwitschia nutritionally unsuitable for other Pyrrhocoridae (Figure 2). Here, inter- and intraspecific differences in gut microbiota may at least partially explain the feeding abilities of Pr. angolensis.

From our field sampling, we found that bacterial communities associated with Welwitschia-feeding populations of Pr. angolensis were less diverse than those of Malvaceae-feeding populations and were biased towards specific bacterial taxa (Figure 4); additionally, we found similar patterns of bacterial abundance when we experimentally fed them the respective diets in the laboratory (Figure 7). Populations feeding on Welwitschia are probably constrained to this host plant due to the insects’ inability to fly, the plants’ isolation, and the limited number of other plants capable of growing in these hot and arid regions. Two mutually nonexclusive scenarios may explain the pronounced differences in bacterial community composition and diversity between Welwitschia- and Malvaceae-feeding populations: (i) the host plants determine the microbial community that the bugs are exposed to, and the restricted diet of Welwitschia seeds offers a lower diversity of microbes; and/or (ii) the qualitative and quantitative shifts in bacterial community composition are selectively favoured due to functional benefits for the host. We did not detect the core symbiotic bacteria in the diets fed to our laboratory population (Figure S4), indicating that the most abundant bacteria can be maintained without constant reacquisition. Given that other pyrrhocorids cannot (Figure 2) and do not naturally feed on Welwitschia, bacterial communities are similar among distant populations that feed on similar diets (Figure 4a), core bacteria are stable over the long term in the laboratory (Figure 5; to the ASV level, Figure S3) and bacterial relative abundance changes with experimental diets in patterns consistent with field populations (Figure 7), scenario (ii) appears to be more likely. Because the microbial taxa present in Welwitschia populations are mostly similar to those found in Malvaceae populations (the probable ancestral hosts), we suggest that the bacterial associations may have helped to pre-adapt the bugs to the host plant switch. It would be interesting to compare the feeding abilities of other Probergrothius species with Pr. angolensis, as their gut microbial communities are more similar to each other than to those of other Pyrrhocoridae (Sudakaran et al., 2015).

Bacterial communities of Welwitschia populations were especially biased towards high relative abundance of Commensalibacter (in adults) and Enterococcus (in nymphs; Figure 4), possibly reflecting different nutritional requirements across life stages. Commensalibacter, which was by far the most prominent bacterium in most adults, is a recently described genus (Kim et al., 2012) that has so far only been found in insects such as bees, butterflies, fruit flies and seed bugs (Figure S5). We also found variation in Commensalibacter strains, showing that two specific ASVs (ASV-1 and ASV-2) infected more individuals, and in higher abundance, in Welwitschia populations (Figure S3) and another strain (ASV-3) was only found in Malvaceae populations (Figure S3). This suggests that Commensalibacter’s importance could be strain-specific. Not much is currently known about these bacteria, but their placement as the sister taxon to Acetobacter (Figure S5), and association with other insects with high-sugar diets (Roh et al., 2008; Servin-Garcidueñas, Sánchez-Quinto, & Martínez-Romero, 2014), suggests that they play roles in carbohydrate metabolism (Crotti et al., 2010). Welwitschia populations contained one bacterial taxon that was almost completely absent in Malvaceae populations, an unidentified Holosporaceae symbiont (Figure 4). Other members of this bacterial family are known as intracellular parasites of single-celled eukaryotes like paramecia, and potentially trypanosomes which are common parasites of pyrrhocorids and other Heteroptera (Dohra, Tanaka, Suzuki, Fujishima, & Suzuki, 2014; Salem, Onchuru, Bauer, & Kaltenpoth, 2015; Schaefer & Ahmad, 2000). Hence, this is probably not a nutritional symbiont of the bug, but may be associated with parasitic protists in its gut.

The Malvaceae populations of Pr. angolensis contained most of the bacterial genera that were found in Welwitschia populations, but were more heterogeneous and diverse, overall, and contained several additional genera that were absent or very rare in Welwitschia populations (e.g., Clostridioides, Dysgononas, Bacteroides, Lactobacillus, Citrobacter, Proteus and Stenotrophomonas; Figure 4). This additional diversity may reflect different nutritional requirements of feeding on Malvaceae plants or additional bacteria that are encountered while foraging in these more heterogeneous environments.

Consistently present in similar proportions across all populations were Klebsiella and Bartonella bacteria, which may indicate that these bacteria serve a more general nutritional role for the insects. Our isolated strain of Klebsiella was most closely related to Klebsiella oxytoca, including other insect-associated strains, which are known to grow in a variety of insects and plants, and in the environment (Figure S5; Bagley, 1985). In the common house fly, Musca domestica, for example, they are known to inhabit the surface of eggs and suppress the growth of fungi that compete with or are harmful to fly larvae (Lam, Thu, Tsang, Moore, & Gries, 2009). As pyrrhocorids are known to transmit gut bacteria on the egg surface (Kaltenpoh, Winter, & Kleinhammer, 2009; Salem, Florez, Gerardo, & Kaltenpoh, 2015), the symbionts may protect the eggs that are laid in leaf litter or soil substrate against opportunistic fungi (Schaefer & Ahmad, 2000), as has recently been shown in Lagria beetles (Flórez et al., 2017, 2018). In other insects, Klebsiella are commonly found in the gut (Engel & Moran, 2013) and are known
to have wide-ranging metabolic capabilities, including nitrogen fixation, and can utilize diverse food sources (Temme, Zhao, & Voigt, 2012). *Bartonella* are an interesting finding because they have historically been known only as arthropod-vectored mammalian pathogens and only recently have new clades in this genus been found in nonvector species such as honeybees (Kešnerová, Moritz, & Engel, 2016; Segers, Kešnerová, Kosoy, & Engel, 2017). The single *Bartonella* ASV found in *Pr. angolensis* was most similar to uncultured Rhizobiales bacteria of three other seed-feeding heteropteran species in the families Lygaeidae and Oxyacarenidae (Figure S5), suggesting a specialized role in seed digestion. Other gut-associated *Bartonella* species have putative roles in nitrogen metabolism, so they may play a similar role here as well (Segers et al., 2017).

In addition to the bacterial communities, we also profiled fungal communities inside *Pr. angolensis* to examine fungal symbionts and assess the bug’s vector potential for several known Welwitschia plant pathogens (Cooper-Driver et al., 2000; Pekarek, Jacobson, & Donovan, 2006; Whitaker, Pammenter, & Berjak, 2008). We found one yeast, closely related to *Eremothecium* (syn. *Ashbya*), *gossypii*, that was consistently present (Figure 6). This yeast is a known pathogen of several plants, including cotton, and is transmitted by other Pyrrhocoridae such as *Dysdercus* (Schafer & Ahmad, 2000). That it is consistently associated with *Pr. angolensis* as well suggests that it could play a symbiotic role, as yeasts do in other insects (Frago et al., 2012). However, nutritional roles, if any, would only be nonspecific as *Eremothecium* was completely absent in our laboratory colony, apparently having been replaced by two other yeasts, *Meyerozyma* and *Debaromyces*. Several plant-pathogenic filamentous fungi such as *Cladosporium*, *Phoma*, *Aspergillus*, *Fusarium* and other less abundant types were also found, so *Pr. angolensis* may indeed be a vector of these pathogens as previously suggested (Cooper-Driver et al., 2000; Lane, Beales, & Hughes, 2012; Pekarek et al., 2006).

5 | CONCLUSIONS

Many insects benefit from symbiotic bacteria as a source of ecological innovation (Sudakaran et al., 2017). For example, within sap-feeding Hemiptera, the family Adelgidae (coniferous Gymnosperm-feeding) anciently diverged from its sister family, Aphididae (mostly Angiosperm-feeding), acquiring new obligate bacterial symbionts in the process (Toenshoff, Gruber, & Horn, 2012) that probably alleviated nutritional challenges when switching host plants. Even within Aphididae, certain symbiont associations can influence host plant specialization, indicating that bacteria-mediated host shifts occur repeatedly in insect evolution (Henry et al., 2013; Wagner et al., 2015) and can even be strain-specific (Ferrari, Scarborough, & Godfray, 2007). While these are examples of symbiont associations with bacteriome-associated and intracellular endosymbionts, the insect gut is often much more complex in terms of bacterial diversity and function (Douglas, 2016; Engel & Moran, 2013). Even here, however, insects can evolve highly specific associations such as the strong association of *Burkholderia* bacteria in specialized midgut crypts of Largidae, the sister family to Pyrrhocoridae (Gordon et al., 2016; Takeshita et al., 2015), as well as several other Heteroptera (Kikuchi et al., 2011). We found that the gut microbiota of *Pr. angolensis* differs consistently between populations feeding on two distinct host plant taxa, indicating that bacterial symbionts may have been important in shifting to the unusual diet of Welwitschia seeds. Future studies examining the localization, function and transmission of these bacteria should reveal more about their symbiotic relationships and how they benefit the insect on its novel host plant. More generally, investigating polyphagous insects that maintain consistent, but dynamic, gut microbial communities will be important for understanding how insects adapt to novel food sources. In addition, this will provide valuable information on how invasive species and pests are capable of rapid adaptation in order to successfully establish in novel environments.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

A.J.M. and M.K. conceived and designed the experiments and wrote the manuscript. A.J.M. and T.O.O. performed experiments, collected insects and analysed data. A.J.M. and C.S.I. cloned, isolated and sequenced the bacteria. A.J.M. and M.S.-C. performed gene alignments and reconstructed bacterial and insect phylogenies. H.S. performed DNA extractions and initial 454 pyrosequencing. J.D. performed DNA extractions and initial 454 pyrosequencing. A.J.M. and M.K. conceived and designed the experiments and wrote the manuscript.

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DATA AVAILABILITY STATEMENT

All experimental data generated from insect diet bioassays, gene alignments (insect and bacterial) and Illumina sequencing analyses (ASV abundance table, Qiime2 analysis pipeline, negative control) are available in the Dryad Digital Repository, https://doi.org/10.5061/dryad.zkh18935n (Martinez et al., 2019). DNA gene sequences used in the Pr. angolensis phylogenetic analyses are available on GenBank through accession numbers MN542943–MN543046. 16S rRNA sequences from isolated and cloned Bartonella, Klebsiella, Enterococcus and Commensalibacter bacteria are available on GenBank through accession numbers MN420699–MN420705. 16S rRNA ASVs identified from illumina sequencing of Pr. angolensis collected in Namibia are available on GenBank through accession numbers MNS14430–MNS14588.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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