Expanded geographic distribution and host preference of Anopheles gibbinsi (An. species 6) in northern Zambia

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Abstract

Background

Nchelenge District in northern Zambia suffers from holoendemic malaria transmission despite a decade of yearly indoor residual spraying (IRS) and insecticide treated net (ITN) distributions. One hypothesis for this lack of impact is that some vectors in the area may forage in the early evening or outdoors. *Anopheles gibbinsi* specimens were identified in early evening mosquito collections performed in this study area, and we gleaned further insight into this taxon, including characterizing its genetic identity, feeding preferences, and potential role as a malaria vector.

Methods

Mosquitoes were collected in July and August 2019 by CDC light traps in Nchelenge District in indoor sitting rooms, outdoor gathering spaces, and animal pens from 16:00-22:00. Host detection by PCR, COI and ITS2 PCR, and circumsporozoite (CSP) ELISA were performed on all samples morphologically identified as *An. gibbinsi*, and a subset of specimens were selected for COI and ITS2 sequencing. To determine risk factors for increased abundance of *An. gibbinsi*, a negative binomial generalized linear mixed-effects model was performed with household-level variables of interest.

Results

Comparison of COI and ITS2 *An. gibbinsi* reference sequences to the NCBI database revealed >99% identity to "An. sp. 6" from Kenya. More than 97% of specimens were morphologically and molecularly consistent with *An. gibbinsi*. Specimens were primarily collected in animal pen traps (59.2%), followed by traps outdoors near where humans gather (24.3%), and traps set indoors (16.5%). Host DNA detection revealed a high propensity for goats, but 5% of specimens with detected host DNA had fed on humans. No specimens were positive for *Plasmodium falciparum* sporozoites. Animal pens and inland households >3 km from Lake Mweru were both associated with increased *An. gibbinsi* abundance.

Conclusions

This is the first report of *An. gibbinsi* in Nchelenge District, Zambia. This study provided a species identity for unknown "An. sp. 6" in the NCBI database, which has been implicated in malaria transmission in Kenya. Composite data suggest that this species is largely zoophilic and exophilic, but comes into contact with humans and the malaria parasites they carry. This species should continue to be monitored in Zambia and neighboring countries as a potential malaria vector.

Introduction

After 15 years of global reductions in malaria cases, progress has slowed since 2015, with an estimated 229 million malaria cases in 2019 (1). Progress made has been heterogeneous across Africa, with some countries still experiencing a high burden of malaria, and others approaching elimination. In regions where major vectors have been successfully controlled, species that were once secondary vectors or had remained unrecognized have become an increasing concern, and may be responsible for more transmission than previously documented (2–8). Many of these anopheline species tend to be exophilic or display behavioral
plasticity in their foraging and resting behaviors, and may not choose humans as their preferential host (9, 10). It is unclear if these species always contributed to transmission, or if they fill an open niche when primary vector populations are reduced (4, 5, 8, 10–12). To fully understand the importance of these implicated vectors, we must characterize their role not only when a region is close to elimination but when other well-studied anophelines are primarily driving transmission.

Nchelenge District is located in Luapula Province, northern Zambia and lies along the eastern shore of Lake Mweru. The district is innervated by many streams and tributaries that empty into the lake, creating a marshy ecology for malaria vector breeding sites that drives high year-round malaria transmission (13–16). Well-recognized vectors in the area include Anopheles funestus s.s., with peak abundance during the dry season, and An. gambiae s.s. with persistent low-level abundance. Both species reproduce year-round in this region (14). High transmission in Nchelenge District has continued even after annual indoor residual house spraying (IRS) and multiple mass insecticide treated net (ITN) campaigns across the district, leading to the hypothesis that vectors may be biting outside or before people go under their mosquito nets at night (15–18).

To better characterize additional potential vectors, mosquito collections were performed to identify early-foraging anopheline species in Nchelenge District, Zambia, during the cool dry season in 2019. A large proportion of specimens collected as part of this study were Anopheles gibbinsi Evans, a species which has not previously been reported in Zambia. Literature on this species is sparse, though it has been documented in the highlands of eastern Africa, from Ethiopia down to the Democratic Republic of the Congo (DRC) (2, 19–23). Importantly, 7.7% of morphologically-identified An. gibbinsi captured in an early-foraging study in Kenya were determined to be positive for Plasmodium falciparum sporozoites, suggesting that this species is a vector of malaria (2). In this study, we gained further insight into this understudied species, including characterizing its genetic identity, feeding preferences, and potential role as a malaria vector.

Methods

Study area and household selection:

Data were collected in July and August 2019 in Nchelenge District, Zambia (Fig. 1). This region experiences three seasons: a rainy season from December to April, a cold dry season from May to August, and a hot dry season from September to November (14). Nchelenge District lies along Lake Mweru and has several streams and tributaries that persist during the dry season, creating an expanse of marshy mosquito breeding sites.

Twenty-four households were selected for this study: twelve located < 3 km from the lake's shore and twelve located > 3km from the lake (lakeside and inland, respectively). Sampled households were required to have at least one animal pen outside of the main sleeping structure, and inland households were required to be located within 0.6 km of a major stream.

Environmental covariates:

During the study visits, household coordinates were uploaded into ArcGIS Pro (ESRI, Redlands, CA, USA). Streams were previously mapped and categorized (17), and distance to the nearest major stream was calculated using the “near” tool in ArcGIS Pro (ESRI, Redlands, CA, USA).

Entomological sampling:
Three lakeside and three inland households were sampled each day, and a Latin-square method was used to rotate the trap placement in each sampled household (24). Mosquitoes were collected using miniature CDC Light Traps (John W. Hock Co., Gainesville) from 16:00 to 22:00. The head of household was provided a watch at the time of set up and was asked to turn the trap on at 16:00 and tie the collection bag at 22:00. Traps were hung between 1.5–1.8 m above the ground, either in the indoor sitting room of a household, an outdoor area where people gather in the evening, or outdoors next to an animal pen. Other than the incandescent light on the trap and any people or animals that spent time near the trap, no other lures were used. Surveys regarding household features and human behavior were conducted at the time of enrollment and additionally for each trap collection. The head of household answered all the enrollment questions, and if they were not present for the follow-up questionnaire, the spouse or eldest child responded.

**Mosquito processing:**

Captured mosquitoes were killed by freezing, morphologically identified using Gillies and Coetzee (25), and stored individually in a microfuge tube on silica gel to desiccate in the field. Desiccated samples were returned to the Johns Hopkins Bloomberg School of Public Health (BSPH) in Baltimore, Maryland, USA, where each specimen was split into head/thorax and abdomen. Prior to splitting, 17 samples that were morphologically identified as *An. gibbinsi* were sent to the University of the Witwatersrand to confirm morphological identity.

DNA from female anopheline mosquito abdomens were crushed in lysis buffer using a Qiagen TissueLyser II (Qiagen, Hilden, Germany) and extracted using an automated DNA extraction method with a QIAcube HT (Qiagen, Hilden, Germany) at Purdue University (26). After DNA extraction, each specimen underwent a PCR assay to amplify a fragment of the internal transcribed spacer region 2 (ITS2) of the nuclear genome as described previously (27–29). A random representative subset of 5.4% of *An. gibbinsi* specimens were selected for Sanger sequencing of the ITS2 target and the Barcode of Life fragment of the cytochrome oxidase I (COI) gene as previously described (27–29). Sequencing for specimens returned to BSPH was conducted at the Johns Hopkins Medical Institutions (JHMI) Synthesis and Sequencing Facility. Forward and reverse sequences were imported into Geneious Prime (version 2021.2.2, Biomatters, Ltd, Auckland, New Zealand, https://www.geneious.com), trimmed to remove low-quality reads and primer sequences, and aligned to create a consensus sequence for each specimen. Consensus sequences were compared to the National Center for Biotechnology Information (NCBI) database and reference samples using BLASTn, and final identifications were confirmed if they had >99% identity to an NCBI sequence. Data were submitted to NCBI's GenBank and accession numbers were acquired for both ITS2 (OM459737-OM459768) and COI (OM456780-OM456806) sequences.

### Host Detection Analyses

A host DNA detection assay was created to determine host preference. Primers from Kent & Norris 2005 (30), Izadpanah et al. 2018 (31), and Kumar et al. 2015 (32) were combined into a multiplexed PCR assay to detect individual and mixed blood meals from human, cow, pig, dog, chicken, and goat DNA, producing differential product sizes for each host animal (supplementary table 1). Each 25 µL PCR reaction consisted of 1x buffer, 1.0 mM dNTPs, 0.625 units of Taq polymerase (New England Biolabs #M0273S), 50 pmol of each primer, and 1.0 µL extracted abdomen DNA (supplementary table 1). Thermocycler conditions consisted of an initial denaturation of 5 minutes at 95°C, followed by 35 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45
Moleculär konfirmation

Drei Proben, die alle identifizierenden Merkmale (d.h., keine fehlenden Tarsen, alle Schuppen vorhanden, Flügel und Proboszis intakt) morphologisch konfirmiert wurden als *An. gibbinsi* am BSPH und durch Photographien an die University of the Witwatersrand (Supplementärfigur 1). COI und ITS2-PCR wurden auf diesen Proben durchgeführt und sequenziert. Der Produktgrößen (inklusive Primer) vom ITS2-PCR für alle drei Proben war 507 basenpaare, und der COI Produktgrößen war 709 basenpaare. Alle drei ITS2-Sequenzen waren 100% identisch zueinander, und COI-Sequenzen waren 98.9% identisch zueinander. Die NCBI BLAST-Resultat für alle ITS2 und COI-Sequenzen war "*An. sp. 6*" mit >99% Identität für jede Probe (Supplementärtable 2). Diese drei Proben wurden als *An. gibbinsi* Referenzproben für die restlichen Studien (OM459761-OM459763, OM56804-OM56806).

Von den 17 Proben, die an die University of the Witwatersrand versandt wurden, sieben waren morphologisch konfirmiert als *An. gibbinsi*. Zwei Proben wurden identifiziert als *An. marshallii* Komplex, eines wurde als *An. funestus* identifiziert, und sechs waren nicht zu konfirmieren aufgrund der Verlust der Beine. ITS2-PCR und Sequenzierte war erfolgreich auf 5 von 7, die waren konfirmiert als *An. gibbinsi*, und alle passten die Referenzproben mit 100% Identität.

Morphologische Genauigkeit und Standortpräferenz

Vierhundert und fünfzehn *An. gibbinsi* wurden morphologisch identifiziert. Ein ITS2-PCR wurde auf allen 453 Proben, und 443 (97.8%) produzierten einen 507bp Band auf elektrophoretischer Analyse, bestätigend die *An. gibbinsi* Identifikation. Fünf (1.1%) Proben wurden molekular identifiziert als *An. funestus s.s.*, zwei (0.4%) Proben hatten ein 439bp ITS2-PCR amplicon Größe und waren unidentifiziert auf Spezies-Niveau nach Sequenzierung, und drei (0.7%)
samples failed amplification after three attempts. Twenty-four An. gibbinsi samples (5.4%) representing all three trap types were selected to undergo ITS2 and COI fragment sequencing to confirm species identity. All ITS2 sequences matched the reference samples with 100% identity, and COI sequences matched reference samples with > 99% identity. All specimens had a NCBI BLAST result of “An. sp. 6” with > 99% identity for both COI and ITS2 fragments (supplementary table 2).

**Household characteristics**

*Anopheles gibbinsi* were collected from 15 of 24 (62.5%) study households, primarily in traps set at animal pens (n = 261, 59.2%), followed by traps outdoors near where humans gather (n = 107, 24.3%), and traps set indoors (n = 73, 16.5%) (Fig. 1).

**Host preference and parasite detection**

A PCR assay to detect human, goat, cow, dog, pig, and chicken DNA was performed on 417/443 (94.1%) molecularly confirmed *An. gibbinsi* samples to assess host preference. Twenty-six samples were excluded because they did not have an intact abdomen. Host DNA was detected in 83 (19.9%) samples. Sixty three of 417 (15.1%) samples were recorded as visually blood fed during morphology, and host DNA was detected in 54 (85.7%) of those specimens (supplementary table 3). Additionally, host DNA was detected in 29/354 (8.2%) that were not recorded as visually blooded (supplementary table 3). Of the *An. gibbinsi* samples positive for host DNA, goat DNA was detected in 71/83 (85.5%) (Table 1). Other hosts detected were pig (n = 7, 8.2%), human (n = 4, 4.8%), and dog (n = 1, 1.2%). The trap type with the highest proportion of specimens with host DNA were from traps placed near animal pens (76/246, 30.8%), followed by traps placed indoors (5/63, 7.9%), and lastly from traps placed near gathering locations (2/103, 1.9%) (Table 1). CSP ELISAs were performed on all 443 samples, but none were positive for sporozoites.

<table>
<thead>
<tr>
<th>Trap type</th>
<th>Dog</th>
<th>Goat</th>
<th>Human</th>
<th>Pig</th>
<th>No band</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal</td>
<td>0</td>
<td>69</td>
<td>0</td>
<td>7</td>
<td>170</td>
<td>246</td>
</tr>
<tr>
<td>Outdoor gathering</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>101</td>
<td>103</td>
</tr>
<tr>
<td>Indoor</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>63</td>
<td>68</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>71</td>
<td>4</td>
<td>7</td>
<td>334</td>
<td>417</td>
</tr>
</tbody>
</table>

**Risk factor analysis**

In the univariate analysis, household level risk factors associated with a higher abundance of *An. gibbinsi* were inland households, animal pens, using an open well or stream/pond for a water source, households with natural wall materials, and not receiving IRS in the previous campaign (October 2018) (Table 2). Increased distance from large streams and increased number of rooms in a household were associated with decreased abundance of *An. gibbinsi* (Table 2). Many of these variables were correlated. For example, inland households were associated with decreased distance to streams, smaller households, and using a stream or pond as a water source, so only one of these variables was included in the final multivariate model. The multivariate model with the lowest AIC included only household location and trap location, and revealed inland households and animal pen traps remained associated with increased counts of *An. gibbinsi* (Table 2).
Table 2
Univariate and multivariate results from negative binomial generalized linear mixed-effects model performed with # *An. gibbinsi* per trap as the outcome.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Multivariate analysis</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IRR</td>
<td>SE</td>
<td>95% CI</td>
<td>p-value</td>
<td>IRR</td>
<td>SE</td>
<td>95% CI</td>
<td>p-value</td>
</tr>
<tr>
<td>HH location lakeside</td>
<td>84.60</td>
<td>2.20</td>
<td>[20.67, 538.13]</td>
<td>&lt; 0.001 ***</td>
<td>86.50</td>
<td>2.25</td>
<td>[20.28, 587.06]</td>
<td>&lt; 0.001 ***</td>
</tr>
<tr>
<td>inland</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trap type indoor</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>outdoor gathering</td>
<td>0.98</td>
<td>1.35</td>
<td>[0.52, 1.85]</td>
<td>0.96 ns</td>
<td>1.00</td>
<td>1.38</td>
<td>[0.53, 1.86]</td>
<td>0.989 ns</td>
</tr>
<tr>
<td>animal pen</td>
<td>3.18</td>
<td>1.36</td>
<td>[1.75, 5.83]</td>
<td>&lt; 0.001 ***</td>
<td>3.13</td>
<td>1.36</td>
<td>[1.73, 5.72]</td>
<td>&lt; 0.001 ***</td>
</tr>
<tr>
<td>Distance to stream (km)</td>
<td>0.41</td>
<td>1.41</td>
<td>[0.17, 0.77]</td>
<td>0.01 **</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td># Rooms in HH</td>
<td>0.43</td>
<td>1.48</td>
<td>[0.18, 0.93]</td>
<td>0.03 *</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td># People in HH</td>
<td>0.75</td>
<td>1.27</td>
<td>[0.43, 1.19]</td>
<td>0.22 ns</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proportion sleeping under net</td>
<td>0.75</td>
<td>5.27</td>
<td>[0.02, 28.41]</td>
<td>0.86 ns</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IRS status yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IRS status no</td>
<td>12.7</td>
<td>2.78</td>
<td>[1.73, 136.51]</td>
<td>0.01 **</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water source bore hole</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>open well</td>
<td>17.5</td>
<td>2.84</td>
<td>[2.26, 135.94]</td>
<td>0.01 **</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>steam or pond</td>
<td>46</td>
<td>3</td>
<td>[5.35, 396.04]</td>
<td>&lt; 0.001 ***</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wall materials bricks</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>concrete</td>
<td>2.68</td>
<td>4.81</td>
<td>[0.12, 58.28]</td>
<td>0.53 ns</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>natural</td>
<td>28.7</td>
<td>3.69</td>
<td>[2.21, 371.22]</td>
<td>0.01 **</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Floor materials finished</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>natural</td>
<td>1.43</td>
<td>3.27</td>
<td>[0.1, 17.03]</td>
<td>0.761 ns</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Roof materials grass</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Variable</td>
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<td></td>
<td></td>
<td>Multivariate analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
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<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td></td>
<td>IRR</td>
<td>SE</td>
<td>95% CI</td>
<td>p-value</td>
<td>IRR</td>
<td>SE</td>
<td>95% CI</td>
<td>p-value</td>
</tr>
<tr>
<td>metal</td>
<td>0.99</td>
<td>3.74</td>
<td>[0, 21.95]</td>
<td>0.997</td>
<td>ns</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eaves</td>
<td>closed</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>open</td>
<td>11.6</td>
<td>[0.8, 168.05]</td>
<td>0.073</td>
</tr>
<tr>
<td></td>
<td>partial</td>
<td>0.42</td>
<td>[0, 45.2]</td>
<td>0.718</td>
<td>ns</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cooking materials</td>
<td>charcoal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>coal &amp; charcoal</td>
<td>5.49</td>
<td>[0.34, 160.66]</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>wood</td>
<td>0.52</td>
<td>[0.03, 9.04]</td>
<td>0.63</td>
<td>ns</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fire burned last night</td>
<td>no</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>0.78</td>
<td>[0.42, 1.47]</td>
<td>0.43</td>
</tr>
<tr>
<td>HH owns cats</td>
<td>no</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>3.38</td>
<td>[0.16, 87.9]</td>
<td>0.41</td>
</tr>
<tr>
<td>HH owns chickens</td>
<td>no</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>0.88</td>
<td>[0.06, 12.18]</td>
<td>0.92</td>
</tr>
<tr>
<td>HH owns dogs</td>
<td>no</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>0.11</td>
<td>[0.01, 1.3]</td>
<td>0.08</td>
</tr>
<tr>
<td>HH owns ducks</td>
<td>no</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>0.19</td>
<td>[0.01, 3.63]</td>
<td>0.26</td>
</tr>
<tr>
<td>HH owns goats</td>
<td>no</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>1.09</td>
<td>[0.04, 25.09]</td>
<td>0.96</td>
</tr>
<tr>
<td>HH owns pigs</td>
<td>no</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>0.7</td>
<td>[0.06, 9.16]</td>
<td>0.76</td>
</tr>
</tbody>
</table>

HH = household; * = p value 0.01–0.05; ** = p value 0.001–0.01; *** = p value < 0.001
Discussion

Prior to this study, An. gibbinsi had not been reported in Zambia, very little was known about this species’ foraging behaviors, and no genetic data were associated with this taxon. Perhaps the most unexpected finding is that the ITS2 and COI fragments sequenced from morphologically confirmed An. gibbinsi matched with 100% identity to “An. sp. 6” data in GenBank from other published work (8, 18). Identifying An. sp. 6 as An. gibbinsi provides more context for this understudied taxon, as findings from the existing literature can be linked to create a composite understanding of its behavior and vector potential. For example, An. sp. 6 was molecularly identified in Nchelenge District in a study from 2016, but morphology did not identify it as An. gibbinsi, due to damage to the specimen (18). In Kenya, specimens morphologically identified as both An. gambiae and An. funestus were only molecularly identified as An. sp. 6 (34), illustrating the challenges of identifying these uncommon and often unknown taxa (8). Generating reference sequences for morphologically confirmed specimens will be invaluable as Anopheles species identification continues to incorporate high-throughput molecular techniques and genomic data.

In this study, the most common species misidentification was with An. funestus (1.1%) followed by an unidentified species (0.4%). This is similar to published reports that found 16/25 (64%) of An. sp. 6 morphologically identified as An. funestus, and 9/25 (36%) identified as An. gambiae (34). Misidentification of anophelines is a common problem when using only morphology, especially when samples are damaged from trapping methods. This underscores the need for more training in morphological identification, and for the development of reliable genetic references for comparison across anopheline taxa.

In this study, 60% of An. gibbinsi specimens were caught near animal pens, and goat DNA was detected in 85.5% of all An. gibbinsi specimens with detectable host DNA. Additionally, traps near animal pens had the highest proportion of An. gibbinsi with host blood compared to trap placements near humans or sheltered/indoor locations, suggesting that this species is largely zoophilic and exophilic. However, 16.3% (n = 73) of specimens were caught indoors, and 5.4% (n = 4) of those were found with a human blood meal. This is similar to findings from the Kenyan highlands where 2/11 (18.%) of An. sp. 6 harbored human blood meals (34). While this species appears to be largely zoophilic and exophilic, opportunistic feeding on humans and occasionally indoors may not be unusual, as similarly reported for other secondary and understudied malaria vectors including An. coustani, An. rupes, and An. squamosus (4, 6, 10, 11).

Given the high proportion of goat blood meals in the samples, it is unsurprising that animal pens were associated with a higher abundance of An. gibbinsi than traps placed indoors or near where people gather outdoors in both the univariate and multivariate analysis. Inland households, increased proximity to streams, and using an open well/pond were also associated with higher counts of An. gibbinsi. These suggest that An. gibbinsi may be using slow moving streams and rivers as breeding sites in Nchelenge District during the dry season. However, considering that inland households were specifically selected because of their proximity to potential breeding sites, it is possible there is another explanation for these associations that was not captured in this study. Additionally, walls made from natural materials were also associated with higher An. gibbinsi counts. Natural wall materials and using a stream or pond as a water source compared to a bore hole may indicative of lower socioeconomic status or temporary housing, which may also impact mosquito densities.
This study did not detect any *An. gibbinsi* specimens positive for *P. falciparum* sporozoites, but parasite-positive *An. sp. 6* (2/27, 7.4%) were reported from the Kenyan highlands found using a multiplexed qPCR method (34). Additionally, another study from the Kenyan highlands found 7.7% of morphologically-identified *An. gibbinsi* positive for sporozoites by CSP ELISA (2). Given the high transmission in Nchelenge District and potential for *An. gibbinsi* to serve as a vector, it is important that this species be included in ongoing and future malaria surveillance.

**Conclusion**

This study documented *An. gibbinsi* as an anopheline species present in the dry season of 2019 in Nchelenge District, Zambia – the first report of this anopheline species from Zambia. Comparison of COI and ITS2 sequences to NCBI’s GenBank database revealed > 99% identity to *An. sp. 6*, which has been implicated in malaria transmission in Kenya (2, 34). Most specimens were captured near animal pens, and host DNA detection revealed a propensity for goats. Although this finding may be skewed by the collection method, composite data suggest that this species is largely zoophilic and exophilic. However, 5% of specimens with detected host DNA had fed on humans, indicating that this potential vector species is likely to ingest human malaria parasites. The vector competence of *An. gibbinsi*, as supported by reports from Kenya, suggest that this species should continue to be monitored in Nchelenge District. Importantly, this study also provides genetic references for *An. gibbinsi*, which will help inform future studies as molecular identification and verification become more common in malaria entomology.

**Abbreviations**

IRS – indoor residual spray

ITN – insecticide treated net

CSP – circumsporozoite protein

CDC LT – center for disease control light trap

DRC – Democratic Republic of Congo

BSPH – Johns Hopkins Bloomberg School of Public Health

ITS2 – internal transcribed spacer 2

COI – cytochrome oxidase I

PCR – polymerase chain reaction

JHMI – Johns Hopkins Medical Institute

MR4 – Malaria Research and Reference Reagent Resource Center

ELISA – enzyme-linked immunoabsorbant assay
Declarations

Ethics approval and consent to participate:

Ethical clearance and approval were obtained from the Institutional Review Boards of the Tropical Diseases Research Center in Zambia and the Johns Hopkins Bloomberg School of Public Health in Baltimore, Maryland. Informed consent for household participation in the study was given by the head of household at the time of enrollment.

Animal Ethics Declaration

Not applicable

Consent for publication:

Not applicable

Availability of data and materials:

The datasets used in the present study are not publicly available to protect the confidentiality and privacy of study participants, but are available from the corresponding author upon appropriate reasonable request and approval from the corresponding national research and ethics committee.

Competing interests:

The authors declare that they have no competing interests

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Authors' contributions:

MEG, DEN, and JCS conceived and designed the study. MEG and MM coordinated and supervised field collections performed by JSL, DM, and MEG. RSK and MEG completed all laboratory assays, and MEG performed the statistical analysis. MC morphologically identified samples sent to University of the Witwatersrand, and LLK and YMD prepared and sequenced those samples. MEG, DEN, WJM, RSK, MC, and LLK drafted the manuscript, and all authors have read and approved the final manuscript.
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References


Figures
Figure 1

Top panels indicate the geographic location of the study site. Yellow circles in the bottom panel represent the total counts of *An. gibbinsi* captured at each household throughout the study period. Households with no *An. gibbinsi* are represented by red triangles.

Supplementary Files

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