

# High resolution species assignment of *Anopheles* mosquitoes using *k*-mer distances on targeted sequences

Marilou Boddé<sup>1,2,\*</sup>, Alex Makunin<sup>2</sup>, Diego Ayala<sup>3</sup>, Lemonde Bouafa<sup>3</sup>, Abdoulaye Diabaté<sup>4</sup>, Uwem Friday Ekpo<sup>5</sup>, Mahamadi Kientega<sup>4</sup>, Gilbert Le Goff<sup>3</sup>, Boris K. Makanga<sup>6</sup>, Marc F. Ngangue<sup>7</sup>, Olaitan Olamide Omitola<sup>5</sup>, Nil Rahola<sup>3</sup>, Frederic Tripet<sup>8</sup>, Richard Durbin<sup>1,2</sup>, Mara K. N. Lawniczak<sup>2,\*</sup>

1 Department of Genetics, University of Cambridge, Downing Street, Cambridge, CB2 3EH, UK

2 Wellcome Sanger Institute, Hinxton, CB10 1SA, UK

3 Institut de Recherche pour le Développement, MIVEGEC, Univ. Montpellier, CNRS, IRD, Montpellier, 34394, France

4 Institut de Recherche en Sciences de la Santé, Direction Régionale de l'Ouest, 399 Avenue de la Liberté, (+226) 20981880, Bobo-Dioulasso, Burkina Faso

5 Federal University of Agriculture Abeokuta, Alabata 110001, Abeokuta, Ogun State, Nigeria

6 Institut de Recherche en Ecologie Tropicale, BP13354, Libreville, Gabon

7 Centre International de Recherches Medicales de Franceville, Franceville, Gabon

8 Centre for Applied Entomology and Parasitology, Keele University, Newcastle, ST5 5BG, UK

\* [mmb52@camb.ac.uk](mailto:mmb52@camb.ac.uk), [mara@sanger.ac.uk](mailto:mara@sanger.ac.uk)

## Abstract

The ANOSPP amplicon panel is a genus-wide targeted sequencing panel to facilitate large-scale monitoring of *Anopheles* species diversity. Combining information from the 62 nuclear amplicons present in the ANOSPP panel allows for a more nuanced species assignment than single gene (e.g. COI) barcoding, which is desirable in the light of permeable species boundaries. Here, we present NNoVAE, a method using Nearest Neighbours (NN) and Variational Autoencoders (VAE), which we apply to *k*-mers resulting from the ANOSPP amplicon sequences in order to hierarchically assign species identity. The NN step assigns a sample to a species-group by comparing the *k*-mers arising from each haplotype's amplicon sequence to a reference database. The VAE step is required to distinguish between closely related species, and also has sufficient resolution to reveal population structure within species. In tests on independent samples with over 80% amplicon coverage, NNoVAE correctly classifies to species level 98% of samples within the *An. gambiae* complex and 89% of samples outside the complex. We apply NNoVAE to over two thousand new samples from Burkina Faso and Gabon, identifying unexpected species in Gabon. NNoVAE presents an approach that may be of value

to other targeted sequencing panels, and is a method that will be used to survey *Anopheles* species diversity and *Plasmodium* transmission patterns through space and time on a large scale, with plans to analyse half a million mosquitoes in the next five years.

## Introduction

The *Anopheles* genus contains dozens of mosquito species that are the vectors for the *Plasmodium* parasites which cause human malaria, and are thus of global public health interest. The genus contains nearly 500 formally described species (Harbach and Kitching 2016), which span more than 100 million years of evolution (Marinotti et al. 2013). Only a subset of these species has the ability to transmit human malaria; however this vectorial capacity is not limited to a specific part of the species tree, but found throughout the genus phylogeny. Many species are members of closely related species complexes or groups, which are morphologically indistinguishable and share large amounts of genetic variation due to their ability to hybridise in areas of sympatry (*Anopheles gambiae* 1000 Genomes Consortium et al. 2017; Harbach and Kitching 2016). Accurate species identification of *Anopheles* mosquitoes is difficult yet of crucial importance. This is because even closely related species may differ in vectorial capacity, insecticide resistance status and behavioural traits, all of which can influence the efficacy of malaria control efforts (White, Collins, and Besansky 2011). Novel control efforts like gene drive are likely to be implemented this decade for *An. gambiae*, and the success of these efforts will need to be closely monitored. A thorough understanding of the geographic and temporal distribution of different *Anopheles* species and the potential extent of gene flow between species within the same complex is a necessary condition to implement gene drive technology as a malaria control tool.

Currently, the typical process of species identification for *Anopheles* mosquitoes starts by assigning them to species complexes or groups using morphological keys (Gillies and De Meillon 1968; Gillies and Coetzee 1987; Rattanarithkul and Panthusiri 1994; Coetzee 2020; Irish et al. 2020). These morphological keys are usually specific to geographical regions and hence require in-depth and up-to-date knowledge of the species ranges. Moreover, the keys are specific to certain life-stages and may require one to grow larvae to a later stage or even adulthood. Because the morphological features distinguishing one group of species from another can be very nuanced, the accuracy of morphological classification depends on the level of experience and expertise of the person carrying out the identification. Species inside species complexes can be morphologically indistinguishable from each other at the adult stage and hence molecular assays are required for precise species identification. The most commonly used method is a PCR-based species diagnostic assay, targeting the highly variable internal transcribed spacer (ITS2) or similarly variable genomic regions (Cohuet et al. 2003; Scott, Brogdon, and Collins 1993; Fanello, Santolamazza, and della Torre 2002; Wilkins, Howell, and Benedict 2006), although other approaches exist, for example based on mass spectrometry (Nabet et al. 2021). The PCR assays require the use of primers specific to the species complex or group, hence higher level morphological misclassification can lead to failure to generate PCR product or even erroneous species classification (Erlank, Koekemoer, and Coetzee 2018). Mutations in primer or restriction sites can also lead to PCR failures. Moreover, in the case of

hybrids or cryptic species, species identification based on a single marker can result in overconfident assignment to a single species, lacking the nuance that is desirable in this case. There exist panels targeting multiple loci, however, these are also specifically designed to work on a single species complex (Rongnoparut et al. 1996; Lanzaro et al. 1995; Wang-Sattler et al. 2007; Santolamazza et al. 2008).

To help overcome these challenges, a multilocus amplicon panel called ANOSPP (for “**AN**opheles **SP**ecies and **P**lasmodium”) was previously designed to amplify loci from any individual from any Anopheline species (Makunin et al. 2022). In brief, the panel targets 62 loci in the generic *Anopheles* nuclear genome, spread over all chromosome arms, including exonic, intronic as well as intergenic regions. Additionally, it targets two conserved loci on the generic *Plasmodium* mitochondrial genome, to simultaneously evaluate *Plasmodium* presence and species for each individual mosquito. Sequence data from up to 62 nuclear loci targeted by the ANOSPP panel for each sequenced specimen increases the resolution to distinguish closely related species, and to flag potential hybrid or contaminated samples as well as cryptic species. Moreover, the multilocus approach opens the possibility of population genetic and structure analyses for single or closely related species.

The ANOSPP panel has been developed to improve accuracy and depth of information as well as to drive down costs and time required to carry out vector species surveillance (Makunin et al. 2022). Accordingly, all that is required is to identify the individual mosquito as an Anopheline (as opposed to a Culicine), which requires minimal expertise as it is based on the length of the maxillary palps. Furthermore, the panel can use an extremely small aliquot of DNA (<1% of whole mosquito extraction) extracted from each mosquito using a cheap, nondestructive, high-throughput workflow (Makunin et al. 2022). Each mosquito is stored in the well of a 96 well plate in ethanol, the non-destructive lysis buffer is added, incubated overnight, and then removed. Ethanol is then added again to the mosquito carcass to preserve the mosquito and enable morphological evaluation of any individual post-sequencing. A dilution of the lysate is made, which is then PCR amplified with a cocktail of 64 primer pairs in a single well through a two-step process (Makunin et al. 2022). A single Illumina library is generated containing amplified and bar-coded material pooled from 768 samples, then sequenced on a single Illumina MiSeq lane.

The ANOSPP panel originally used a species assignment method based on alignment distances, using static sequence similarity thresholds fitted per target (Makunin et al. 2022). Using this method, amplicons contain sufficient information to distinguish between the majority of the 56 species represented in the original dataset; only samples within some species complexes could not be accurately assigned. However, this method was only tested on a small dataset and the test set was also used as the training set to form the haplotype clusters. Additionally, some targets were extremely divergent between different groups of samples in the dataset and the distances for these targets appear to be sensitive to the choice of alignment algorithm. Here we present a new species assignment method that we call NNoVAE, which is a *k*-mer based method consisting of an initial step using Nearest Neighbours (NN) that identifies samples down to a species or, in some cases, a species complex, and a second step using a Variational Autoencoder (VAE) for species identification within a species complex. The VAE in

the second step is specifically trained on the species complex to which test samples get assigned by the NN step. The complex-specific VAE is required because the similarity between closely related species is too close to detect with a general purpose method like the NN step. Both assignment steps in NNoVAE work on the same  $k$ -mer tables.

NCBI GenBank's BLAST (Altschul et al. 1997) can be used to assess the similarity of any sequence to those within any INSDC database such as NCBI GenBank (Benson et al. 2018). BLAST works on a single query at a time, and does not assign species directly, but rather reports the best hits of the query sequence alongside  $p$ -values based on the alignment score corrected for the size of the database. Similarly, the standard search methods for BOLD (Ratnasingham and Hebert 2007) performs species assignment based on a threshold of percent identity between the test sample and the samples in the reference database. The threshold is informed by extensive knowledge of the divergence of the single marker cytochrome oxidase subunit I (COI) between different species. In addition, the BOLD analysis toolkit contains a NN method to explore the relationship between multiple species in the database. Like BLAST and the BOLD toolkit, the NN step of NNoVAE uses sequence-based distances between the haplotypes of test samples and samples in a reference database; however this is performed across all amplicons and uses  $k$ -mer distances instead of alignments. By using  $k$ -mers, we avoid the issues associated with alignment of highly divergent sequences and moreover are able in a natural way to use short indels and small structural variants as well as SNPs. NNoVAE aims to assign species identity and simultaneously collect information about the relationship of the test sample to different species or species-groups in the database, which is particularly useful when the test sample is of a species not represented in the database. Moreover, NNoVAE combines the information from the 62 different target regions in ANOSPP to build confidence in the species assignments, or reflect uncertainty in the species assignments where appropriate.

NNoVAE resolves species identity within the *An. gambiae* complex using a VAE trained on samples from this complex. A variational autoencoder is a machine learning method that learns structure in high-dimensional data by encoding it into a low-dimensional space and subsequently generating simulated data from the low-dimensional encodings (Kingma and Welling 2013). VAEs have previously been used for species delineation in spiders (Derkarabetian et al. 2019) and visualisation of population structure in *Anopheles* and humans (Battey, Coffing, and Kern 2021). Both these studies used sequence alignments containing much more genomic sequence than the amplicon panel provides. In contrast, NNoVAE is  $k$ -mer based, making it robust to alignment ambiguity and enabling it to efficiently make use of all the available sequencing data.

With the application of the NNoVAE method to data resulting from the ANOSPP panel, we aim to create a robust and efficient platform for molecular species identification within the entire *Anopheles* genus. We present a method that can assign individuals to any of the 62 species currently represented in the reference database as well as taxonomically place species not yet represented. We will include more samples from more species in the reference database as they are sequenced by the amplicon panel, so as to represent the full diversity of the *Anopheles* genus. NNoVAE also indicates the uncertainty of the assignment and provides information on

other closely related species in the dataset, so in particular can flag potential hybrids. It can also be applied to whole genome shotgun (WGS) data by computationally extracting the amplicon target regions, allowing to integrate WGS reference panels with amplicon-sequenced field samples.

## Methods

### Data processing

#### Panel sequences

The amplicon sequencing data are processed as described previously (Makunin et al. 2022). In brief, the fastq files containing the reads are split into one file per target by cutadapt v2.5 (Martin 2011), which uses the primer sequences to do so. Cutadapt also filters for read pairs where both the forward and reverse read match the appropriate primer and trims the primers. Next, DADA2 v1.10 (Callahan et al. 2016) is used to reconstruct sample haplotypes from these read pairs and they are filtered using a custom script to include only haplotypes supported by at least 10 read pairs and with at least 0.1 haplotype frequency per sample-amplicon pair.

#### Reference genomes

*In silico* amplicon extraction from reference genomes is done as described previously (Makunin et al. 2022). Targets are extracted by matching the primer sequences in the reference genome using SeekDeep v2.6 command 'genTargetInfoFromGenomes' (Hathaway et al. 2018).

#### Publicly available data

Where short read whole genome sequence data are publicly available for *Anopheles* mosquitoes, we use these data to pull out target haplotypes to add to our reference index. If the genomic coordinates of the primers are known (e.g. the reads are aligned to a publicly available reference genome), we extract the reads overlapping the primer or target sites for each amplicon separately from BAM files and convert these to fastq files, using samtools v1.9 (Li et al. 2009). These are used as input for fermi-lite (Li 2015), which creates an assembly graph. The unitigs from the assembly graph are cleaned up by cutadapt v3.1 (Martin 2011): the sequences outside the primer sites are trimmed, while the sequences matching the primers are retained and the unitigs are oriented according to the primers. Next, the unitigs are merged using the information from the assembly graph with a custom python script, which relies on MAFFT v7.475 (Kato and Standley 2013) for sequence alignment. In the final step, the primers are trimmed from the resulting haplotypes by cutadapt and any haplotypes that do not have primer sequences on both ends are removed, which helps to get rid of contamination. If the genomic coordinates of the primers are not known, we align the samples to the most appropriate reference genome, identify the genomic coordinates of the primer sites if not yet known and follow the steps above. This pipeline is implemented in Snakemake 5.30.2 (Mölder et al. 2021). Snakefile and scripts are available on GitHub.

## Data structure

The resulting haplotypes are stored in a table with columns recording the sample name, target, haplotype sequence, read count of supporting reads (for amplicon data only), fraction of supporting reads (for amplicon data only). So each row corresponds to a unique haplotype for a sample target combination, hence samples that are heterozygous at a certain target will have two rows for the same target.

## Implementation

Species labels are assessed and species-groups constructed using custom Python scripts implemented in python 3.8 (Van Rossum and Drake 2009). The VAE is implemented in keras 2.3 (Chollet and Others 2015) using a custom python script. Convex hull construction and distance computations rely on scipy 1.6 (Virtanen et al. 2020) and pygel3d 0.2 (Bærentzen 2018). Plots are created with matplotlib 3.3 (Hunter 2007) and seaborn 0.11 (Waskom 2021). All scripts and environments are available on GitHub.

## K-mers

Alignments of amplicon target sequences from highly diverged species are often poor and it is difficult to define a ‘fair’ distance metric based on these alignments. Moreover, there is not a straightforward way to account for small indels and structural variants with alignment based distances. *K*-mer based distances naturally incorporate indels and structural variation and account for highly diverged sequences in an objective way and provide a solution to the problems arising from relying on alignments. Therefore, our species assignment method uses *k*-mers to support better comparisons between the sequences in the database and the sequences of the test sample.

There is a trade-off in the choice of *k*. For large *k* there is little tolerance for errors, while for small *k* there is a high chance that the same *k*-mer is found in multiple locations in the sequence. For example, in a 149bp sequence, 5 evenly spread SNPs result in no 25-mers matching the reference. On the other hand, the chance that all 4-mers are unique in a sequence of the same length is incredibly small ( $<10^{-22}$ ). Based on these trade-offs, we selected 8-mers as a reasonable length. The total sequence length of the amplicon panel targets for the current *An. gambiae* PEST reference genome sequence AgamP4 is 9928 bp, with a mean target length of 160 bp. There are 65536 unique 8-mers, so the chance that all 8-mers within a target are unique is 84% on average. Across the nearly 10 kb of amplified sequence, the chance that all 8-mers are unique is vanishingly small, but the expected number of unique 8-mers is approximately 8533 (sd 46) and the expected number of non-unique 8-mers is approximately 680 (sd 22).

The methods we present here work with *k*-mer tables created from each haplotype from each target. A *k*-mer table consists of  $4^k$  columns, each corresponding to a unique *k*-mer. To translate

a sequence to a  $k$ -mer table, we record in each column how often the corresponding  $k$ -mer occurs in the sequence. This results in a sparse table (the sparsity of course depends on the choice of  $k$ ) with non-negative integer entries.

The  $k$ -mer distance between two sequences is defined as follows. Translate both sequences to  $k$ -mer tables as described above and call these  $q_1$  and  $q_2$ . Then the  $k$ -mer distance between them is given by  $d_k(q_1, q_2) = \frac{\sum |q_1 - q_2|}{\sum |q_1 + q_2|}$ , i.e. the number of non-matching  $k$ -mers divided by the total number of  $k$ -mers in both sequences. The normalisation is required to correct for a bias attributing smaller distances to shorter sequences.

## Results and Discussion

### Reference database construction

Species assignment methods commonly work by comparing a query sequence to a reference database (Ratnasingham and Hebert 2007; Benson et al. 2018). The completeness and quality of the reference database heavily influence the accuracy of the assignment method. The reference database we constructed consists of well-curated samples sequenced with the panel, *in silico* extracted reference genomes, and *in silico* extracted whole genome short read data. As we expect that the reference database will expand to include additional species and populations over time, we assign a version number to the database. The reference database described here, which we call NNv1, contains 186 samples, representing 62 species spread over 4 subgenera. The dataset from (Makunin et al. 2022) forms the backbone of the reference panel. In addition, six species in the *An. gambiae* complex have been included from publicly available whole genome data (The Anopheles gambiae 1000 Genomes Consortium 2021; Fontaine et al. 2015) in order to increase the resolution in a group of hard to distinguish species. To maintain the advantages of multi-locus assignment, we required samples to have at least 10 targets amplified to be included in the reference database. Ideally, the database would contain several specimens per species to represent within species variation. This is particularly important for species with a wide geographical range.

The amplicon panel is designed to improve accuracy of species assignments over morphological or single-marker methods. However, the species labels supplied by our sample partners were in most cases obtained by the latter methods, hence it was necessary to reconfirm them. All label information for the reference database NNv1 is listed in Supplementary Table 1, and the assignment of species labels is discussed in more detail (Supplementary Information Section 1), but we present an outline of the principles we used here. For most samples sequenced by the amplicon panel, two molecular barcodes (COI and ITS2) were also sequenced and compared to the sequences available in BOLD (Ratnasingham and Hebert 2007) and NCBI (Benson et al. 2018). Here, we used the barcode information as well as the pairwise  $k$ -mer distances between samples in the reference database NNv1 to generate a consensus species label for each sample. A few samples show inconsistencies between their

partner labels, molecular barcodes and amplicon assignments; these samples were also removed or flagged as overly diverged in Makunin et al. (2022). For these samples the distances to samples of the same species label is much larger than the distance to some other samples in the database, suggesting they were mislabelled. In some cases there is additional evidence from molecular barcodes and it was possible to relabel them to the species they match. Other samples are clearly different from samples with the same species label, but do not clearly belong to any other species in the database. These are labelled as the species-group they belong to appended by ‘\_sp1’, ‘\_sp2’, etc. Hopefully, by extending the reference database in the future, we can get a better understanding of which species such samples represent. For some closely related species, the different species labels were supported neither by the pairwise-distances nor by the barcodes. Samples from these species are assigned to a group of closely related species and their consensus labels end in ‘\_c’ to notify that they are part of a complex of species that cannot be distinguished by the nearest neighbour method. None of the *in silico* extracted samples showed inconsistencies with pair-wise  $k$ -mer distances and hence all retained their published labels.

## Species-groups construction

The species-groups are defined based on the pairwise  $k$ -mer distances between samples in the reference database NNv1 and make use of the consensus species labels discussed above. The  $k$ -mer distance between two samples,  $s_1$  and  $s_2$ , is defined as

$$d_{s,k}(s_1, s_2) = \frac{1}{|T_1 \cap T_2|} \sum_{t \in T_1 \cap T_2} \sum_{q_1 \in Q_{1,t}} \sum_{q_2 \in Q_{2,t}} \frac{d_k(q_1, q_2)}{|Q_{1,t}| |Q_{2,t}|},$$
 where  $T_i$  is the set of targets amplified in sample  $i$

and  $Q_{i,t}$  is the set of unique haplotypes of sample  $i$  at target  $t$ . So in words, for a given target the  $k$ -mer distance between two samples is the mean  $k$ -mer distance between all pairs of haplotypes from the two samples; e.g. if sample  $s_1$  is homozygous and  $s_2$  heterozygous at target  $t$ , then the  $k$ -mer distance at target  $t$  between these samples is the mean of the  $k$ -mer distance of the haplotype from  $s_1$  compared to the first haplotype from  $s_2$  and the  $k$ -mer distance of the haplotype from  $s_1$  compared to the second haplotype from  $s_2$ . If a sample has more than two alleles at a single target, we take into account all haplotypes according to the above definition. The  $k$ -mer distance between two samples is defined as the average of the  $k$ -mer distance between these samples at all the targets that were amplified in both samples.

Figure 1 shows the pairwise  $k$ -mer distances between all samples in the reference database. The samples are ordered roughly by phylogeny (as in the tree in Figure 2) and this results in a visible structure in the distance plot. One can observe dark triangles below the diagonal, reflecting that samples of the same or closely related species have a smaller  $k$ -mer distance to each other than to other samples. This effect repeats itself on different scales, mirroring the multi-level structure in the phylogenetic tree.





reference database NNv1 into species-groups, such that the  $\delta$ -mer distance between each pair of samples within the group is smaller than the threshold and the  $\delta$ -mer distance from any sample within the group to any sample outside the group is larger than the threshold. The motivating assumption is that the samples in the same species-group share more recent ancestry with each other than with samples that are members of different species-groups. While several thresholds satisfy the partitioning condition, they are most useful for the purpose of species classification when they generate species-groups that (approximately) correspond to classification at an established taxonomic level. Not surprisingly, there are no thresholds that perfectly satisfy the partitioning condition and are completely concordant with current taxonomy, but several thresholds give groups that are close to taxonomic entities and given the paucity of molecular data for some of these species, it may be that the taxonomic entities are less phylogenetically accurate than the  $k$ -mer based groupings. In exploring different thresholds (Supplementary Information Section 2), we selected three levels that best matched described taxonomic entities. The species-groups at each of the threshold levels we've selected are listed in Supplementary Table 1.

For very low thresholds, each sample will form its own species-group, which is not informative. For a threshold of 0.1 on the sample  $\delta$ -mer distance, most species-groups satisfy or nearly satisfy the partitioning condition, and each species-group contains a single species or multiple species from a known species complex or group, e.g. *An. gambiae* and *An. coluzzii* form a fine-level species-group together. We refer to the species-groups at the 0.1 level as the fine level species-groups and they are most useful for species assignment, because they provide the highest resolution. A threshold of 0.3 merges samples representing species from some well-known complexes, like the entire *An. gambiae* complex, into a single species-group. Similarly, many of the species for which we currently only have a single representative in the reference database NNv1, are merged into larger species-groups. We refer to this level of species-groups as the intermediate level, which provides insight into the degree of similarity between different fine level species-groups. Additionally, the intermediate level species-groups can be informative when we sequence a sample whose species is not represented in the database because the species assignment at the intermediate level places the sample within its most closely related species-group in the database. Thresholds higher than 0.3 tend to violate the partitioning condition to a greater extent, but it is desirable to include a coarse level classification to get an approximate taxonomic position of unrepresented species and of more diverged species. We selected 0.51 as a threshold for the coarse level classification because this gives reasonably clear species-groups that roughly correspond to taxonomic series. However, it is not perfect, as it groups together the *Myzomyia* and *Neocellia* series in the *Cellia* subgenus and it splits the *Neomyzomyia* series into three distinct species-groups. A similar effect was observed in Makunin et al. (2022), where the *Neomyzomyia* series did not form a monophyletic clade.

At the fine level, most species-groups contain all samples from a single species, but there are some exceptions. For some species, e.g. *An. nili* and *An. hyrcanus*, the samples are split into multiple fine-level species-groups, because they appear much more distinct from each other than you would expect in a single species (see Supplementary Information Sections 1 and 2 for

more detailed discussion). In our assignment, these will be treated as distinct species, highlighting to entomologists and taxonomists that further work to refine species in these groups is needed. Conversely, there are also species-groups that contain samples from multiple different species. These do not represent a single species, but instead they represent a complex of closely related species. Species inside species complexes often share a lot of genetic variation and the  $k$ -mer distance based method that we discuss here does not have sufficient resolution to reliably distinguish between them. The species-groups at the fine level that contain more than a single species are the *An. marshallii* group (contains *An. hancocki*, *An. brohieri* and *An. demeilloni*), *An. gambiae/coluzzii*, the *An. sondaicus* complex (contains *An. sondaicus* and *An. epiroticus*) and the *An. coustani* group (contains *An. coustani*, *An. ziemanni*, *An. tenebrosus* and *An. paludis*).

## Nearest neighbour assignment

The first step of the hierarchical assignment method performs nearest neighbour assignments to samples in the reference database at the three different levels of species-groups introduced above. The assignment is initially done independently at each target, for each sample, computing assignment proportions for the species-groups at the chosen level, normalised such that they sum up to one over all species-groups. The resulting per-target assignment proportions are then averaged over all targets, resulting in the overall sample assignment proportions at the chosen level. If the sample assignment proportion is at least 0.8 for one species-group, the sample is classified as a member of that group. If the classification threshold of 0.8 is not met at the chosen level, the sample remains unassigned at that level.

For example, to assign a sample  $s$  at the coarse level, we translate its target sequence corresponding to target 1 to an 8-mer count table, denoted as  $q_{s,1}$ . Next, we compute its 8-mer distance,  $d_8(q_{s,1}, q_{r,1})$ , to every target sequence in the reference database corresponding to target 1, i.e. to every  $q_{r,1}$ . The nearest neighbours of the test sequence  $q_{s,1}$  are those sequences in the database that minimise the 8-mer distance between themselves and  $q_{s,1}$ . In other words, the nearest neighbour sequences of  $q_{s,1}$  are the target 1 sequences in the database with the highest percentage of matching 8-mers. The nearest neighbour sequence of  $q_{s,1}$  can be a sequence that occurs in a single sample in the reference database, or the nearest neighbour sequences can be the same sequence occurring in multiple samples in the database, or the nearest neighbour sequences can be distinct sequences that have the same distance to  $q_{s,1}$ .

Now we bring in the species-groups. For each species-group we record the frequency of nearest neighbour sequences. This can be thought of as an ‘allele-frequency’ when we classify each target sequence as either a ‘nearest neighbour allele’ or not a ‘nearest neighbour allele’. But just like allele frequency, it takes into account the zygosity of the samples. The nearest neighbour frequencies are normalised, such that they are equal to one when summed over all species-groups. These quantities are the per-target assignment proportions.

This procedure is repeated for every amplified target in the test sample. Finally the per-target assignment proportions are averaged over all successfully amplified targets to give the overall assignment proportions for sample  $s$  at the coarse level. If there is a species-group with an assignment proportion of at least 0.8, the sample is classified as a member of this group, otherwise it remains unassigned at this level. Assignments to the intermediate and fine levels are made in the same fashion, starting from the same nearest neighbour assignments, but based on the relevant species-group memberships.

The per-target assignment proportions are based on the frequency of nearest neighbour sequences in the species-groups and not simply on the count of nearest neighbour sequences. The use of frequencies corrects for the different sizes of the species-groups. Suppose a nearest neighbour sequence occurs in 2 out of 10 samples of species-group A and in 1 out of 2 samples in species-group B (and assume all samples are homozygous). By using counts, we would attribute  $2/(2+1) = 0.67$  and  $1/(2+1) = 0.33$  assignment proportion to species-group A and B, respectively. But if we did this, species-group A would only have a higher assignment proportion because it contains more samples. What we are really interested in, is how similar the target sequence of the test sample is to the target sequences in the species-groups. So by using the nearest neighbour frequencies, the assignment proportions are  $(2/10)/(2/10+1/2) = 0.29$  and  $(1/2)/(2/10+1/2) = 0.71$  for species-group A and B, respectively.

The nearest neighbour frequencies observed for a given target of a given sample at a given assignment level are normalised to obtain the per-target assignment proportions. This normalisation ensures that every target is weighted equally. Without the normalisation, the weight of a target would be determined by a combination of the overall frequency of the nearest neighbour sequences and their distribution amongst the species-groups, whilst we are more interested in the distribution than the total frequency.

Targets that did not amplify in the sample are simply ignored. There are different reasons why a certain target does not get amplified in a sample. It might be that the primer binding sites for the target are too diverged or altogether absent in the test sample's genome. Or the target might not be amplified due to technical reasons like poor DNA quality. In the former case, we are implicitly using the information contained in the missingness, because we restrict our attention to the targets that did amplify in the sample, which should be the same targets that amplified in the samples of the same species contained in the reference database. In the latter case, as long as the missingness is randomly affecting the targets, ignoring missing targets does not bias the assignment proportions.

So far we have assumed that the test sample was homozygous at each target. To generalise to the heterozygous case, we compute the per-target assignment proportions separately for both target sequences and average them for the final per-target assignment proportions. It does occasionally happen that a sample has more than two different target sequences. This can be due to errors in the PCR amplification or sequencing, contamination by other samples, or a certain target region might be duplicated in the genome of some species. In the NNv1 reference database on average 1.2% of targets per sample have more than two different sequences; in

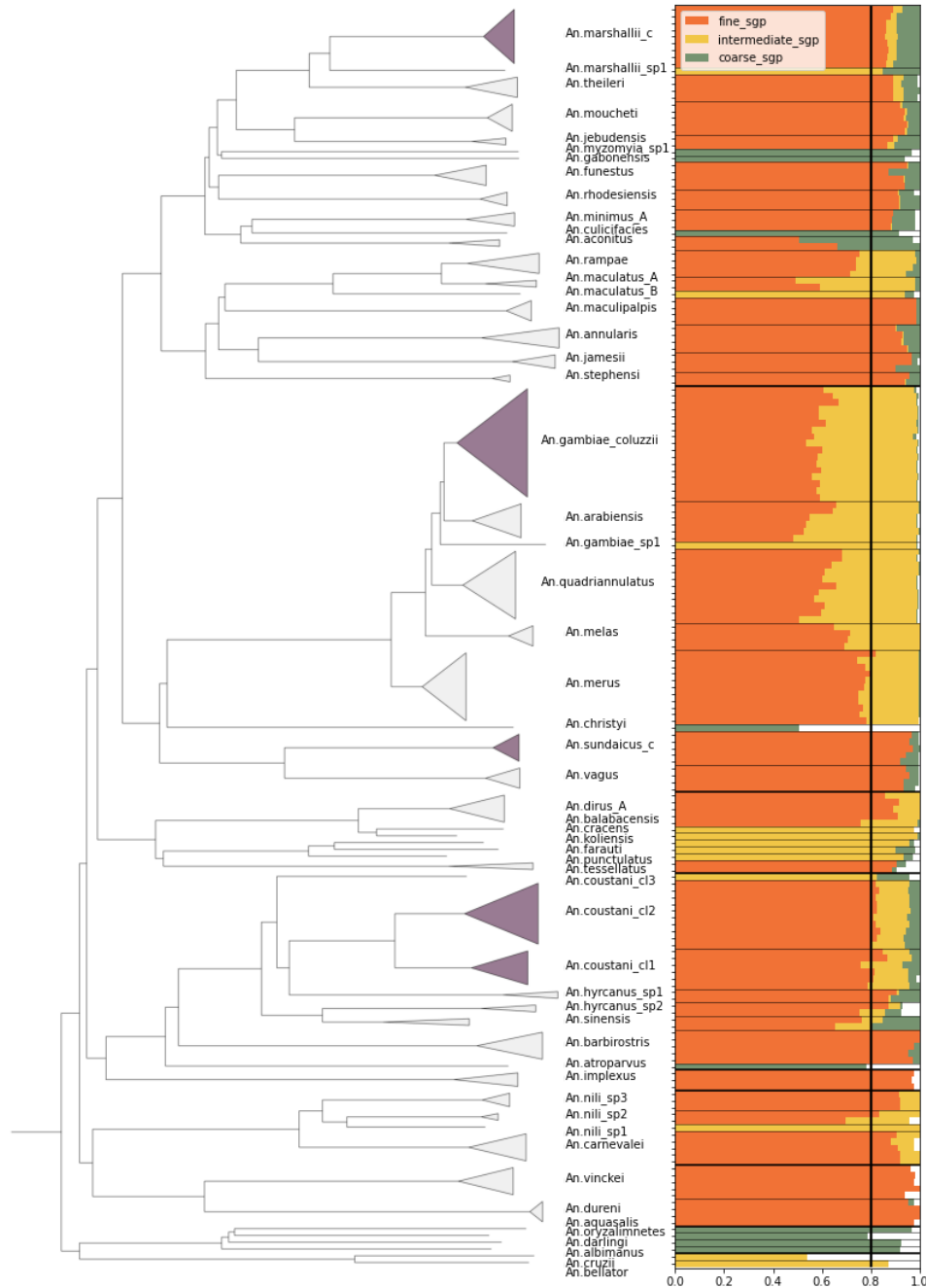
the query datasets discussed later in this article this percentage ranged from 0.3% to 2.3%. We deemed this small enough to simply extend the per-target assignment proportion computation to include targets with more than two different sequences, by taking the average assignment proportions over different sequences as for the heterozygous case.

Figure 2 shows two examples of the nearest neighbour assignment of a test sample. The test samples are Amou-3-2 and Agam-35, an *An. moucheti* and *An. gambiae* individual, respectively. For Amou-3-2 we see that for most targets, the nearest neighbour sequence is found in all four *An. moucheti* samples in the reference database NNv1. For some targets, the nearest neighbour sequence is only carried by a subset of the *An. moucheti* samples in the database, whilst for other targets the nearest neighbour sequence is also carried by individuals of other species. For Agam-35 the heatmap shows that, for many targets, the nearest neighbour sequences are not only found in *An\_gambiae\_coluzzii* samples, but also in samples from other species in the *An. gambiae* complex. There are only two matches to samples outside the *Pyrethophorus* series, not shown here. This results in a high-confidence assignment to the *Pyrethophorus* series at the coarse level as well as a high-confidence assignment to the *An. gambiae* complex at the intermediate level. At the fine level, the largest assignment proportion is to the *An\_gambiae\_coluzzii* species-group, but it does not meet the 0.8 classification threshold because of the relatively high assignment proportions to other species-groups within the *An. gambiae* complex. So at the fine level, this sample cannot be classified with sufficient confidence to a single species. Later, we will present a method to resolve the species identities of samples within the *An. gambiae* complex.



**Figure 2: Nearest neighbour assignment example.** **Left panel:** The heatmap shows the nearest neighbours of sample Amou-2-3, an *An. moucheiti* specimen, at its different targets. For clarity, not all samples in the reference database have been displayed, only those in the *Myzomyia* and *Neocellia* series (except Amou-2-3). The samples from the reference database are arranged along the x-axis and the targets along the y-axis. An entry is coloured pink if the corresponding sample from the reference database has a nearest neighbour sequence at the corresponding target. Peach entries indicate that the corresponding sample from the reference database does not carry a nearest neighbour sequence at the corresponding target. If either the test sample or the reference sample did not amplify at the corresponding target, the entry is white. The bars at the bottom show the overall assignment proportions for the displayed species-groups, only assignment proportions of at least 1% are shown. From top to bottom the assignment proportions are for the fine, intermediate and coarse level. For the three-letter code abbreviations of species-groups, see Supplementary Table 1. The numeric abbreviations stand for 1: *An. marshallii complex sp1*, 2: *Myzomyia sp1*, 3: *An. gabonensis*, 4: *An. culicifacies*, 5: *An. maculatus B*; none of these species-groups represent more than 1% of the assignment. **Right panel:** The heatmap showing the nearest neighbours of sample Agam-35, an *An. gambiae* specimen. Not all samples in the reference database are displayed, only those in the *Pyretophorus* series (except Agam-35) as well as five samples from the *Neocellia* series. The numeric abbreviations stand for 6: *An. gambiae complex sp1* (0.06 assignment proportion), 7: *An. christyi* and MNs stands for *Myzomyia\_Neocellia\_series*.

The species-group assignment has been tested on the reference database itself, by dropping out one sample at a time. The majority of samples could be assigned to the correct species-group at the fine level when using a threshold of 0.8 assignment proportion, see Figure 3, Supplementary Tables 2-4, Supplementary Section 3. To provide context, we have included a phylogenetic tree constructed from pairwise 8-mer distances using FastME (Lefort, Desper, and Gascuel 2015) and displayed using TreeViewer (Bianchini, n.d.). If we ignore the samples that form a species-group on their own, because we do not yet have sufficient representation for those species, 61.8% of samples are assigned correctly at the fine level, and 98.8% and 100% at the intermediate and coarse level respectively. In most cases, the fine level species-groups consist of a single species, although in some cases they comprise multiple species. The jump in assignment success from the fine to the intermediate level is mostly caused by the *An. gambiae* complex, which is well-represented in the reference database. Most samples within the complex can be assigned to the correct fine level species-group to some extent, but they only meet the assignment threshold at the intermediate level species-group assignment, where all samples in the *An. gambiae* complex are grouped together. This effect is seen in a few other groups as well, and motivates the VAE part of our assignment procedure (described below).



**Figure 3:** Species-group assignment accuracy on reference database NNv1. Samples were dropped out of the database one at a time to test the assignment accuracy. **Left:** phylogenetic tree of the samples in the reference database NNv1 constructed from pairwise 8-mer distances using fastme. Samples are labelled by their fine level species-group label. Dark-shaded clades are instances of species-groups that contain more than one species. **Right:** Samples are placed along the vertical axis, ordered by the species tree. The bars represent the assignment proportion to the correct species-group and the colours indicate the species-group level. As an example, the first sample is assigned to the correct species-group with a proportion of 0.88 at the fine level, with a proportion of 0.91 at the intermediate level and with a proportion of 0.99 at the coarse level. The thin horizontal lines indicate the different species-groups at the fine level and the thick horizontal lines at the coarse level. The separation of the species-groups at the intermediate level has not been displayed for clarity. The vertical line represents the assignment threshold of 0.8.

For the same group of samples, the average correct assignment proportion per sample at the coarse level is 99.4%. This shows that for samples of species that are well-represented in the reference database, there is a near perfect assignment at the coarse level. And in fact, the average correct assignment proportion of 95.8% at the intermediate level shows that these assignments are also generally with high confidence. At the fine level the confidence starts to break down for some samples, in particular the *An. gambiae* complex, which is responsible for many samples in the reference database. Then the average correct assignment per sample is 81.3%. This shows that an additional classification method for species complexes is desirable.

In NNv1, 21 species-groups at the fine level consist of a single sample. These cannot be assigned to the correct species-group in the drop-out assignment experiment. 12 of these samples are members of a larger species-group at the intermediate level and 11 of them can be assigned at the intermediate level, the other sample is not assigned at the intermediate, nor at the coarse level. The remaining nine samples only become a member of a larger species group at the coarse level. Six of them can be classified at this level, the other three remain unclassified.

The four unclassified samples are *An. christyi*, *An. atroparvus*, *An. oryzalimnetes* and *An. cruzii*. All of these are quite diverged from everything else in the reference database and as such do not exhibit a strong matching to any of the coarse level species-groups. In particular, the *Kerteszia* and *Nyssorhynchus* subgenus are underrepresented, both in number of species and number of samples, but also the basal species in for instance the *Pyretophorus* series are not well represented and are too diverged from the other species in this series to exhibit strong similarity to the other samples from this series. In the case of *An. cruzii* in the *Kerteszia* subgenus it is actually impossible to assign it to its coarse level species group with the current threshold of 0.8, because of its 26 amplified targets, only 15 are also amplified in the single other sample in the reference database from the *Kerteszia* subgenus, which results in a theoretical maximum assignment to the correct species-group of 0.58. Note, this is the only species-group at the coarse level for which the theoretical maximum correct assignment for a sample in the reference database is smaller than the threshold. But it again underlines the necessity to extend the reference database, both in number of species and number of samples per species.

When a sample is of a species not represented in the reference database, three things can happen. If its species is much more closely related to a single species in the database than to all the others, it will likely be assigned to the species it is related to. Alternatively, if the database contains multiple closely related species, it will be assigned at a higher level to the group that contains all these closely related species. If the sample is highly diverged from all species represented in the database, its nearest neighbours will essentially be chosen at random, and the assignment threshold will not be met. This emphasises the importance of extending the reference database, both by increasing the number of species represented and by increasing the number of samples per species, with a particular focus on capturing the within-species diversity (e.g. representing the geographic species range, representing all karyotypes when polymorphic chromosomal inversions are present).



The species-groups with a single representative can be used to explore the three possible scenarios for when the test sample belongs to a species not present in the reference database: assignment to a closely related species, assignment to a group of species at the intermediate or coarse level, or no assignment.

The first scenario is represented by *An.bellator*. The only other representative of the *Kerteszia* subgenus in our reference dataset is a sample representing *An. cruzii*. At the fine level species-groups, the *An. bellator* sample is assigned for 0.875 to *An. cruzii* and hence meets the threshold to be classified at the fine level. So it can happen that a sample is classified to the wrong species, if this species is the only reasonably close species in the database. This scenario is more likely for diverged groups of species with little representation in the database, for example the *Nyssorhynchus* and *Kerteszia* subgenera.

An example of the second scenario is the sample Amar-3-1, in the *Myzomyia* series. At the fine level it has an assignment proportion of 0.474 to the *An\_marshallii\_complex* and 0.378 to the *An. theileri* species-group. All other species-groups have an assignment proportion of less than 0.06. So this sample is more related to these two species-groups than to anything else in the reference database, but it does not belong to either of them. At the intermediate level it has an assignment proportion of 0.850 to the *An\_marshallii\_group* species-group, which is the *An\_marshallii\_complex* and *An. theileri* species-group combined. Now it meets the threshold and it will be classified as a member of the *An\_marshallii\_group* species-group.

*An. christyi* is an example of a sufficiently diverged sample that does not reach high assignment proportions for a single species-group at any level. At the coarse level, it is assigned to the *Pyrethophorus\_series* with 0.506 and the *Myzomyia\_Neocellia\_series* with 0.392 and other species-groups have much lower assignment proportions. Hence, it is not possible to classify this sample as a member of any species-group. Adding more samples from this and other underrepresented species to the database, would increase its power to classify samples from those species.

## Gambiae complex classifier datasets

The nearest neighbour approach is not able to confidently distinguish between closely related species that share a lot of genetic variation, but it does identify samples of those species as members of a species complex at the intermediate assignment level. To resolve the species identity inside these species complexes, we use a variational autoencoder approach specifically trained for the complex under consideration. We demonstrate this method for the *An. gambiae* complex, both because many of our samples fall within this complex and it is medically relevant to be able to distinguish them, and also more practically, because we have access to a large dataset of species-labelled samples. The classifier we present here contains 7 out of 8 formally named species in the complex (Coetzee et al. 2013), as well as two putative cryptic species (Tennessen et al. 2021; Barrón et al. 2019). Compared to NNv1, three additional species are represented in this classifier to present as much of the diversity of the *An. gambiae* complex as

possible. We expect that this method can be applied to other species complexes for which large species-labelled datasets are available.

The *An. gambiae* complex classifier has been constructed using a training set (GCref v1) and a validation set (GCval v1) of species-labelled samples. We included as many described species in the *An. gambiae* complex as we could find or generate sequence data for. For species with wide geographic ranges and a large amount of genomic data available, we also aimed to represent the diverse geography where possible.

Both GCref v1 and GCval v1 consist of amplicon sequences and *in silico extracted* published samples (Fontaine et al. 2015; Neafsey et al. 2015; Tennessen et al. 2021; The Anopheles gambiae 1000 Genomes Consortium 2021). The samples from Nigeria and Madagascar were not species-labelled, but they were unambiguously classified by an earlier version of this classifier and we included these samples because Nigeria and Madagascar fill geographic gaps in our sampling dataset. The species represented in GCref v1 are *An. gambiae* (406), *An. coluzzii* (222), *An. arabiensis* (94), *An. quadriannulatus* (11), *An. melas* (3), *An. merus* (6), *An. bwambae* (3), *An. tengrela* (38) and putatively *An. fontenillei* (4). These samples are generally high coverage: 97% of samples have at least 55 of 62 targets amplified. The average number of targets tends to be lower for the samples representing species other than *An. gambiae*, *An. coluzzii* or *An. arabiensis*, which are also those species represented by fewer samples, but the geographic ranges of these other species are also much more restricted so the samples we do have are likely good representatives of the species. The species represented in GCval v1 are *An. gambiae* (80), *An. coluzzii* (15), *An. arabiensis* (30), *An. melas* (1), *An. merus* (5) and *An. tengrela* (12). The average number of amplicons for the species other than *An. gambiae*, *An. coluzzii* or *An. arabiensis* is lower than in GCref v1 set. Given that for those species, there is only a small number of samples available, we decided to use ones with at least 45 targets amplified in GCref v1 and the ones with at least 30 targets in GCval v1. Sample information for these datasets can be found in Supplementary Tables 5 and 6.

The input for the VAE is one 8-mer count table per sample, summed over all targets. If a test sample is heterozygous at a given target, we translate each of its haplotypes to an 8-mer count table and sum them to get the test sample's 8-mer count table for the corresponding target. If a test sample is homozygous at a given target, we translate its haplotype to an 8-mer count table and double the counts to obtain its 8-mer count table at the corresponding target. The counts are doubled in order to represent the target sequences as diploid sequences, and not introduce artificial differences in the total number of 8-mers between homozygous and heterozygous target sites. If the test sample has more than two different haplotypes at a given target, two haplotypes are chosen at random and the sample is treated as a heterozygote. It happens on average in less than 1% of the amplified targets that there are more than two different sequences, so we expect that this inexact way of dealing with those cases does not have a major impact on the results. Because we model the 8-mer counts as the observations of a Poisson distribution, the counts have to be integers, hence we cannot average over all observed alleles as in the nearest neighbour method. If a given target did not amplify in the test sample, the associated 8-mer count table will just contain zeroes, equivalent to simply ignoring missing

data. To obtain the VAE input, we sum the 8-mer tables over all 62 targets. This results in a single table per sample, with  $65,536 (=4^8)$  integer entries, roughly summing to twice the number of basepairs covered by the amplified targets, so a little under 20,000 for a sample in which all targets amplified.

## Variational Autoencoder

The within-complex assignment is based around a variational auto-encoder. The VAE consists of an encoder, a latent space projection and a decoder. The specific design we used was inspired by popVAE (Battey, Coffing, and Kern 2021). The encoder is a fully-connected neural network that takes high-dimensional data as input and encodes that as a point in a latent space of much lower dimension. The decoder is also a fully-connected neural network, and it takes as input a point in the latent space and outputs 'simulated' data of the same dimensions as the input data. The VAE learns a 'good' encoding by adjusting the weights in the encoder and decoder to obtain an output similar to the original input. To prevent overfitting, the input of the decoder is not the exact output of the encoder, but a nearby point in latent space. Furthermore, the loss function used to update the encoder and decoder weights contains a regularisation term on the latent space, in addition to the term measuring the similarity of the decoder output and the original input. Because of the introduced sampling noise and the regularisation constraint, the most efficient way to encode the data is to represent samples that are similar in the high-dimensional data by nearby points in the low-dimensional latent space. In summary, we expect that species identity shapes the structure in the 8-mer count tables and that the VAE projects this structure to the low-dimensional latent space, resulting in clustering by species in the latent space.

In our case the encoder input is the 8-mer count table of the training set, so a table of dimension  $n \times 65536$  with non-negative integer entries, where  $n$  is the number of samples in the training set. The output of the encoder is a set of  $2d$  parameters for each sample, where  $d$  is the dimension of the latent space. For each dimension, one parameter corresponds to the mean position in latent space and one parameter corresponds to the variance of the position in latent space. In our case we use a three-dimensional latent space ( $d = 3$ ). The input of the decoder is a position in latent space for each sample, sampled from the distribution determined by the encoder output. The decoder output is an  $n \times 65536$  dimensional table of strictly positive entries, however, unlike the input table, the entries are not necessarily integers.

The loss function is the sum of two terms: one measuring the difference between the input and output data and one acting as a regulariser on the latent space. The relative weight of these terms can be adjusted. If we model the count tables as observations of independent Poisson variables, the difference between the input and output can be measured as the Kullback-Leibler divergence (KL divergence) of the Poisson distribution with the means given by the output from the Poisson distribution with the means given by the input. The KL divergence is the same up to a constant as the negative of the Poisson loglikelihood with the input as the observed counts and the output as the means. So minimising the KL divergence is equivalent to maximising the loglikelihood with respect to the output. The difference term of the loss function is obtained by

summing over all unique  $\delta$ -mers. The theoretical minimum of the difference term of the loss function is zero, and this is attained if the output is exactly the same as the input. However, this theoretical minimum cannot be attained in practice, because the sparsity of the input implies that there will be entries equalling zero and the activation function of the decoder generating the output results in strictly positive entries.

The regularisation term of the loss function is based on the KL divergence of the normal distribution parameterised by the encoder output from a standard normal distribution, i.e.  $N(0, 1)$ . The regulariser is computed for each latent space dimension separately. Again, the theoretical minimum equals zero and is attained when the mean outputted by the encoder is zero and the variance outputted by the encoder is one. The regularisation term of the loss function is defined as the KL divergence summed over the latent space dimensions. This effectively enforces the distribution specified by the encoder output to look like a multi-dimensional Gaussian distribution with mean zero, variance one and covariance zero. In layman's terms, it pulls the projected positions of the samples in latent space towards the origin and establishes a natural scale for them. The regulariser prevents overfitting by making it expensive for the encoder to place samples far away from the origin. The loss function used to train the VAE is the weighted sum of the similarity term and the regularisation term described above, with a parameter  $w$  that controls the relative strength of the two terms.

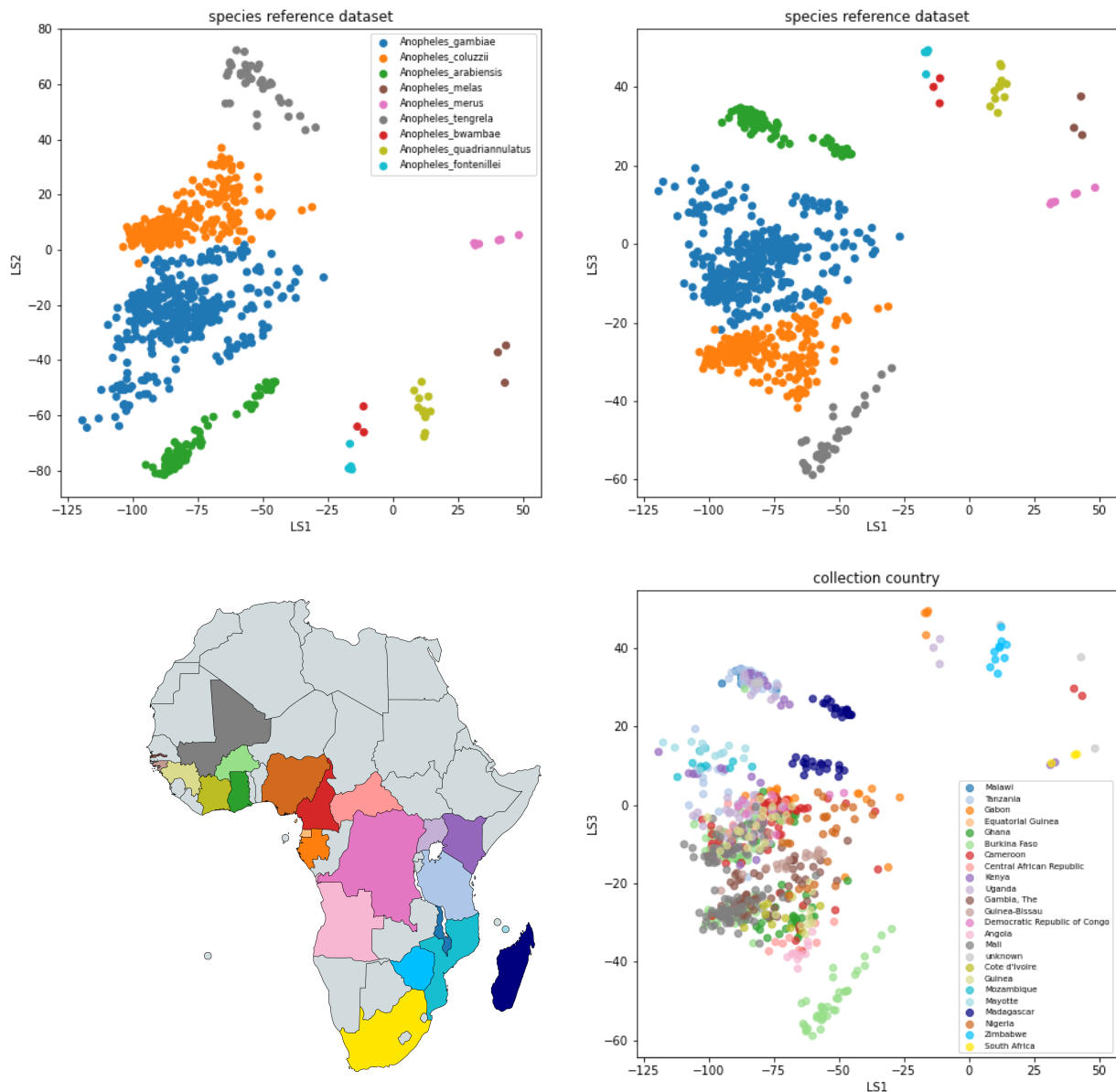
We set most of the parameters involved in training the VAE by comparing the latent-space projections for different parameter values, using a subset of GCref v1 containing only samples from *An. gambiae*, *An. coluzzii* and *An. arabiensis*. The criteria we used to pick parameter values were species classification accuracy of the reference set and the validation set, using assignments based on convex hulls (described below), visible within-species structure, and, as a secondary criterion, useful visualisation. Further detail on the choice of parameter values is provided in Supplementary Information Section 4. The latent space projection is also affected by the training dataset. We observed that the presence or absence of most countries does not affect the classification, except for the Gambia and Guinea-Bissau. When these countries are removed, the accuracy to distinguish between *An. gambiae* and *An. coluzzii* considerably reduces. This is not surprising, since these samples lie on the boundary of the *An. gambiae* and *An. coluzzii* clusters and as such are crucial for assigning those species. The complete results can be found in Supplementary Information Section 5.

## Within-complex species classification

We use the trained and tuned VAE to assign species as follows. We input the summed  $\delta$ -mer table of the test samples into the encoder of the VAE. The encoder outputs a position in latent space for each sample. Importantly, the VAE is agnostic to species labels; the species assignment happens based on the position in latent space of the test samples in relation to the latent space positions of the species-labelled reference dataset GCVAEv1.

The top two panels of Figure 4 show the latent space projection of GCVAEv1. While most species form nicely isolated clusters, *An. gambiae* and *An. coluzzii* border each other closely.

Interestingly, the boundary is formed by mosquitoes from The Gambia and Guinea-Bissau. These mosquitoes are labelled as *An. gambiae* by conventional molecular barcoding, but they cannot be confidently assigned to either *An. gambiae* or *An. coluzzii* using over 500 ancestry informative markers (AIMs) or whole genome PCA (Anopheles gambiae 1000 Genomes Consortium et al. 2017; Clarkson et al. 2020). The clusters containing *An. bwambiae* and the putative new species *An. fontenillei* are placed very close to each other, and can also not be reliably distinguished. These species are closely related, but up until now they have only been discovered in Uganda and Gabon, respectively, and so, since they do not seem to have overlapping geographic species ranges (Barrón, 2019), the species identity of samples falling into either of these two clusters can be resolved by their geographic origin.



**Figure 4:** VAE projection of the *gambiae* complex reference dataset. Top panels: the samples are represented by dots at the inferred mean position in three-dimensional latent space and coloured by their species label. The left

panel shows latent dimension 1 versus latent dimension 2 and the right panel shows latent dimension 1 versus latent dimension 3. Bottom panel: the same projection as above, but here the samples are coloured by the country of collection revealing structure related to geography.

We perform species classification using the convex hulls of species clusters. A convex hull is the mathematical notion of the smallest convex set containing all points of interest. A nice metaphor is to imagine that you are wrapping all points corresponding to samples of a single species together in such a way that requires the minimal amount of wrapping paper. We constructed one convex hull for each species represented in the dataset, using latent space positions of the samples from GCref v1 as well as of 363 additional species-labelled samples that were not used in training the VAE, to account for possible effects caused by projecting the samples to the latent space (sample to be found information in Supplementary Table 7). For our classification procedure it is important that the convex hulls of different species do not overlap. When constructing convex hulls from the full sample set, only the convex hulls corresponding to *An. gambiae* and *An. coluzzii* overlap. We trimmed these hulls by iteratively removing samples from the set of points used to construct them until they didn't overlap. In total we removed 17 *An. gambiae* and 6 *An. coluzzii* samples. The samples from *An. bwambae* and *An. fontenillei* are combined in one convex hull because they are so close together in latent space.

The classification of new samples happens as follows. If the latent space position of the test sample falls inside a convex hull, the sample is classified as that species. If the latent space position of the test sample falls outside all convex hulls, there are two options. If the sample is much closer to one convex hull than to all others, it is classified as the species corresponding to the hull it is closest to. To be precise, this happens if the euclidean distance to the closest convex hull is at least 7 times smaller than the distance to all other convex hulls. This allows for 'fuzzy' boundaries of the convex hulls that are proportional to the separation between the different hulls. We fitted the parameter value 7 on the dataset from Gabon to reflect the assumption that *An. tengrela* is not believed to be found in Gabon. If the latent space position of the test sample falls outside all convex hulls and outside their fuzzy boundaries, the sample is assigned 'uncertain' followed by the labels of all the species whose convex hulls are within a radius of 7 times the distance to the closest convex hull, in order of proximity. This assignment reflects the uncertainty in our classification of samples that fall in an area in latent space where no species-labelled samples fall. At the same time it gives information on our best guess or guesses for the species identity and leaves open the possibility to modify the assignments based on prior knowledge, e.g. species ranges or habitat restrictions.

There is one exception to the above assignment procedure: if the closest two convex hulls are from *An. gambiae* and *An. coluzzii* and the euclidean distance to both of these hulls is less than 14 then the test sample is classified as *uncertain\_gambiae\_coluzzii* or *uncertain\_coluzzii\_gambiae*, depending on which convex hull is closer, because we cannot reliably distinguish between these species in this part of the latent space.

In addition to structure driven by species, the latent space projection of GCref v1 also exhibits geographical structure. Within the *An. gambiae* species cluster, there is a distinct subcluster of samples from Madagascar. Similarly, there is a distinct subcluster of samples from Madagascar

in the *An. arabiensis* species cluster. As mentioned before, the boundary between the *An. gambiae* and *An. coluzzii* species clusters is formed by samples from the far West of Africa. These samples also stand out as a separate group in a study on whole genome data (Caputo et al. 2021; Clarkson et al. 2020). There also appears to be a cluster of East African *An. gambiae* samples that are distinct from the main cluster of *An. gambiae* as well as the Madagascar samples. It is promising to see signatures of geographic structure within species from the amplicon panel data, because this suggests that the panel can also be useful to explore population structure within species.

## VAE classification accuracy

We applied the species assignment procedure to GCval v1 (Figure 5). 134 out of 142 samples (94.4%) samples are assigned to a single species and 132 of those (98.5%) are assigned to the species concordant with their species label. One sample labelled as *An. coluzzii* is classified as *An. gambiae* and one sample labelled as *An. gambiae* is classified as *An. coluzzii*. For all eight samples classified as uncertain, the reference species label is among the set of assigned labels. Seven of the samples classified as uncertain had fewer than 45 targets and we know that the proportion of missing targets affects the position in latent space of the projected samples. The other sample classified as uncertain falls in the space between *An. gambiae* and *An. coluzzii*. Further information can be found in Supplementary Table 6.

## Case studies

### Ag1000G whole genome sequenced samples that are too diverged from the reference genome

The Ag1000G project removes samples from its analysis that appear not to be *An. gambiae*, *An. coluzzii* or *An. arabiensis* based on their divergence from the PEST (*An. gambiae*) reference genome. We ran NNoVAE on all samples that fail the divergence filter from data releases v3 and v3.1 through v3.5. In these datasets, 212 of nearly 10,000 samples were filtered due to high divergence from the PEST reference genome; we assign 166 of those to *An. funestus* at the fine level and 17 to the *An. gambiae* complex at the intermediate level. Furthermore, we assign one sample to *An. nili gp sp3* and one to *An. jebudensis* at the fine level, and one to *An. marshallii* group at the intermediate level. There are 20 samples that get assigned only at the coarse level to the *Myzomyia Neocellia* series and six samples do not get assigned at any level. See Supplementary Table 8 for all sample and assignment information.

All 17 samples that are assigned to the *An. gambiae* complex had at least 50 targets. We assigned 5 samples to *An. merus*, 11 to *An. melas* and 1 to *Uncertain\_melas\_quadriannulatus* (Figure 5). The geographic origin of the samples assigned to *An. merus* and *An. melas* is compatible with the known ranges of these species (Wiebe et al. 2017).

Two of the unassigned samples stand out by their assignments: one sample appears to be from the *An. gambiae* complex but contaminated by an *An. funestus* sample; the second appears to

be a member of the *Culex* genus rather than the *Anopheles* genus, based on mitochondrial analysis (data not provided). For this sample we extracted only 13 targets. The remaining unassigned samples and those only assigned at the coarse level have at least 42 targets. They can be split into four groups of samples that are similar to each other in assignment proportions and mitochondrially. Because of the high amplicon recovery rate and the similarity of assignment proportions, often found in different countries, we believe that these samples represent species that are not present in NNv1. We aim to confirm the species identity for these groups by morphology and genomic comparison to publicly available data and, assuming this is successful, to include them in the next update of the reference database..

## Burkina Faso

We collected 950 mosquitoes from three different locations in Burkina Faso. All individuals were morphologically assigned to the *An. gambiae* complex. For 905 individuals (95.3%) we obtained at least 10 amplified targets, the minimum number required for NN assignment. 901 samples are indeed assigned to the *An.gambiae complex* at the intermediate level, 2 samples are assigned to *An. nili group sp3* at the fine level, one sample could only be assigned at the coarse level to the *Myzomyia Neocellia* series and one sample could not be assigned at any level. The latter two samples require more detailed morphological investigation, which is made possible by the non-destructive extraction approach we used on these mosquitoes.

For 770 of the samples assigned to the *An. gambiae complex* (85.5%) we obtain at least 50 amplified targets, the minimum number required for VAE assignment. We find three species in this dataset: *An. gambiae*, *An. coluzzii* and *An. arabiensis* (Figure 5). Most samples (91.6%) could be assigned to a single species, but we also find some samples labelled as uncertain shared between *An. gambiae* and one other species. The two samples that fall in the space between the *An. gambiae* and *An. arabiensis* reference samples are remarkably far away from all other samples. We cannot exclude the possibility that contamination between samples plays a role here, especially because these samples were stored with 10 individuals in ethanol in a single 1.5 mL tube in the years before sequencing. All metadata and assignment information can be found in Supplementary Table 9.

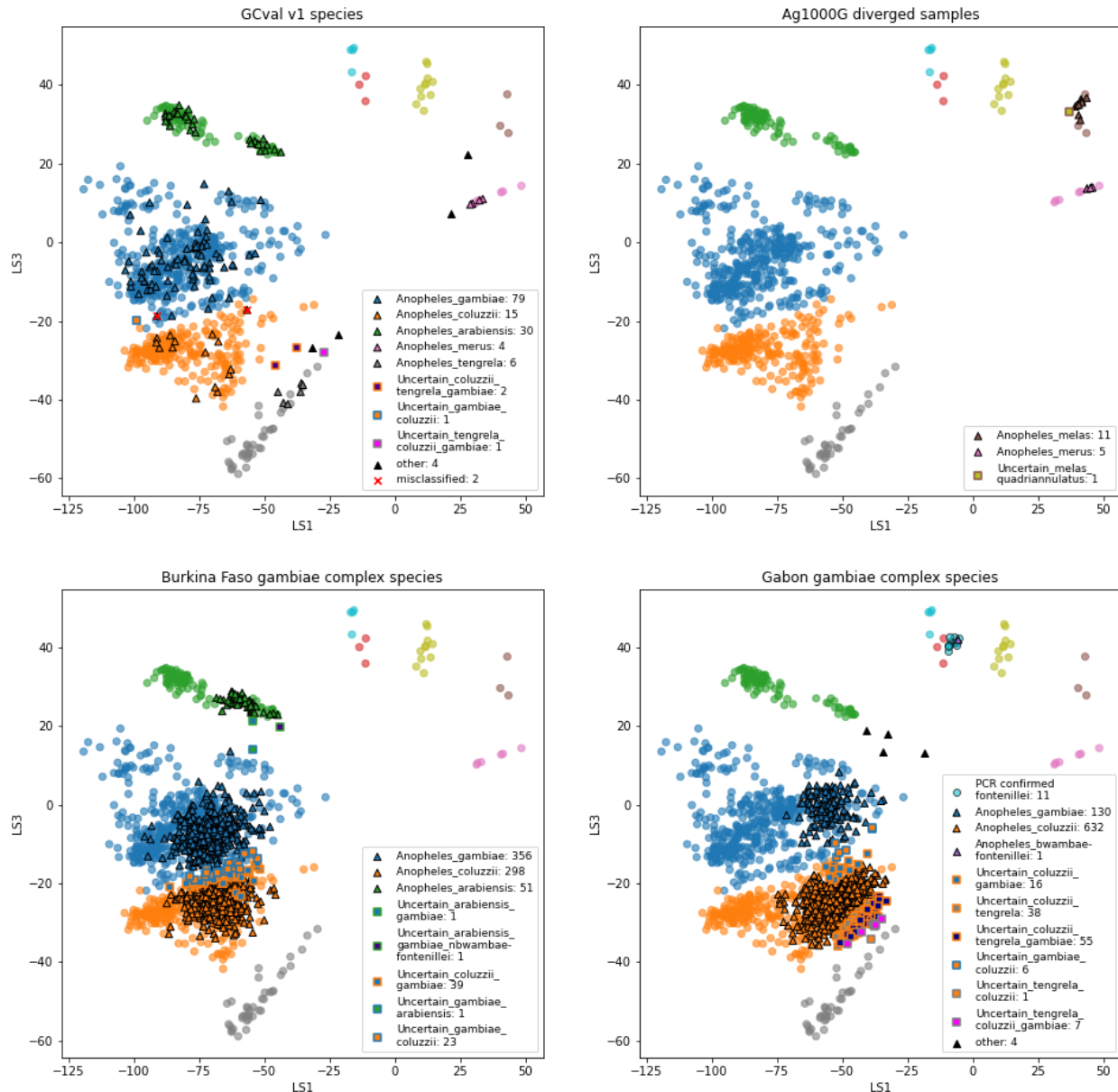
## Gabon

We collected 1,056 mosquitoes by human landing catch from different locations in Lopé village in Gabon. All individuals were morphologically identified as members of the *An. gambiae* complex. 11 mosquitoes were identified as *An. fontenillei* by a species diagnostic PCR (Fanello, Santolamazza, and della Torre 2002) and those clustered with the *An. fontenillei* and *An. bwambae* samples in the VAE projection as expected. Those samples were used to construct the convex hulls. For 1002 (95.9%) of the unidentified samples we obtain at least 10 targets and these are classified by the NN step of NNvAE. The vast majority (993 samples; 99.1%) is assigned to the *An. gambiae complex*. The other species we find in this dataset are *An. funestus* (2 individuals) and *An. coustani complex* (2 individuals), plus 5 individuals that could



not be assigned at any level. The 5 unassigned individuals had a high proportion of multi-allelic targets, suggesting contamination may have occurred at some point in the process.

Of the 993 mosquitoes assigned to the *An. gambiae* complex, 890 (89.6%) have sufficiently many targets to be run through the VAE (Figure 5). Most of these are assigned to a single species: 632 to *An. coluzzii*, 130 *An. gambiae* and 1 *An. fontenillei* (the latter is actually assigned to the combined convex hull of *An. fontenillei* and *An. bwambae*, but are assumed to be *An. fontenillei* because of the non-overlapping species ranges). *An. fontenillei* was first discovered in the forest of La Lopé National Park, 10-15km south of the sampling locations presented in this study (Barrón et al. 2019). Although the species strongly prefers forested habitats, it is not surprising to find one of them in the village given the small distance. It is noticeable that a large number of samples fall in between the *An. coluzzii* and *An. tengreia* clusters. Judging from the projection, we suspect that the whole cluster is *An. coluzzii*, but it may be interesting to explore what drives the variation within this large cluster and why so many samples are projected to an area where none of our reference samples are. Four other samples are projected close to the *An. arabiensis* cluster, although they are assigned to a large set of species labels. Independent PCR species diagnostics confirmed these four samples as *An. arabiensis* (Fanello, Santolamazza, and della Torre 2002). This was surprising because *An. arabiensis* has not been observed in Gabon before, despite extensive sampling efforts. The fact that we see only four such specimens in this set of more than 1,000 with sufficient data, demonstrates that whatever species these mosquitoes are, they are probably quite rare or less likely to be caught by human landing catches. We plan to generate whole genome sequencing data for these individuals to investigate their species and their relationship to *An. arabiensis* from other geographic locations and to other sympatric species. All sample information and assignment results are listed in Supplementary Table 10.



**Figure 5:** VAE projections of **A)** validation set GCval v1 **B)** diverged samples from Ag1000G **C)** sample set from Burkina Faso **D)** sample set from Gabon. The samples from the reference set GCref v1 are displayed as half transparent circles, coloured by species as in Figure 4. The samples from each of the projected sample sets are coloured by their assigned labels. The numbers behind each label corresponds to the number of samples in that category. Samples with more than 3 species-labels are listed as 'other'. The two samples from GCval v1 for which the species label does not match the assigned species are marked with a red cross.

## Conclusion

In this paper we presented NNoVAE, a method for robust species identification for the entire *Anopheles* genus from multilocus targeted amplicon sequencing data. This integrated approach removes the need for sorting the specimens into species groups or complexes based on

morphology, which is labour intensive and error prone, particularly in the case of damaged mosquitoes, for instance when collected with CDC light traps. The NN step can distinguish between most species in our reference database. But equally importantly, it gives an indication of the uncertainty of the assignment, using the same thresholds for the entire genus, enabling us to quantify confidence in assignment in a meaningful way allowing for comparison between all species groups. For individuals from species not yet represented in the reference dataset, we can often assign to a species-group at the intermediate level (corresponding to taxonomic species groups or complexes) or at the coarse level (corresponding to taxonomic series or subgenera). A few samples did not meet the threshold to be assigned at the coarse level, but the assignment results do give an indication of the position of these samples in the phylogeny. Initial explorations of the mitochondrial sequences for those samples do not indicate a close match to publicly available mitochondrial data of any *Anopheles* species. We hope to resolve the species identity of these samples by extending our reference database and collaborating with morphological experts, but until then we retain the groups of unresolved species to compare future samples against them.

The NN step alone struggles to differentiate closely related species within species complexes. For the *An. gambiae* complex we developed a high resolution species identification method based on a variational autoencoder. This VAE step should be easily extendable to other species complexes for which a sufficient amount of species-labelled data is available, which we expect to be the case soon for *An. funestus* and *An. coustani*. The VAE can accurately distinguish between eight species in the *An. gambiae* complex; only *An. bwambae* and *An. fontillei* are too close together in the projection to reliably separate them, but geographic origin helps with this. The species assignments for the VAE are currently quite conservative; only if the VAE projection of the test sample falls within the cloud of training samples from a single species, or much more close to it than to any other cluster, is it assigned to that species. Otherwise it gets assigned all the species labels of nearby clusters. This way, we flag potential outliers or unexpected species, as for the *An. arabiensis* in Gabon, but entomologists can still decide to exclude certain species labels if they are sure that they are not appropriate for their collection location and time, for instance the *An. tengrela* label in Gabon.

The VAE projection of the *An. gambiae* complex also shows some population structure within species clusters. Some of the structure reflects the geography of the collection locations, e.g. Madagascar stands out as a separate subcluster both for *An. gambiae* and *An. arabiensis*. However, the sample sets from Burkina Faso and Gabon show that samples from the same location can be projected to different positions in latent space and it would be interesting to investigate what is driving this observed diversity.

NNoVAE relies on a reference database and therefore the accuracy of the species assignments also depends on the quality and completeness of the reference database. We expect that version 2 of the reference database will contain approximately fifty additional species, as well as more individuals of species that are underrepresented in NNv1 and we are seeking further well-characterised samples.

NNoVAE can characterise vector populations in a uniform way across the *Anopheles* genus and as such contributes to our understanding of *Anopheles* species composition, population structure, species ranges, and transmission potential. The combination of ANOSPP and NNoVAE offers a cheaper, more robust, more informative, and more reliable way to carry out malaria vector surveillance that we hope will be embraced over the coming years by the medical entomology community and National Malaria Control Programs.

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## Author contributions

Mara K.N. Lawniczak conceived of the project with input from Marilou Boddé and Richard Durbin. Marilou Boddé carried out the analytical work with input from Mara K.N. Lawniczak, Richard Durbin and Alex Makunin. Mosquito samples were provided by Diego Ayala, Lemonde Bouafou, Abdoulaye Diabaté, Uwem Friday Ekpo, Mahamadi Kientega, Gilbert Le Goff, Boris Makanga, Marc F. Ngangue, Olaitan Olamide Omitola, Nil Rahola and Frederic Tripet. The manuscript was written by Marilou Boddé with input from Mara K.N. Lawniczak and Richard Durbin. All authors read and approved the manuscript.

## Data availability

Raw sequencing data will be made available on ENA (accession to be confirmed). Pipelines and analysis code, together with processed target haplotypes are available on GitHub: <https://github.com/mariloubodde/NNoVAE>.

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