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Genomic profiling of climate adaptation in *Aedes aegypti* along an altitudinal gradient in Nepal indicates nongradual expansion of the disease vector

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Abstract

Driven by globalization, urbanization and climate change, the distribution range of invasive vector species has expanded to previously colder ecoregions. To reduce health-threatening impacts on humans, insect vectors are extensively studied. Population genomics can reveal the genomic basis of adaptation and help to identify emerging trends of vector expansion. By applying whole genome analyses and genotype-environment associations to populations of the main dengue vector *Aedes aegypti*, sampled along an altitudinal gradient in Nepal (200–1300 m), we identify putatively adaptive traits and describe the species' genomic footprint of climate adaptation to colder ecoregions. We found two differentiated clusters with significantly different allele frequencies in genes associated to climate adaptation between the highland population (1300 m) and all other lowland populations (≤800 m). We revealed non-synonymous mutations in 13 of the candidate genes associated to either altitude, precipitation or cold tolerance and identified an isolation-by-environment differentiation pattern. Other than the expected gradual differentiation along the altitudinal gradient, our results reveal a distinct genomic differentiation of the highland population. Local high-altitude adaptation could be one explanation of the population's phenotypic cold tolerance. Carrying alleles relevant for survival under colder climate increases the likelihood of this highland population to a worldwide expansion into other colder ecoregions.

KEYWORDS
climate change genomics, latent factor mixed model, range expansion, whole genome pooled sequencing, yellow fever mosquito

Ruth Müller and Ann-Marie Waldvogel considered joint senior authors.

1 | INTRODUCTION

Biodiversity, the diversity and abundance of organisms, their genes and ecosystems, is the foundation of human health and well-being by providing essential ecosystem services. However, vector-borne diseases (VBDs), arising from inter-relationships between pathogens, invertebrate vectors and host species, are also part of biodiversity (Marselle et al., 2021). VBDs account for 17% of all infectious diseases worldwide (WHO, 2020) and are thus detrimental to human health. Even though vectors play an important role in pollination, one might describe VBDs and especially vector species as the dark side of biodiversity (Lahondère et al., 2020).

Climate warming is generally expected to promote the expansion of ectothermic insects to cooler ecoregions (Kraemer et al., 2019; Liu-Helmersson et al., 2019; Reuss et al., 2018; Samuel et al., 2016). This is not only explained by the simple fact that rising temperatures will decrease temperature barriers currently shielding cooler ecoregions, thus allowing for climate niche tracking (Thomas, 2010; Waldvogel, Feldmeyer, et al., 2020). Climate warming will also selectively challenge range-edge populations, thereby continuously priming adaptive changes along environmental gradients (Gibson et al., 2009; Hargreaves & Eckert, 2019). This is even more important for anthropogenically dispersed species. After the introduction into a new environment, invasive populations are unlikely at their fitness optimum, that is, they are usually experiencing strong environmental selection whilst additionally suffering from reduced genetic diversity and accordingly reduced adaptive potential (Bennett et al., 2021b).

Climatic clines influence insect population divergence as shown in *Anopheles gambiae* (Cheng et al., 2012), *Drosophila melanogaster* (Bergland et al., 2016; Kolaczkowski et al., 2011; Rane et al., 2015) and also in *Aedes aegypti* (Bennett et al., 2021b). Invasive species that expand their ranges along clines are expected to locally adapt (Sherpa, Guéguen, et al., 2019). For instance, *Ae. albopictus* adapted genetically and morphometrically to Northern latitudes prior to its successful worldwide expansion (Sherpa, Blum, & Després, 2019). Further investigations of cold tolerance in the native range of *Ae. albopictus* support thermal adaptation across a temperature cline (Sherpa et al., 2022). *D. melanogaster* has also been shown to carry beneficial alleles for the survival under temperate and tropical conditions prior to their invasion in North America and Australia (Bergland et al., 2016). Thus, the interpretation and identification of genomic signatures of "climate adaptation" can be regarded as special case of classical local adaptation, since environmental heterogeneity or ideally the gradual variation of climate along environmental gradients will result in gradual or at least environmentally correlated signatures of selection (Waldvogel et al., 2018).

Dengue fever expanded over the last decades and is predicted to spread further (Messina et al., 2019; Murray et al., 2013); more than 390 million people are at a risk of a dengue infection (Bhatt et al., 2013). The spread of the disease via its main vector species *Ae. aegypti* (Linnaeus, 1762) was facilitated through globalization, urbanization and climate change (Gubler, 2011; Kraemer et al., 2019; Wilson et al., 2020). For *Ae. aegypti* it is documented that populations invaded novel habitats by following their spatially expanding climate niche (Garzón et al., 2021; Liu-Helmersson et al., 2019). Therefore, their continued spread in the future is likely (Iwamura et al., 2020; Trájer, 2021). Further expansion to cooler ecoregions such as Europe, however, will additionally require adaptation to the environmental conditions there (Kramer et al., 2020; Sherpa, Blum, & Després, 2019). Evidence of photoperiod-induced dormancy in a temperate population of *Ae. aegypti* is an example that indicates the adaptive potential to climates with colder winter periods (Fischer et al., 2019). It is, however, less clear whether range-edge populations carry sufficient adaptive potential for further acceleration of their expansion process.

To recognize emerging trends in adaptive traits of *Ae. aegypti* to cooler ecoregions, the study of the species while it is currently spreading along climatic transects with ongoing disease expansion (e.g., Dengue) in the Hindu Kush Himalayan (HKH) country is a powerful model system (Darsie et al., 1990; Dhimal et al., 2014; Dhimal, Bhandari, et al., 2021; Dhimal, Gautam, et al., 2015; Dhimal, Kramer, et al., 2021; Malla et al., 2008; Peters & Dewar, 1956; Phuyal et al., 2020; Rijal et al., 2021; Thakuri et al., 2019). Adaptation of *Ae. aegypti* populations to colder climates, associated traits and gene sets are, however, scarcely investigated (Schmidt et al., 2021). In addition, genetic admixture of populations from different origins in the expansion areas can benefit invaders by mitigating negative effects of their introduction and masking deleterious alleles and/or generating new allelic combinations creating novel phenotypes as raw material for selection and rapid adaptation (Sherpa, Blum, Capblancq, et al., 2019).

Accordingly, the overwintering potential in a highland population (1300 m) in the HKH region can be lower compared to the lowlands (≤800 m; Kramer et al., 2020; Kramer, Pfeiffer, et al., 2021). Adaptation of the mosquito populations to higher altitudes might thus be facilitated by climate change. Population genomics can help to examine the role and speed of climate on adaptation in the course of the species expansion (Waldvogel, Feldmeyer, et al., 2020). Here, we tested if gradual climate heterogeneity along an altitudinal gradient is reflected in patterns of genomic differentiation of natural *Ae. aegypti* populations sampled along an altitudinal gradient, henceforth referred to as pattern of "climate adaptation". We studied climate adaptation in *Ae. aegypti* field populations sampled along a prominent climate gradient of Nepal, using the currently most commonly applied genotype-environment association (GEA) tool (LFMM, Figure 1; abbreviations are given in Appendix S1: Table S1; Waldvogel, Schreiber, et al., 2020).

2 | MATERIALS AND METHODS

2.1 | Collection of mosquitos

We sampled *Ae. aegypti* populations, each with a minimum of 96 individuals, from four sampling sites: Chitwan (CH200, 200 m asl), Dhading (DH600, 600 m asl), Dharke (DK800, 800 m asl) and

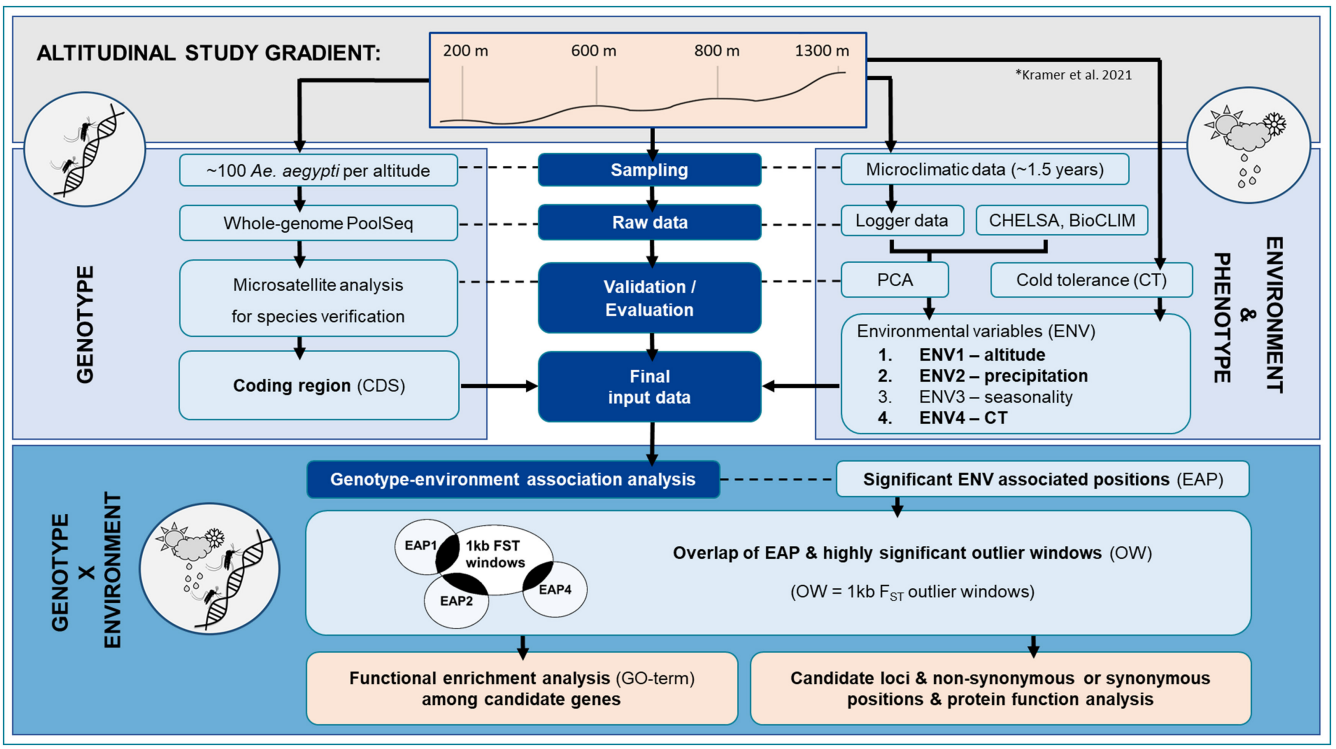

FIGURE 1 Study design to analyse climate adaptation of natural *Ae. aegypti* populations along an altitudinal gradient. CDS, coding region; CT, cold tolerance data normalized to controls (Kramer, Pfeiffer, et al., 2021); ENV, environmental variable; EAP, significant ENV associated positions; OW, significant 1 kb F_{ST} outlier windows.

Kathmandu (KT1300, 1300 m asl). The sampling sites are distributed along an altitudinal, temperature gradient in Central Nepal (Kramer, Baral, et al., 2021; Kramer, Pfeiffer, et al., 2021; Figure 1) and connected via a motorway (Chitwan ➔ Dhading [side valley; road distance:~97 km] ➔ Dharke [~57 km] ➔ Kathmandu [~31 km]). Mosquitoes were identified by a taxonomist following the guidelines described in Darsie et al., 1990 (Sampling details in Appendix S1, Table S2). For DNA isolation (Qiagen DNeasy blood and tissue kit), two legs of each adult were pooled per population. To control the quantity of DNA, Qubit Fluorometer (Invitrogen) measurements were performed.

2.2 | Whole-genome sequencing

Four pooled DNA samples were sequenced on an Illumina HiSeq to yield 150 bp paired-end pooled sequencing (Pool-Seq) whole genome data (Figure 1). The ratio of ≥96 individuals per population and targeted coverage of ~20–30× per pool was chosen to allow an accurate estimation of genome-wide allele frequencies (Fabian et al., 2012; Futschik & Schlötterer, 2010). Pool-Seq genome data were quality trimmed and separately preprocessed using the wrapper script autotrim.pl (Waldvogel et al., 2018), which integrates Trimmomatic (Bolger et al., 2014) and fastqc (Andrews, 2010).

2.3 | Analysis of subspecies

To link our population genomic analyses to prior microsatellite work and to identify potential subspecies as they are described for *Ae. aegypti* (Gloria-Soria et al., 2016), we developed a workflow to assess microsatellite (μsats) diversity from genome-wide Pool-Seq data. For this analysis explicitly, the trimmed files were mapped to the unmasked reference genome of *Ae. aegypti* (Matthews et al., 2018) using NextGenMap (ngm; Sedlazeck et al., 2013). Accounting for the possible presence of subspecies of *Ae. aegypti* (dominant African subspecies: *Ae. aegypti formosus*; outside of Africa: *Ae. aegypti aegypti* (Gloria-Soria et al., 2016) in the samples, ngm was used since this mapper is independent of the amount of genomic polymorphism present in reads (Sedlazeck et al., 2013). Each read of genome-wide Pool-Seq data belonging to one individual chromosome (diploid individuals), provides the required haplotype-specific data to analyse population structure using μsats. First, 12 μsats were identified (A1, A9, AC1, AC2, AC4, AC5, AG2, AG4, B2, B3, CT2, AG1; Gloria-Soria et al., 2016), located and extracted along the reference genome via the in_silico_PCR.pl script (https://github.com/egonozer/in_silico_pcr) and making use of established primers (Brown et al., 2011; Slotman et al., 2007). AG1 could not be identified along the reference genome and was therefore excluded from the analysis. Following the identified coordinates of the reference genome, μsats alignments were

extracted from mapped bam files using samtools (Li et al., 2009). Each μsat alignment was realigned to the extracted μsat reference sequence and, if available, to reference sequences of the respective μsats (Slotman et al., 2007). Alignments were edited using GENEIOUS PRIME 2019.2.1. Repeated elements were identified either using the μsats reference or the MISA-web tool (Beier et al., 2017). As a measure of quality filtering, realigned sequences (single sequences = haplotypes) were included only if each μsat covered at least 2 bp before the start and behind the end of μsat region. Gaps and duplicates were removed and start and end positions of sequences were set to Ns to fix the alignment structure when saving the data in fasta format. Counting repeated elements (in bp) per μsats and individual, their frequencies per population were calculated. Using this population frequency data, 50 individuals were simulated with a custom Python script under the Hardy–Weinberg equilibrium to make our data comparable to individual frequency data. Individuals were only simulated if a minimum amount of four reads was present at a μsat.

To compare this data with a worldwide set of populations and to test for the presence/absence of subspecies in Nepal, the same workflow was followed using publically available genome-wide data of four laboratory populations (likely *Ae. aegypti formosus*: West Africa—likely from Freetown-Sierra Leone; likely *Ae. aegypti aegypti*: Australia—Innisfail, USA—Clovis, Costa Rica—Puntarenas) comprising each 30 females (individual sequencing; Matthews et al., 2018; Pritchard et al., 2000; Accession number: SRX3413563-SRX3413566). Only μsats with a coverage higher than or equal to four individuals were used for the further analysis (used μsats: A9, AC1, AC4, AG2, B2, B3). The population from the USA was excluded due to low individual coverage of this specific data set (Appendix S1: Table S3). Using the Bayesian clustering method implemented in the software STRUCTURE version 2.3.4 (Pritchard et al., 2000), the population structure as described in Gloria-Soria et al. (2016) was assessed. Each conducted run assumed an admixture model and correlated allele frequencies with a burnin of 250,000 iterations with in addition 750,000 repetitions. To test differences between the African and all other populations, the structure analysis was performed with $K = 2$ (compare with (Gloria-Soria et al., 2016) with 10 iterations. To summarize STRUCTURE results of the 10 iterations per K and plot consistent cluster colouring CLUMPAK was used (Kopelman et al., 2015). Exclusively to assess differences amongst the populations of Nepal $K = 1$–4 was calculated.

2.4 | Population differentiation

Estimation of population differentiation using the genome wide single nucleotide polymorphism (SNP) data followed the pipeline of *PoPoolation2* (Kofler, Pandey, et al., 2011) and Waldvogel et al. (2018). Before mapping, overlapping read pairs were assembled using PEAR (Zhang et al., 2014). This was necessary in order to make use of the full data set, though only a small proportion of reads were found to overlap, while avoiding erroneous allele frequency estimates in overlapping regions. Assembled and unassembled reads were mapped to the available reference genome (masked version) of *Ae. aegypti* (Matthews et al., 2018) using bwa mem (Li & Durbin, 2009). Duplicates were removed using picard tools (Broad Institute, 2019) and all bases below a minimum mapping quality of 10 were discarded (samtools; Li et al., 2009). With PoPoolation (Kofler, Orozco-terWengel, et al., 2011) population specific parameters such as the nucleotide diversity (π; genome-wide per site and in 1 kb window, exon-wide per site) and the population mutation parameter were estimated (θ; genome-wide in 1 kb window). The effective population size (N_e) was calculated using genome-wide θ estimates as follows: $N_e = \frac{\theta}{4\mu}$. The genome wide mutation rate (μ) of *Chironomus riparius* was used for the N_e calculation (Oppold & Pfenninger, 2017). For comparative analyses between populations, the pipeline PoPoolation2 was followed (Kofler, Pandey, & Schlötterer, 2011). In brief, pairwise F_{ST} values (fst-sliding.pl) of all population pairs in a sliding window of 1 kb along the subsampled sync-file were calculated. The upper 1% tail of the F_{ST} distribution was defined as threshold for non-neutral differentiation, as this has been shown to provide a conservative threshold for a robust drift expectation (Waldvogel et al., 2018). In addition, for each 1 kb-window Fisher's p-values (fisher-test.pl) were calculated and the Benjamini-Hochberg correction against multiple testing to all p-values was performed. We defined highly significant outlier windows (OW) to be those windows that remained significant after FDR correction ($q < 0.01$). Circos tool was used to graphically illustrate the distribution of OWs along the genome (Krzywinski et al., 2009). As described for the OW estimation we additionally calculated highly significant outlier positions for each population (OP) per site. In addition, to test for genome-wide isolation by distance patterns, a Mantel test with 23 permutations (complete enumeration) in R/VEGAN (Oksanen et al., 2020) between the genome-wide mean F_{ST} values and the geographical distance was calculated. Next to the described F_{ST} outlier detection method of significant positions we also used BAYPASS version 2.1 (Gautier, 2015; Günther & Coop, 2013) to identify significant outliers in allele frequencies of our Nepalese populations (Appendix S1: Information 1).

2.5 | Environmental data

The following environmental data of *Aedes* sampling sites were analysed: (i) microclimate data/logger data (temperature data; Appendix S1: Tables S4 and S5), (ii) high-resolution data from CHELSA of 1979–2013 (Appendix S1: Table S6), and (iii) Bioclim variables (Karger et al., 2017; data source: (Karger et al., 2022); 30 arcsec, ~1 km from CHELSA version 1.2; Appendix S1: Table S6). HOBO data loggers (type UX100-011A, ONSET) were installed indoors in houses with no heating or air condition and bad isolation and outdoors at shaded artificial places (e.g., near households) at sampling sites from 11/2017 to 03/2019. Loggers were additionally installed at 1800 and 2050 m asl (Ranipauwa = RP1800, 1800 m asl; Dhunche = DU2050, 2050 m asl). In DK800, HOBO loggers were missing and the data of the sampling site were interpolated from logger data obtained along the gradient between 200–2050 m asl using linear regression (PRISM, version 7, GraphPad Software Inc.). To

reduce confounding covariation in the environmental data set a principal component analysis (PCA) was run.

2.6 | Genotype-environment association

To analyse how the genomic differentiation is potentially correlated with environmental variation across sites, a genotype-environment association analysis was performed using latent factor mixed model (LFMM) in the frame of the LEA R-package (Caye et al., 2019). The Pool-Seq approach does not account for pool size (Waldvogel et al., 2018) and thus 20 pseudo-individual allele frequency spectra were inferred by simulating observed allele frequencies at each locus referring to the BAYENV approach (Günther & Coop, 2013). In accordance, for each locus environmental factors were replicated 20 times. Considering the large genome size of *Ae. aegypti* as well as the main target to identify candidate genes in downstream analysis only the coding regions (CDS) were included. Three PCA components and the cold tolerance (normalized mean survivorship after cold exposure to −2°C for 8 days to controls; CT; Kramer, Pfeiffer, et al., 2021) were used as environmental input variables (ENV) for the GEA (Appendix S1: Table S7). We ran the LFMM function lfmm_ridge with a latent factor of $K = 4$ (reflecting number of populations; algorithm = analytical). *p*-values were calibrated by computing the median and median absolute deviation (MAD) of the *z* scores using the "lfmm_test" function (Appendix S1: Table S7). We ran LFMM twice for different combinations of environmental input variables: (1) PCA1—altitude (ENV1), (2) PCA2—precipitation (ENV2), PCA3—seasonality (ENV3) plus PCA4—CT (ENV4, Appendix S1: Tables S6 and S7, Figures S1–S5). Resulting output *p*-values were FDR corrected and positions with $q < 0.01$ defined as significant ENV associated positions (EAP). SNPs were considered as candidate if identified by both GEA (EAPs) and population-based (OWs) approaches. Differences in allele frequencies of the candidate SNPs (EAP-OW positions) along the gradient were analysed per ENV using Prism (version 7, GraphPad Software Inc.). In order to identify highly significant positions for climate adaptation, we verified if EAP-OW are additionally present in those OPs.

Next to LFMM we in addition run BAYPASS version 2.2 (Gautier, 2015) to identify candidate genes for climate adaptation. In brief, as for LFMM we only included allele frequencies of the CDS and as environmental input ENV1–ENV4 was used as described above. The threshold to identify significant associations was set according to the Jeffrey's rule (Jeffreys, 1961) and only SNPs with a Bayes factor > 20 were considered as candidate SNPs. LFMM candidate genes/SNPs were compared with BayPass results.

2.7 | Functional enrichment associated with climate adaptation

For a functional enrichment analysis of candidate genes, all genes containing EAP-OW positions were annotated using the coordinates of protein coding genes of the *Ae. aegypti* reference genome (Matthews et al., 2018). The reference genome was then used in InterProscan (Quevillon et al., 2005) to obtain gene ontology information as reference for the subsequent functional enrichment analysis. Gene ontology (GO) terms significantly enriched in EAP-OW genes were then analysed using the topGO R package (Alexa & Rahnenführer, 2016) in the category "biological processes", with the weight01 algorithm and Fisher statistics. Enriched GO terms with a $p < .05$ were further assessed (Waldvogel et al., 2018).

To analyse if base substitutions at SNPs lead to synonymous or nonsynonymous mutations in the amino acid sequence of candidate genes including LFMM and BayPass results, TBG-TOOLS version 0.2 (https://github.com/Croxa/tbg-tools Schoennenbeck et al., 2021) was used. The characteristic of the amino acid present, before and after the base exchange was also assessed (Löffler et al., 2007; Voet et al., 2008). Knowledge on the biological function of candidate genes containing nonsynonymous mutations was collected from literature and databases. We performed a literature survey in Google Scholar by using the candidate gene name (or/and the locus tag) in combination with the following terms: (1) *Aedes aegypti*, (2) *Aedes*, (3) mosquito, and (4) insect. Furthermore, we extracted candidate gene IDs containing nonsynonymous and synonymous mutations and searched for their function using UniProt, NCBI, and Vectorbase. The procedure was likewise repeated also for candidate genes containing synonymous mutations but only the locus tag and the species name was used as a search term.

2.8 | Genomic signatures of local adaptation not related to climate

Next to climate adaptation, we searched for candidate genes indicating strong local adaptation. Therefore, we defined candidate genes/positions laying in the CDS that were not overlapping with an EAP (significant ENV associated positions) but were present in an OW and additionally overlapped with an OP (OW-OP), as candidates for local adaptation not related to climate (population based outlier detection). Potential candidate genes for local adaption that are involved in insecticide resistance or vector competence were especially focused. Additionally, we compared candidate SNPs/genes of the Nepalese population with a recent study that investigated genomic signs of "local environmental adaptation" in populations from Panama (17 genes; Bennett et al., 2021b). Allele frequency differences of detoxification genes (as listed by Faucon et al., 2017) and vector competence genes associated with resistance to DENV1 or/and DENV3 infection (top 0.001% most significant SNPs described by Dickson et al., 2020) were compared to our data set for (1) being part of the CDS, (2) having an overlap with OW-OP and (3) showing a nonsynonymous or synonymous mutation (TBG-TOOLS version 0.2; https://github.com/Croxa/tbg-tools; Schoennenbeck et al., 2021). Differences in allele frequencies at candidate SNPs between populations were visualized in a heat map each (PRISM, version 7, GraphPad Software Inc.).

We located the kdr (knockdown resistance) mutations V1016G, F1534C, and S989P in the reference genome and extracted the sequences of each sorted bam-file per population by using the in_silico_PCR.pl script (https://github.com/egonozer/in_silico_pcr) and the primers given by Endersby-Harshman et al. (2020). Extracted sequences were processed in Geneious Prime 2019.2.1 and excact genome positions of the *kdr* mutations were calculated. Allele frequencies at the position of the *kdr* mutations were extracted from the sync-file and overlaps of *kdr* mutations with OW as well as OP were checked. The combined occurrence of the *kdr* mutations in the populations sampled along the altitudinal gradient was investigated using allele frequency differences in a Bayesian multivariate response model (details in Appendix S1: Information 1).

3 | RESULTS

3.1 | Subspecies analysis

The STRUCTURE analysis at μsat loci of 50 simulated diploid genotypes based on allele frequencies from our Nepalese Pool-Seq data confirms that our genomic data sets only consist of one *Ae. aegypti* subspecies. All 10 runs with STRUCTURE using $K = 2$ with six μsats in a comparison to other populations worldwide (West Africa, Costa Rica, Australia- Innisfail; Matthews et al., 2018) indicate that the African population is different from the Nepalese populations (Figure 2a; Appendix S1: Figure S6). Due to lower coverage of the population from KT1300 of Nepal, only five μsats were included (AC1 excluded) and the Australian population was restricted to three μsats (A9, AC1 and B3 were excluded). When comparing Nepalese populations amongst each other (10/10 runs with K2-11 μsats) higher similarities between the lowland (CH200, DH600 and DK800) populations are present (Figure 2b; Appendix S1: Figure S6).

3.2 | Population differentiation and diversity

Mean coverage ranges from 17.86–22.46 (Appendix S1: Table S8). Nucleotide diversity (π) is smaller in exonic regions compared to the genome-wide average (per site) and all populations show a similarly low π with an average of 0.0127 in 1 kb windows. The low-altitude population CH200 has the highest population mutation rate (θ), however, there is no increasing trend towards higher altitude. Concerning the effective population size (N_e), there is a trend towards decreasing values along the altitudinal gradient, however smallest N_e is found in DH600 (Appendix S1: Table S8). Genome-wide mean pairwise F_{ST} range between 0.05–0.067 (Table 1) indicating low levels of genomic differentiation and high relatedness amongst populations (Figure 3; Appendix S1: Figure S7). Moreover, the Mantel test revealed no signs of isolation by distance ($p = .67$, $r = -.27$).

3.3 | Environmental data

The CHELSA data shows a gradual decrease of mean, minimum and maximum temperature along the altitudinal gradient (Appendix S1: Figure S8). The microclimate data shows higher variability throughout the seasons with a decreasing trend of mean and minimum temperature with increasing altitude but higher variability in the maximum temperature (Appendix S1: Figure S9). CHELSA data reveals a nongradual precipitation pattern from 200–1300 m with only minor differences between sites (Appendix S1: Figure S10). The first three components of the PCA are mainly related to the following environmental factors: PCA1—altitude (70.86%; ENV1), PCA2—precipitation (27.45%; ENV2) and PCA3—seasonality (1.69%; ENV3; Appendix S1: Table S7, Figures S1–S4). While ENV1 mainly comprises the environmental variable altitude and ENV3 temperature seasonality, ENV2 especially comprises variables related to the precipitation of the monsoon season and less pronounced altitude and temperature seasonality. This potentially highlights a link of ENV2 to humidity (Appendix S1: Figures S1–S3).

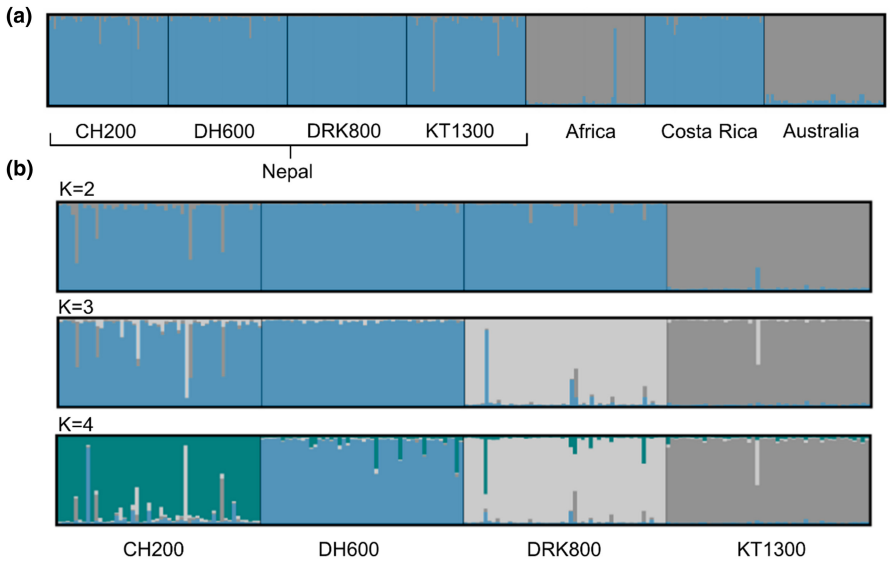

FIGURE 2 Global (a) and local (b) genetic structure of *Aedes aegypti* populations. Comparison of four populations from Nepal (a) with populations from Africa, Costa Rica and Australia (Matthews et al., 2018) using six microsatellite regions ($K = 2$) and (b) with each other using 11 microsatellite regions ($K = 2$–4; Appendix S1: Figure S6). Altitude of sampling sites of *Ae. aegypti* populations in Central Nepal: CH200 = 200 m asl (Chitwan), DH600 = 600 m asl (Dhading), DK800 = 800 m asl (Dharke), KT1300 = 1300 m asl (Kathmandu).

TABLE 1 Mean F_{ST} value and number of candidate SNPs/genes for climate and local adaptation. Altitude of sampling sites of *Ae. aegypti* populations: CH200, 200 m asl; DH600, 600 m asl; DK800, 800 m asl; KT1300, 1300 m asl. Climate adaption: Outline of stringent signatures of climate selection (ENV1—altitude, ENV2—precipitation, ENV4 = cold tolerance) by overlapping outlier windows of highly significant population differentiation (OW; 1 kb-window) with EAPs (GEA gene list) for each population comparison. Local adaptation: Overlap between F_{ST} 1 kb-window outlier analysis (OW) and the F_{ST} 1b-window outlier analysis (OP) excluding SNPs and genes from the EAP-OW analysis (OW-OP). Numbers given per position and per gene hit: Integrated-hits/unique-hits/nonsynonymous. If not marked otherwise, all unique hits are also present in the upper 1% tail of the site-specific F_{ST} distribution (OP)

	CH200-DH600	CH200-DK800	CH200-KT1300	DH600-DK800	DH200-KT1300	DK800-KT1300	Total
Mean F_{ST}	0.05784	0.04996	0.05567	0.06251	0.06676	0.05822	–
OW-OP							
Highly significant site-specific positions	1251	1171	1250	1331	1400	1189	6303
Number of genes (without duplicate)	277	280	284	269	286	263	1200
OW							
Highly significant 1 kb-windows	2919	2911	2928	2940	2931	2922	–
Number of genes (without duplicates)	535	576	579	501	551	550	–
Overlap of OW and EAP- number of positions							
ENV1	0	0	4	1	4	2	11/9/2
ENV2	2	2	19	1	15	20	59/40[a]/12
ENV4	0	0	8	1	7	4	20/14/3
Overlap of OW and EAP- number of genes							
ENV1	0	0	5	1	3	1	10/8
ENV2	2	2	15	1	13	14	47/32
ENV4	0	0	8	1	6	3	18/13

[a]Nine positions are not present in the upper 1% tail of the site-specific F_{ST} distribution, including five nonsynonymous SNPs.

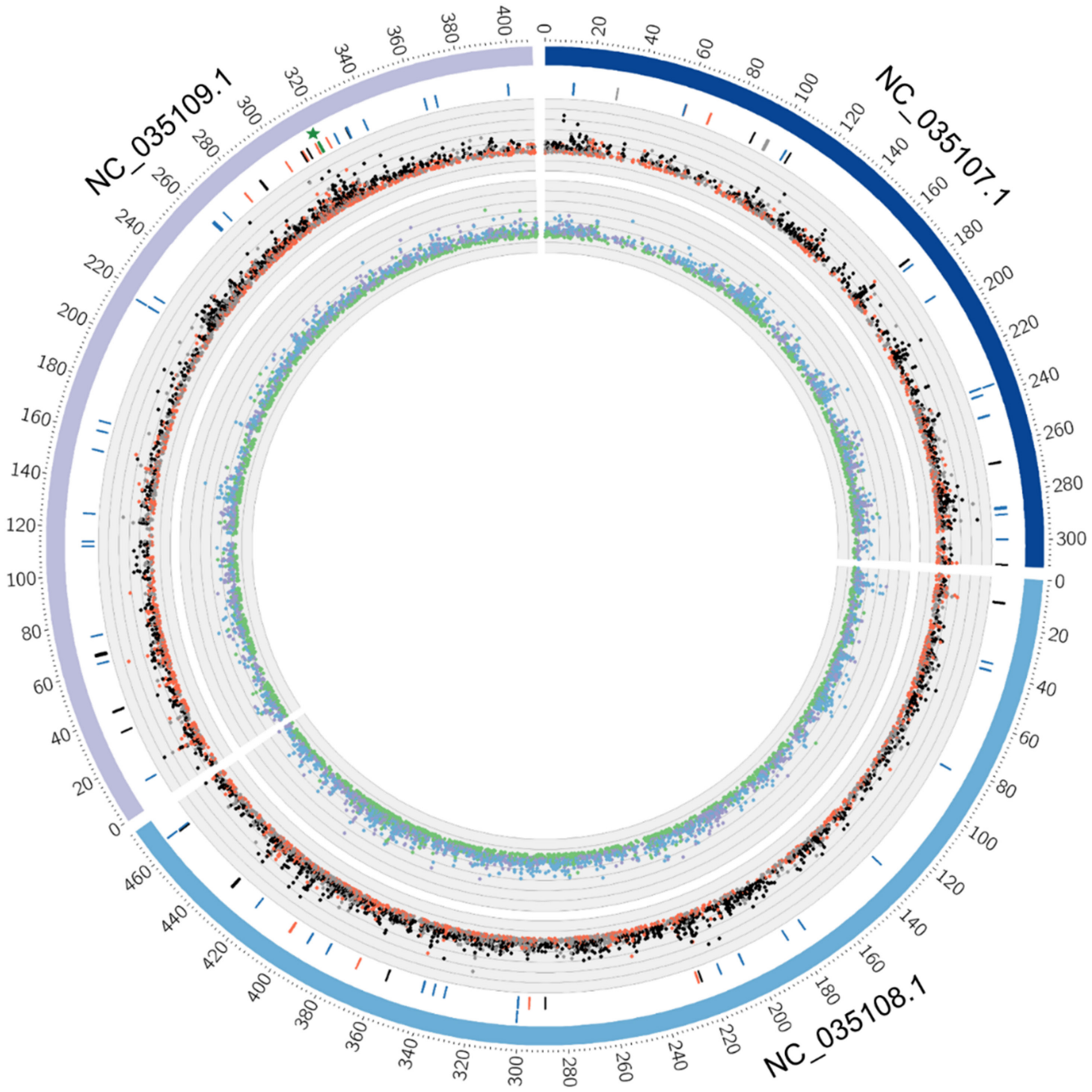

FIGURE 3 Genome wide pairwise F_{ST} distribution per 1 kb-windows (OW) of Nepalese *Aedes aegypti* populations. The three chromosomes of the *Ae. aegypti* genome are represented in the outermost circle. From innermost to outermost circle: (a) the innermost circle shows the pairwise F_{ST} distribution (range: 0–0.7) in 1 kb windows between the lowland populations (purple: CH200 vs. DH600; green: CH200 vs. DK800; light-blue: DH600 vs. DK800), (b) the middle circle shows the comparison between the lowland populations and the KT1300 (red: CH200 vs. KT1300; black: DH600 vs. KT1300; grey: DK800 vs. KT1300), (c) the white circle gives the position of all EAP-OW genes (black), the candidate genes containing nonsynonymous mutations (red), the detoxification genes containing significant positions (blue), the voltage-gated sodium channel (green and a green star) and the vector competence genes (grey). Altitude of sampling sites of *Ae. aegypti* populations: CH200, 200 m asl; DH600, 600 m asl; DK800, 800 m asl; KT1300, 1300 m asl.

3.4 | Genotype-environment association

The LFMM analysis reveals 47 SNPs within 46 genes associated to ENV1 (associated with altitude), 216 SNPs within 172 genes associated to ENV2 (associated with precipitation), zero SNPs associated to ENV3 (associated with seasonality) and 69 SNPs within 64 genes associated to ENV4 (cold tolerance) (Table 1; Appendix S1: Figure S5). After our stringent filtering when overlapping significant ENV associated positions (EAPs) with highly significant F_{ST} OW (1 kb-window; Figure 1) 9 SNPs within eight genes associated to altitude (ENV1) are present. We accordingly retain 40 SNPs within 32 genes associated to precipitation (ENV2) and 14 SNPs within 13 genes associated to cold tolerance

(ENV4; Table 2). All EAP-OW (overlap of EAP with OW) SNPs are also present in highly significant outlier positions per site (OP) except nine SNPs associated with precipitation (Table 1; Appendix S2: Tables S1–S4). Observed allele frequencies plotted against the altitudinal gradient of population origins reveal a major difference in allele frequency of candidate loci in KT1300 compared to all other lowland populations (CH200, DH600, DK800; Figure 4). Amongst all 186 unique candidate genes for climate adaptation (EAP) identified in the LFMM analysis 2 were also found in the BayPass analysis. BayPass confirmed gradual allele frequency changes in association to ENV3- seasonality and ENV4- cold tolerance, but nonassociation to ENV1- altitude and ENV2- precipitation (Appendix S2: Table S5).

3.5 | Functional enrichment associated to climate adaptation

The investigated populations of *Ae. aegypti* across the Nepalese altitudinal gradient reveal 33 candidate genes (EAP-OW) with signatures of climate selection (temperature [ENV1]: 8, precipitation [ENV2]: 31, cold tolerance [ENV4]: 13). Functional analysis of genes that are associated with ENV1, ENV2 and ENV4 yielded multiple enriched GO terms (Appendix S1: Table S10).

Two SNPs located in EAP-OW genes (overlap of EAP with OW) associated with altitude, 12 SNPs in EAP-OW genes associated with precipitation and three SNPs in EAP-OW associated with cold tolerance are nonsynonymous (Appendix S1: Figure S11) and thus we further assessed their functions (Table 2). Amongst those EAP-OW SNPs, 12 genes are associated to different ENVs including three uncharacterized genes (Table 2). The functions of the nine characterized genes containing an EAP-OW SNP can be separated into (1) immune response, (2) life-cycle, (3) insecticide resistance, and (4) protein regulation (all details: Appendix S1: Table S9). The characteristics (such as: polar, nonpolar, basic, acidic) of the amino acids before and after the base exchange demonstrate differences in five genes (Table 2). These changes can likely lead to a change in the protein structure or function.

The functional analysis of EAP-OW genes containing synonymous mutations associated to the different ENVs were separated into the same groups as described (Appendix S1: Table S9; Appendix S2: Tables S6–S8). The gene "coatomer subunit beta" contains two SNPs, of which one is associated with cold tolerance (ENV4) and constitutes a synonymous mutation. The other SNP constitutes a nonsynonymous mutation and is associated with precipitation (ENV2).

3.6 | Genomic signatures of local adaptation not related to climate

By overlapping the OW (highly significant outlier window) and OP (highly significant outlier positions; OW-OP), 1171–1400 SNPs in 263–286 candidate genes as signatures of local adaptation are identified per population comparison (Table 1). There is no overlap between candidate genes for "local environmental adaptation" identified by Bennett et al. (2021b) and candidate genes for climate adaptation obtained by the LFMM analysis (EAP-OW or EAP) but one candidate gene associated with ENV3 of the BayPass analysis overlaps with a candidate gene identified by Bennett (AAEL007657—"putative vitellogenin receptor"; Appendix S2: Table S5). Moreover, two candidate genes for local adaptation (OW-OP) show an overlap with the candidate genes of Bennett which, however, were identified with different methods (Appendix S2: Table S9). The first gene (AAEL007657—"putative vitellogenin receptor") significantly differs between the DH600 populations and all other populations, whereas the second (AAEL002683—"xanthine dehydrogenase") significantly differs only between the CH200 and DH600 populations.

In total, 200 significant SNPs in 53 detoxification genes are associated to local adaptation (Appendix S1: Figure S12; Appendix S2: Table S10). These SNPs significantly differ between populations within the OP as well as the OW (Figure 3). Out of the 200 SNPs, 113 SNPs in 30 genes constitute a nonsynonymous mutation. The allele frequency distribution at these candidate loci were compared in a heat map revealing a slightly different pattern of frequency distribution in the KT1300 population (Appendix S1: Figure S12). An opposite trend of allele frequency distributions is present between the KT1300 population and CH200 population as well as DK800.

In total, five SNPs in four genes are involved in vector competence (Appendix S1: Figure S12). Three SNPs in two genes ("protein scarlet", "leucine-rich repeat-containing protein 40") overlap with OW-OP and have been earlier associated with DENV-1 infection by Dickson et al. (2020). Two of these three SNPs are nonsynonymous SNPs. The OW-OP overlapping SNPs that are associated with DENV-3 infection in the two genes "cadherin-86C" and "integrin alpha-PS1" are synonymous SNPs (Appendix S1: Figure S12; Figure 3).

Along the altitudinal temperature gradient, knockdown resistance (*kdr*) mutations slightly differ between populations. *Ae. aegypti* populations carry *kdr* mutations majorly in the biggest urban sites (KT1300 followed by CH200). The V1016G mutations differ the most between populations with the wildtype (GGA) most prominently in CH200 (0.32) and KT1300 (0.44). The F1534C mutation (TGC) is major in KT1300 (0.31) compared to all other populations and no difference between populations is present in the S989P mutation (Table 3, Figure 3; Appendix S1: Figure S13). None of the *kdr* mutations overlap with a significant OW/OP. Accordingly, they do not contribute to patterns of population differentiation. For the Bayesian approach, we excluded the S989P mutation, since no difference between populations was present. The Bayesian approach for comparison of the allelic combinations F1534C and V1016G shows that there is no effect of altitude on the respective allele frequencies (Appendix S1: Figure S13).

4 | DISCUSSION

Within this study we investigate patterns of genomic differentiation in *Ae. aegypti* populations sampled along an altitudinal climate

TABLE 2 Nonsynonymous substitutions of EAP-OWs that indicate significant involvement of genes in climate adaptation. The genomic position, base, alternative base, amino acid (AA) exchange, association to respective environmental variables (ENV1, altitude; ENV2, precipitation; ENV4, cold tolerance) and the annotated candidate gene are given. Significantly enriched GO terms (Appendix S1: Table S10) are mentioned if they can be linked to the candidate gene using Uniprot. Characteristic of amino acid before and after alternative base exchange at nonsynonymous SNP position are also given (Löffler et al., 2007; Voet et al., 2008)

Chromosome	Position	Base	Alternative base	AA exchange	Triplet position	Amino acid characteristic	ENV1	ENV2	ENV4	Gene	Enriched GO-terms
NC_035107.1	59,746,123	G	T	P➔H	2	nonpolar ➔ polar (basic)		X		adenylate cyclase type 9[a]	
NC_035107.1	70,557,897	T	C	I➔V	1	nonpolar ➔ nonpolar	X	X	X	proto-oncogene tyrosine-protein kinase ROS	transmembrane receptor protein tyrosine kinase signalling pathway and protein phosphorylation
NC_035108.1	223,930,033	G	A	A➔V	2	nonpolar ➔ nonpolar		X		homeobox protein araucan	
NC_035108.1	295,810,879	G	A	E➔K	1	polar (acidic) ➔ polar (basic)		X		uncharacterized protein LOC5566519[a]	
NC_035108.1	370,218,447	G	A	V➔I	1	nonpolar ➔ nonpolar	X	X	X	breast cancer antioestrogen resistance protein 3	small GTPase mediated signal transduction
NC_035108.1	402,025,916	T	A	H➔Q	3	polar (basic) ➔ polar		X		toll-like receptor Tollo[a]	
NC_035109.1	278,880,294	G	T	E➔D	3	polar (acidic) ➔ polar (acidic)		X		zinc finger CCCH domain-containing protein 13[a]	
NC_035109.1	300,326,627	T	A	I➔K	2	nonpolar ➔ polar(basic)		X	X	uncharacterized protein LOC5574261	
NC_035109.1	307,981,403	A	T	F➔I	1	nonpolar ➔ nonpolar		X		probable peptide chain release factor C12orf65, mitochondrial[a]	
NC_035109.1	308,648,742	G	A	V➔I	1	nonpolar ➔ nonpolar		X		tubulin-specific chaperone D	
NC_035109.1	314,722,675	G	A	A➔T	1	nonpolar ➔ polar		X		coatomer subunit beta'	
NC_035109.1	319,909,869	C	G	L➔V	1	nonpolar ➔ nonpolar		X		uncharacterized protein LOC5578603	

aNot present in the upper 1% tail of the site-specific F_{ST} distribution.

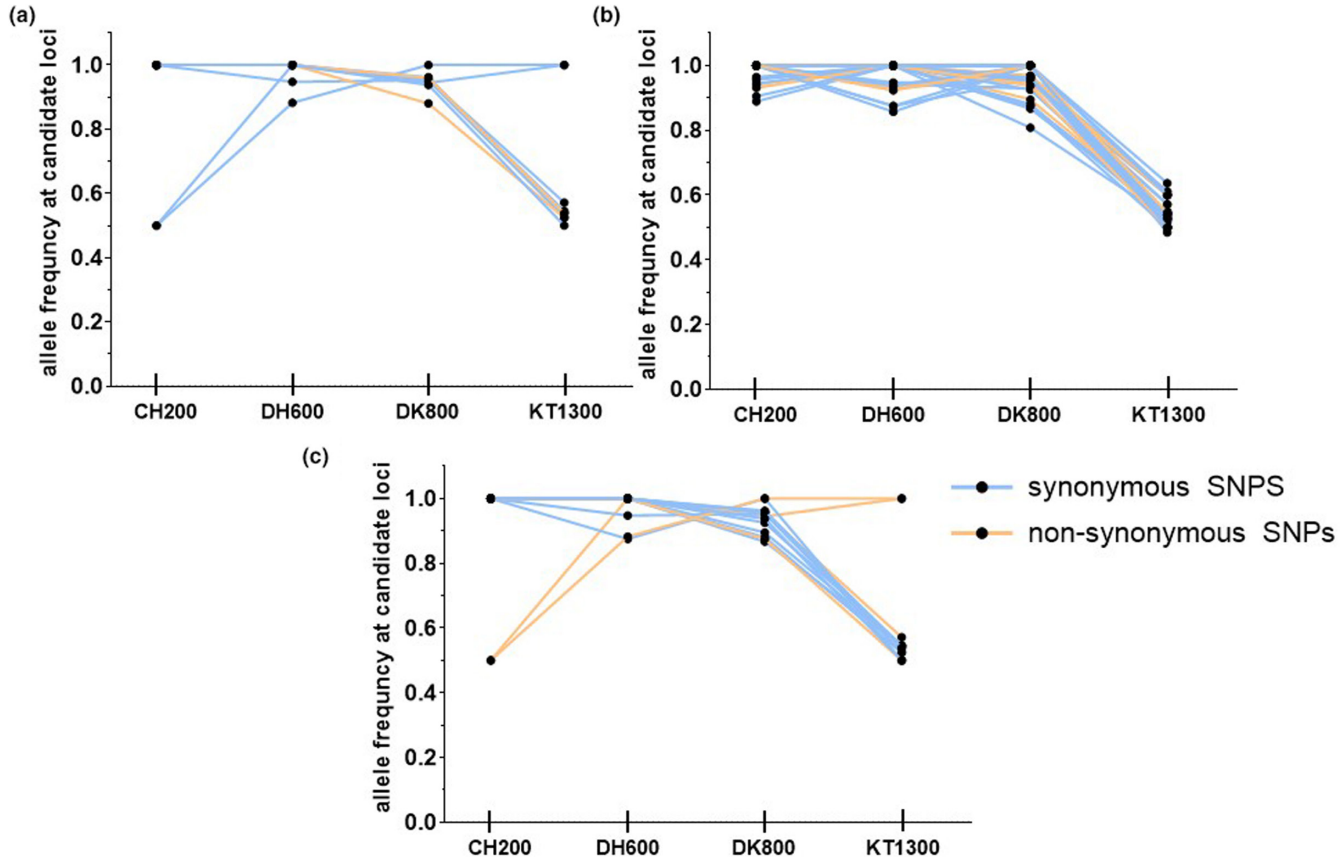

FIGURE 4 Allele frequencies of candidate loci (EAP-OW) plotted against the altitudinal gradient of their population origin. Candidate loci associated with (a) ENV1—altitude, (b) ENV2—precipitation, (c) ENV4 = cold tolerance. Details on nonsynonymous SNPs in Table 2.

gradient in the Hindukush Himalayan region and reveal an outstanding differentiation pattern in the population sampled from the highest altitude (1300 m asl, Kathmandu) compared to the lowland populations (≤800 m asl). This finding contradicts our original hypothesis of a continuous variation and might speak against a gradual expansion process of the disease vector towards higher altitudes. By demonstrating a distinct genomic footprint of climate adaptation in *Ae. aegypti*, our study assists to close the knowledge gap on adaptive traits and associated gene sets on climate adaptation of *Ae. aegypti* (Schmidt et al., 2021), while signatures of nonclimate local adaptation reveal a broad functional basis of the species.

4.1 | Nepalese *Ae. aegypti* populations belong to one subspecies

In comparison to worldwide *Ae. aegypti* populations, we show that all examined Nepalese populations belong to one subspecies, probably *Ae. aegypti aegypti* (Figure 2). This distinction was essential to verify that allele frequency differences were analysed on the population but not the interspecific level. In general, it is important to distinguish the subspecies due to their proposed difference in vector competence (Sylla et al., 2009), even though these interspecific effects seem to depend on virus genotypes (Dickson et al., 2014) and environmental factors (Black IV et al., 2002; Sim & Hibberd, 2016). Additionally, it is important to differentiate between the subspecies because of their different host preference for humans or animals (McBride, 2016).

4.2 | Patterns of genomic differentiation imply isolation of populations by environment

Other than the expected pattern of gradual variation along the altitudinal temperature gradient, we found significant allele frequency differences at candidate loci for climate adaptation only between the Kathmandu (KT1300) population and all other lowland populations (≤800 m; Figure 4). Thus, lowland populations versus the highland population form two differentiated clusters. This nongradual pattern of genomic differentiation along the altitudinal gradient can have alternative, though not necessarily mutually exclusive, reasons. Since, the capital of Nepal (Kathmandu), is the central trading point of the country, population differentiation might derive from differences in population history such as a differential invasion history of the Kathmandu population. Alternatively, with regard to the environmental conditions along the altitudinal gradient assessed in this study, the significant differentiation in climate-associated outlier loci might be indicative for local high-altitude adaptation.

TABLE 3 Allele frequency and allelic variant of *kdr* mutations with exact genome positions

Mutation ID	Chromosome	Position	Amino acid code	CH200	DH600	DK800	KT1300
Allele frequency							
S989P	NC_035109.1	315,984,077	TCC—wild-type	1	1	1	1
			CCC—mutant	0	0	0	0
V1016G	NC_035109.1	315,983,762	GTA—wild-type	0.68	0.93	0.78	0.56
			GGA—mutant	0.32	0.07	0.22	0.44
F1534C	NC_035109.1	315,939,224	TTC—wild-type	0.94	0.88	0.91	0.69
			TGC—mutant	0.06	0.12	0.09	0.31
Allelic variant (total count)							
S989P	NC_035109.1	315,984,077	TCC—wild-type	26	22	23	26
			CCC—mutant	0	0	0	0
V1016G	NC_035109.1	315,983,762	GTA—wild-type	15	14	14	10
			GGA—mutant	7	1	4	8
F1534C	NC_035109.1	315,939,224	TTC—wild-type	17	15	10	9
			TGC—mutant	1	2	1	4

Note: CH200, 200 m asl; DH600, 600 m asl; DK800, 800 m asl; KT1300, 1300 m asl.

Genetic differences between Kathmandu and the lowlands could result from differential invasion history of *Ae. aegypti* in Central Nepal. With the given data of our study it is not possible to resolve the invasion history of the investigated populations. Nevertheless, existing knowledge on the dispersal of the mosquitoes throughout the country can provide an early understanding of the invasion process and help formulating hypotheses for follow-up projects. The active dispersal capacity of *Ae. aegypti* is low and was reported as up to 730 m in the field (Marcantonio et al., 2019; Muir & Kay, 1998; Staunton et al., 2019; Verdonschot & Besse-Lototskaya, 2014). Thus, the vector expands its distribution range passively. *Aedes* mosquito eggs get dispersed either by the transportation of eggs in used vehicle tires (Dhimal, Ahrens, et al., 2015) or through hitch-hiking of adult mosquitoes via human transportation such as aircrafts and vehicles (Huber et al., 2004; Ibáñez-Justicia, 2020). *Ae. aegypti* was first recorded in Southern Nepal in 2006 (Malla et al., 2008) and since then spread rapidly throughout the country following different introduction routes along the gradient (Dhimal et al., 2014; Dhimal, Gautam, et al., 2015; Kramer, 2021; Kramer, Baral, et al., 2021). In Kathmandu, *Ae. aegypti* was recorded for the first time in 2009 (Gautam et al., 2009). The sampling sites from Chitwan (CH200) to Kathmandu (KT1300) are connected via multiple introduction roads from India (or Asia). However, since Kathmandu is the capital of Nepal and the only international airport is located there, it is thus the primary destination for any long-distance transport. This might have resulted in repeated invasion events of *Ae. aegypti* from outside of Nepal into Kathmandu. Given the clustered pattern of population differentiation between lowlands and highland populations, multiple differential or repeated invasion events across the gradient are likely. Moreover, travel and transportation routes are not unidirectional in Nepal and invasion from Kathmandu to the lowlands is also possible. More in depth analyses involving genome-wide individual-based analysis of the population structure and admixture will be necessary to finally resolve the invasion history along the gradient.

Within the focus of our study, the pattern of local high-altitude adaptation exclusively in the highland population without a gradual pattern along the altitudinal gradient has probably been shaped by distinct differences in environmental and climate conditions between Kathmandu and lowland population sites. Kathmandu's climate is the coldest along the gradient, but also experiencing the harshest increase in temperature due to urbanization, a so-called heat island effect (Karl, 1988; Kramer, 2021; Mitchell Jr., 1961; Poudel et al., 2020; Thakuri et al., 2019; Appendix S1: Figures S8 and S9). Furthermore, Kathmandu represents the coldest climate where subzero temperatures as cold as −2°C were present during the last years (Kramer, 2021; unpublished data: Phuyal et al., 2022). We can thus conclude, that the Kathmandu climate is extreme, under strongest change, and different from the climate conditions in the lowlands, thus setting differential conditions eventually driving the isolation by environment (IBE) pattern between Kathmandu and the lowlands. Since genetic differentiation of the investigated *Ae. aegypti* populations is independent of geographic distance (see Mantel's test) but increases with environmental differences (Figure 4), we conclude IBE over isolation by distance (IBD; Wang & Bradburd, 2014). Moreover, amongst all environmental factors of our genotype association analysis, fewest loci of significant differentiation have been found to be associated to altitude (ENV1), indicating that differences between populations are not majorly described by altitudinal geographic differences. Thus, these are optimum conditions for the identification of signatures of local adaptation without confounding demographic effects (Hartke et al., 2021; Hoban et al., 2016). While the evolution of IBD is related to the interplay of genetic drift and movement, IBE is usually related to the adaptability to environmental selection pressures (Jiang et al., 2019; Orsini et al., 2013). Extreme and distinct environmental and climate conditions in Kathmandu, thus,

are likely to exert strong selection pressure on the highland population. The ecologically driven high-altitude adaptation is probably priming the Kathmandu population for further successful expansion into cooler habitats. When established successfully at first, populations promote the invasion via new introduction routes into the invaded range, as defined by the bridgehead effect (summarized by Sherpa, Blum, Capblancq, et al., 2019). In Nepal, *Ae. aegypti* is present up to 2100 m altitude above mean sea level but far less abundant at altitudes above 1300 m (Dhimal et al., 2014; Dhimal, Gautam, et al., 2015; Kramer, 2021; Kramer, Baral, et al., 2021). It is unclear, if individuals present above 1300 m are seasonal migrants each year or belong to the permanently established population within the region. Thus, the established Kathmandu population can be defined as range-edge population along the investigated gradient.

The non-gradual pattern of genomic differentiation in *Ae. aegypti* across Nepal indicates the potential for the invasion into cooler habitats for different, mutually not exclusive reasons. Strong environmental filtering and selection is promoting high-altitude adaptation (see next section) in a population that has either been reaching the range-edge, thus priming the adaptation to colder climate or it has already been carrying relevant alleles due to the introduction via alternative invasion events compared to populations in the lowlands. The observed genomic differentiation may eventually lead to the formation of two *Ae. aegypti* lineages in Nepal, with temperate *Ae. aegypti* populations evolving along the altitudinal, as well as latitudinal gradient and a highland population with further cold tolerance adaptation. Thus, the cold tolerance and hence the fitness advantage of the high altitude population in Nepal (Kramer, Pfeiffer, et al., 2021) may further increase (Bennett et al., 2021b), as also indicated by the establishment of a more cold resistant population of *Ae. aegypti* in a temperate region in Argentina (Buenos Aires; de Majo et al., 2019; Fischer et al., 2019; Garzón et al., 2021). Such a phenotype would increase the introduction risk of *Ae. aegypti* into new, previously too cold ecoregions with dengue naïve human populations.

4.3 | Signatures of climate adaptation in *Ae. aegypti* are genomically widespread and involve few genes

We uncovered the genomic footprint of climate adaptation in *Ae. aegypti*. Similar investigations were performed in different insect species, for example, the harlequin fly (Waldvogel et al., 2018) and two cryptic ant species (Hartke et al., 2021). The investigated *Ae. aegypti* populations across the Nepalese altitudinal gradient reveal a set of 33 candidate genes (EAP-OW) that are genomically wide-spread and carry signatures of climate selection, equaling ~0.2% of protein-coding genes. This is far less than the identified genomic footprint of climate adaptation (i.e., adaptation to temperature and precipitation) in the harlequin fly *Chironomus riparius* involving 1.2% of protein-coding genes (Waldvogel et al., 2018). Differences in sampling design might explain this variation, as the altitudinal sampling gradient in Central Nepal comprised small to intermediate geographic distance, whereas Waldvogel et al. (2018) sampled the fly populations at larger (>200 km) distances across a continental climate gradient. The here presented short-distance sampling design along a well-defined climate gradient reduces the likelihood of false-positive signals of undetected environmental variables if compared to larger scale designs incorporating higher cross-correlating heterogeneity.

For some candidate genes (EAP-OW), it was possible to identify nonsynonymous SNPs. Nonsynonymous mutations may be associated with functional protein differences of phenotypic effect (Schoennenbeck et al., 2021). The 12 candidate genes (EAP-OW) for climate adaptation did carry nonsynonymous mutations (Table 2, Appendix S1: Table S9, Information 2), such as the "toll-like receptor Tollo". This gene was already studied in *Ae. aegypti* and plays a role in the immune response, and particularly in the antidengue defence (e.g., Ramirez & Dimopoulos, 2010; details in Appendix S1: Table S9). The nonsynonymous mutations in this candidate gene lead to an amino acid with changed characteristics (Table 2). Since synonymous mutations may influence splicing, RNA stability, RNA folding, translation or cotranslational protein folding, candidate genes (EAP-OW) containing synonymous mutations were also checked for their biological function (Sharma et al., 2019; for details see Appendix S2: Tables S6–S8). The two genes "segmentation protein Fushi tarazu" and "Nasrat" are involved in the survival and later successful hatching of eggs and associated with precipitation (see more details in Appendix S2: Tables S7 and S8; Field, 2015; Simington et al., 2020). The association with precipitation adds up since precipitation has an impact on survival and later successful hatching of eggs. Two genes identified by the LFMM approach were also found to be associated to different environmental variables using the BayPass tool, the two genes "uncharacterized LOC5566116" and the "sine oculis-binding protein homologue" seem to be of high importance for climate adaptation in *Ae. aegypti* (Appendix S2: Tables S2–S5).

4.4 | Signatures of local nonclimate adaptation reveal a broad functional basis in *Ae. aegypti*

Other than gradual climate selection regimes, local selection pressures act on populations only in their specific habitat. Accordingly, there are SNPs that are not associated to the climatic gradients but still highly divergent between some or all *Ae. aegypti* populations (OW-OP). These SNPs are candidates for local nonclimate adaptation. Approximately 8.2% of protein-coding genes, i.e. 1200 genes, show signatures of local selection. Similarly, 7.6% of genes were found to be locally adapted in *C. riparius* (Waldvogel et al., 2018). Two of the identified candidate genes for local adaptation were already found to play a role in local adaptation of *Ae. aegypti* in Panama (Bennett et al., 2021b). Due to the identification of these two genes in *Ae. aegypti* populations from different countries, the two genes ("putative vitellogenin receptor" and "xanthine dehydrogenase", for more details see Appendix S1: Information 2) appear to play an important role in local adaptation of this species. Moreover, the candidate gene "putative vitellogenin receptor" was also obtained using

the BayPass approach (Bennett et al., 2021b; Appendix S2: Table S5). For verification, knock-out studies testing the molecular function of the *Ae. aegypti* candidate genes containing different SNPs at given positions are highly recommended.

Amongst all candidate genes for local adaptation, we spotlight two traits that are important from a medical VBD perspective, namely insecticide resistance and vector competence. The insecticide resistance of *Ae. aegypti* determines the success of vector control programs (Faucon et al., 2017). Most variations with the detoxification enzymes are probably not functionally associated with insecticide resistance. Instead, some are the consequence of strong selection pressure, hence only some reflect selection of a variant showing an increased metabolic activity against insecticides (Faucon et al., 2017). However, *kdr* mutations such as V1016G, F1534C and S989P are known to lead to pyrethroid insecticide resistance in *Ae. aegypti* (summary in Faucon et al., 2017). In accordance with Kawada (Kawada et al., 2020), in Nepal the *kdr* mutations F1534C and V1016G are present with varying frequencies and the S989P mutation was not present in all study populations (Table 3, Figure 3). Within the CH200 and KT1300 population, there is a trend of increased *kdr* mutations. It can be hypothesized that this trend is present since fogging of insecticides (e.g. deltamethrin) mainly occurs in urban areas. Thus, *kdr* mutations may be more present in urban areas such as CH200 and KT1300 compared to less urban regions such as DK800 and DH600 (Kawada et al., 2020). Kawada showed at least for CH200 and KT1300 their susceptibility to pyrethroids. However, none of the *kdr* mutations are found to overlap with a significant OW/OP and accordingly did not contribute to patterns of population differentiation. Given that the Nepalese populations showed an intermediate to high resistance to pyrethroids, but only small amounts of insecticides are used in Nepal compared to other Asian countries (Kawada et al., 2020), this indicates a reduced selection pressure on *kdr* mutations in Nepal. The genetic presence of *kdr* mutations might derive from the introduction of *Ae. aegypti* populations from neighbouring countries (Kawada et al., 2020). The vector competence of *Ae. aegypti* determines the efficiency of dengue transmission to humans and thus it is important to understand this trait at a local level. SNPs associated with DENV-1 and/or DENV-3 infection were found in all populations and likely play a role in dengue resistance of *Ae. aegypti* in Central Nepal. This assumption is supported by reported DENV type-specific resistance of a population from Gabon (Dickson et al., 2020). However, the verification of SNPs and their functional meaning in the identified candidate genes for insecticide resistance and dengue vector competence merits definitely further research.

4.5 | Implications for climate adaptation

Genomic differentiation of the highland population compared to the lowland populations supports the notion that *Ae. aegypti* is able to adapt to colder climates. It will require additional investigations including genome-wide individual resequencing data to understand if the highland population shows signatures of local cold climate adaptation at the range-edge or whether the relevant alleles have been introduced through an alternative invasion event when compared to the lowland populations. In any case, the identified alleles of the highland population are likely relevant for their invasion to colder regions. In general, it is of major importance to track the trends of climate adaptation not only in emerging viruses (de Almeida et al., 2021), but also in the respective vector populations especially. On the most basic level, differentially adapted populations, be it to climate or local conditions, could have different abilities to transmit arbovirus diseases (Bennett et al., 2021b). With our study we demonstrate that effective monitoring of vector populations using NGS strategies allows for the interpretation of emerging expansion trends. The here applied approach of population sampling and sequencing proved to be a powerful and cost-effective methodology to assist the comprehensive monitoring and mapping of the vector species *Ae. aegypti* (Rinker et al., 2016). Patterns of population differentiation, genomically as well as physiologically, deliver important evolutionary and ecological information to be integrated into vector distribution models under climate change scenarios, especially in cooler ecoregions (Kearney et al., 2009). Current distribution models predicting the future distribution of vector populations should incorporate the adaptive response of species for more precise predictions (Bennett et al., 2021b). Genomic diversity and, thus, biodiversity by means of adaptation and simultaneously climate warming is likely to increase the risk of expansion of *Ae. aegypti* worldwide to colder ecoregions. With the increasing distribution range of the vectors worldwide as well as in Nepal and the HKH region in particular (Iwamura et al., 2020; Kraemer et al., 2019; Trájer, 2021) also the spread of VBDs will increase (worldwide: e.g., dengue: Messina et al., 2019; Nepal: Acharya et al., 2018), underlining that parts of biodiversity can be detrimental to human health. For efficient vector control, it is important to consider that locally adapted populations could impact control efforts that are based on gene drive system, but adaptive genes could also be targets for population control using gene editing strategies (Bennett et al., 2021a; Gabrieli et al., 2014; Hancock et al., 2016).

Results obtained in this study could potentially be used for the inference of the adaptive response of *Ae. aegypti* to colder ecoregions worldwide. The health systems in cooler ecoregions need to prepare for future VBD outbreaks and develop surveillance strategies to prevent the establishment of dengue vectors. To identify emerging trends within the adaptation of *Ae. aegypti* to new environments, we recommend to investigate populations in Nepal from higher altitude as well as populations along altitudinal and latitudinal clines worldwide. Moreover, next to reciprocal transplant experiments in a safe experimental set up (Bennett et al., 2021a, 2021b), molecular investigations of the function of the candidate genes, the verification of the association of candidate genes with different environmental variables and differences in vector competence between the KT1300 populations and lowland populations should be verified.

AUTHOR CONTRIBUTIONS

Isabelle Marie Kramer, Meghnath Dhimal, Ishan Gautam, Pramod Shreshta, Sunita Baral and Ruth Müller sampled the mosquito

populations. Pramod Shreshta entomologically identified the mosquito species. Isabelle Marie Kramer and Ann-Marie Waldvogel majorly analysed the data. Markus Pfenninger, Barbara Feldmeyer, Juliane Hartke, Axel Magdeburg, Bodo Ahrens and Ruth Müller assisted in the data analysis. Isabelle Marie Kramer, Barbara Feldmeyer, Parbati Phuyal, Juliane Hartke, Ruth Müller and Ann-Marie Waldvogel visualized the data. Meghnath Dhimal, Ruth Müller and Ann-Marie Waldvogel conceptualized the study. Markus Pfenninger, David A. Groneberg and Ruth Müller provided study resources. Ruth Müller and Ann-Marie Waldvogel supervised the study. Ruth Müller and Axel Magdeburg were responsible for the study administration and Ruth Müller also for the funding acquisition. Isabelle Marie Kramer and Ann-Marie Waldvogel wrote the original draft. Markus Pfenninger, Barbara Feldmeyer, Meghnath Dhimal, Ishan Gautam, Pramod Shreshta, Sunita Baral, Parbati Phuyal, Juliane Hartke, Axel Magdeburg, David A. Groneberg, Bodo Ahrens and Ruth Müller reviewed and edited the original draft. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The PoolSeq-data sets supporting the conclusions of this article have been made available in the ENA- European Nucleotide Archive under the following accession number: PRJEB56779.

BENEFIT-SHARING STATEMENT

With a memoradum of understanding between the Nepal Health Research Council and the Institute of Occupational, Social and Environmental Medicine at the Goethe University Frankfurt, we are committed to international scientific partnerships, as well as institutional capacity building. All collaborators working on this study are included as coauthors, and the results of research have been shared with the health ministry of the study country (Nepal Health Research Council). Moreover, the field collection of mosquitoes was authorized by the Nepal Health Research Council (application number 1058) and the transport of the mosquitoes from Nepal to Germany was in accordance with the Material Transfer Agreement no. 381/2017.

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