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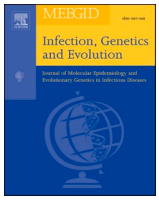

Is Côte D'Ivoire a new high hybridization zone for the two major malaria vectors, *Anopheles coluzzii* and *An. gambiae* (Diptera, Culicidae)?

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ABSTRACT

Anopheles gambiae and *An. coluzzii* are very closely related and recently differentiated species representing the main malaria vectors in the Afrotropical region and responsible of up to >3 infective bites/person/night in Côte D'Ivoire, where prevention and control has stagnated in recent years. The aim of the present study was to genetically and ecologically characterize *An. gambiae* and *An. coluzzii* populations from two villages of Côte D'Ivoire, lying in the coastal forest belt and 250 km inland in the Guinean savannah mosaic belt, respectively. Results reveal high frequencies of both species in both study sites and high frequencies of hybrids (4–33%) along the whole year of sampling. Consistently with observations for the well-known high hybridization zone at the far-west of the species range, hybrid frequencies were higher in the coastal village and highest when the two species occurred at more balanced frequencies, supporting the "frequency-dependent hybridization" ecological speciation theory. Pilot genotyping revealed signatures of genomic admixture in both chromosome-X and −3. Coupled with previous reports of hybrids in the region, the results point to the coastal region of Côte D'Ivoire as a possible regions of high hybridization. Preliminary characterization of parameters relevant for malaria transmission and control (e.g. possibly higher sporozoite rates and indoor biting preferences in hybrids than in the parental species) highlight the possible relevance of the breakdown of reproductive barriers between *An. gambiae* and *An. coluzzii* not only in the field of ecological evolution, but also in malaria epidemiology and control.

1. Introduction

Malaria is a leading cause of morbidity and mortality in Côte D'Ivoire (PMI 2018-2019, 2019). Progresses in malaria prevention and control has stagnated in recent years in the country, as well as in other neighboring west African countries, with the estimated number of cases increasing 15.8% between 2015 and 2018 (from 260 to 300 cases per 1000 population) (World Health Organization, 2020). Among factors responsible of the high malaria burden in the region is the very efficient vectorial system accountable for up to >3 infective bites/person/night (PMI 2018-2019, 2019; PMI, 2020). This system is largely constituted by the two most synanthropic species of the *An. gambiae* complex (Diptera, Culicidae), i.e. *An. coluzzii* Coetzee & Wilkerson, 2013 and *An. gambiae* Giles, 1900. The latter strongly predominates in northern forest-savannahs and Sudanese savannahs, while *An. coluzzii* predominates in the south-western forested region (Edi et al., 2014). The two species are found at more balanced frequencies in south eastern evergreen and deciduous forested region (PMI 2018-2019, 2019; PMI, 2020). Extensive insecticide resistance is observed throughout the country, with populations showing resistance to all 4 classes of insecticides (Oumbouke et al., 2020).

Anopheles gambiae and *An. coluzzii* are very closely related and

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recently differentiated species, yet they present distinct ecological adaptations, particularly at the pre-imaginal stage, with *An. gambiae* dominating in temporary rain-dependent and *An. coluzzii* in human-made permanent breeding sites, (Lehmann and Diabate, 2008). They were first recognized based on fixed differences in the chromosome-X linked rDNA region (still currently used for molecular identification) and named as molecular forms M and S (Della Torre et al., 2001) for which different mechanisms promoting reproductive isolation have been proposed (Diabaté et al., 2009; Pennetier et al., 2010). Later, despite evidence of imperfect assortative mating, these were raised to formal species (Coetzee et al., 2013), based on evidence showing that their genomes contain regions of differentiation resulting in exclusive taxonomic clustering across much of their shared geographical range (Turner et al., 2005; Lawniczak et al., 2010; Neafsey et al., 2010; White et al., 2010; Reidenbach et al., 2012;; Weetman et al., 2012;);. Since then, results from genomic and ecological studies have raised *An. gambiae* and *An. coluzzii* to models of ecological speciation, revealing a rich mosaic of different ancestries in the two species, shaped by geography, ecology and speciation and highlighting evidence of strong intra-specific sub-structuring at the western and eastern extremes of their range (The Anopheles gambiae 1000 Genomes Consortium, 2017, 2020). Paracentric chromosomal inversions, mostly shared by the two species, further complicate the scenario, and contribute to adaptation to marginal niches and to intra-specific sub-structuring (Coluzzi et al., 2002) . Despite their major role in malaria transmission in the sub-Saharan region and the potential epidemiological consequences (White et al., 2011), the intra-specific sub-structuring and the degree of hybridization and introgression between *An. gambiae* and *An. coluzzii* are still not clearly depicted in large part of their range.

Even though only few putative adult hybrids between the two species are being detected continent-wise, several examples of periodic breakdowns of pre- and/or post-mating barriers, resulting in extensive hybridization and in detectable levels of adaptive introgression and current gene flow, have been reported (Pombi et al., 2017). Very importantly from the perspective of malaria control, introgressive hybridization from *An. gambiae* to *An. coluzzii* of alleles associated to resistance to pyrethroid insecticides has been shown to have occurred multiple times in the last decades, rapidly increasing target site resistance in the recipient species, *An. coluzzii* (Pinto et al., 2007; Hanemaaijer et al., 2018).

According to the "frequency-dependent hybridization" ecological speciation theory (Kirkpatrick, 2001; Seehausen, 2004; Stankowski, 2013), the prevalence of hybrids along their sympatric range is expected to be higher where their degree of inter-specific contact is maximal. This is certainly the case for *An. coluzzii* and *An. gambiae* in the coastal region from The Gambia and Guinea Bissau at the western extreme of the species' range (hereafter referred to as far-west) (Pombi et al., 2017), where the two species were predicted to occur at relatively high frequencies (Tene Fossog et al., 2015). This region of apparently high hybridization was first revealed by the finding of individuals characterized by heterozygous X-linked IGS-diagnostic markers (hereafter IGS-hybrids) at frequencies significantly higher (>20%; Caputo et al., 2008; Oliveira et al., 2008; Niang et al., 2014) than those usually observed in the rest of the range (<2%; Pombi et al., 2017). Further studies exploiting also other genomic markers suggest a stable breakdown of reproductive isolation leading to massive introgression and intra-specific genomic partitioning of far-west coastal populations (Caputo et al., 2011; Marsden et al., 2011; Lee et al., 2013; Nwakanma et al., 2013; Caputo et al., 2014). Genomic studies on field populations have revealed a sub-structuring of *An. gambiae* populations in the region (The Anopheles gambiae 1000 Genomes Consortium, 2017, 2020), leading to the hypothesis of the existence of a coastal putative "hybrid form" (carrying an *A. gambiae* chromosome-X centromere and *An. coluzzii*-like autosomes) separated from inland *An. gambiae* by a central region dominated by *An. coluzzii* (Vicente et al., 2017) . Data also highlighted potential for spatio-temporal stability of the putative coastal hybrid form and evidence of resilience against introgression of medically important loci and traits, as well as of greater zoophilic tendency.

In addition to the far-west, the other region in West Africa predicted to harbour relatively high frequencies of *An. coluzzii* and *An. gambiae*, and thus possibly representing a second high- hybridization zone, is the forested region between south-east Côte D'Ivoire and south-west Ghana (Tene Fossog et al., 2015). Intriguingly, multiple records of IGS-hybrids have been reported in Côte D'Ivoire since 2012: in the evergreen forested coastal region (hybrid frequency = 2.5–11%; Edi et al., 2017; PMI 2018-2019, 2019; Mouhamadou et al., 2019; Meiwald et al., 2020) and in central western forested region (4.3% Man area region, at the borders with Guinea; Assogba et al., 2018), as well as hundreds of kilometers northwards, i.e. in rice fields in Yamoussoukro area (H = 1.8–7.7%; Chouaïbou et al., 2017; Assogba et al., 2018; PMI 2018-2019, 2019) and in urban/peri-urban sites in the forest-savanna mosaic belt in Bouakè area (H = 0.6–5.4%; Assogba et al., 2018; Oumbouke et al., 2020) ~210 km and ~ 300 km from the coast, respectively. Moreover, an overall frequency of 2.1% of IGS-hybrids was reported in 10 sites across the different eco-climatic zones (Fodjo et al., 2018). Noteworthy, all the above reports refer to adult females emerged from larvae collected in the field in order to assess susceptibility to insecticide by in vivo bioassays. To the best of our knowledge, the only report of an adult IGS-hybrid collected in the country dates back to 1998, when the two species were still referred to as M and S molecular forms (H = 1.1%, Bouake area; Della Torre et al., 2005).

The aim of the present study was to characterize *An. gambiae* and *An. coluzzii* adult populations from a coastal and an inland village of Côte D'Ivoire where both species were expected to occur at relatively high frequencies, and to identify possible signatures of genomic admixture. Results highlight frequencies of adult *An. gambiae/An. coluzzii* hybrids along the whole year even higher than those observed in the far-west high hybridization region, strongly supporting the "frequency-dependent hybridization" hypothesis, and open the opportunity to preliminary characterize the hybrids with respect to parameters relevant for malaria epidemiology and control.

2. Materials and method

2.1. Entomological collections

Mosquito collections were carried out in two villages in Côte D'Ivoire (Fig. 1A), where both *An. coluzzii* and *An. gambiae* are reported to be present (PMI 2018-2019, 2019; PMI, 2020).

Ayamé village Piste IV (GPS: 5°28′ N 3°12′W, hereafter coastal village) is located in the proximity of a large artificial lake and hydroelectric basin in the south-eastern region of Aboisso, 100 km east of the capital city Abidjan and 50 km from the coast. The region lies within the evergreen forest mosaic belt and is characterized by an equatorial transition climate, with annual rainfall ranging between 1300 and 2400 mm, with a long rainy season in March–July and a shorter one in September–December. The region is hilly and covered with dense moist forest and cacao or coffee plantations.

Petessou village (GPS: 8°6′ N 5°28′W; hereafter inland village) is situated at about 300 asl, 11 km south from the city of Bouaké and > 250 km from the coast. The village is located nearby a permanent watercourse maintaining a large area of shallows used for rice farming and market gardening, representing suitable breeding sites for mosquitoes (Zogo et al., 2019). The region lies within the Guinean savannah mosaic belt and is characterized by a tropical humid climate, with annual rainfall ranging between 1000 and 1600 mm. Dry season lasts from November to February and rainy season from March to October.

Mosquito collections were carried out in December 2018, March, May and October 2019 for four consecutive nights/month by CDC-light traps located inside and outside 10 randomly selected houses. Traps were located nearby a person sleeping under bed-net both indoors and outdoors. Ethical approval for the study was granted by Ministère de la

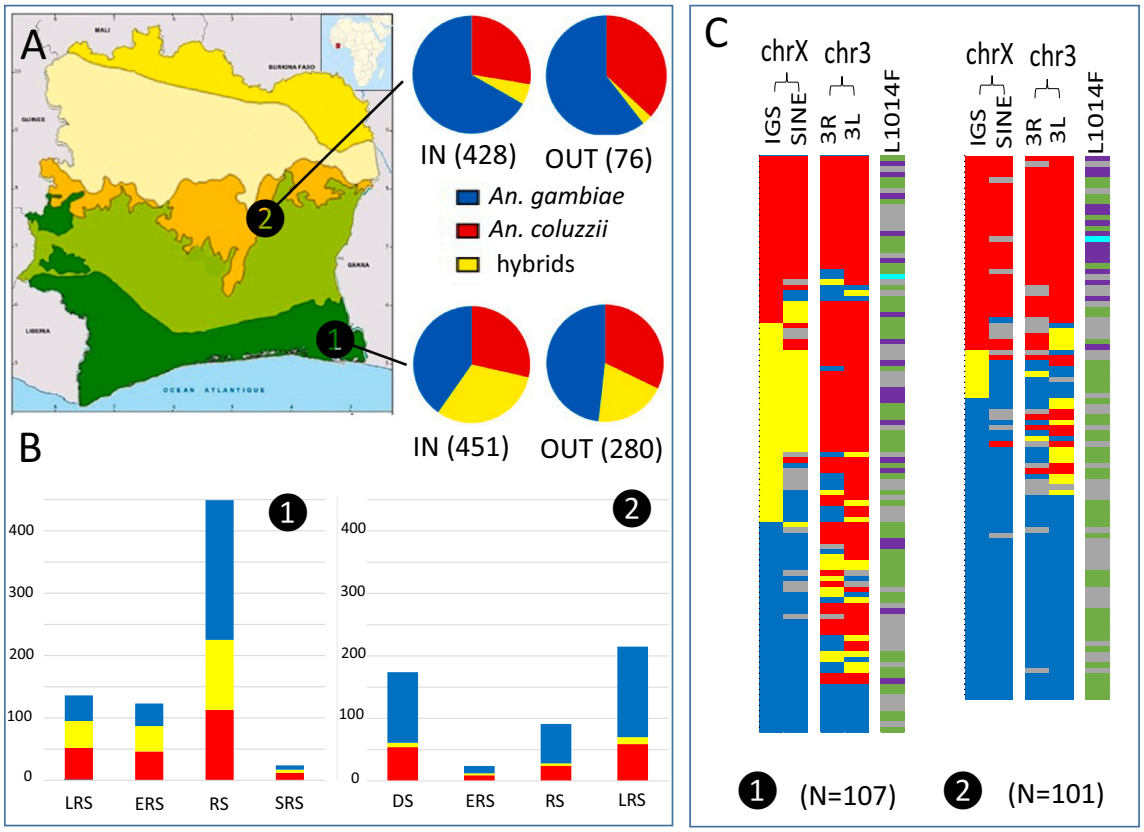

Fig. 1. *Anopheles coluzzii* (red), *An. gambiae* (blue) and IGS-hybrids (yellow) in indoor and outdoor collections in two villages in Côte D'Ivoire (A) and in the monthly collections between December 2018 and October 2019 (B). Coastal village: LRS = Late Rainy Season (5–8 December 2018), ERS = Early Rainy Season (6–9 March 2019), RS = Rainy Season (15–18 May 2019), SRS = Short Rainy Season (9–12 October 2019). Inland village: DS = Dry Season (12–15 December 2018), ERS = Early Rainy Season (12–15 March 2019), RS = Rainy Season (26–29 May 2019), LRS = Late Rainy Season (16–19 October 2019). Dark green in the map = evergreen forest; green = deciduous forest; orange = forest-savannah mosaic bet; Yellow = Sudanese savannah (modified from Edi et al. 2017). C = Genotyping results of the coastal and inland populations, relative to: two X-centromeric diagnostic markers (IGS and SINE), two Ancestry Informative Markers (sensu AG1000G 2017) on chromosome-3 (3R and 3 L), and the L1014F mutation in the *vgsc* gene on chromosomal arm 2 L (light blue = 1014 L homozygous susceptible; green = 1014F homozygous resistant; purple = 1014 L/1014F heterozygous; grey = unsuccessfully genotyped locus). Only specimens for which at least 2 of the 4 species-specific markers were successfully genotyped are included. Each row represents an individual mosquito and columns represent genotyped markers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Santé et de l'Hygiene Publique (Comité National D'Ethique de la Recherche, CNER, 023–18/1VISHP/CNER, Abidjan 3/4/2018).

Sampled mosquitoes were morphologically identified as *An. gambiae* sensu lato (s.l.) based on Gillies and Coetzee (1987) and were preserved in tubes with silica gel.

2.2. Molecular identification and genotyping

DNA was extracted from head and thorax of *An. gambiae* s.l. females following the protocol by (Rider et al., 2012) and used as template for molecular analyses. All collected specimens were genotyped for diagnostic SNPs in the IGS-rDNA region by Santolamazza et al. (2004) or Wilkins et al. (2006) PCR-approaches. Individuals showing a IGS-heterozygous genotype are referred hereafter as IGS-hybrids. *Plasmodium falciparum* (Welch, 1897) (Apicomplexa, Plasmodiidae) presence was assessed by a TaqMan approach (Bass et al., 2008).

A subset of random selected specimens was also genotyped for:

1) presence of *An. coluzzii*-specific insertion of a Short Interspersed Nuclear Element (SINE) in the centromeric region of chromosome-X (Santolamazza et al., 2008; hereafter referred to as SINE-X).
2) two Ancestral Informative Markers (sensu Ag1000G, 2017) on chromosomal arms 3R (3R:42848) and 3L (3L:129051) by the Tetra-arms-PCR protocol developed by Caputo et al. (2021).
3) L1014F (kdr-West) mutation in the voltage-gated-sodium-channel (*vgsc*) gene, associated with insecticide-resistance (Bass et al., 2007).
4) 2Rb, 2Rc and 2La paracentric chromosomal inversions by the molecular karyotyping PCR-approaches developed by Montanez-Gonzalez et al. (2020); Montanez-Gonzalez et al. (2021) and White et al. (2007), respectively.

2.3. Statistical analyses

The mean number of mosquito/person/night (m/p/n) was estimated based on Generalized Linear Mixed Models (GLMMs) assuming that this corresponds to the number of collected mosquitoes/trap/night (Kilama et al., 2014). A negative binomial distribution for the response variable (i.e. counts of mosquitoes per night), with mean μ and dispersion parameter θ was chosen to predict m/p/n. Two models were fitted: GLMM-1 considers as covariates the villages, the months of collection and their interaction. GLMM-2 considers as covariates the species, the village, and their interaction. A random effect structure was included in both models to account for the individual variability of each house.

Plasmodium falciparum sporozoite rate (SR) was estimated by GLMM. Assuming that the response variable (presence and absence of *P. falciparum* DNA in the head+thorax of processed specimens) follows a binomial distribution, two models were fitted: GLMM-3 considers as covariates the villages, the months of collection and their interaction. GLMM-4 considers the species, the village and their interaction. A

random effect structure was included in both models to account for the individual variability among sampled houses.

The Entomological Inoculation Rate (EIR) was computed as the product of the estimated m/p/n and the estimated SR, under the assumption that collected specimens represent the biting fraction of the mosquito population.

The relationship between the relative frequency *An. coluzzii* and *An. gambiae* and the relative frequency of IGS-hybrid genotypes in each village was assessed by Generalized Additive Models (GAM-1). Assuming that the frequency of IGS-hybrids follows a binomial distribution, GAM-1 was fitted with the relative frequency of hybrid genotypes as response variable and the relative frequency of *An. coluzzii* and *An. gambiae* taxa and the villages as covariates.

Anopheles coluzzii, An. gambiae and IGS-hybrid genotypes indoor/outdoor abundance was estimated by GLMM-5 including as covariates the village, the month of collection, the indoor/outdoor trap location, and their interaction. We fitted a model for each species separately. Since *An. coluzzii* and *An. gambiae* abundance data using a Poisson distribution resulted over-dispersed, we considered a Negative Binomial distribution with mean μ and dispersion parameter ϑ, and the count of specimens/night/house and trapping location (indoor/outdoor) in the two villages as response variable. On the other hand, overdispersion was not detected for IGS-hybrid genotypes when assuming a Poisson distribution with mean λ, where the response variable was the number of hybrid genotypes/night/house and indoor/outdoor location in the two village. Initially, the full model included village, month, and trap location, and all their interaction as independent variables. A random effect structure was included in both models to account for the individual variability of each house. A model selection procedure was applied to all GLMM-5 (Burnham and Anderson, 2004). Subsequently, the Akaike Information Criteria (AICc) was used to rank all submodules, then the best parsimonious model was considered between the subset of models having a difference (delta) in AICc <4. Due to convergence problem, the maximum number of iterations of the glmer. nb function in the lme4 package has been increased to 10.000.

Frequency of paracentric chromosomal inversions was estimated by GLM-6 only in the inland village, due to low inversion frequencies in the coastal village. Assuming that the response variable "inversion frequency" follows a binomial distribution, the full model considers as covariates *An. coluzzii* and *An. gambiae* (no hybrid genotypes were considered, due to low sample), location and their interaction. We decided whether to include or not the interaction terms by comparing the models by AIC. The model was fitted for each paracentric chromosomal inversion separately.

R statistical software version 3.6.3 (R Core Team, 2019) and packages lme4 (Bates et al., 2015), MuMIn (Bartoń, 2019), mgcv (Wood et al., 2016), tydiverse (Wickham, 2017) and MASS (Venables and Ripley, 2002) were used for all statistical analysis.

3. Results

Seven-hundred forty-four *An. gambiae s.l.* females were collected in the coastal village, with a mean number of m/p/n ranging from 3.4 in May (95% CI 2.2–5.3, ϑ =0.45) to 0.2 in October (95% CI 0.1–0.3, ϑ =0.45). The total number of *An. gambiae s.l.* females collected in the inland village was 505, with an m/p/n ranging from 1.3 in October (95% CI 0.8–2.05, ϑ =0.45) to 0.15 in March (95% CI 0.005–0.27, ϑ =0.45) (GLMM-1, Table S0).

Plasmodium falciparum detection was successful for 729 and 476 specimens from the coastal and the inland village, respectively. *Plasmodium falciparum* SR ranged from 13.6% in March (95% CI 8.1–22.0) to 4.1% in October (95% CI 0.06–24.3) in the coastal village, and from 16% in March (95% CI 5.8–36.8) to 2% (95% CI 0.07–5.5) in October in the inland village. No significant differences were observed between the two villages (except in the inland village where SR was significantly lower in October than in December 2018; p-value<0.001 and March 2019; p-value = 0.026) (GLMM-3; Table S1, Fig. S1). The average EIR ranged between 0.16 (March)-0.007 (October) in the coastal village, and between 0.05 (December)-0.02 (March) in the inland one. Overall, the estimated average of EIR was 0.07 and 0.03 infectious bites/person/night in the coastal and inland village, respectively.

Overall, 1235 specimens were successfully genotyped for species-specific SNPs in the IGS-rDNA region: *An. coluzzii* (N = 368), *An. gambiae* (N = 641) were found at frequencies of 30.4% and 42.1% in the coastal village and 29% and 66.1% in the inland one, respectively (Fig. 1A; Table S2). IGS-hybrids (N = 226; Fig. 1B) were found at overall frequency of 27.5% in the coastal village (where frequencies ranged between 20.8% in October and 33.3% in March), and of 5% in the inland village (where frequencies ranged between 4% in Dec and 12.5% in March).

In the coastal village, results of GAM-1 show a curvilinear relationship between IGS-hybrid frequencies and *An. coluzzii* and *An. gambiae* relative frequencies (p-value<0.0001; Table S3; Fig. 2), indicating a higher IGS-hybrid prevalence when the two taxa occur at balanced frequencies. No statistical association is observed in the inland village.

3.1. Phenotypic characterization of Anopheles coluzzii, An. gambiae and of IGS-hybrids

3.1.1. Temporal dynamics and indoor/outdoor preferences

According to covariates selection in GLMM-5 the mean predicted abundance of *An. coluzzii*, *An. gambiae* and IGS-hybrids is correlated to village, month of collection and indoor/outdoor trapping location and includes the interactions between village and month of collection, as well as between village and indoor/outdoor trapping location (Fig. 3; Tables S4, S5, S6).

The analysis of the interaction between villages and months of collection highlights significant differences in species seasonality in the two villages (p-value =0.002) (Table S4; S5), with peaks of abundance at the peak of the rainy season in the coast (May: *An. coluzzii* = 2.8; *An. gambiae* = 8.1) and at the end of the rainy season inland (October: *An. coluzzii* = 1.9; *An. gambiae* = 4.2). The same pattern is predicted for IGS-hybrids in the coastal village (peak in May = 3.8), although the predicted mean abundance of hybrids is neglectable in the inland village except in October (0.54; p-value =0.009) (Table S6).

On the other hand, the analysis of the interaction between villages and indoor/outdoor trapping location highlights significantly higher mean predicted abundance of the two species in indoor vs outdoor collections in the inland villages (p-value<0.0001). In the coastal one only the mean predicted abundance of IGS-hybrids is predicted to be significantly higher indoors (p-value = 0.0002) (Table S6). IGS-hybrids abundance in indoor vs outdoor trapping location collections in the inland village was not computed due to the very low sample size.

3.1.2. Mean females/person/night

According to GLMM-2 model, the mean number of *An. coluzzii*, IGS-hybrids and *An. gambiae*/person/night (m/p/n) is 0.90, 0.85 and 1.26 in the coastal, and 0.59, 0.10 and 1.28 in the inland village, respectively (Table 1). In addition, the model highlights higher IGS-hybrids/p/n than *An. coluzzii*/p/n (p < 0.0001) in the coastal village and lower IGS-hybrids/p/n than mean numbers of both species in the inland one (p < 0.001; Table S7).

3.1.3. Sporozoite and entomological inoculation rates

According to GLMM-4, *P. falciparum*-SRs *An. coluzzii* and *An. gambiae* are 9% and 6% in the coastal village and 6% and 4% in the inland one (Table 1), with no significant differences between the two species. On the hand, SR is higher in IGS-hybrids than in the two species in the inland village (SR = 16% in 23 hybrids), while no significant differences are shown in the coastal village (Table S8, Fig. S2).

EIR values range between 0.06 and 0.08 and 0.001–0.05 infective bites/night in the coastal and inland village, respectively (Table 1).

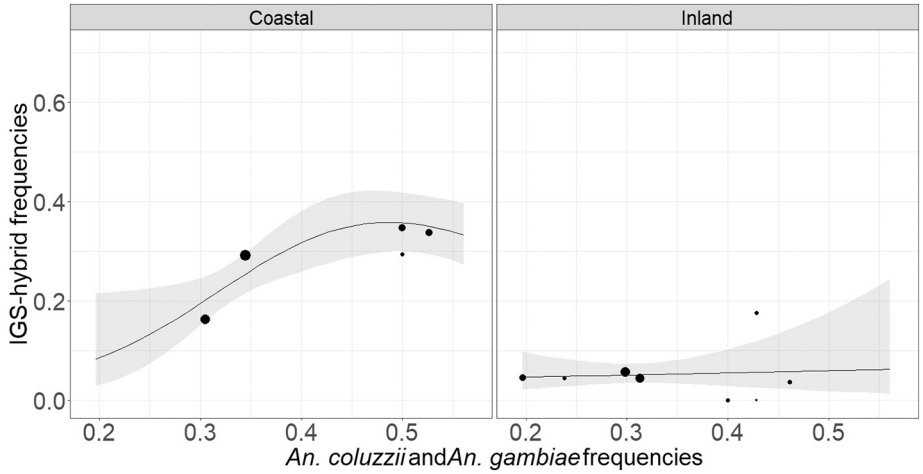

Fig. 2. Predicted relative frequency of IGS-hybrids (lines) as a function of *Anopheles coluzzii* and *An. gambiae* frequencies in a coastal (left) and inland (right) village in Côte D'Ivoire (GAM-1). Shaded areas = 95% confidence intervals. Dots = sample size classes from 50 to 250 mosquito/collection.

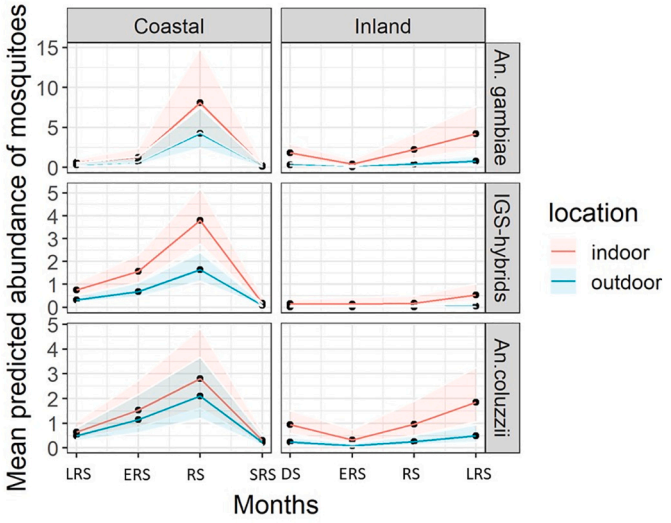

Fig. 3. Mean predicted abundance of *Anopheles coluzzii, An. gambiae* and IGS-hybrid females collected in a coastal and inland village in Côte D'Ivoire between December 2018 and October 2019. Coastal village: LRS = Late Rainy Season (5–8 December 2018), ERS = Early Rainy Season (6–9 March 2019), RS = Rainy Season (15–18 May 2019), SRS = Short Rainy Season (9–12 October 2019). Inland village: DS = Dry Season (12–15 December 2018), ERS = Early Rainy Season (12–15 March 2019), RS = Rainy Season (26–29 May 2019), LRS = Late Rainy Season (16–19 October 2019). Black dots = mean number of specimens/collection. Red and blue lines connect the predicted fit from the regression models on data collected indoors and outdoors, respectively. Shaded areas around the predicted lines = 95% confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Genotypic characterization of *An. coluzzii, An. gambiae* and IGS-hybrids

3.2.1. Chromosome-X SINE genotyping

A total of 241 specimens were genotyped for the presence of the *An. coluzzii*-specific SINE-X insertion in the centromeric region of chromosome-X (Santolamazza et al., 2008). The concordance between IGS and SINE-X genotyping is 75% in the coastal (N genotyped = 139) and 88% in the inland sample (N genotyped = 102) (Table S9). The most frequently observed mismatch (i.e. 20 out of 47 specimens with mismatching genotyping results) involved IGS-hybrids identified by SINE-X genotyping as *An.gambiae*. SINE-X heterozygous genotypes were detected in the coastal village only (35/139); of these 11.4%, 20% and 68.6% have been identified as *An. coluzzii, An. gambiae* and IGS-hybrid specimens, respectively.

Table 1
Mean number of mosquito/person/night (m/p/n), *Plasmodium falciparum* Sporozoites Rates (SR) and Entomological Inoculation rate (EIRs) in members of the *Anopheles gambiae* complex in a coastal and inland village in Côte D'Ivoire. N = total numbers of mosquitoes examined for the presence of *Plasmodium falciparum* over the whole sampling season; 95% CI = confidence intervals. Θ = dispersion parameter in the GLMM-2 0.35.

Village	Species	N	m/p/n (95% CI)	SR (95% CI)	EIR
Coastal	*An. gambiae*	303	1.3 (0.8–1.9)	0.06 (0.03–0.1)	0.07
	IGS-hybrids	199	0.8 (0.3–1.4)	0.07 (0.04–0.13)	0.06
	An. coluzzii	222	0.9 (0.6–1.4)	0.09 (0.05–0.15)	0.08
	An. gambiae sl	729	1 (0.7–1.5)	0.07 (0.05–0.11)	0.076
Inland	*An. gambiae*	315	1.3 (0.8–1.9)	0.04 (0.02–0.8)	0.05
	IGS-hybrids	23	0.1 (0.1–0.2)	0.16 (0.5–0.37)	0.001
	An. coluzzii	137	0.6 (0.4–0.9)	0.06 (0.03–0.12)	0.03
	An. gambiae sl	476	0.6 (0.4–0.9)	0.05 (0.03–0.09)	0.035

3.2.2. Chromosome-3 genotyping

A total of 188 specimens (out of 218 processed ones) were successfully genotyped for the two Ancestry Informative Markers (AIMs, sensu (The A. gambiae 1000 Genomes Consortium, 2017), one on chromosomal arm-3R (3R: 42848) and one on -3L (3L: 129051) (Fig. 1C; Table 2 and S10) (Caputo et al., 2021). In the coastal village, 61.2% of the

Table 2
Characterization of chromosome-3 genotype in *Anopheles coluzzii* (CO), *An. gambiae* (GA) and hybrids (IGS—H) from a coastal and an inland village of Côte D'Ivoire. Chr X (IGS) = diagnostic SNPs in the IGS rDNA region (Wilkins et al., 2006, and Santolamazza et al., 2011); CO-Chr3, GA-Chr3 = specimens with concordant homozygous *An. coluzzii* or *An. gambiae* chromosome-3 genotypes; Chr3-H = specimens with chromosome-3 markers either discordant or concordantly heterozygotes.

	CHR X (IGS)	CO-Chr3	Chr3-H	GA-Chr3	Total
Coastal	*An. coluzzii*	**25**	4	2	31
	IGS-H	27	**10**	0	37
	An. gambiae	11	15	**9**	35
	Total	**63**	**29**	**11**	**103**
Inland	*An. coluzzii*	**27**	3	0	30
	IGS-H	1	**2**	4	7
	An. gambiae	3	9	**36**	48
	Total	**31**	**14**	**40**	**85**

specimens (63/103) carry *An. coluzzii*-specific alleles at both chromosome-3 loci and 10.7% (11/103) the *An. gambiae*-specific alleles. In the inland village, 36.5% (31/85) and 47.1% (40/85) specimens carry either *An. coluzzii* or *An. gambiae*-specific alleles, respectively. Individuals characterized by a heterozygous genotype for both chromosome-3 loci were only found in the coastal village (3/103). Genotypes characterized by discordant 3R/3 L genotypes were found at frequencies of 25.2% (26/103) and 16.5% (14/85), respectively. These were mostly characterized by a homozygous 3R-GA genotype.

3.2.3. Association between chromosome-X and −3 genotypes

Inconsistent IGS and chromosome-3 genotypes (non in bold in Table 2) are significantly more frequent in the coastal (57%; 59/103) than in the inland village (23.5%; 20/85) ($p < 0.001$). In the coastal village, 80.6% (25/31) of *An. coluzzii* and only 25.7% (9/35) of *An. gambiae* show consistent association between IGS-species diagnostic marker and both chromosome-3 markers ($p < 0.0001$) (Fig. 1C; Table 2). In the inland village, 90% (27/30) of *An. coluzzii* and 75% (36/48) of *An. gambiae* show consistently associated CO-Chr3 and GA-Chr3 genotypes (p = n.s) (Fig. 1C; Table 2).

The overall picture does not change substantially if specimens are identified either by SINE-X or by both IGS and SINE-X diagnostics (Table S11). However, it is interesting to note that in the coastal village 18 out of 19 individuals characterized by an IGS and SINE-X heterozygous genotype, indicative of F1 hybrids (Santolamazza et al., 2011), are characterized by a "*coluzzii*-like" chromosome-3 genotype (Fig. 1C).

3.2.4. L1014F genotyping

Mutation L1014F was genotyped in 120 and 115 specimens from the coastal and the inland village respectively. Frequency of the 1014F allele was >90% in *An. gambiae* in the costal (N alleles = 65/72) and in the inland (121/122) village, respectively, and > 80% in hybrids (i.e. 69/80 and 12/12, respectively) (Table S12). In *An. coluzzii* higher frequency of the 1014F is observed in the coastal (83%, 73/88) than inland (68%, 65/96) village ($\chi^2 = 4.907$, *p*-value = 0.02). Only 5 *An. coluzzii* (3 from the inland and 2 from the coastal village) and 1 *An. gambiae* (from the coastal village) specimens carry the susceptible 1014 L allele in homozygosis. The frequency of 1014F allele is significantly higher in *An. gambiae* than in *An. coluzzii* in the inland village (Fisher exact test, p-value<0.0001).

3.2.5. Paracentric chromosomal inversion molecular karyotyping

Paracentric chromosomal inversions were molecularly karyotyped in 120 and 209 specimens in the coastal and inland village respectively, with 94%, 98% and 98% success rate for 2Rb, 2Rc and 2La inversions, respectively (Table S13). Higher frequencies of 2Rb and 2La inversions are observed in the inland village compared to the coastal one in either species, as well as in IGS-hybrids (*p*-value<0.005) (Table S13, Fig. S3). The 2Rc inversion polymorphism is found at frequencies up to 9% in the three groups in the two villages, with no significant differences between groups or villages. Probability of occurrence of 2Rb and 2La inversion frequencies in the inland village, as estimated by GLM-6, is statistically higher in *An. gambiae* than in *An. coluzzii* both indoors and outdoors (Table S14, S15 and Fig. S4).

4. Discussion

This study confirms expectation of relatively balanced frequencies of *An. coluzzii* and *An. gambiae* in the forested coastal region of Côte D'Ivoire, as well as in the south-east Guinean forest-savannah mosaic belt, 250 km inland (Fig. 1). The chromosomal polymorphism pattern of the coastal population shows the expected "forest" karyotype (i.e. almost complete standard karyotype), while the inland population is characterized by a "savanna-like" karyotype (i.e. frequencies 2Rb and 2La inversion up to 32 and 55%, respectively). The latter are higher in *An. gambiae*, confirming a different role of inversions in ecological adaptation in the two species (Coluzzi et al., 2002).

High frequencies of IGS-hybrids are observed during the whole year, ranging from 21% up to 33% in the coastal site and from 4% to 12.5% in the inland one. These data - coupled with other IGS-hybrid reports in larval samples [(from 0.6% to 10.9% since 2012; (Marsden et al., 2011; Lee et al., 2013; Nwakanma et al., 2013; Caputo et al., 2014; Tene Fossog et al., 2015)]- suggest that reproductive isolation between *An. coluzzii* and *An. gambiae* is not complete in Côte D'Ivoire, neither in the forested southern region, nor in the forest-savanna mosaic belt. A similar pattern has been reported so far only at the western extreme of the two species range (i.e. in the "far- west", Caputo et al., 2011) where, consistently with present data, hybridization was higher in the coast than inland.

Previous and present reports of relatively high frequencies of both species and hybrids in Côte D'Ivoire are fully consistent with the predictions of high frequencies of the two species in the forested region between south-east Côte D'Ivoire and south-west Ghana (Tene Fossog et al., 2015), as well as with expectations to find high prevalence of hybrids where their degree of inter-specific contact is maximal (Pombi et al., 2017). Present data add a temporal perspective to the spatial scenario. In fact, the frequency of IGS-hybrids in the coastal site is significantly higher during the rainy season when *An. coluzzii* and *An. gambiae* frequencies are more balanced (Fig. 2). Lower absolute abundance of IGS-hybrids did not allow highlighting the same trend in the inland village.

Temporary break-downs of reproductive barriers leading to introgressive hybridization of adaptive insecticide resistant *kdr* alleles has been shown to have occurred multiple times out of the far-west region. This did not produce any discernable long term impact on the species' genomes, other than in regions associated to genes involved in insecticide resistance, e.g. the VGSC gene region on chromosome-2 and a dramatic increase in the resistant *kdr* allele frequency in most west African *An. coluzzii* populations (Lee et al., 2013; Clarkson et al., 2014). In Côte D'Ivoire, introgressive hybridization of the 1014F allele from *An. gambiae* to *An. coluzzii* likely predates 2004 (Edi et al., 2014) and the allele is today reported at very high frequencies in both species in the whole country (PMI 2018-2019, 2019; PMI, 2020), as well as in both species in both study sites (≥68%). This suggests that the observed pattern of genomic admixture is not associated to recent adaptive introgression of 1014F allele.

The relatively large IGS-hybrid sample size and the balanced frequency of *An. coluzzii* and *An. gambiae* in the coastal village allowed a preliminary characterization of parameters relevant for malaria transmission and control in the three groups. No significant differences between IGS-hybrids and the two species are observed with regard to Human Biting Rates (0.8–1.3 mosquitoes/person/nigh) and Sporozoite Rates (6–9%). The only exception refers to the significantly higher Sporozoite Rate observed in IGS-hybrids from the inland site (16%), but this should be taken with caution, due to the limited sample size ($N = 26$). Overall, Sporozoite Rates are in the range of those reported by Adja et al., 2011; Zogo et al., 2019; Zoh et al., 2020, while Human Biting Rates and Entomological Inoculation rates are lower in our study compared to assessments based on Human Landing Catches (HLC), possibly due to lower efficacy of trap collections compare to HLC [e.g. (Adja et al., 2021)]. In addition to these epidemiologically relevant parameters, we investigated the three groups' indoor/outdoor biting preferences. *Anopheles coluzzii* and *An. gambiae* exhibit a strong endophagy only under the inland eco-climatic conditions, while in the coastal village only IGS-hybrids are collected significantly more indoors.

It is important to remind that in the far-west region heterozygous patterns of IGS-diagnostic markers were shown not to be appropriate proxies of F1 hybrids, as commonly assumed for the rest of the species range (Santolamazza et al., 2011) . Previous studies on far-west male populations showed that a heterozygous IGS pattern may be the product of recombination and gene conversion within the multicopy ribosomal region (Caputo et al., 2016) . To investigate whether this is also the case

in Côte D'Ivoire, we used additional species-specific PCR-based assays for further characterizing the collected samples. First, we genotyped the SINE-X insertion (in proximity of the IGS ribosomal region on chromosome-X centromere) which is *An. coluzzii*-specific in most of the two species range (Santolamazza et al., 2008). Results show presence of specimens heterozygous for SINE-X insertion only in the coastal village. Most of these specimens (69%) are characterized by an IGS-heterozygous pattern and may represent putative F1-hybrids (Caputo et al., 2016; Santolamazza et al., 2008) . Second, we genotyped two Ancestry Informative Markers on either arms of chromosome-3 (3R and 3 L), which were shown to be capable to ascertain admixture similarly to a multilocus approach (Caputo et al., 2021). Results show low frequency of autosomal admixture in *An. coluzzii* IGS-identified specimens in both villages (15%), and a higher one in *An. gambiae* (46%). Highest admixture is observed in coastal *An. gambiae* (74%), where "pure" *An. gambiae* individuals (i.e. 9 specimens with concordant IGS and chromosome 3 markers) are largely outnumbered by individuals apparently characterized by an *An. gambiae*-like chromosome-X genotype and an admixed chromosome-3 one ($N = 26$). This pattern suggests asymmetric autosomal introgression from *An. coluzzii* to *An. gambiae*, as already hypothesized in the far-west region (Marsden et al., 2011). However, while in The Gambia and Guinea Bissau most putative F1-hybrids are characterized by polymorphic chromosome-3 loci (Caputo et al., 2021), in the coastal village individuals characterized by IGS/SINE-X heterozygous patterns are apparently characterized by *An. coluzzii*-like autosomes.

5. Conclusions

The present results based on longitudinal field sampling in two sites in east Côte D'Ivoire fits with previous observations and lead to identify this area as a putative area of high hybridization between the two main malaria vectors in the country, *An. coluzzii* and *An. gambiae*. Hybridization appears to be higher in the coastal evergreen forested region, but is substantial also in the Guinea-savannah belt, at least up to 250 km inland. Interestingly, south-east Côte D'Ivoire and south-west Ghana were predicted to be the larger area in the Gulf of Guinea where *An. coluzzii* maintains high relative frequencies with respect to *An. gambiae* (Tene Fossog et al., 2015). Overall, the scenario very well fits the hypothesis that extrinsic post-mating selection against hybrids is lower and hybrid fitness is higher under environmental conditions where/when neither of the parental taxa predominates, as predicted by the ecological speciation theory (Kirkpatrick, 2001; Seehausen, 2004; Stankowski, 2013).

Further studies are needed to characterize the biological basis of the break-down of interspecific reproductive barriers (both premating, e.g. swarm segregation and mate recognition, and post-mating, e.g. larval selection) in the region and the eco-geographic distribution, seasonality, ecology and behavior of hybrid individuals. These studies, however, are hampered by the difficulty in defining the taxonomic units to be studied. Here we analyzed epidemiologically relevant phenotypic characters (i.e. seasonality, human biting rate, sporozoite rate, biting behavior) based on IGS-PCR diagnostics. However, preliminary genomic characterization of the target populations provides evidence of a complex pattern of admixture on both chromosome-X and − 3, which deserves deeper investigations.

The existence of a high hybridization area in Côte D'Ivoire does not only have a great interest for those interested in the evolution of the *An. gambiae* complex and, more in general, on ecological evolution, but may also have implications in malaria epidemiology and control. Preliminary phenotypic characterization of IGS-hybrids highlights sporozoite rates comparable to (or possibly higher than) those of *An. coluzzii* and *An. gambiae*. Data are clearly insufficient to assess their vectorial capacity, but suggest that that longevity is not reduced in adult hybrids, further supporting their high fitness. Finally, high levels of gene-flow between the two species - as preliminary revealed by the signatures of admixture in the populations analyzed - are very relevant from the perspective of assessing the outcomes of gene-drive based vector control strategies. Future epidemiological assessment and malaria control plans in Côte D'Ivoire - and in other regions in the Gulf of Guinea where both species are predicted to coexist at relatively high frequencies - will need to take into careful account the results of this (and, hopefully) further deeper studies.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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