Oxamniquine resistance alleles are widespread in Old World *Schistosoma mansoni* and predate drug deployment

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Short title: Standing variation for oxamniquine resistance in schistosomes

ABSTRACT

Do mutations required for adaptation occur *de novo*, or are they segregating within populations as standing genetic variation? This question is key to understanding adaptive change in nature, and has important practical consequences for the evolution of drug resistance. We provide evidence that alleles conferring resistance to oxamniquine (OXA), an antischistosomal drug, are widespread in natural parasite populations under minimal drug pressure and predate OXA deployment. OXA has been used since the 1970s to treat *Schistosoma mansoni* infections in the New World where *S. mansoni* established during the slave trade. Recessive loss-of-function mutations within a parasite sulfotransferase (SmSULT-OR) underlie resistance, and several verified resistance mutations, including a deletion (p.E142del), have been identified in the New World. Here we investigate sequence variation in *SmSULT-OR* in *S. mansoni* from the Old World, where OXA has seen minimal usage. We sequenced exomes of 204 *S. mansoni* parasites from West Africa, East Africa and the Middle East, and scored variants in *SmSULT-OR* and flanking regions. We identified 39 non-synonymous SNPs, 4 deletions, 1 duplication and 1 premature stop codon in the *SmSULT-OR* coding sequence, including one confirmed resistance deletion (p.E142del). We expressed recombinant proteins and used an *in vitro* OXA activation assay to functionally validate the OXA-resistance phenotype for four predicted OXA-resistance mutations. Three aspects of the data are of particular interest: (i) segregating OXA-resistance alleles are widespread in Old World populations (4.29 – 14.91% frequency), despite minimal OXA usage, (ii) two OXA-resistance mutations (p.W120R, p.N171IfsX28) are particularly common (>5%) in East African and Middle-Eastern populations, (iii) the p.E142del allele has identical flanking SNPs in both West Africa and Puerto Rico, suggesting that parasites bearing this allele colonized the New World during the slave trade and therefore predate OXA deployment. We conclude that standing variation for OXA resistance is widespread in *S. mansoni*.

AUTHOR SUMMARY

It has been argued that drug resistance is unlikely to spread rapidly in helminth parasites infecting humans. This is based, at least in part, on the premise that resistance mutations are rare or absent within populations prior to treatment, and take a long time to reach appreciable frequencies because helminth parasite generation time is long. This argument is critically dependent on the starting frequency of resistance alleles – if high levels of "standing variation" for resistance are present prior to deployment of treatment, resistance may spread rapidly. We examined frequencies of oxamniquine resistance alleles present in *Schistosoma mansoni* from Africa and the Middle East where oxamniquine has seen minimal use. We found that oxamniquine resistance alleles are widespread in the Old World, ranging from 4.29% in the Middle East to 14.91% in East African parasite populations. Furthermore, we show that resistance alleles from West African and the Caribbean schistosomes share a common origin, suggesting that these alleles travelled to the New World with *S. mansoni* during the transatlantic slave trade. Together, these results demonstrate extensive standing variation for oxamniquine resistance. Our results have important implications for both drug treatment policies and drug development efforts, and demonstrate the power of molecular surveillance approaches for guiding helminth control.

INTRODUCTION

The rate at which drug resistance alleles (or any other beneficial alleles) spread within populations in response to a drug treatment (or any other selection pressure) is critically dependent on the starting frequency of resistance alleles in the population when new drugs are deployed (1,2). If no such alleles are present when a novel drug is introduced, then there is a waiting time for resistance alleles to arise. Furthermore, because the starting allele frequency will be $1/(2N_e)$, where N_e is the effective population size, the vast majority of resistance alleles that arise will be lost due to genetic drift and fail to establish (2,3) (Fig. 1). The barrier to establishment is particularly severe for recessive traits because these will be present in heterozygotes at low frequency and therefore not exposed to selection. This effect, otherwise known as "Haldanes's sieve" (4), may be particularly relevant for drug resistance evolution in diploid pathogens, because resistance mutations in drug targets typically result in phenotypic resistance only when two copies are present. However, if resistance alleles are already segregating as standing variation in pathogen populations when drug treatment is initiated, then there is no waiting time for mutations to arise. Furthermore, there is a high probability of fixation and spread is rapid because many resistance alleles are present in homozygous recessive genotypes and therefore exposed to selection. Hence, whether resistance alleles (or alleles for other traits) arise *de novo* or are segregating as standing variation, is a central question in evolutionary biology and public health, and key to predicting the effective "shelf life" of new drug treatments (3,5).

There is growing evidence for resistance to anti-helminthics in several helminths infecting humans, including *Onchocerca volvulus* (6), the filarial nematode causing river blindness, and in soil transmitted helminths (hookworm, whipworm and *Ascaris* roundworms) (7) which cumulatively infect over one billion people worldwide (8). However, perhaps the best understood is oxamniquine resistance in schistosome blood flukes, for which the mechanism of drug action and the genetic and molecular basis for drug resistance are now known. Oxamniquine (OXA) kills *Schistosoma mansoni*, but is ineffective against two other major schistosome species infecting humans (*S. haematobium* and *S. japonicum*) (9). OXA is a pro-drug that is activated by a schistosome sulfotransferase (SmSULT-OR) encoded on chromosome

6 (10). Loss-of-function mutations within SmSULT-OR result in resistance: only parasites that are homozygous for resistance alleles are phenotypically resistant (OXA-R). We initially identified the locus and mutation, a single amino acid deletion (p.E142del), underlying OXA-R using a genetic cross involving a resistant parasite line selected from a parasite isolate from a Puerto Rican patient in 1971 (11). Additionally we identified a second mutation (p.C35R) in *S. mansoni* obtained from an incurable Brazilian patient (12). These two mutations result in disruption of the active site of the SmSULT-OR and inability to activate OXA (10). Both p.E142del and p.C35R, and two additional mutations resulting in truncated proteins, were subsequently identified in a field survey of SmSULT-OR genetic variation of parasites from a single Brazilian village (13). The combined allele frequency of these four resistant alleles was 1.85%.

OXA was widely used in the New World, where only *S. mansoni* is present, during the 1970s to early 2000's (14), so the OXA-R alleles we observed in Brazil (13) could conceivably have resulted from drug selection. In contrast, OXA saw minimal usage in Africa where both *S. haematobium* and *S. mansoni* are present. In South America, an estimated 6 million doses of OXA were used to treat the 7 million people infected with *S. mansoni* prior to 1987 (15). In contrast, 3 million doses had been used in Africa (Egypt, Ethiopia, Ivory Coast, Kenya, Malagasy, Malawi, Rwanda-Burundi, South Africa, Sudan, Tanzania, Uganda, Zambia, Zaire) and the Middle East (Arabian penensula) (15), where an estimated ~60 million people were infected with *S. mansoni* (16). Hence, OXA selection was minimal in Africa compared with South America. The central aim of this paper is to determine whether *de novo* mutation or standing variation best explains OXA-R in *S. mansoni* populations. To do this we examined *SmSULT-OR* genetic variation in Old World parasite populations where OXA treatment has been minimal.

RESULTS

1. Samples

We sequenced exomes from 92 miracidia from Tanzania (n=57), Niger (n=10), and Senegal (n=25) from the SCAN collection at the Natural History Museum (17), with each miracidium derived from a different patient. In addition, we included 112 samples collected from Oman: these included 86 single worms and 26 pools of cercariae from single infected snails. The Omani samples were derived from 11 human infections, 2 naturally infected rodents, and 26 naturally infected snails. We compared these Old World parasite samples with: (i) the OXA-R parasite from Puerto Rico (HR) carrying the causative p.E142del mutation identified previously (10), for which whole genome sequence is available, and (ii) published *SmSULT-OR* sequences from a single Brazilian location (n=189) (13).

2. New and known mutations identified in the *SmSULT-OR* gene

We identified a total of 85 *SmSULT-OR* mutations across all three populations (West Africa, East Africa and Middle East), including 76 coding mutations and 9 non-coding mutations (Supp. table 1). Exon 1 carried 24 mutations. These included 13 non-synonymous single nucleotide polymorphisms (SNPs), 8 synonymous SNPs, one duplication, one deletion, and one premature stop codon. Exon 2 carried 52 mutations: 26 non-synonymous SNPs, 23 synonymous and three deletions. There were no differences in the density of mutations in the two exons (Fisher's exact test, d.f.=1, p=0.09). The overall transition/transversion ratio was 1.94. Forty-three percent (37/85 mutations) showed an average allele frequency greater than 5%.

Among the 85 mutations, we have previously found three coding and one non-coding mutations in the New World (Caribbean and Brazil): the substitution p.P67L (g.200C>T), the deletion p.E142del (g.4348_4350delGAA), the substitution p.L256W (g.4691T>G) and the 3' untranslated region (UTR) substitution g.4720C>T (10,13). Just one of these mutations (p.E142del) encodes a confirmed OXA-R allele. Frequencies of all mutations in each population are shown in the supp. table 1.

We measured nucleotide diversity (π) in both New and Old World populations. Nucleotide diversity was the highest in Oman ($\pi = 0.02424 \pm 2.4 \times 10^{-4}$) followed by East Africa ($\pi = 0.00195 \pm 2 \times 10^{-4}$), West Africa ($\pi = 0.00126 \pm 1.7 \times 10^{-4}$) and Brazil ($\pi = 0.00021 \pm 0.3 \times 10^{-4}$). If drug pressure drives the changes at *SmSULT-OR*, we might expect to see signals of positive selection. We examined sequence conservation by measuring proportions of non-synonymous difference per non-synonymous site relative to synonymous changes per synonymous site (K_a/K_s) between *S. mansoni* and a *S. rodhaini* outgroup. K_a/K_s ranged from 0.20 – 0.24 suggesting that changes at non-synonymous sites are 4-5 times less abundant than at synonymous sites, consistent with weak purifying selection. Similarly, the ratio of non-synonymous polymorphisms per non-synonymous site relative to synonymous polymorphisms per synonymous site (pN/pS) ranged from 0.111 to 1.36 and none of these values were significantly greater than unity (Table 1). These tests did not provide evidence that *SmSULT-OR* is under positive selection.

3. *In vitro* tests and *in silico* evaluation of impact of mutations

We used three approaches to identify *SmSULT-OR* mutations that are likely to result in OXA-R.

a. Visual assessment

Some mutations have obvious deleterious effect on the protein by introducing premature stop codons. This is the case for three mutations identified in *SmSULT-OR*. These mutations will generate truncated proteins that do not contain the active site. Other mutations, such as p.L179P, p.P225S or p.W120R, were either close enough to the OXA or the 3'-phosphoadenosine-5'-phosphosulfate (PAPS) binding sites or showed potential steric hindrance or structural deformation likely to have an effect on protein activity. Other mutations, such as p.A74T, p.P106S or p.Q176R, were on the surface of the protein, distant to any active site, and therefore unlikely to have an impact.

b. Functional assay of OXA binding

We used an *in vitro* OXA activation assay to functionally assay the impact of mutations on SmSULT-OR activity for four newly discovered mutations and the two known OXA-R mutations

(Fig. 3). The known resistant mutations, p.C35R and p.E142del, showed from zero to very low level of OXA activation, as expected (10). p.L179P showed a similarly low level of activation. The mutations p.S160L and p.P225S showed intermediate OXA activation: we conservatively classed these as OXA sensitive alleles. p.P106S showed similar activity to the wild type and p.S160L. We were able to produce recombinant protein p.W120R but all our attempts to fold this protein were unsuccessful. This mutation clearly has a dramatic impact on protein stability as predicted by visual inspection and thermodynamic modelling results (below). We considered this mutation to be OXA-R.

c. Thermodynamic modelling

We used thermodynamic modelling to evaluate the potential impact of substitutions on protein stability (indels such as p.E142del cannot be modelled). The difference in free enthalpy ($\Delta\Delta G$) ranged from -2.942 to 19.826 for the 37 mutations (Fig. 4). Among those, p.L179P and p.C35R which have the greatest impact on OXA activation (Fig. 3) showed the second and third highest $\Delta\Delta G$, respectively. p.W120R, for which we were unable to fold recombinant protein, showed the highest $\Delta\Delta G$, consistent with a dramatic impact on stability. p.S160L and p.P225S showed intermediate $\Delta\Delta G$ consistent with the results of the OXA activation assay.

In total, we identified 7 independent OXA-R mutations in Old and New World parasite populations examined using these three approaches (Table 2, Fig. 2). These included 3 indels and 4 amino acid mutations.

4. Frequency of OXA-R alleles

Having determined which of the mutations identified are likely to cause OXA-R, we measured the frequency of resistant variants and the frequency of resistant parasites in each population (Table 2, Fig. 5). To accurately identify resistant alleles and parasites, we phased our variant calling data on the first 3 Mb of the chromosome 6 (corresponding to 30,812 variable sites). West African samples carried only two resistant variants (p.S12X and p.E142del). East African samples carried five resistant variants, two of them at a frequency over 0.05 (p.W120R and

p.N171IfsX28). The p.W120R mutation was also found at high frequency in Middle East (0.065). We found the highest frequency of OXA-R alleles in East Africa (14.91%, 17/114), followed by the Middle East (6.25%, 14/224), West Africa (4.29%, 3/70), and Brazil (1.85%, 7/378).

Most parasites carried OXA-R alleles in the heterozygous state and are predicted to be OXA sensitive. Heterozygous OXA-sensitive parasites were found in West and East Africa (8.6% and 24.6%, respectively), Middle East (13.4%) and in Brazil (1.06%). Parasites that are homozygous for OXA-R alleles at *SmSULT-OR* (but not necessarily carrying the same resistant variant) are OXA-R. We found homozygous parasites predicted to be phenotypically OXA-R at a frequency of 5.26% (3/57) in East Africa and 1.06% (2/189) in South America only (Fig. 5).

5. Haplotype analysis of *SmSULT-OR* and flanking regions

We used our phased data to investigate the haplotypes surrounding our resistant variant (p.E142del) in the Old and New World (Caribbean) (Table 1) and to investigate whether these resistant alleles derived from a common ancestor. This is of interest because it would suggest that these alleles predate the slave trade. We identified an identical 102.5 kb haplotype block around p.E142del shared between the Caribbean sample and one of the West African samples from Niger (Fig. 6A). This haplotype block contained 399 SNPs that varied in at least one of the 205 parasites examined (all samples except those from Brazil where haplotype data were not available). We used a minimum spanning network to investigate haplotype relationships using the 399 bi-allelic variants found in this 102.5 kb block across all phased haplotypes (Fig. 6B). The haplotypes associated with the p.E142del OXA-R alleles found in both Puerto Rico and Niger clustered together, consistent with a common origin for West African and Caribbean p.E142del OXA-R mutations.

The p.E142del mutation was also identified in Brazil (13) but we sequenced the *SmSULT-OR* exons only, rather than complete exomes, for these Brazilian samples preventing reconstruction of extended haplotypes. This Brazilian p.E142del was also found associated with two mutations (p.P67L and g.4720C>T) found in West Africa but without the p.L256W found in the Caribbean and Nigerien samples. Similarly, the p.E142del was also found in a Tanzanian

sample but this sample has a distinct haplotype from those found in West Africa (Fig. 6). These results suggest either that there are several independent origins of East and West African p.E142del mutations, or alternatively, that the p.E142del is extremely old and has distinctive flanking haplotypes resulting from recombination and mutation.

DISCUSSION

1. Evidence for standing genetic variation for OXA resistance

Two lines of evidence support the view that standing variation is the source of OXA-R alleles in schistosomes. First, we have indirect evidence: OXA-R alleles are geographically widespread in Africa and the Middle East despite limited treatment with OXA (or hycanthone, its structural analog; (18)) in these regions. Furthermore, that OXA-R alleles are found at high frequency in East Africa, where there has been minimal use of OXA, while OXA-R alleles are found at low frequency in Brazil, where OXA has been used extensively, is also consistent with the idea that mutation and drift, rather than selection, explain the patterns of variation observed. The second line of evidence is more direct: we showed that an OXA-R mutation, p.E142del, with an identical 102.5 kb flanking haplotype, is sampled from both West African and Caribbean schistosomes. This result strongly suggests that p.E142del alleles were present in West Africa prior to the transatlantic slave trade (1501-1867) (19), and were transferred to the New World on ships carrying West African slaves. Hence, OXA-R alleles were segregating within *S. mansoni* populations at least 470 years before deployment of OXA in the 1970s (20).

Our molecular data are backed up by clinical observations from the early use of OXA. Resistant parasites were detected in Brazil in the 1970s (21,22), before any mass drug administration (14). Similar observations of *S. mansoni* infected patients resistant to OXA treatment were made in East Africa (23). The existence of OXA-R parasites was confirmed experimentally by infecting mice with parasites isolated from patients that were parasite positive following drug treatment (21–23). Together these observations support the existence of segregating OXA-R alleles in schistosome populations before OXA deployment for parasite control.

2. Differences in OXA treatment efficacy between East and West Africa

Given the variation in OXA-R frequencies that we observe in different geographical samples, it is interesting to examine the literature on clinical treatment efficacy of OXA for treating

schistosomiasis patients. Early trials of OXA to treat patients with intestinal schistosomiasis resulted in multiple treatment failures in Egypt, East Africa and South Africa (23–27). As a consequence, the WHO recommended use of higher doses of OXA in East Africa, compared with West Africa (28). Human host metabolism did not explain this lack of efficacy because the OXA availability in blood was the same or higher in the East African patient populations than in West African or South American populations (29,30). Therefore, the presence of resistant parasites was the most likely explanation. OXA-R parasites were identified in Kenya by treating mice infected with parasites from patients showing poor treatment response (23). We observed a 14.91% frequency of OXA-R alleles (and 5.26% frequency of homozygous OXA-R parasites) in East Africa compared with 4.29% frequency of OXA-R alleles (and no homozygous OXA-R parasites) in West Africa. Our results provide a molecular explanation for the poor treatment response observed in East Africa, relative to other locations.

3. Implications for development of OXA derivatives

OXA kills *S. mansoni* but not *S. haematobium* or *S. japonicum*. OXA derivatives are currently under development to obtain more potent molecules acting against all three schistosome species. In fact, OXA derivatives that kill adult worms of the three main species of schistosomes infecting humans *in vitro* have now been developed (31). The low frequency of homozygous OXA-R parasites in *S. mansoni* suggests that OXA derivatives will result in imperfect cure rate for this species, particularly in East Africa. However, the primary purpose of generating OXA derivatives is to treat *S. haematobium* and *S. japonicum*, perhaps as a partner drug for praziquantel (PZQ). Examining natural variation in homologous sulfotransferase genes from these two species (*ShSULT-OR* and *SjSULT-OR*) to better understand the potential for resistance evolution will be critical if OXA derivatives are to be developed to control these parasites.

4. Implications of standing variation for drug resistance evolution

Standing variation in drug resistance genes has a major impact on how fast resistance evolves (2). If resistant alleles are already present in the population prior to treatment, drug resistance has the potential to spread much more rapidly because there is no waiting time for a resistance mutation to appear *de novo* (Fig. 7A) (3). An increasing number of examples support this view. The nematode *Caenorhabditis elegans* carries a large diversity of β-tubulin alleles allowing worms to be resistant to benzimidazoles (32). Similarly, the extremely high level of genetic diversity in the parasitic nematode *Haemonchus contortus* is responsible for the rapid emergence of resistance to benzimidazoles and other drug classes: resistance was usually detected within 10 years of drug introduction, consistent with standing variation or recurrent mutation (33,34). In the filarial worm *Onchocerca volvulus*, standing variation appears to be involved in resistance to ivermectin (6). Standing variation is also involved in herbicide resistance in plants: resistance alleles from the weed *Alopecurus myosuroides* were identified in plant collection almost 100 years before herbicides were used (35). Antibiotic resistance provides particularly dramatic examples of standing variation: plasmids encoding multidrug resistance were found in frozen bacteria isolated from the permafrost demonstrating that antibiotic resistance plasmids were already present thousands of years ago (36,37).

Drug resistance in parasitic helminths infecting livestock or humans is now recognized as a potential concern for control efforts, but was neglected for a long time using the argument that the slow reproduction rate of worms would greatly delay resistance evolution (38). The observation that resistance alleles exist as standing variation within multiple pathogen or parasite populations in the absence of drug treatment suggests previous optimism about the shelf life of drugs was poorly founded. If resistance alleles are already at relatively high frequency in populations when new drugs are deployed, resistance may evolve extremely rapidly (3,5).

In the case of OXA resistance, using an N_e estimate of 65,000 (39), OXA-R alleles spread from 15% starting frequency to fixation in 170 generations in 100% of simulations when selection is strong (s=0.1). Assuming a generation time between 3 months and 1 year, this is equivalent to a period of 42.5 to 170 years (Fig. 7A). However, if OXA-R alleles were evolving *de novo*, simulations indicate that fixation will occur rarely (0.04% of the simulations) and this

would take 1,197 generations (corresponding to a period of 299.25 to 1,197 years) from the time a mutation arises. We can use the observed frequency of OXA-R alleles segregating in East African populations to make predictions about the spread of phenotypically resistant parasites (i.e. those homozygous for OXA-R alleles) (Fig 7B). If we use a frequency of 10% resistant parasites to denote unacceptable efficacy (this threshold is used for antimalarial drugs (40)) then this threshold will be reached in 19 to 444 generations (4.75 to 444 years) using a range of selection coefficients (from 0.01 to 0.2). The selection coefficients driving spread of drug resistance alleles in schistosomes is not known, but the range we have used covers measured selection coefficients driving drug resistance spread in another human parasite (*Plasmodium falciparum*) (41,42). These simulations suggest that, while OXA-R allele frequencies are relatively high in East Africa, OXA derivatives could still be extremely effective for schistosome control in the short-medium term, particularly if deployed as a combination therapy, partnered with a second drug with a different mode of action (e.g. PZQ).

Our simple models for predicting spread of OXA-R alleles under drug selection ignore fitness costs of OXA-R alleles, which might limit the rate of spread. The fitness cost of inactive SmSULT-OR in the absence of drug treatment still needs to be assessed accurately (13) but the occurrence of natural homozygotes for defective alleles suggests limited costs. The exact role of this enzyme in schistosome biology is not known. Sulfotransferases transfer the sulfo group from a donor (usually PAPS) to the substrate, resulting in inactivating or solubilizing of the substrate (43). Substrates can be endogenous signal molecules (hormones, neurotransmitters) or exogenous chemicals. In the latter case, sulfotransferases play a role in detoxifying natural or synthetic toxins (43), but sometimes they do the opposite by activating pro-drugs as observed with the anti-tumor drug N-benzyl indolecarbinols (44) or OXA (10,45). Because the same enzyme can sulfate both endogenous and exogenous compounds, it is often difficult to determine their exact biological role. In schistosomes, we speculate that SmSULT-OR could have roles in regulating endogenous molecules as well as detoxifying chemicals present in their environment (*i.e.* in the feces, water, snail or human blood, etc.).

5. Molecular markers for resistance surveillance

Molecular markers of OXA-R now allow efficient monitoring of the distribution of OXA-R alleles in schistosome populations. This approach is widely used for other parasites and pathogens such as malaria parasite (46) and HIV (47). For schistosome, collections such as SCAN which contain thousands of parasite samples from multiple locations (17) will be extremely valuable for this work: regular sampling in different endemic regions will help to update the resistance landscape. However, not all identified *SmSULT-OR* mutations will lead to resistance, so it is critical that molecular screening is paired with functional evaluation or computational prediction, as we have done here.

PZQ is the only drug currently available to treat schistosomiasis and effort in mass drug administration has recently been expanded 10 fold (48), with a target of administering 250 million treatments per year, increasing selective pressure for PZQ resistance. We suspect that standing variation for PZQ resistance is also likely, because this trait is easy to select in the laboratory. This has been done by several groups using independent laboratory populations, and resistance has spread within very few generations (49–54), strongly suggesting that resistance alleles were already present in these laboratory populations. Furthermore, the laboratory populations used for selection were isolated before PZQ was available (23,55) and so were not exposed to drug selection. Molecular markers for monitoring the frequency of PZQ-R in natural parasite populations would be extremely valuable, as demonstrated by our work on OXA-R.

MATERIALS AND METHODS

1. Ethics statement

a. African samples

Samples from Senegal and Niger were collected as part of the EU-CONTRAST project (56), a multidisciplinary alliance to optimize schistosomiasis control and transmission surveillance in sub-Saharan Africa, as detailed in Webster *et al.* (57) with ethical approval granted by ethical committees of the Ministry of Health (Dakar, Senegal), and the Niger National Ethical Committee (Niamey, Niger) with additional ethical approval obtained from the St Mary's Hospital Local Ethics Research Committee, R&D office (part of the Imperial College Research Ethics Committee (ICREC; EC no. 03.36. R&D no. 03/SB/033E)), in combination with the ongoing CONTRAST and Schistosomiasis Control Initiative (SCI) activities.

For the Tanzanian samples collected as part of the Schistosomiasis Consortium for Operational Research and Evaluation (SCORE), ethical approvals were granted by the Imperial College Research Ethics Committee and the SCI Ethical approval (EC no. 03.36. R&D no. 03/SB/033E); the National Institute for Medical Research (NIMR, reference no. NIMR/HQ/R.8a/Vol. IX/1022); University of Georgia Institutional Review Boards, Athens, GA (2011-10353-1).

Following routine procedures in the field, the objectives of the study were first explained to the local village chiefs and political and religious authorities who gave their consent to conduct the study. Written consent for the schoolchildren to participate in longitudinal monitoring of the national control programme for schistosomiasis was given by head teachers, and/or village chiefs where there were no schools, due to the fact that in African villages, written consent of the child's guardian is often very difficult to obtain owing to the associated impoverished conditions and often low literacy. Each individual child also gave verbal consent before recruitment. Following sampling, a praziquantel treatment (40 $mg.kg^{-1}$) was offered to infected participants.

b. Omani samples

We obtained ethical clearance from the Sultan Qaboos University and the Ministry of Health of Oman to use positive stool samples for schistosomiasis collected by the Ministry of Health of Oman during epidemiological screening. Ethical approval was given by the Medical Research and Ethical Committee (MREC) of the Non-Communicable Disease Control Section of the Directorate General of Health Affairs, Headquarters, Ministry of Health, Oman (no. MH/DGHA/DSDC/NCD/R&S/167/01).

We obtained ethical approvals of animal studies in France from the French Ministère de l'Éducation Nationale, de la Recherche et de la Technologie, from the French Ministère de l'Agriculture et de la Pêche (agreement no. A 66040), and from the French Direction Départementale de la Protection des Populations (no. C 66-136-01 with prefectoral order no. 2012-201-0008). Certificate for animal experimentation was given to H.M. (authorization no. C 66.11.01; articles no. R 214-87, R 214-122 and R 215-10). Housing, breeding and animal care followed the guidelines of the French CNRS. The different protocols used in this study had been approved by the French veterinary agency from the DRAAF Languedoc-Roussillon (Direction Régionale de l'Alimentation, de l'Agriculture et de la Forêt), Montpellier, France (authorization no. 007083).

2. Sampling

a. African samples

S. mansoni miracidia were collected from individual patients in three different West African countries in 2007: 27 patients from two locations in Senegal, and 17 patients from two locations in Niger (Supp. table 2) (57,58). Additionally, *S. mansoni* miracidia were collected as part of the SCORE program from 64 children in seven villages on the shores of Lake Victoria in Tanzania (East Africa) in January 2012 (Supp. table 2) (59).

Collection of miracidia was performed as previously described (57,60). Briefly, individual stool samples from positive patients were homogenized through a mesh and washed through with water. The content was then transferred in a Pitchford funnel assembly, washed with

425 additional water, and the filtered homogenate was drained into a Petri dish. The Petri dish
426 containing the homogenate was then left in bright ambient light (not direct sunlight) to allow
427 hatching of miracidia. Miracidia were visualized under a dissecting microscope and individually
428 captured in 3-5 µL of water using a micropipette. Miracidia were then pipetted individually onto
429 Whatman FTA cards for DNA preservation. Cards were allowed to dry for 1 hour and then
430 stored for future research (57,58,60).

431 b. Omani samples

432 The Omani schistosome samples were collected during 8 field trips, from 2001 to 2015, in
433 different areas in Dhofar, Oman and originated from either *Homo sapiens*, *Rattus rattus* or
434 *Biomphalaria pfeifferi* (Supp. table 2): 11 patients from 4 localities, 2 rats from 2 localities, 26
435 snails from 3 localities.

436 We obtained schistosome adult worms from naturally infected rats and from laboratory
437 infected mice. The mice were infected with cercariae from naturally infected snails or from
438 laboratory snails infected with miracidia isolated from stool samples. We collected miracidia
439 from stool samples as described in Moné *et al.* (61). We exposed *Biomphalaria pfeifferi* snails to
440 miracidia as described in Mouahid *et al.* (62). We maintained laboratory and naturally infected
441 snails at constant temperature (26°C) and balanced photoperiod (12h light / 12h dark) and fed
442 them with fresh lettuce *ad libitum*. Prior to infection, we anaesthetized the mice by
443 intraperitoneal injection of an anesthetic solution (0.01 $mL.g^{-1}$ of mouse body weight). We
444 prepared the anaesthetic solution using 0.5 mL of Rompun (20 $mg.mL^{-1}$; Bayer) and 1 mL of
445 Imalgène (100 $mg.mL^{-1}$; Rhône Mérieux) diluted in 8.5 mL of autoclaved NaCl 8.5 ‰. Abdomens
446 of anesthetized mice were shaved and exposed during 1 hour to cercariae shed from laboratory
447 or naturally infected snails. Cercariae from naturally infected snails were kept in 95° ethanol
448 when the number of cercariae was too low for mouse infection. Laboratory infected mice or
449 naturally infected rats were euthanized by intraperitoneal injection of sodium pentobarbital
450 solution (1.1 mL diluted in 10 mL of ethanol 10%). We recovered adult worms by perfusion (63);
451 the worms were washed in NaCl 8.5 ‰, and single worms (female or male) were fixed in 95°

ethanol and preserved at -20°C. Worms and cercariae were washed in 1X TE buffer for 1 hour prior to DNA extraction.

3. DNA processing and library preparation

We amplified DNA and sequenced exomes from single miracidia preserved on FTA cards (1 miracidium per patient) for African samples or from DNA extracted from worms or cercariae for Omani samples. FTA-preserved samples were processed following our published protocol (64). Briefly we punched a 2 mm disc containing the miracidium from the FTA card, washed it with the FTA Purification Reagent (GE Healthcare Life Sciences), rinsed it twice with TE^{-1} buffer, and finally dried it. DNA from single worms and cercariae were extracted using the DNeasy Blood and Tissue kit (Qiagen) following the tissue protocol with an incubation time of 2h at 56°C and an elution in 200 µL. Samples were quantified using the Qubit dsDNA HS assay kit (Invitrogen).

We then performed whole genome amplification (WGA) on each FTA punch or on 2 µL or 4 µL of DNA solution using the Illustra GenomiPhi V2 DNA Amplification kit (GE Healthcare Life Sciences). Amplified DNA was purified with the SigmaSpin™ Sequencing reaction Clean-up (Sigma-Aldrich), following the manufacturer protocol. We quantified purified samples using the Qubit dsDNA BR assay (Invitrogen). DNA samples that failed WGA were cleaned up and concentrated using the Genomic DNA Clean & Concentrator kit (Zymo Research) following the manufacturer protocol and these samples with concentrated DNA were used to perform a new WGA.

Because WGA reactions are non-specific, we performed qPCR on each sample from FTA cards to quantify proportion of schistosome DNA (i.e., check for an excess of amplified DNA from the environment (water, fecal matter, etc.)). We excluded samples that showed less than 100 schistosome genomes copies in 20 ng of DNA which were not suitable for subsequent exome capture. We assessed genome copies by quantifyng the *S. mansoni α-tubulin* single copy gene (64).

We prepared exome capture libraries on the selected African samples and on all the successfully amplified Omani samples using the pre-capture pooling method of the SureSelect XT^2 Target Enrichment System (Agilent) (64). Finally, we sequenced libraries on a HiSeq 2500 and data were demultiplexed using the Casava pipeline.

4. Alignment, variant calling and phasing

We aligned sequences as described in Le Clec'h *et al.* (64). Briefly, we aligned data against the v5 *S. mansoni* genome using BWA and SAMtools, realigned around indels using GATK, PCR duplicates were marked using picard and Q-score were recalibrated using GATK.

To identify variants in the *SmSULT-OR* (Smp_089320) gene, we performed a variant calling using FreeBayes (v1.1.0-46-g8d2b3a0) (65) in the first 3 Mb of the chromosome 6. We used a minimum base quality (-q) of 20 and a minimum mapping quality (-m) of 30. We included mutation sites identified previously (10,13) in order to genotype these sites specifically for further comparison. We defined 4 populations: Caribbean (HR), West Africa (SN and NE), East Africa (TZ) and Middle-East (OM). We excluded variants supported by less than 4 reads using VCFtools (v0.1.14) (66). We then used vcf-subset from VCFtools to remove sites with reference alleles only. We adjusted variant positions using vt normalize (v0.5772-60f436c3) (67). We finally used Beagle (v4.1) (68) to first estimate genotypes and to then phase data. Unphased variant calling data and scripts used to generate analyze the data are available on Zenodo (https://doi.org/10.5281/zenodo.2850876).

5. Genetics analysis

We performed all the genetics analysis using phased exome data. We handled the VCF data in R (R v3.5.0 (69)) using the vcfR package (v1.8.0.9) (70). We determined the longest haplotype block from the samples carrying the p.E142del using a custom R code after filtering invariable sites in these samples. We built the haplotype network using the R package poppr (v2.8.1) (71,72) after selecting variants within the longest haplotype coordinates showing no more than

505 20% of missing data. We computed the frequency of the OXA-R allele using a custom R script
506 and functional annotation obtained through a custom bash script.

507 We generated haplotype sequences of the coding sequence only for the Old World samples
508 using the updated phased VCF data exported from R and the BCFtools (v1.2) (73). We
509 generated haplotypes of the South American samples from previous genotyping using a custom
510 bash script. We included the *SmSULT-OR* homologue sequence from *S. rodhaini* previously
511 identified (13). We aligned the haplotypes using Clustal Omega (v1.2.2) (74). We determined
512 nucleotide diversity, number of synonymous and non-synonymous sites using DnaSP software
513 (v6.12.01) (75). Populations were defined as described in the previous section. Scripts used to
514 analyze the data are available on Zenodo.

515

516 **6. Recombinant SmSULT-OR protein production and OXA activation assay**

517 We produced recombinant SmSULT-OR proteins following Chevalier *et al.* (13). Briefly,
518 mutations were introduced in the cloned *SmSULT-OR* gene sequence (Smp_089320; GenBank
519 accession no. HE601629.1) and introduced into *Escherichia coli* for protein production. Proteins
520 were extracted from bacterial culture and purified on an affinity chromatography column. His-
521 tag was removed using Tobacco etch virus (TEV) protease, the solution was dialyzed overnight
522 and passed through affinity chromatography again to remove His-tag and TEV protease. The
523 sample was loaded onto a GE-pre-packed Q anion exchange column and eluted, and the pooled
524 fractions were dialyzed overnight. The protein was finally concentrated at 10 $mg.mL^{-1}$.

525 We tested the impact of a subset of the mutations observed on OXA activation using an *in*
526 *vitro* assay (13). We tested the ability of recombinant SmSULT-OR proteins to activate OXA in a
527 protease inhibitor cocktail with sheared *S. mansoni* gDNA as a final target for the tritiated OXA
528 pre-mixed with ATP, $MgCl_2$ and PAPS co-factor. After 2.5 h of incubation at 37°C, the reaction
529 was stopped and DNA was extracted three times. Radioactivity in the remaining aqueous phase
530 containing the DNA and in water (blank) was counted in a liquid scintillation spectrometer.
531 Blank values were subtracted from sample values. We performed three independent reactions
532 for each recombinant protein.

7. Evaluation of impact of mutations on protein stability

We first visually assessed the scored mutations on the SmSULT-OR protein structure (PDB code 4MUB (10)) using the mutagenesis function of PyMol software (v2.3.0; Schrödinger, LLC).

We then used the Rosetta package (v3.9) to test the impact of scored mutations on protein stability. We used the ddg_monomer application to compute the difference in free enthalpy ($\Delta\Delta G$) between the mutated (amino acid substitutions only) and the wild-type protein. The higher the $\Delta\Delta G$, the more unstable is the mutated protein. For this, we prepared ligand files (OAQ and A3P) by downloading SDF files containing all ligand structures from SmSULT-OR crystals available from the Protein Data Bank website. We modified SDF files to add hydrogens using Openbabel (v2.4.0) (76), and generated params files using molfile_to_params.py from Rosetta. We used the structure of SmSULT-OR to generate a constraint file using the minimize_with_cst application and the convert_to_cst_file.sh script. We removed ligand information from the constraint file. For each mutation, we finally generated a resfile and ran the ddg_monomer application with 50 iterations and options adapted from the protocol 16 of Kellogg *et al.* (77). Scripts used to analyze the data are available on Zenodo.

8. Statistical analysis

We conducted statistical analyzes using R (v3.5.0) (69). We performed simulations using a modified version of the driftR simulator (78). The OXA activation assay data were tested for normality using a Shapiro-Wilk and compared by a parametric ANOVA followed by a tukey post-hoc test.

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TABLES

Table 1 – Summary statistics for sequence variation in *SmSULT-OR*.

Location	Nucleotide diversity ($\pi \pm$ standard deviation)	Dn	Pn	Ds	Ps	N	S	pN/pS	Ka/Ks
South America (Brazil)[a]	$0.00021 \pm 0.3 \times 10^{-4}$	17	4	23	0	596.19	162.81	N/C	0.20184
West Africa (Senegal / Niger)	$0.00126 \pm 1.7 \times 10^{-4}$	18	10	23	2	599.02	162.98	1.36	0.21293
East Africa (Tanzania)	$0.00195 \pm 2 \times 10^{-4}$	18	18	23	9	591.83	161.17	0.545	0.21312
Middle East (Oman)	$0.02424 \pm 2.4 \times 10^{-4}$	10	15	11	36	598.91	160.09	0.111	0.243
Overall	0.01224 ± N/C	10	39	11	40	583.59	157.41	0.263	0.24521

[a] Data from Brazil from a previous study (13) added for comparison.

Dn, Ds: Number of fixed non-synonymous and synonymous differences between species (*S. mansoni* and *S. rodhaini*)

Pn, Ps: Number of polymorphic non-synonymous and synonymous differences within species (*S. mansoni*)

N: Number of non-synonymous sites

S: Number of synonymous sites

pN/pS=(Pn/N)/(Ps/S)

Ka/Ks=(Dn/N)/(Ds/S)

N/C: not calculable

Table 2 – OXA-R mutations scored in the *Schistosoma mansoni SmSULT-OR* gene and their frequency in the New and Old World.

Nucleic acid mutation	Amino acid mutation	Mutation frequency			
		South America (Brazil)[a] n=189	West Africa (Senegal / Niger) n=25 / n=10	East Africa (Tanzania) n=57	Middle East (Oman) n=112
c.3dupGTTTATCCATAATG	p.I2VfsX3	-	-	0.0096	-
c.35C>A	p.S12X	-	0.014	-	-
c.103T>C	p.C35R	0.0027	-	-	-
c.358T>C	p.W120R	-	-	0.0510	0.065
c.424_426delGAA	p.E142del	0.008	0.035	0.0098	-
c.510delT	p.N171IfsX28	-	-	0.0625	-
c.536T>C	p.L179P	-	-	0.0089	-
Total frequency		0.0107	0.044	0.1418	0.065

The code used for nucleic acid mutations indicates the sequence type (c = coding), the position, and the mutation type (X>Y = substitution of X by Y, insN = insertion of N, delN = deletion of N, dup = duplication). The code used for amino acid mutations indicates the sequence type (p = protein), the reference amino acid, the position, and finally the alternative amino acid, and when frame shift (fs) occurs, the position of the stop codon (X) after the mutation. For details about the nomenclature, see Ogino *et al.* (79).

[a] Data from Brazil were obtained in a previous study (13) and added for comparison.

816 **FIGURES**

817 **Fig. 1 – Drug resistance evolution from standing variation or *de novo* mutation**. Drug
818 resistance alleles spread rapidly and reach fixation with a high probability when drug resistance
819 alleles are already present as standing variation. In contrast, if resistance alleles are absent
820 when treatment is initiated, resistance mutation must arise *de novo*, so there is a waiting time
821 before the resistance alleles appear, and most resistance alleles are lost by genetic drift, so the
822 probability of establishment and fixation is low.

823

824 **Fig. 2 – Mapping of the resistance mutations on the gene sequence and structure of**
825 ***Schistosoma mansoni* SmSULT-OR sulfotransferase.** Exon 1 and exon 2 are represented in
826 orange and beige, respectively. Single nucleotide polymorphisms and insertion/deletion events
827 are represented in cyan and magenta, respectively. (A) Linear representation of the *SmSULT-OR*
828 gene showing the relative position of the mutations and their translation in amino acid
829 sequences. (B) Positions of mutations on the SmSULT-OR protein. Oxamniquine is represented
830 in yellow, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) co-factor is represented in green.

831

832 **Fig. 3 – Enzymatic activity of recombinant *Schistosoma mansoni* SmSULT-OR sulfotransferase**
833 **expressed from different allelic variants.** This *in vitro* oxamniquine activation assay quantifies
834 DNA-oxamniquine complexes by scintillation (counts per minute). Bars show the mean of three
835 replicates, while error bars are S.E.M. Enzyme carrying known loss-of-function mutations, such
836 as p.C35R or p.E142del, as well as a newly identified variant (p.L179P) showed no or low
837 oxamniquine activation, while two newly identified variants (p.S160L and p.P225S) showed
838 intermediate activation. The newly identified p.P106S did not impair oxamniquine activation.
839 The different letters indicate significant differences in oxamniquine activation means (Tukey's
840 HSD, $p < 0.05$).

841

842 **Fig. 4 – *In silico* evaluation of mutations on protein stability.** We computed the difference in
843 free enthalpy ($\Delta\Delta G$) between the mutated and the wild type proteins. The higher the $\Delta\Delta G$, the
844 more unstable is the mutated protein. Only single amino acid changes within the resolved
845 crystal structure were examined. For completeness, we included mutations from our current
846 dataset and from previous studies (10,13). Grey bars correspond to known sensitive alleles. Red
847 bars correspond to validated resistant alleles. Grey labels correspond to mutations identified
848 previously from South America (13).

849

850 **Fig. 5 – World map showing proportion of resistance and sensitive alleles in South America**
851 **(Brazil), West Africa (Senegal and Niger), East Africa (Tanzania) and Middle East (Oman).**
852 Number of homozygous (hmz) and heterozygous (htz) parasites for resistant alleles, and total
853 number of parasites sampled (n) are shown below the pie charts. East Africa showed the
854 highest frequency of resistant alleles and resistant parasites. Data from Brazil from a previous
855 study (13) was added for comparison.

Fig. 6 – Common origin of the p.E142del mutation in the Old and New World. (A) Haplotype variation in all the samples bearing p.E142del from Caribbean (HR9), Niger (NE) and Tanzania (TZ) across a 102.5 kb region of chr. 6. Each row represents a chromosome, HR9 was used as reference, blank squares reflect HR9 allele state, and black squares correspond to the alternative allele. Relative bp position to p.E142del (0 bp) is shown on the x-axis. The first and last variants showed on the block correspond to the break of the haplotype block. The Caribbean sample (HR9) and a Nigerien sample (Sm.NE_Di158.1) share an identical haplotype block of 102.5 kb. (B) Minimum spanning network of 410 haplotypes of the 102.5 kb region previously identified. The network was built using 399 bi-allelic variants. Node size is proportional to sample size (smallest node: n=1; biggest node n=50). Nodes with samples carrying p.E142del are circled in blue. Caribbean and West African haplotypes carrying p.E142del clustered together indicating a common origin. The p.E142del from East Africa has different flanking haplotypes.

Fig. 7 – Impact of starting allele frequency on OXA-R allele change. (A) Monte Carlo simulation over 1,500 generations using strong selection (selection coefficient (*s*) = 0.1) on a population size (N) of 65,000 with a starting allele frequency (p(R)) of 0.15 for standing variation or 1/(2N) for new mutation. These simulations underestimate time to fixation for true *de novo* mutation, because we do not account for the waiting time for resistance to appear which is dependant on the rate of drug resistance mutations and N_e (3). The starting frequency of 0.15 corresponds to the frequencies of OXA-resistance alleles observed in Kenya. (B) Change in frequency of resistant parasites (i.e. homozygotes for OXA-R alleles) from standing variation under a range of selection coefficients. The dashed line corresponds to the two thresholds (10% and 20%) at which treatment efficacy would be compromised. The numbers at the dashedlines correspond to the parasite generations needed to cross these thresholds. These predictions are deterministic, because we expect minimal stochastic variation when starting resistance allele frequencies are high.

884 **SUPPLEMENTARY TABLES**

885

886 **Supp. table 1 – Mutations scored in samples from West Africa (Senegal and Niger), East Africa**
887 **(Tanzania) and Middle East (Oman).**

888

889 **Supp. table 2 – Sampling information for the schistosome material used.**

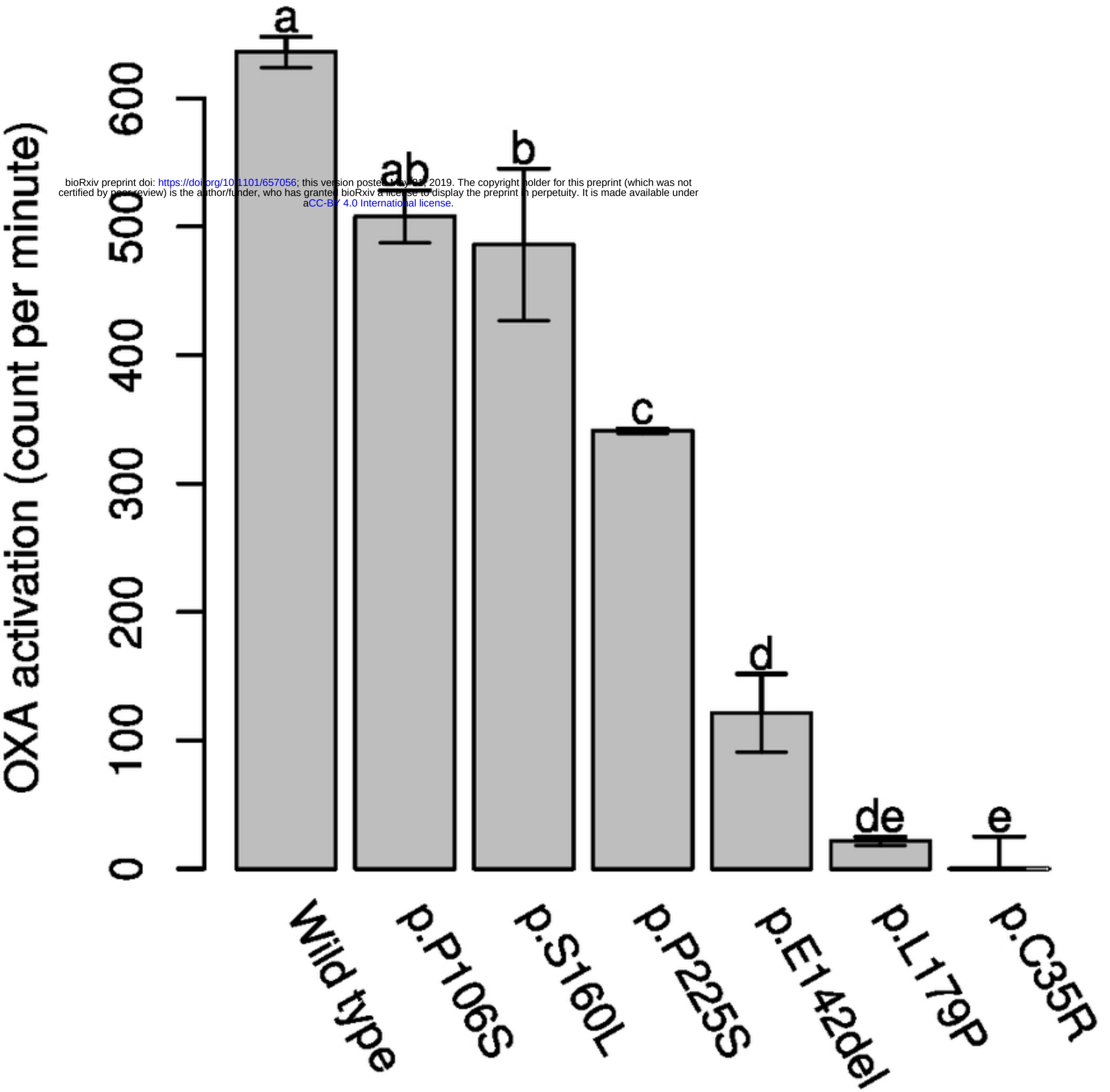


Figure 3

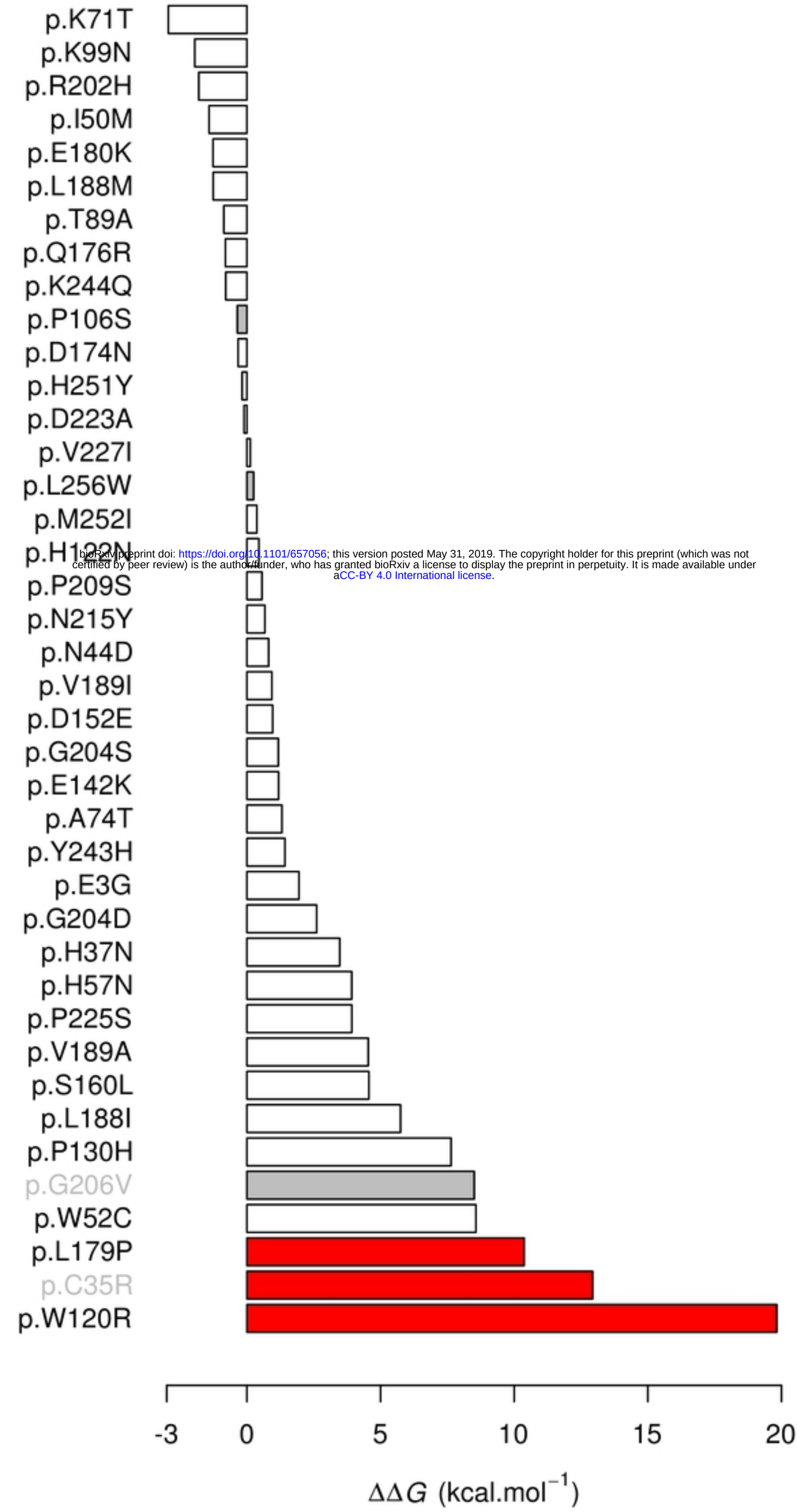
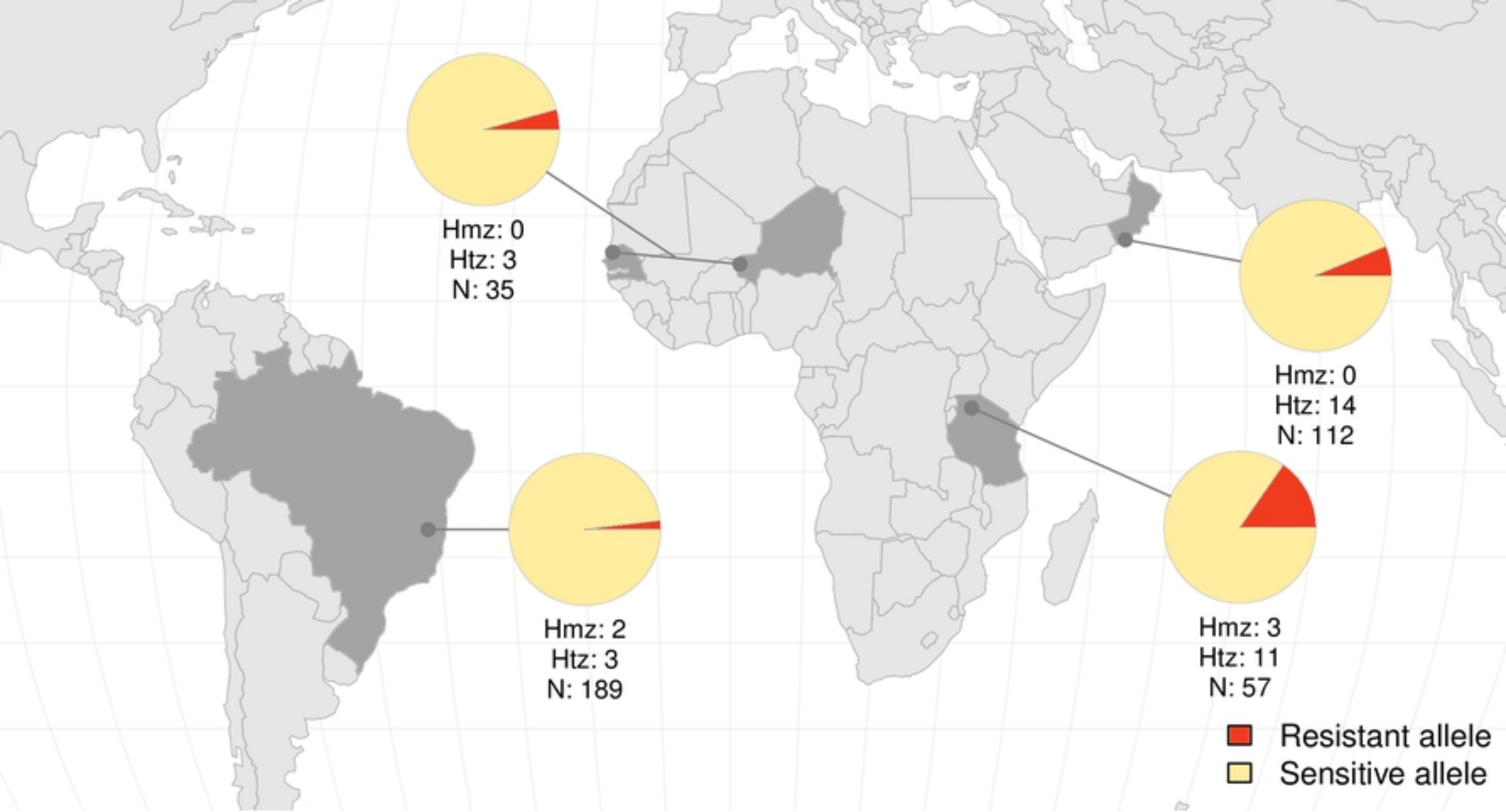


Figure 4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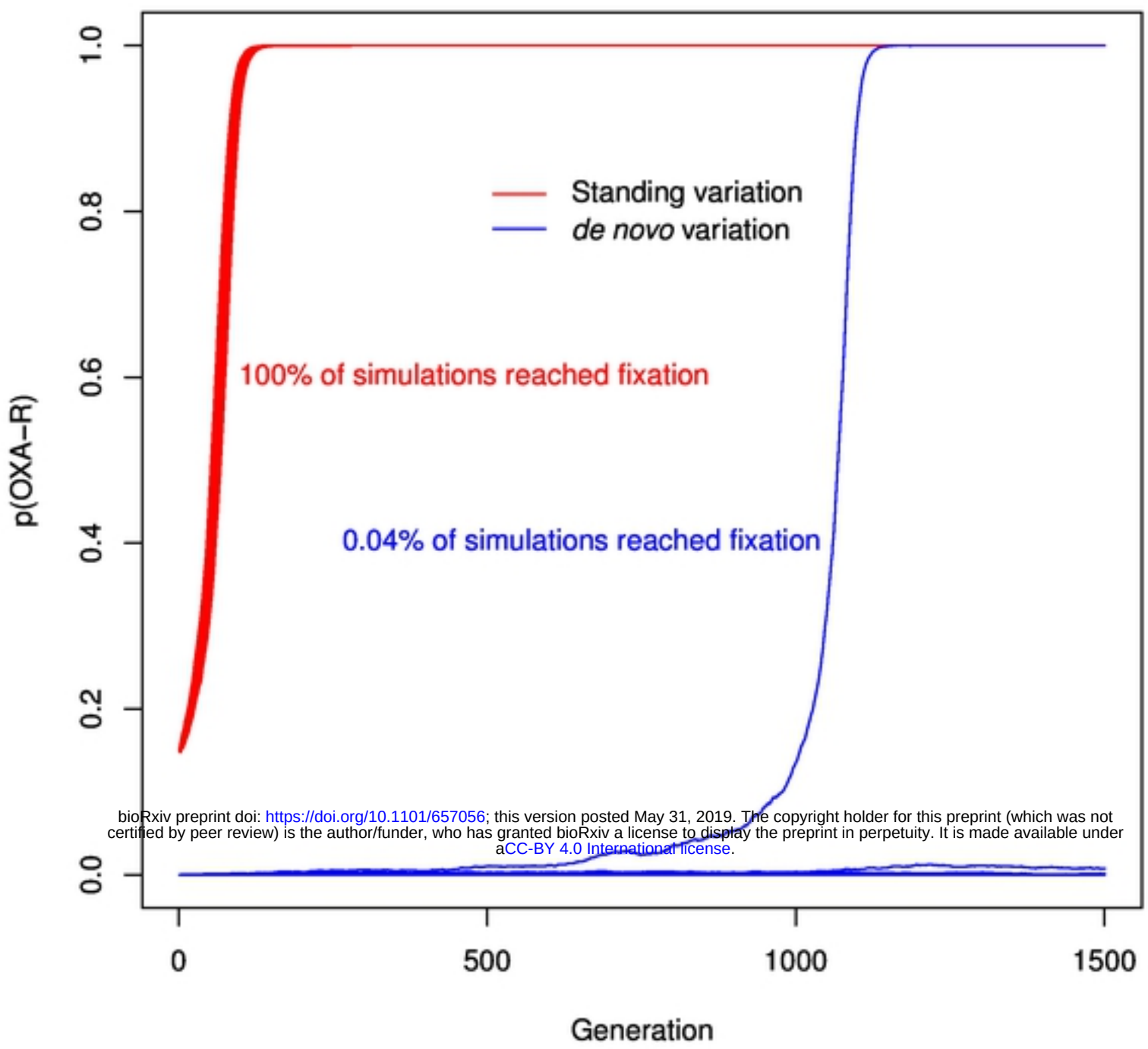
Htz: 3
N: 35
Hmz: 0
Htz: 14
N: 112
Hmz: 2
Htz: 3
N: 189
Hmz: 3
Htz: 11
N: 57
Resistant allele
Sensitive allele

Figure 5

A



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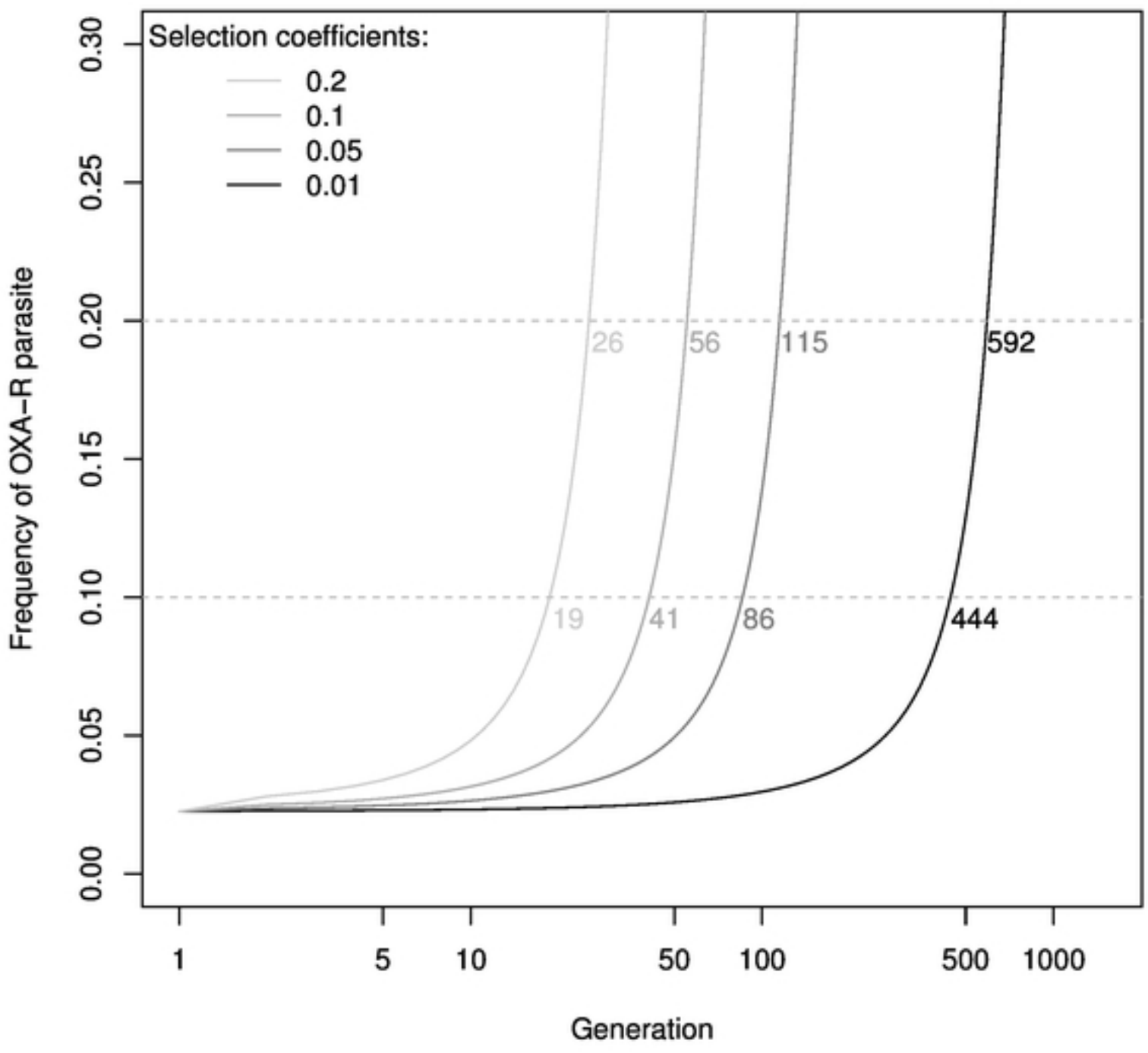


Figure 7

Standing variation

Mutation **present** before selection

Starting allele frequency = p(resistant allele)

→ **No** waiting time

Drug resistance evolves rapidly

High probability of fixation

De novo variation

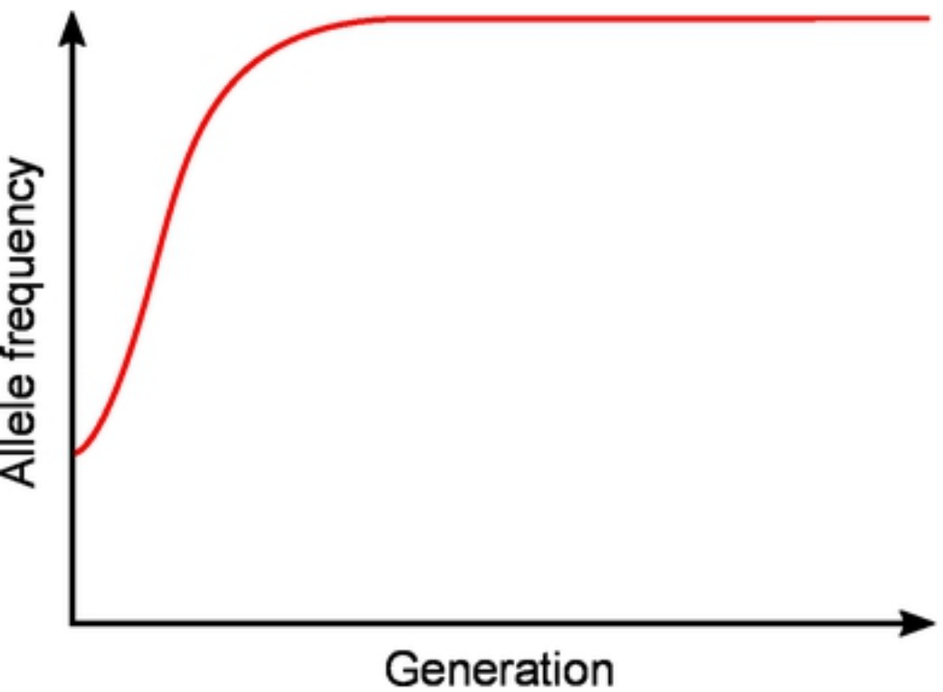
Mutation **must appear** before selection

Starting allele frequency = 1/(2Ne)

→ Waiting time

Drug resistance evolves slowly

Low probability of fixation



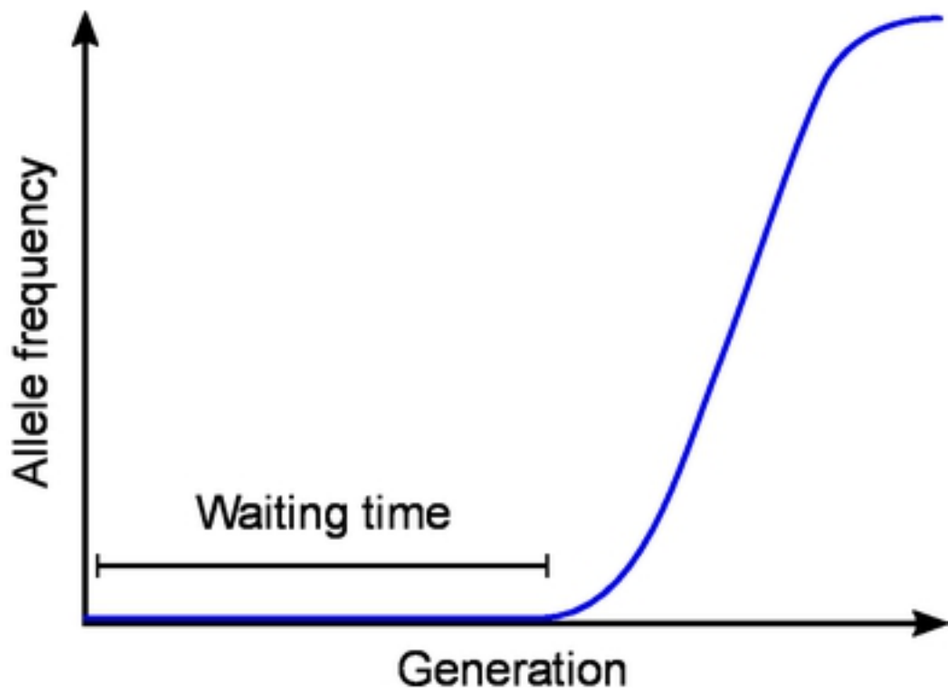


Figure 1

A

+1

Exon 1

Exon 2

+4,790

Nucleic acid mutations

c.3dupGTTTATCCATAATG

c.35C>A

c.358T>C

c.424_426delGAA

c.510delT

c.536T>C

Amino acid mutations

p.I2VfsX3

p.S12X

p.W120R

p.E142del

p.N171IfsX28

p.L179P

B

p.E142del

p.N171IfsX28

p.W120R

p.I2VfsX3

p.S12X

p.L179P

+90°

Figure 2

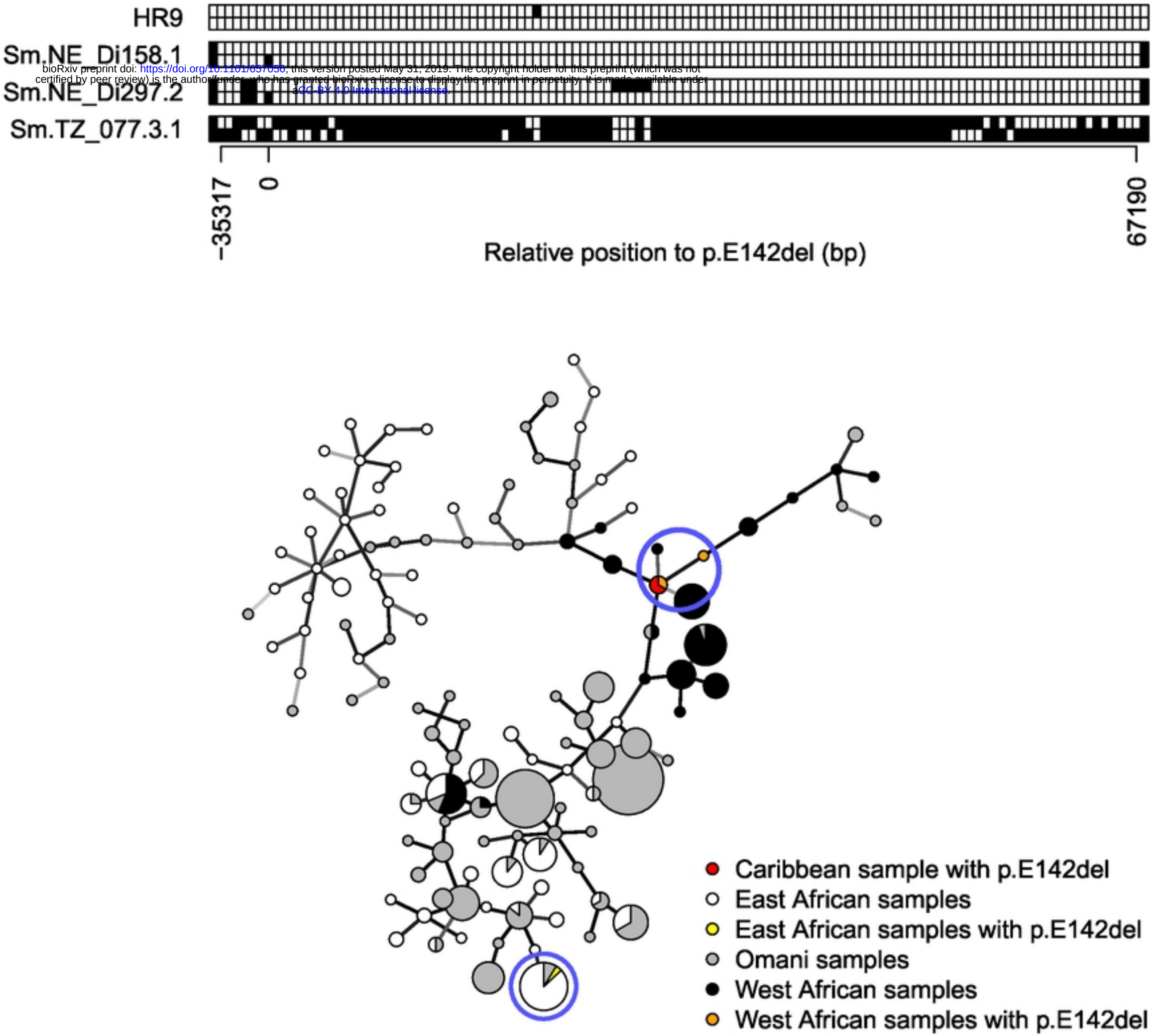


Figure 6