Female mosquitoes are major vectors of human disease and the most dangerous are those that preferentially bite humans. A ‘domestic’ form of the mosquito *Aedes aegypti* has evolved to specialize in biting humans and is the main worldwide vector of dengue, yellow fever, and chikungunya viruses. The domestic form coexists with an ancestral, ‘forest’ form that prefers non-human animals and is found along the coast of Kenya. We collected the two forms, established laboratory colonies, and document striking divergence in preference for human versus non-human animal odour. We further show that the evolution of preference for human odour in domestic mosquitoes is tightly linked to increases in the expression and ligand-sensitivity of the odorant receptor *AegOr4*, which we found recognizes a compound present at high levels in human odour. Our results provide a rare example of a gene contributing to behavioural evolution and provide insight into how disease-vectoring mosquitoes came to specialize on humans.

Blood-feeding as a behavioural adaptation is exceedingly rare in insects. Of the one million to ten million insect species on earth, only ~10,000 feed on the blood of live animals. Among these, only about 100 species blood-feed preferentially on humans. When biting insects evolve to prefer humans, they can spread diseases such as malaria and dengue fever with devastating efficiency. The mosquito *Aedes aegypti* provides one of the best examples of specialization on humans. It originated as a wild, animal-biting species in the forested areas of sub-Saharan Africa, where the subspecies *Ae. aegypti formosus* is still often found living in forests and biting non-human animals today. In contrast, the derived

---

**Figure 1 | Field collection of forest and domestic forms of *Ae. aegypti* in Rabai, Kenya.** a. Map of Rabai, Kenya showing collection sites, with six colonies labelled. b, c. Typical indoor (b) and outdoor (c) water containers or traps. d. Examples of characteristic differences in body colour between domestic (left) and forest (right) females, selected from the extremes of variation. e-h. Summary of colour variation in scales (e, g) and cuticle (f, h) among laboratory colonies tested after 3–7 (e, f) and 5–11 generations (g, h), including colonies from Thailand and Uganda. Cartoons at the bottom of e–i indicate in red where morphology was sampled. See also Extended Data Fig. 1. Data are plotted as mean ± s.e.m. (n = 10–15 mosquitoes per colony).

i. Extent of white scaling on the first abdominal tergite (n = 10 mosquitoes per colony). In all figures, black and brown indicate forest and domestic colonies, respectively, except K14 in brown with black outline to represent its mixed morphology.
non-African subspecies Ae. aegypti aegypti has evolved to specialize in biting humans and thus has become the main worldwide vector of dengue and yellow fevers2–4.

The evolutionary adaptations that help subspecies Ae. aegypti exploit humans are most clearly seen where it has been reintroduced along the coast of East Africa and is known as the ’domestic’ form. Researchers investigating the outbreak of an unknown illness in Tanganyika in 1952 discovered homes heavily populated by brown-pigmented ’domestic’ mosquitoes. Subsequent work in the Rabai region of Kenya in the 1960s and 1970s showed that domestic mosquitoes readily entered homes5, preferred to lay eggs in nutrient-poor river and rain water stored in containers indoors6,7, were resistant to starvation as larvae6, and had evolved a strong preference for biting humans5,8–11. Black-pigmented populations of the native African subspecies formosus, known in Rabai as the ‘forest’ form, were found just hundreds of metres away, avoiding homes, laying their eggs in tree holes and rock pools outdoors, and preferring to bite non-human animals. These differences translated into marked divergence in capacity to spread human diseases, including chikungunya, the unknown illness from 1952, yellow fever, prevalent in Africa and South America since the sixteenth century, and dengue fever, a disease currently infecting almost 400 million people around the world each year12.

Remarkably, the domestic and forest forms in Rabai remained separate in nature but were interfertile in captivity8, providing a rare opportunity to investigate the genetic basis and evolution of traits that adapt mosquitoes to humans. Here we find that human host preference in domestic mosquitoes is strongly correlated with functional genetic variation in an odorant receptor, Or4, which recognizes a component of human body odour.

Domestic and forest forms continue to coexist
Forest and domestic mosquitoes were last documented in Rabai, Kenya in the 1970s, and we returned there in 2009 to determine whether they still exist. We collected Ae. aegypti larvae and pupae in water-storage containers inside approximately one in every five homes visited (Fig. 1a, b). We also collected eggs, larvae, and pupae of several mosquito species, including Ae. aegypti, outdoors in natural and artificial containers in villages and nearby forest (Fig. 1a, c). From these collections, we established 29 laboratory colonies, each descending from fewer than 20 males and females collected in the same house or outdoor location (Fig. 1a).

Previous reports described differences in body colour between the forms1,6,13. Indeed, females from all outdoor colonies and some indoor colonies were black, resembling forest mosquitoes (Fig. 1d). Those from the remaining indoor colonies were brown, resembling domestic mosquitoes (Fig. 1d). Differences in thorax colour were maintained across multiple laboratory generations (Fig. 1e–h; Extended Data Fig. 1). Black and brown colonies also differed in abdominal scaling (Fig. 1i). A single indoor colony, K14, included individuals with a mix of black and brown traits (Fig. 1e–i). Black mosquitoes resembled a subspecies formosus colony from inland Africa (Uganda), whereas brown mosquitoes resembled a subspecies aegypti colony from Asia (Thailand) (Fig. 1e–i). In light of these morphological differences, and genetic differences among the field-collected progenitors of our colonies13, we hereafter refer to black and brown colonies as forest and domestic, respectively. In summary, mosquitoes fitting the morphological description of the two forms continue to coexist in Rabai, Kenya, 35 years after they were last documented.

Domestic mosquitoes strongly prefer humans
We used three assays to characterize the preference of forest and domestic forms for humans versus non-human animals. We offered guinea-pig as a non-human host because it is among the diverse hosts to which mosquito bites are known to elicit an immune response10,13. In a biting assay in which females are exposed directly to live hosts (Fig. 2a), forest females preferred the guinea-pig (Fig. 2b) and domestic females weakly preferred the human (Fig. 2b). Domestic females were approximately twice as likely to respond overall (Fig. 2c), possibly reflecting adaptation to indoor environments, and by extension laboratory settings13. When host cues were presented in an olfactometer (Fig. 2d), forest and domestic females again showed significantly different preferences and response rates (Fig. 2e, f), with domestic females displaying a strong preference for humans. All colonies fell into two discrete behavioural clusters corresponding precisely to the
forest and domestic designations made on the basis of morphology (Fig. 2g). Behaviourally, forest colonies resembled subspecies *formosus* from Uganda, and domestic colonies resembled subspecies *aegypti* from Thailand.

We further confirmed these results with host-scented nylon sleeves supplemented with equal amounts of carbon dioxide (CO₂), a potent activator of mosquito host-seeking (Fig. 2h). Three domestic colonies retained their strong preference for humans, whereas three forest colonies ranged from no preference to moderate preference for guinea-pig (Fig. 2h).

To extend our results to other non-human hosts, we also assessed the preference of a small subset of colonies for human versus chicken in the live host assay, obtaining qualitatively similar results (Fig. 2i). Our findings confirm that domestic mosquitoes have evolved a marked preference for human body odour.

**Human preference is associated with OR expression**

Novel chemosensory preferences in insects are sometimes accompanied by changes in the peripheral chemosensory system. We reasoned that altered gene expression in antennae may contribute to preference, and profiled differential gene expression in this major olfactory organ using RNA sequencing (RNA-seq). To identify general differences between forms, we compared three forest versus three domestic colonies (Fig. 2h).

To determine which of these differences are genetically associated with host preference, we crossed two representative colonies and compared pools of strongly human-preferring versus guinea-pig-preferring F2 hybrids (Fig. 3a, b).

A total of 959 antennal genes were differentially expressed in colonies (Fig. 3c, e), 46 genes were differentially expressed in F2 pools (Fig. 3d, e), and 14 genes were differentially expressed in the same direction in both comparisons (Fig. 3c–e and Supplementary Table 1). Odorant receptors (ORs), a family of insect chemosensory receptors, were significantly over-represented among differentially expressed genes (Fig. 3f, P < 0.0001). Two other families of chemosensory genes, the ionotropic receptors...
(IRs)\textsuperscript{23} and odorant-binding proteins (OBPs)\textsuperscript{24} were less enriched or not enriched, respectively (Fig. 3f). A selective role for the OR pathway in helping mosquitoes distinguish among hosts is consistent with previous work in a laboratory strain of Ae. aegypti\textsuperscript{25}.

Of the 14 genes significant in both colony and F2 comparisons, two were ORs (Fig. 3f), Or4 and Or103. Both were upregulated in human-preferring mosquitoes (Fig. 3g, solid lines). Or4 was also the second most highly expressed ligand-selective OR in the antennae of domestic females overall (Fig. 3g), and we chose this gene for further study.

**Or4 recognizes the human odorant sulcatone**

An olfactory receptor could modulate host preference by mediating attraction or repulsion to specific host odours, so we asked whether Or4 is activated by a component of human or guinea-pig odour. We expressed Or4 heterologously in a Drosophila olfactory neuron lacking a ligand-selective OR\textsuperscript{26}, and tested responses to fractionated host odour from guinea-pigs and humans (Fig. 4a). Or4 did not respond to any fraction of guinea-pig odour (data not shown), but responded consistently to a fraction of human odour corresponding to 6-methyl-5-hepten-2-one, commonly called sulcatone (Fig. 4b).

Sulcatone is a volatile odorant repeatedly identified in human body odour\textsuperscript{27–30}. Although sulcatone is also emitted by a variety of other animals\textsuperscript{31–34} and plants\textsuperscript{35–37}, it appears to reach uniquely high levels in humans (Fig. 4c–e). It was abundant in the odour of nylon sleeves worn by five humans, but undetectable or at low concentration in unworn sleeves or sleeves worn by four guinea-pigs (Fig. 4d, c). For reference, another widespread volatile, benzaldehyde, did not differ significantly between samples (Fig. 4c, d). We also found approximately four times more sulcatone in the body odour of live humans than in the odour of a live chicken or the hair of horses, cows, and sheep (Fig. 4e). Collectively, these results suggest that increased expression of Or4 may help mosquitoes distinguish humans from non-human animals by conferring sensitivity to sulcatone. Interestingly, the malaria mosquito Anopheles gambiae has at least 4 ORs that are strongly activated by sulcatone\textsuperscript{38}, but none is closely related to AaegOr4 (ref. 39). These two species diverged from each other approximately 150 million years ago and evolved independently to specialize in biting humans\textsuperscript{40}.

**Preference linked to Or4 sensitivity and expression**

Evolution of preference for human hosts could occur not only via changes in Or4 expression, but also via changes in the Or4 coding region that affect protein function. Natural variation in chemoreceptor proteins has previously been shown to alter ligand-sensitivity\textsuperscript{41} and odor perception\textsuperscript{42}. We found extensive variation in Or4, with seven major alleles present in the two parent colonies and F2 hybrids (Fig. 5a–e; Extended Data Fig. 3). The domestic parent, K14, was dominated by the closely related A and B alleles (Fig. 5a, b) and a highly divergent G allele (Fig. 5a, b). The K27 forest parent, in contrast, harboured 5 distinct alleles at low to moderate frequency (Fig. 5a, b). RNA-seq data from 8 additional colonies suggest that these patterns apply globally. Human-preferring colonies derived from Kenya, Thailand, USA, and West Africa were dominated by A-like alleles, whereas animal-preferring colonies from Kenya and Uganda were all highly variable (Extended Data Fig. 2). Although all alleles were present in F2 mosquitoes, they were inherited at different frequencies by human-preferring and guinea-pig-preferring individuals (Fig. 5c, d). Moreover, F2s tended to carry alleles characteristic of the parent with similar preference (compare Fig. 5b and 5d), suggesting that some aspect of allele-specific function affects preference.

We asked how allelic variation affects Or4 receptor function. The protein-coding sequence of Or4 is remarkably variable among alleles with differences in 13 of 406 residues on average, and 26 residues in the
most extreme case (Fig. 5a; Extended Data Fig. 3). Given this high level of variation, we conducted a molecular analysis to confirm that all alleles correspond to a single copy gene (Extended Data Fig. 4). We tested the function of each allele in Drosophila and found that A, B, C, F, and G were highly sensitive to sulcatone, whereas D and E were much less sensitive (Fig. 5c). Variation in spontaneous activity mirrored the variation in odour-evoked activity (Fig. 5g).

We also asked whether Or4 alleles vary in expression and could thus help explain the upregulation of this locus in human-prefering mosquitoes. We reanalysed the RNA-seq data, parsing gene expression according to major alleles in each F2 pool and normalizing by allele frequency to isolate levels of allele-specific expression. Two major conclusions emerged. First, all alleles are expressed at higher levels when carried by human-prefering F2s than when carried by guinea-pig-prefering F2s (Fig. 5h, compare red to blue). This difference suggests that a genetic element unlinked from Or4 contributes to upregulation in human-prefering mosquitoes. Second, the rank order of gene expression of each allele was preserved, regardless of whether it was carried by human-prefering or guinea-pig-prefering mosquitoes (Fig. 5i, compare alleles within red or blue). For example, B is always expressed at the highest level and F at the lowest level. These consistent and significant differences among alleles suggest that genetic elements linked to Or4 and varying among alleles also contribute to changes in expression.

To determine which characteristics of Or4 alleles are tied to behaviour, we asked whether ligand-sensitivity and/or expression can account for host preference-based differential inheritance of alleles (Fig. 5d). Remarkably, both factors had significant effects on relative allele frequency in F2s and together explained 92% of the variation (Fig. 5i). This striking dependence suggests that expression and sensitivity to sulcatone have independent and additive effects on preference. Moreover, strong human preference appears to require alleles with both high sensitivity and expression. For example, E is one of the most highly expressed alleles, yet it has weak sensitivity to sulcatone and is biased towards guinea-pig-prefering mosquitoes (Fig. 5i). Conversely, allele F has high sensitivity to sulcatone, yet it is expressed at extremely low levels and is also slightly biased towards guinea-pig-prefering mosquitoes (Fig. 5i).

Discussion

We have re-established the Rabai forest and domestic mosquito study system for investigation of the striking evolutionary adaptations that help Drosophila females find, bite, and thereby spread disease to humans. We show that preference for humans is tightly linked to increases in both the expression and ligand-sensitivity of odorant receptor Or4. These changes may help mosquitoes distinguish humans from non-human animals by increasing behavioural sensitivity to the signature human odorant sulcatone. Interestingly, sulcatone has been described as a mosquito repellent when added to human odour at certain concentrations and sometimes as an attractant when added at low concentrations or delivered alone. This raises the intriguing possibility that while a baseline level of sulcatone signals humanness, mosquitoes may prefer humans that have lower levels of sulcatone over those with high levels. In other words, as is true for many odours, it is possible to have too much of a good thing. We further note that sulcatone is unlikely to be the only odorant that makes us smell human, nor Or4 the only gene contributing to human preference in domestic Ae. aegypti. Guinea-pig odour perfumed with sulcatone was not preferred over the odour of guinea-pig alone by human-prefering mosquitoes (Extended Data Fig. 5). We strongly suspect that evolutionary changes at other loci, including some of the candidates from our antennal RNA-seq analysis, may also play a role.

Our results also provide insight into the molecular basis of behavioural evolution. Despite exciting progress in this area, specific examples of genetic changes associated with behavioural change are extremely rare. Previous authors documented changes in the peripheral olfactory system of organisms with novel host preference, but direct links between these changes and behaviour have been missing. We have established a clear genetic association between such changes and behaviour. This work begins to unravel the molecular genetic basis of an important evolutionary shift in insect host preference. More generally, such host shifts not only impact the efficiency of mosquitoes as vectors of infectious disease, but contribute to the economic damage caused by agricultural pests and play a key role in the formation of new species.
28. Labows, J., Preti, G., Hoelzle, E., Leyden, J. & Kligman, A. Analysis of human axillary volatiles: compounds of exogenous origin. J. Chromatogr. 163, 294–299 (1979).
29. Bernier, U. R., Kline, D. L., Barnard, D. R., Schreck, C. E. & Yost, R. A. Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for the yellow fever mosquito (Aedes aegypti). Anal. Chem. 72, 747–756 (2000).
30. Syed, Z. & Leal, W. S. Acute olfactory response of Culex mosquitoes to a human- and bird-derived attractant. Proc. Natl Acad. Sci. USA 106, 18803–18808 (2009).
31. Birkett, M. A. et al. The role of volatile semiochemicals in mediating host location and selection by nuisance and disease-transmitting mosquito species. Med. Vet. Entomol. 18, 313–322 (2004).
32. Jumane, Z., Gries, R., Unruh, T., Rowland, E. & Gries, G. Identification of the larval aggregation pheromone of codling moth, Cydia pomonella. J. Chem. Ecol. 31, 911–924 (2005).
33. Nielsen, B. L. L., Jérome, N., Saint-Albin, A., Rampin, O. & Maurin, Y. Behavioural response of sexually naive and experienced male rats to the smell of 6-methyl-5-hepten-2-one and female rat faces. Physiol. Behav. 120, 150–155 (2013).
34. Stoeltier, M., Maier, T. S., Tolasch, T. & Steidle, J. L. M. Foreign-language skills inrove-beetles? Evidence for chemical mimicry of ant alarm pheromones in myrmecophilous Pella beetles (Coleoptera: Staphylinidae). J. Chem. Ecol. 33, 1382–1392 (2007).
35. Rines, H. W., French, R. C. & Daasch, L. W. Nonanal and 6-methyl-5-hepten-2-one: endogenous germination stimulators of undecuploids of Puccinia graminis var. tritici and other rusts. J. Agric. Food Chem. 22, 96–100 (1974).
36. Socaci, S. et al. Chromometric discrimination of different tomato cultivars based on their volatile fingerprint in relation to lycopene and total phenolics content. Phytochem. Anal. 25, 161–169 (2013).
37. Webster, B. et al. Identification of volatile compounds used in host location by the black bean aphid, Aphis fabae. J. Chem. Ecol. 34, 1153–1161 (2008).
38. Carey, A. F., Wang, G., Su, C. Y., Zwiebel, L. J. & Carlson, J. R. Odorant receptor alleles are at GenBank (accession numbers KF801614, KF801615 and KF801617–KF801621). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests.}

**Supplementary Information** is available in the online version of the paper.

**Acknowledgements** We thank M. K. N. Lawmizicak, K. J. Lee, M. N. Nitabach, and the Vosshall laboratory for discussion and comments on the manuscript; J. E. Brown and J. R. Powell for discussion and coordination of field collections; W. Takken for advice regarding many aspects of this work; J.-P. Mutebi, B. Miller, and A. Ponlawat for live specimens from Uganda and Thailand; D. Beck, K. Nygaard, K. Prakash, and L. Seelholzer for expert technical assistance. We also thank X. Chen for pre-publication access to a draft Ae. albopictus genome assembly, and J. Liesch for access to Orlando strain RNA-seq data. We received valuable advice on collecting and working with forest and domestic forms of Ae. aegypti from M. Trpis, J. L. Peterson, and P. Lounibos, and on the design and use of two-port olfactometers from U. Bernier and V. Sherman. This work was funded in part by a grant to R. Axel and L.B.V. from the Foundation for the National Institutes of Health through the Grand Challenges in Global Health Initiative. This work was supported in part by the following National Institutes of Health grants: K99 award from NIDCD to C.S.M. (DC012069), an NIAD Vectorbase DBP subcontract to L.B.V. (HHSN272200900039C), and a CTSA award from NCATS (UL1TR000043). R.I. received support from the Swedish Research Council and SLU: Insect Chemical Ecology and Evolution (IC-E3). L.B.V. is an investigator of the Howard Hughes Medical Institute.

**Author Contributions** C.S.M. and L.B.V. conceived the study. C.S.M. participated in the execution and analysis of all aspects of the study. J.L. helped coordinate mosquito collection in Rabai, Kenya under the supervision of R.S. S.A.S. helped design and carry out the morphological analyses presented in Fig. 1e–f. F.B. helped clone, analyze, and genotype mosquitoes for the Od4 alleles presented in Fig. 5a–d, and construct transgenic Drosophila lines for use in single sensillum recordings. A.B.O. and R.I. designed, conducted, and analysed the GC–SSR and GC–MS experiments presented in Fig. 4 and carried out pilot experiments comprising dose–response curves and spontaneous activity analysis of alleles A and E, similar to those presented in Fig. 5e–g. C.S.M. and L.B.V. designed all other experiments, interpreted the results, designed the figures, and wrote the paper.

**Author Information** Raw RNA-seq data are available for download at the NCBI Sequence Read Archive (accession number SRP035216). Coding sequences of Aeg-GR alleles are at GenBank (accession numbers KF801614, KF801615 and KF801617–KF801621). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to L.B.V. (Leslie.Vosshall@rockefeller.edu).
METHODS

Ethics and regulatory information. Mosquitoes were collected and exported from Kenya with approval of the director of the Kenya Medical Research Institute under the study approved by the Scientific Steering Committee and Ethical Review Committee (SSC No. 1679). Live mosquito eggs were imported to the USA with permits issued by the United States Department of Agriculture and the Centers for Disease Control and Prevention. The use of non-human animals in host preference tests at The Rockefeller University was approved and monitored by The Rockefeller University Institutional Animal Care and Use Committee (Protocol 11487). The participation of humans in blood-feeding mosquitoes during routine colony maintenance and as subjects in host preference tests at The Rockefeller University was approved and monitored by The Rockefeller University Institutional Review Board (IRB protocol LVO-0652). The protection of human subjects and ethical work with animals at the Swedish University of Agricultural Sciences (SLU) was in accordance with the Central Ethical Review Board and the Committee for Laboratory Animal Science in Sweden, respectively. All human subjects gave their informed consent to participate in the work carried out at The Rockefeller University and at SLU.

Field collection and creation of laboratory colonies. We collected mosquito (Ae. aegypti) eggs, larvae, and pupae in indoor and outdoor habitats in the Rabai region of Kenya in January 2009 (39° 34′–36′ E, 3° 55′–57′ S). Indoor collections in the Rabai region of Kenya (Fig. 1a) were made, with the verbal permission of homeowners, by visually scanning artificial containers used to store water with flashlights and removing larvae and pupae with a nylon sieve. Artificial containers harboring mosquitoes included plastic buckets, metal jerry cans, and traditional earthenware pots (Fig. 1b). Outdoor collections were made in both village environments and nearby forest fragments along the Kombeni River (Fig. 1a) in two ways. First, larvae and pupae were removed from artificial containers left outdoors using a sieve, and from natural containers such as tree holes (Fig. 1c, top) using a turkey baster or small plastic pipette. In some cases, tree holes retained water from the last rains, while in others we introduced well water two days before collection to induce hatching of dormant eggs. Second, freshly laid eggs were collected in oviposition traps of ‘soil water’ prepared by incubating deionised water with commercial potting soil cups (Solo cup company) lined with seed-germination paper and filled with 30 ml of water. Mosquito eggs did not hatch in open cups (Fig. 1c, bottom) or left on the ground for 3 days. Traps comprised black plastic cups (13 cm diameter, 15 cm tall) half-filled with water and lined with coarse brown seed-germination paper (76 pound, Anchor paper) (Fig. 1c, bottom). Adult females attracted to the cups laid their eggs on the wet paper, which was then removed and dried to prevent embryos from hatching. When mosquitoes were collected as larvae or pupae, individuals originating in the same home/container or cluster of nearby homes/containers were reared to adulthood, mated with each other, blood-fed, and induced to oviposit in a field laboratory so that eggs of the first laboratory generation could be dried and exported to the USA. A total of 29 laboratory colonies were established (K1–K29), each founded by between 1 and 14 females (median 4) collected within 0–50 m of each other in the field (Fig. 1a) and mated to males collected from the same area. The only exception was K27, which was established using males from a forest tree hole that yielded no females. We therefore mated g1–g2 females from 16 colonies, 1–3 trials per colony and assessed the significance of the difference between forest and domestic colonies using two-sided, non-parametric tests, where the mean for each colony served as a single data point.

Nylon sleeve olfactometer assay. We tested g1–g2 females from 16 colonies, 1–3 trials per colony and assessed the significance of the difference between forest and domestic colonies using two-sided, non-parametric tests, where the mean for each colony served as a single data point.

Live host olfactometer assay. We used a live host olfactometer assay to test mosquito preference for human versus chicken. A preference index equal to the number of mosquitoes that blood-fed on the human minus the number that blood-fed on the guinea-pig divided by the total number of mosquitoes that blood-fed on either host. Overall response was the fraction of mosquitoes that blood-fed on either host. We tested g1–g2 females from 16 colonies, 1–3 trials per colony and assessed the significance of the difference between forest and domestic colonies using two-sided, non-parametric tests, where each colony served as a single data point.

Biting assay. This assay was used to test mosquito preference for human versus guinea-pig (Fig. 2a) and was based on a previously described landing assay. For each trial, we allowed approximately 50 females to acclimate overnight in a large custom-made cage (61 × 61 × 91.5 cm) constructed with aluminium screening on 3 sides and clear vinyl on the fourth side for easy viewing (BioQuip). The following morning, we simultaneously introduced a human arm (33-year-old female) and an anaesthetized guinea-pig (pigmented strain, one of two females, 2–6 months old) through cloth sleeves at opposite ends of the cage and rested them on the floor of the cage −60 cm apart (Fig. 2a), and recorded the number of mosquitoes that blood-fed within 10 min. We defined blood-feeding as landing on the host, inserting the proboscis, and drawing enough blood into the abdomen that it was visible to the naked eye of the observer. Preference index was calculated as number of mosquitoes that blood-fed on the human minus the number that blood-fed on the guinea-pig divided by the total number of mosquitoes that blood-fed on either host. Overall response was the fraction of mosquitoes that blood-fed on either host. We tested g1–g2 females from 16 colonies, 1–3 trials per colony and assessed the significance of the difference between forest and domestic colonies using two-sided, non-parametric tests, where each colony served as a single data point.

Colony maintenance and insect rearing. Mosquitoes were maintained at 25–28 °C with 70–80% relative humidity under a 14 h light: 10 h dark cycle (light on at 8 a.m.). Eggs were hatched by submerging in a broth containing deoxygenated deionised water and powder Tetramin tropical fish food (Tetra). Larvae were cultured in deionised water and fed Tetramin tablets. Adults were maintained in large plastic cages (30 × 30 × 30 cm, BioQuip) and given unlimited access to 10% sucrose. In each generation, 75–250 adult females from each colony were blood-fed on a human volunteer. To minimise the potential for natural selection on host preference, a human arm was offered 1–2 times each day until 90% or more of the females had taken a full blood-meal. Eggs were collected in 96 ml black plastic soufflé cups (Solo cup company) lined with seed-germination paper and filled with 30 ml of ‘soil water’ prepared by incubating deionised water with commercial potting soil at room temperature in an open vessel for 1–10 weeks. Eggs were dried to prevent hatching and stored at 18 °C, 85% relative humidity for 6–12 months. Eggs from domestic colonies K1–K6 were less tolerant to drying and storage than eggs from forest colonies. They were therefore hatched after only 1–4 months, resulting in shorter generation times.

Morphological analysis. We characterized the scale and cuticle colour of mosquitoes from 14 colonies in 2010 (g3 to g7; n = 10–15 mosquitoes per colony), and a partially overlapping set of 8 colonies in 2012 (g5 to g11; n = 10–15 mosquitoes per colony). We collected adult female mosquitoes within 48 h of eclosion and stored them at −20 °C for up to 4 weeks. Immediately upon removal from the freezer, we inserted an insect pin laterally through the thorax and positioned each specimen using a pinned specimen manipulator (Rose Entomology) one at a time under a microscope (Nikon Eclipse SMZ1500) fitted with a ring light and a digital camera (Nikon digital sight DS-2MV controlled by NIS-Elements F v3.0 software). We took photographs in two characteristic positions, one highlighting the dark and light scales that decorate the scutum (Extended Data Fig. 1a) and the other showing the bare cuticle of the postnotum on the posterior face of the thorax (plus the first 3–4 segments of the abdomen (Extended Data Fig. 1b). We measured scale and cuticle colour on the dark parts of the scutum and postnotum, respectively, in Adobe Photoshop (v. CS6, Adobe Systems Inc.) by sampling RGB values at four characteristic positions using the colour picker tool (Extended Data Fig. 1a, b), and converted RGB numbers to hue, saturation, value (HSV) using R software (v. 2.1.50, http://www.r-project.org/). The light environment and camera settings were held constant for the duration of each analysis, but differed slightly between analyses, such that values from 2010 and 2012 are not directly comparable.

We assessed the extent of white scaling on the first abdominal tergite in the 2012 analysis using an ordinal scale from 1 to 5 (Extended Data Fig. 1c) as follows: 1, up to a few scattered white scales; 2, small patch of white scales at midline; 3, contiguous patch of white scales at midline stretching from top to bottom of tergite and covering up to 60% of visible area; 4, contiguous patch of white scales covering 60–90% of visible area; 5, contiguous patch of white scales covering >90% of visible area. These scores correspond roughly to letters F through K in the scheme of McClelland et al. A single experimenter scored all mosquitoes blind to their identity.

Host preference assays. Mosquitoes used in host preference assays were adult females 1–3 weeks post eclosion that had been given the opportunity to mate, but had not taken a blood-meal. Females were sorted briefly under cold anaesthesia (−4 °C) and blood-fed on access to food or water 16–24 h before testing.

Biting assay. This assay was used to test mosquito preference for human versus guinea-pig (Fig. 2a) and was based on a previously described landing assay. For each trial, we allowed approximately 50 females to acclimate overnight in a large custom-made cage (61 × 61 × 91.5 cm) constructed with aluminium screening on 3 sides and clear vinyl on the fourth side for easy viewing (BioQuip). The following morning, we simultaneously introduced a human arm (33-year-old female) and an anaesthetized guinea-pig (pigmented strain, one of two females, 2–6 months old) through cloth sleeves at opposite ends of the cage and rested them on the floor of the cage −60 cm apart (Fig. 2a), and recorded the number of mosquitoes that blood-fed within 10 min. We defined blood-feeding as landing on the host, inserting the proboscis, and drawing enough blood into the abdomen that it was visible to the naked eye of the observer. Preference index was calculated as number of mosquitoes that blood-fed on the human minus the number that blood-fed on the guinea-pig divided by the total number of mosquitoes that blood-fed on either host. Overall response was the fraction of mosquitoes that blood-fed on either host. We tested g1–g2 females from 16 colonies, 1–3 trials per colony and assessed the significance of the difference between forest and domestic colonies using two-sided, non-parametric tests, where each colony served as a single data point.

The comfort of the animal was assured by rest and feeding periods outside of the olfactometer. Neither the animals nor the human volunteer were bitten by mosquitoes during this assay. We screened g1–g5 females from 26 colonies, 1–7 trials per colony in the human versus guinea-pig comparison and a subset of 5 colonies, 1 trial per colony for human versus chicken. A preference index equal to the number of mosquitoes entering the human trap minus those that entered the animal trap divided by the total number of mosquitoes entering either trap was calculated. Overall response was the fraction of mosquitoes that entered either trap. We assessed the significance of the difference between forest and domestic colonies using two-sided, non-parametric tests, where the mean for each colony served as a single data point.

Neighbourhood olfactometer assay. We tested the potential for live host odours to influence mosquito host-finding in the olfactometer to isolate the effects of specific host odours on mosquito preference in the context of a controlled amount of CO2 (Fig. 2h, top). This assay was conducted as described27, except that instead of pushing carbon-filtered air through the olfactometer with pumps, ambient air was gently pulled over the nylon
sleeves and into the main compartment with a fan in exactly the same way as the live host assay. Both air streams were supplemented with an equal amount of CO₂, resulting in a final concentration of 0.2–0.3%. Human- and guinea-pig-scented sleeves comprised 35-cm long sections of women’s sheer nylon stockings that had been worn on a human arm (one of two females 22–35 years old) or guinea-pig torso (pigmented strain, one of two females, 6–24 months old) for 24 h during normal daily activity. Conditioned sleeves were stored at 20 °C for up to a month before use, and individual sleeves were used in only one trial per colony. We tested g2–g8 females from each of the 6 forest colonies, 4–17 trials per colony. We quantified preference and overall response as described for the live host olfactometer assay. We used a one-way ANOVA followed by Tukey’s test to assess significance of variation among colonies.

Sulcatone perfuming experiments. The olfactometer apparatus was modified from the main figures as follows: the air stream was carbon-filtered and simultaneously pushed by a pump and pulled by an exhaust fan. The stream first mixed with CO₂ and picked up the odour of a guinea-pig-worn nylon sleeve in a large cylinder before entering a plastic bag and splitting into two streams. The two streams then passed through two smaller cylinders containing a 55 mm diameter filter circle (Whatman) loaded with 50 μl of either solvent (paraffin oil) or 10−5 sulcatone, a concentration chosen because this is within the range of concentrations eluted from human-worn Sulcatone perfuming experiments.

We quantified preference and overall response as described for the live host olfactometer assay. We used a one-way ANOVA followed by Tukey’s test to assess significance of variation among colonies.

Isothermal rolls were loaded with 50 μl of either solvent (paraffin oil) or 10−5 sulcatone, a concentration chosen because this is within the range of concentrations eluted from human-worn Sulcatone perfuming experiments.
chicken, 100 ng for other animals). Extracts from the 20 individual humans were stored before the addition of the internal standard. Final collections were concentrated under a gentle stream of nitrogen before analysis.

Gas chromatography-coupled single sensillum recording (GC–SSR). We used GC–SSRs as described to screen human and guinea-pig odour for individual volatiles that activated Or4 allergens, which was heterologously expressed in the Drosophila ab3A neuron (Fig. 4a, b). We separated volatiles from host odour extracts using an Agilent 6890 gas chromatograph (GC) (Agilent Technology) fitted with a fused silica capillary column (30 m × 0.25 mm) coated with non-polar HP-5 stationary phase (d.f. 0.25 μm). and using hydrogen gas as the mobile phase (45 cm s⁻¹).

Aliquots of the extracts (5–7.5 μl) were injected splitless for 30 s, with the injector maintained at 225°C. The GC oven temperature was programmed to 30°C (3 min hold), followed by a ramp of 8°C min⁻¹ to 225°C, and held isothermal for 10 min. The GC was fitted with a make-up gas fed (4 psi N₂) four-way cross (Graphpack 3D/2 Crosspiece Sulfinert, Gerstel) at the end of the column, delivering half of the make-up gas to a glass tube (6 mm i.d.).

We performed 3 replicates of GC–SSR for each of the following host odour collections: human body headspace, human-worn sleeve headspace, guinea-pig-worn sleeve headspace. Compounds were considered bioactive if their elution corresponded to changes in ab3A neuron activity in all 3 replicates. Bioactive compounds were then identified via gas chromatography–mass spectrometry (GC–MS) as described below.

Chemical analysis of volatile collections. We used gas chromatography–mass spectrometry (GC–MS) to identify the single bioactive component of human sleeve odour noted in GC–SSR experiments. Human sleeve volatile extract was injected (2 μl) into a combined Agilent 6890N gas chromatograph and 5975 mass spectrometer (Agilent Technology) fitted with an HP-5 column and programmed as follows for the GC–SSR analyses. The active compound was identified by comparison with reference mass spectra in our custom made library and commercially available libraries (NIST05 and Wiley). The putative identification of sulcatone was confirmed by parallel injections of synthetic reference compounds with authentic samples on both libraries (NIST05 and Wiley). The putative identification of sulcatone was confirmed by parallel injections of synthetic reference compounds with authentic samples on both libraries (NIST05 and Wiley).

For each individual, we used a LightScanner (Idaho Technology Inc.) to characterize the melting curves of a 196 bp PCR amplicon from a 3′-block, unlabelled, 21 bp oligonucleotide probe complementary to allele B in the region surrounding the diagnostic SNP. Melting curves were characteristic to genotype, allowing easy discrimination of individuals carrying 0, 1, or 2 copies of allele B. Reactions were analysed according to the manufacturers instructions, using unlabelled primers and probe: 5′-CAAGGTGTGAATGATCGTTGGTTC-3′ (forward), 5′-ATGCACCTCTTCATCGCTGCC-3′ (reverse). We quantified the relative frequency of each major allele in F2 hybrids using a frequency index equal to frequency in human-preferring F2s minus frequency in guinea-pig-preferring F2s divided by the sum of the frequencies in the two types of F2s.

Confirmation of Or4 as a single copy gene. The Ae. aegypti reference genome contains another olfactory receptor, named Or5, that is 96% identical to the Or4 gene across the coding sequence, leading to a predicted Or5 protein that is 97% identical to the Or4 protein. Although we did not sample any sequences identical to Or5 in our field-derived colonies, some of the alleles we sampled were more similar to Or5 than to Or4, raising the possibility that they actually belong to this second hypothetical locus. To confirm that all sampled alleles segregate at a single locus, we reanalysed our PacBio genotyping data, focusing on the number of alleles carried by individual mosquitoes. As described above, we amplified a diagnostic segment of Or4/Or5 from the genomic DNA of 270 females and sequenced ~150 pieces of DNA from each resulting amplicon. The reads for each mosquito were then assigned to alleles based on sequence. The vast majority of reads for each mosquito corresponded to a single allele (homozygotes) or were evenly split between two alleles (heterozygotes) (Extended Data Fig. 4). No mosquito had substantial numbers of reads assigned to more than two alleles. We conclude that Or4 is a single locus supported by the Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme (2009) (Xiaoguang Chen, personal communication).
with multiple diverse alleles and suspect that the gene annotated as Or5 represents a missassembly of Or4 allelic sequences segregating within the genome reference strain. Or4 transmembrane prediction and snake plots. For Extended Data Fig. 3, we used TMHMM (v.2.0)60 to predict the location of transmembrane domains in the A reference allele of Or4. The output was manually edited to remove a predicted transmembrane domain that occurred in an anomalous position relative to predictions for the olfactory co-receptor Orco and other ligand-selective ORs. Snake plots were generated using TOPO2 (ref. 65).

Single sensillum electrophysiology. We used single sensillum recording (SSR) to characterize the spontaneous activity and odour-evoked responses of 7 major Or4 alleles heterologously expressed in Drosophila ab3A olfactory sensory neurons. Recordings were conducted as described previously61 using 5–11-day-old females and a modified method for immobilizing the third antennal segment. We glued a small wedge-shaped plastic Lego piece (rectangular footprint 15 mm × 10 mm with angled surface rising at 45° to 10 mm above base) to a glass microscope slide such that a coverslip affixed to its upper face with double stick tape would protrude upwards at a fixed angle of 45°. We then immobilised the fly in a pipette tip mounted on a ball of dental wax opposite the coverslip as described60. After bringing the base of the fly’s antennae into contact with the edge of the coverslip, we used two small drops of UV glue (KOA 300, Kexmert) to secure the rim of the cut pipette tip on either side of the protruding head to the edge of the coverslip and cured it for 10 s with a 405 nm violet laser pointer. We then used a glass micropipette to gently lower one antenna towards the angled coverslip until the arista contacted a small drop of UV glue placed directly below, and immediately cured the glue with the laser. These modifications prevented the fly preparation and antenna from drifting away from the coverslip over the course of a recording. Neither transgenic nor wild type ab3 sensilla responded to the odour of the glue.

We prepared serial dilutions of sualactone (C.A.S. 409-02-9, Sigma-Aldrich) v/v in paraffin oil and loaded 30 µl aliquots into odour delivery pipettes on the day of recording as described60. During recording, we applied a continuous stream of charcoal-filtered air to the insect antenna. For each stimulus, we first cleared the odour delivery pipette of accumulated volatiles by redirecting a fraction of the air stream through it and away from the preparation for a 1 s pulse. Fifteen seconds later, we then delivered the stimulus by redirecting air through the pipette for another 1 s pulse, but this time with the tip inserted back into the air stream flowing over the antenna. We used each pipette no more than twice.

We applied dilutions to a single ab3 sensillum per fly in the following order: solvent, 10⁻², 10⁻⁶, 10⁻⁵, 10⁻⁴, 10⁻³, 10⁻². To increase resolution for the most biologically relevant doses, we sometimes stimulated a second sensillum on the same fly by lack of A cell response to ethyl hexanoate (10⁻²) pulse, but this time with the tip inserted back into the air stream flowing over the antenna for 1 s.

For SSR recordings, we used a high-speed, computer-controlled system that allowed us to deliver applications of the stimulus at a fixed time after computer-recorded stimulus onset on the basis of the excitatory response elicited in the ab3B cell by 10⁻² pulse, and adding separate transcript sequences for each major Or4 allele A–G. We then realigned unfiltered RNA-seq reads to this transcriptome using Bowtie2 v.2.1.0 (ref. 68) and estimated expression using eXpress v.1.5 (ref. 69) specifying Or4 alleles as haplotypes of a single locus. We used the Bowtie2 alignment parameters recommended for use with eXpress, allowing an unlimited number of hits per read (–a –rdg 6.5 –rfg 6.5 –score-min 1.0–6.0 –0.4). eXpress then uses a probabilistic model to weight the hits from which it may be derived69. The overall alignment rate was 47–51% for all libraries. For each F2 pool, we estimated expression for the two replicate libraries separately and then averaged them. The replicate estimates for a single pool were generally closer to each other than either was to estimates from the other pool. Expression values for each allele are partly a function of the frequency of that allele among the 110–125 individuals whose antennae were dissected for RNA extraction. We therefore normalized each allele’s expression in a given F2 pool by its frequency among the individuals that made up that pool to generate final estimates of allele-specific expression. We used a two-way ANOVA to test for significant differences between the estimates derived from human-prefering versus guinea-pig-prefering pools and among the estimates for individual alleles.

Statistical analysis. All statistical analyses were performed with JMP software v. 8 (SAS Institute, Inc.) or R software v.2.15.0 (http://www.r-project.org/). For all parametric tests, including t-tests and analyses of variance, data were tested and met the assumptions of normality and homogeneity of variance.
Extended Data Figure 1 | Measuring colour and scaling of adult female *Ae. aegypti* mosquitoes. **a**, Representative photograph used to measure scale colour (Fig. 1e, g). Red dots mark the approximate position of 4 points where the colour of dark scales on the scutum was assessed. **b**, Representative photograph used to measure cuticle colour (Fig. 1f, h). Red dots mark the approximate position of 4 points where the colour of bare cuticle on the circular postnotum was assessed. **c**, Representative photographs used to assess the extent of white scaling on the first abdominal tergite (Fig. 1i), outlined with the red rectangle. Each individual is representative of the scaling score shown at the bottom.
Extended Data Figure 2 | Or4 coding sequence variation in human-preferring and guinea-pig-preferring colonies from around the world.

a, Geographical origin of colonies characterized in b and c. Circle fill colour indicates preference of strains. Circle outline colour indicates origin: Purple, laboratory strain derived from USA; blue, reference genome strain derived from West Africa; orange, Uganda; red, Kenya, green, Thailand. b, Host preference assayed in the live host olfactometer. Data for Thailand, K14, K2, K4, K27, K18, K19, and Uganda are reprinted from Fig. 2g. c, Frequency of non-synonymous single nucleotide polymorphisms (SNPs) in female antennal RNA-seq reads. SNPs are defined as differences from the A reference allele. SNPs with frequency \( \leq 0.1 \) are not shown. Vertical black and red lines indicate SNPs that were present and absent, respectively, in the major alleles subject to functional analysis.
Extended Data Figure 3 | Amino acid differences of major Or4 protein alleles. Dots represent amino acid differences with respect to the genome reference, not an inferred ancestor. Red dots indicate differences that are unique to the given allele. Blue dots indicate differences that are shared among multiple alleles. Snake plots are based on the predicted orientation and location of transmembrane domains. Extracellular loops are oriented up and cytoplasmic loops are oriented down. Allele names are indicated to the left of each snake plot.
Extended Data Figure 4 | Evidence that Or4 is a single copy gene.
a, Histogram showing the number of alleles represented in the Or4-derived PacBio reads obtained for each of 270 parent and F2 hybrid mosquitoes. Alleles were only considered if they received at least 5% of an individual’s reads. b, Histogram showing the fraction of reads from individual mosquitoes assigned to individual alleles. For all 270 mosquitoes, individual alleles were represented by either very few reads (grey bars, inferred to result from allele or barcode assignment errors or polymerase chain reaction contaminants), approximately half the reads (light blue bars, inferred to represent the two alleles in heterozygotes), or over 98% of all reads (dark blue bars, inferred to represent the single allele carried by homozygotes).
Extended Data Figure 5 | Response of human-preferring mosquitoes to sulcatone-scented guinea-pig odour. a, Olfactometer apparatus in which 50 mosquitoes per trial were given a choice between guinea-pig odour/CO₂ mix supplemented with solvent on one side and sulcatone 10⁻⁴ on the other side. b, Corrected preference for sulcatone vs solvent ports is indicated. Data were corrected for the daily average left-right side bias observed across 2–3 solvent vs solvent tests conducted on each day of testing. An index value of 1 indicates strong preference for the sulcatone side, whereas −1 indicates strong preference for the solvent side. Neither mosquito colony showed a preference significantly different from zero (one-sample $t$-test $P=0.76$ for ORL, $P=0.11$ for K14). The trials for each colony were performed across 4–8 days ($n=40$ for ORL and $n=22$ for K14).