1 **Parallel adaptation to higher temperatures in divergent clades of the nematode**
2 ***Pristionchus pacificus***

3

4

5 *Mark Leaver*¹, *Merve Kayhan*^{1,2}, *Angela McGaughran*^{3,4}, *Christian Rodelsperger*³,
6 *Anthony A. Hyman*^{1*}, *Ralf J. Sommer*^{3*}

7

8

9 1 Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse
10 108, 01307, Dresden, Germany.

11 2 Bilkent University, Department of Molecular Biology and Genetics, SB
12 Building, 06800 Ankara, Turkey.

13 3 Max Planck Institute for Developmental Biology, Department of Evolutionary
14 Biology, Spemannstraße 37, 72076, Tübingen, Germany.

15 4 Australian National University, Research School of Biology, Division of Evolution,
16 Ecology and Genetics, Canberra, ACT, 2601, Australia.

17 * Corresponding authors

18 Key words: Parallelism, reversion, adaptation, natural selection, temperature,
19 *Pristionchus pacificus*

20 **Abstract**

21 Studying the effect of temperature on fertility is particularly important in the light of
 22 ongoing climate change. We need to know if organisms can adapt to higher
 23 temperatures and, if so, what are the evolutionary mechanisms behind such
 24 adaptation. Such studies have been hampered by the lack different populations of
 25 sufficient sizes with which to relate the phenotype of temperature tolerance to the
 26 underlying genotypes. Here, we examined temperature adaptation in populations of
 27 the nematode *Pristionchus pacificus*, in which individual strains are able to
 28 successfully reproduce at 30°C. Analysis of the frequency of heat tolerant strains in
 29 different temperature zones on La Réunion supports that this trait is subject to natural
 30 selection. Reconstruction of ancestral states along the phylogeny of highly
 31 differentiated *P. pacificus* clades suggests that heat tolerance evolved multiple times
 32 independently. This is further supported by genome wide association studies showing
 33 that heat tolerance is a polygenic trait and that different loci are used by individual *P.*
 34 *pacificus* clades to develop heat tolerance. More precisely, analysis of allele
 35 frequencies indicated that most genetic markers that are associated with heat tolerance
 36 are only polymorphic in individual clades. While in some *P. pacificus* clades, parallel
 37 evolution of heat tolerance can be explained by ancestral polymorphism or by gene
 38 flow across clades, we observe at least one clearly distinct and independent scenario
 39 where heat tolerance emerged by *de novo* mutation. Thus, temperature tolerance
 40 evolved at least two times independently in the evolutionary history of this species.
 41 Our data suggest that studies of wild populations of *P. pacificus* will reveal distinct
 42 cellular mechanisms driving temperature adaptation.

43 Background

44 A fundamental question in biology is what are the evolutionary mechanisms that drive
 45 the adaptation of organisms to the environmental conditions of their niche. Studying
 46 the evolution of natural populations has shown that common solutions are adopted
 47 during adaptation to similar niches. For example, populations of threespine
 48 sticklebacks have been shown to independently acquire the same phenotype during
 49 repeated adaptation to fresh-water habitats [1,2]. Similarly, during the adaptive
 50 radiation of cichlid fish in the lakes of East Africa, there have been examples of
 51 parallel [3] and convergent evolution, giving rise to striking examples of species
 52 adopting similar body-forms while adapting to the same ecological niche [4]. The
 53 environment, therefore, can be a selective factor that drives the evolution of new
 54 species [5].

55 An important environmental variable is temperature, which fluctuates both on daily
 56 and yearly cycles. Although some information is available about how temperature
 57 affects fitness, especially in ectotherms (cold-blooded organism) [6-8], little is known
 58 about if natural populations adapt to temperature. Furthermore, little is known about
 59 the evolutionary mechanisms behind their adaptation or about the genes that influence
 60 it. Studying adaptation to temperature is particularly important in the light of
 61 increasing evidence for climate change [9].

62 Nematodes, such as *Caenorhabditis elegans*, are excellent model organisms to study
 63 the effect of temperature on the fitness of ectotherms. Temperature is known to affect
 64 their fertility [10,11] and that of its sister species, *C. briggsae* [12]. There is also
 65 evidence that *C. elegans* and *C. briggsae* have adapted to different temperature
 66 niches, with *C. elegans* being more prevalent in temperate regions and *C. briggsae*
 67 being more prevalent in the tropics [12]. However, to understand the dynamics of this
 68 adaptation and its genetic basis, we need to study this in wild isolates of the same
 69 species. Another nematode that has been developed as a model organism for studying
 70 evolutionary biology is *Pristionchus pacificus* [13]. *P. pacificus* is associated with
 71 beetles, with which it has a necromenic relationship. Specifically, it can be found on
 72 beetles in a dormant state called the dauer stage [14,15] and, once the beetle has died,
 73 nematodes re-enter the reproductive cycle and feed on the bacteria growing on the
 74 decomposing body of the beetle [16]. *P. pacificus* is associated with scarab beetles

and stag beetles, and has undergone host switching to utilize different beetle hosts found in novel habitats. This association has enabled the collection of large numbers of natural isolates of *P. pacificus* [14]. As of today, more than 1000 wild isolates of *P. pacificus* have been obtained from around the world [17].

The Indian Ocean island of La Réunion is particularly important for understanding adaptation of *P. pacificus* to different temperature niches, because the island experiences a range of temperatures to which diverse populations of nematodes could have adapted. La Réunion has been colonized many times independently by representatives of all major clades of *P. pacificus* [14,18,19]. These clades of *P. pacificus* (formerly designated A-D) are distinguished by mitochondrial markers [18]. However, analysis of whole genome sequencing data has revealed that these mitochondrial clades actually represent at least six genome-wide clades [20], of which four are predominantly found on La Réunion (A2, B, C, D). The colonization by clade C of La Réunion was most likely achieved via the ancestor of the scarab beetle *Oryctes borbonicus*. Subsequently, clade C strains have spread across most of the western part of the island [14]. *O. borbonicus* is endemic to La Réunion as is *P. pacificus* clade C, suggesting a long history of co-evolution between nematode and beetle [21]. Other beetle species have been introduced to the island more recently. For example, *Maladera affinis* was introduced between 800-1800 [22] and is currently host to clade A strains [14]. Competition between recently arrived and well-established beetle hosts has presumably limited nematode dispersal, leading to distinct, occasionally overlapping populations distributed across the island [23]. Clade B strains are unique to the island and form an isolated population at high altitudes, where they are found in association with the beetle *Amneidus godefroyi* [14,24]. Since colonizing the island, individual clades of *P. pacificus* continue to accumulate genetic and phenotypic variations as they adapt to local conditions [23]. This has led to a high degree of heterogeneity and population structure, particularly in clade C [18]. While strains of different clades differ in about 1% of their sequence, strains of the same clade exhibit lower levels of nucleotide diversity (about 0.1%) comparable to the global diversity of *C. elegans* [20]. Similarly, while recombination frequently occurs within clades, admixture between different clades is rarely observed [18]. Together with evidence for intraspecific competition, this suggests a process of incipient speciation in *P. pacificus* [25,26].

We have previously shown that high temperature differentially affects fertility among *P. pacificus* natural isolates [27]. An isolate from California can no longer give rise to fertile offspring at 30°C, while one from Japan remains fertile at that temperature. The phenotype of the Japanese strain is designated as high temperature tolerant or Htt. We have shown that a single locus on chromosome V in the Japanese strain is responsible for the Htt phenotype and that there is some variation in the phenotype among natural isolates from world-wide locations [27]. However, it is not yet known if this phenotype is subject to natural selection in wild populations. As a volcanic island, La Réunion encompasses a range of ecotypes formed by its diverse geology, biodiversity and weather [23]. More importantly, the island ranges from warm coastal regions that experience temperatures up to 36°C to the cooler slopes of the volcano that experience temperatures up to 22°C. This model system offers the perfect opportunity to test if the Htt phenotype is subject to natural selection in populations of nematodes and, if so, to investigate the evolutionary mechanisms behind such adaptation. Furthermore, we can combine this evolutionary approach with a genetic one to investigate which loci are associated with temperature adaptation.

Here, we assay the temperature tolerance of 289 strains of *P. pacificus*, collected from different sites on the island experiencing a range of temperatures. We show that temperature acts as a selective factor on natural populations of nematodes and further show that high temperature adaptation has evolved at least twice in an example of parallel evolution.

Results

Populations of nematodes on La Réunion are subject to natural selection

We define the Htt phenotype as the ability to give rise to fertile offspring at 30°C. To test if this phenotype is subject to natural selection, we investigated the occurrence of the Htt phenotype in a large collection of natural isolates from La Réunion. A simple test for this is to look for a correlation between the frequency of the trait and the selective factor [28], in this case altitude as a proxy for temperature (Sup. Fig. 1A). We therefore predicted that strains that were isolated at higher altitudes (lower temperatures) should be less likely to be Htt than strains that were collected at lower altitude locations (higher temperatures). To test this, we utilized the existing collection of natural isolates of *P. pacificus* from La Réunion, collected between 2008 and 2011, that are known to represent different genetic populations [18,25]. As there were relatively few strains collected from below 500 m because of sheer cliffs, urban areas and sugar cane farming at lower altitudes, we identified new locations with relatively un-spoilt habitat and collected additional strains between 2012 and 2015 (Sup. Fig. 1B and see methods). Collecting at these sites gave enough *P. pacificus* isolates to complete sampling from sea level (covering a range of maximum daily temperatures from 22-36°C). These strains cover the diversity of genomic clades that are found on the island [25] and are from geographically separated populations that may have adapted to local conditions.

For phenotyping, individual J3 larvae maintained at 20°C were shifted to 30°C then, after seven days of incubation, worms were inspected for their ability to give rise to fertile offspring. Strains were scored either as Htt if they gave fertile offspring or as Ltt if they were infertile or only gave rise to infertile offspring (see methods for details). In total, 21.5% of the 289 strains were Htt (Sup. Table 1). As predicted, low altitude locations tended to have more Htt strains, for example, 76.7% (n=30) of strains from Saint Benoit (SB, 22 m altitude) were Htt (Sup. Table 1). In contrast, no strains from locations above 1000 m were Htt (n=73). To test for a correlation, the occurrence of the Htt phenotype was plotted by altitude (Fig. 1). The percentage of strains giving rise to fertile offspring at 30°C was as high as 77% at the lowest altitude bin and only 6.9% for the bin centered at 1075 m (Fig. 1). A subset of these data fell onto a straight line ($R^2=0.730$), showing a correlation between altitude and high

temperature tolerance. Analysis of variance (Anova, see methods) showed that the correlation between the occurrence of the phenotype and the altitude at which any strain was collected was highly significant ($p\text{-value}=2.2\times 10^{-16}$). These findings show that the occurrence of the Htt phenotype is related to environmental temperature and is therefore likely to be subject to natural selection.

Strains isolated from beetles found at lower altitudes are more likely to be Htt

The beetle species from which nematodes were isolated have different habitat ranges corresponding to their preferred altitudes, as quantified in Sup. Fig. 2. For example, *A. godefroyi* is found only at higher altitudes [24] while other species, including *M. affinis*, prefer lower altitudes [22]. The occurrence of Htt strains isolated from different beetle species should differ depending on the temperatures of the beetle's habitat. Indeed, Htt strains were found more frequently on *M. affinis* and *Aphodius sublividus* (found at low altitudes), but were not found at all on *A. godefroyi* and *O. borbonicus* (found at higher altitudes, inset Fig. 2). Anova also showed that the percentage of Htt strains found on different species of beetles deviated from a random pattern ($p\text{-value}=2.5\times 10^{-11}$).

To eliminate any influence that different beetle hosts may have on the occurrence of Htt strains, we plotted the data of strains isolated from different beetles separately (Fig. 2). The largest number of nematode strains was isolated from *Adoretus sp.* ($n=181/289$), which in contrast to other beetles, is found at a range of altitudes. Indeed, *Adoretus*-derived strains show a linear correlation between altitude and percentage of Htt strains and the correlation between altitude and percentage of Htt strains remains, although weaker than that seen in the whole dataset ($R^2=0.457$, compare Figures 1 and 2). From this it can be concluded that different beetle species carry different frequencies of Htt strains, and that, in the one beetle species found at a range of altitudes (thus, for which the effect of beetle can be factored out), the correlation between altitude and Htt remains.

Strains that belong to clade A are more likely to be Htt

Next, we considered the evolutionary relationship between strains of *P. pacificus* as another potential influencing factor on the frequency of Htt strains. For example,

strains belonging to clade B might respond differently to temperature because they form an isolated population [24] that might be susceptible to the founder effect [29]. Therefore, the percentage of Htt strains belonging to each of the four mitochondrial clades was calculated. Figure 3 shows that clades found at high altitudes have no Htt strains. In contrast, clade A strains, which are more prevalent at lower altitudes, have a higher percentage of Htt strains. Anova showed that the percentage of high temperature tolerant strains found in different clades deviated from that which would be expected purely by chance ($p\text{-value} = 6.6 \times 10^{-14}$). From this it can be concluded that a strain will be more or less likely to be Htt depending on which clade it belongs to.

Of the three influencing factors (beetle, clade and altitude), the influence of beetle can be eliminated by analysing strains isolated from *Adoretus sp.* (Fig. 2), but the influence of clade and altitude remain to be disentangled. In an attempt to distinguish which variable best explained the occurrence of the trait, we performed model selection analysis using Bayesian Information Criterion (BIC). BIC is a statistical tool that selects from a set of models the one that best explains the observed data using a likelihood function [30]. The BIC analysis showed that altitude explained the variation in frequency of the Htt trait better than beetle or clade (0.99 for altitude, 8.7×10^{-11} for clade and 3.0×10^{-6} for beetle: the largest value indicates the variable that best fits the data, see methods for details). This supports our assertion that temperature is likely to be the main influencing factor on the phenotype, as expected from our phenotype assay.

Phylogeny of world-wide strains of P. pacificus suggests parallel evolution of the Htt phenotype

To investigate the evolution of temperature tolerance in a larger collection of *P. pacificus* strains, we combined the current dataset with strains from world-wide locations that we had analyzed previously [27]. SNPs were identified by comparing whole genome data of these strains. These SNPs were then concatenated and the sequence used to construct a phylogenetic tree by the maximum likelihood method with ultrafast bootstrapping [31,32] (Fig. 4). The world-wide diversity of *P. pacificus* consists of six major clades based on genome-wide markers; clades A1, A2, A3, B, C and D, as well as one divergent strain used to root the tree (Fig. 4), consistent with previous work [20,27,33]. Clades C and D consist largely of strains from locations

spread all over La Réunion and nearby island Mauritius; clade B consists exclusively of strains found on La Réunion at locations higher than 2100 m; and clades A1, A2 and A3 consist of strains from worldwide locations, La Réunion and Mauritius. Clade A3 includes the wild type reference strain, RS2333.

Htt strains are sporadically scattered throughout clade C (Fig. 4) suggesting that there were recent and independent acquisitions of high temperature tolerance in strains from La Réunion and Mauritius [27]. There are also Htt strains in clades A1, A2 and A3, including sub-clades with many Htt strains or single strains from diverse locations (Fig. 4). To test if temperature tolerance has arisen multiple times independently, we inferred the phenotype of ancestral strains using a Bayesian algorithm (Fig. 4 and Sup. Fig. 3). This analysis predicts that the last ancestor of all *P. pacificus* strains was likely to be low temperature tolerant. Furthermore, the ancestor of deeply rooted clade B is predicted to have been low temperature tolerant and the same is true for clades C, D and A1 (Fig. 4). In contrast the ancestor of clade A2 is predicted to have been high temperature tolerant (Fig. 4 and Sup. Fig. 3). This suggests that the Htt phenotype is a recent and derived state and could have arisen *de novo* within clades A1, A2, A3 and C, consistent with parallel evolution. The alternative explanation is that the Htt phenotype arose once and was transferred between clades by recombination during outcrossing.

Reversion of strains back to the Ltt phenotype from an Htt ancestor

A closer examination of strains from clade A2 shows that there are many closely related strains that are Htt and form a “hot” sub-clade (Figs. 4). These strains were collected from SB on La Réunion, which has an altitude of 22 m and experiences a maximum annual temperature of 34°C. Interestingly, there is one example of a reversion in this sub-clade: strain RSC028, which is Ltt, was collected at Grand Etang (GE, 527 m), where temperatures are lower than at SB. There are further examples of reversion in clade A2: for example, the event that gives rise to a “cold” sub-clade (Fig. 4 and Sup. Fig. 3). Interestingly, two strains in this sub-clade, JU138 and RS5264, have regained the Htt phenotype. From this, we can conclude that there are examples of reversion of the Htt trait back to the ancestral state as strains adapt to local conditions.

254 *Htt is a polygenic trait*

255 To better understand the evolution of the Htt phenotype, we wanted to identify SNPs
 256 that are associated with the phenotype so that we could later test if their pattern of
 257 inheritance indicated that they had arisen by *de novo* mutation or by recombination
 258 between clades. Genome wide association studies (GWASs) of model organisms have
 259 been used to identify SNPs linked to ecologically important phenotypes [34],
 260 including pH tolerance in *P. pacificus* [25]. Here, we performed GWAS using the
 261 easyGWAS online platform [35] and selected 223 strains for which whole genome
 262 sequence and temperature tolerance data are available. These strains were a subset of
 263 the La Réunion strains as well as strains from world-wide locations. GWAS was
 264 performed using the EMMAX algorithm to account for population structure and a
 265 minor allele frequency cut-off of 10%. The algorithm identified 32117 SNPs after
 266 filtering and inspection of the Q-Q plots revealed evidence for population
 267 stratification (genomic control, $\lambda=0.55$), which could be eliminated by accounting for
 268 the beetle host as a covariate and removing highly divergent strains from the analysis
 269 ($\lambda=0.99$), although this greatly reduced the number of SNPs (4447). Together, the
 270 EMMAX algorithm and the covariate take into account the two influencing factors we
 271 identified as affecting the occurrence of the trait. The hits from our GWAS were
 272 scattered over all six chromosomes with a total of 12 SNPs that were significantly
 273 associated with the Htt phenotype (Sup. Table 2). These SNPs are either in genes that
 274 affect the phenotype or are linked to the causative mutation. This finding suggests that
 275 SNPs in multiple genes influence temperature tolerance, i.e. that Htt is a polygenic
 276 trait.

277 *Clade-specific GWAS and allele-frequency analysis support independent gains*

278 A study of the genetic causes of hypertension [36] showed that polymorphisms linked
 279 to the disease could be better identified by studying individuals in a subpopulation
 280 where there was an increased occurrence of the condition. The equivalent for our
 281 study would be to independently analyse clades A1 and A2 combined because the
 282 percentage of Htt strains is higher in these clades than in the complete dataset. In
 283 comparison, we considered clade C independently because the occurrence of Htt
 284 strains is lower here. GWAS analysis of these strains revealed that these
 285 subpopulations also had low levels of stratification (with $\lambda=1.00$ and $\lambda=1.16$ for clade

A1+A2 and clade C respectively). GWAS of clade A1 and A2 strains gave one cluster of a few SNPs on chromosome V including one that fell above the significance threshold (Sup. Table 2 and results online). In contrast, GWAS of clade C showed a broad region of chromosome I with several SNPs with similar $-\log(p\text{-level})$ that also fell above the threshold. The finding that the different GWASs identify loci on different parts of the genome is consistent with independent acquisitions of the phenotype in clade C and clades A1/A2.

To further test whether different loci have been used by individual clades to evolve heat tolerance, we investigated patterns of fixation of candidate SNPs that were identified by GWAS. If heat tolerance evolved independently in each clade, then the SNPs that are associated with the phenotype should have alleles that are unique to one clade and not polymorphic in other clades. Indeed, most of the identified SNPs are polymorphic in just a single clade (highlighted Sup. Table 3). For example, ChrI:19341896 is polymorphic in clade C (either a T or an A) but always an A in all other clades. This observation and other clade C SNPs (highlighted in blue in Sup. Table 3) supports a *de novo* mutations specific to clade C. This suggesting that there was an independent acquisition of Htt in clade C, and another in clades A1, A2, or A3. Alternatively, if heat tolerance was a polygenic trait that evolved once, signals that are captured by the GWAS should come from SNPs that are polymorphic in all clades exhibiting heat tolerant strains. Indeed, we do see examples of SNPs that are polymorphic in clades with Htt strains (highlighted in yellow in Table 3), for example ChrV:13634723 is polymorphic in clade A1 and clade C (either a T or a C) but does not vary among strains from the other clades. This suggests that recombination might transfer Htt alleles between clades. We also observe examples of SNPs that are polymorphic in one clade, with both alleles found in other clades that are not polymorphic. For example, ChrI:18417120 is polymorphic in clade A1 (either a G or an A) but not in other clades where it is always either a G or an A. The most likely explanation for this is that such positions were polymorphic in the ancestral population, selected for in Htt strains (in clade A1 in the example), and differentially fixed in the other clades. Together, these findings suggest that heat tolerance evolved separately in some clades by *de novo* mutation and/or evolved in other clades by fixation of ancestral polymorphisms and subsequent spread by recombination between clades.

Discussion

Because of ongoing climate change, there is increased interest in the extent and speed by which natural populations can adapt to higher temperatures and the evolutionary mechanisms by which they do so. Here, by comparing the temperature tolerance of 289 strains from La Réunion, we show that natural populations of *P. pacificus* are indeed subject to natural selection and have gained the ability to remain fertile at 30°C. Because we have estimates of the number of generations it took to generate the diversity of each of the major clades [37,38], we can estimate the amount of time it took for this adaptation to occur. For instance, in clade C, within 5.6×10^5 generations, a low temperature tolerant strain had adapted by 2°C to a high temperature tolerant strain. A generation is likely to range between 3 days and 3 months, giving a time frame for adaptation between approximately 4600 and 140000 years (see methods).

Phylogenetic analysis of *P. pacificus* strains suggested that the Htt phenotype has evolved multiple times independently in the different clades in an example of parallel evolution. Alternatively, Htt could have evolved once and either remained polymorphic during the divergence between clades, or transferred between clades by recombination. However, two previous results suggest that this scenario would occur only rarely. First, analysis of mutation accumulation lines indicates that different clades are separated by millions of generations and it seems unlikely that polymorphisms would be maintained for such long periods [38]. In accordance with this, comparison of nucleotide diversity between clades shows very little (<7%) shared variation [20]. Second, analysis of linkage disequilibrium and population structure shows that, while there is frequent recombination within clades, admixture between different clades seems to be rare [18,20]. In summary, the analysis of the evolution of Htt along the phylogeny of *P. pacificus* is compatible with a scenario where Htt has evolved multiple times independently.

We also show examples of reversion back to low temperature tolerance, which occurs most likely by recombination within clades. The presence of reversions suggests that selection is not strong enough to ensure fixation of the phenotype. Alternatively, selection can occur in the other direction, i.e., there might be some balancing selection acting against fixation. Documented examples of reversion in natural populations are

rare and could only be observed in this case because of the large collection of phenotyped strains and a detailed and accurate phylogeny.

GWAS identified SNPs associated with the Htt phenotype that were scattered across the chromosome, suggesting that different mutations can lead to the same phenotype, possibly by affecting different genes in the same pathway that regulates high temperature tolerance. The GWAS of all strains in our study gave 12 SNPs that were significantly associated with the phenotype. This represents SNPs from the world-wide diversity of *P. pacificus* that are either in genes or regulatory regions that directly affect the phenotype, or that are linked to the causative mutation. The GWAS of strains from clades A1 and A2 gave only one SNP associated with the phenotype. This SNP, intragenic between *gst-24* (glutathione S-transferase) and *cyp-29A4* (cytochrome P450, Sup. Table 4) was also found in the GWAS of all clades, showing that the large number of Htt strains in clade A could give a signal strong enough to be statistically significant in the whole dataset. Interestingly, this SNP is on chromosome V and is close to the region that we identified in pair-wise mapping of the Htt phenotype in a clade A1 Japanese strain (RS5194) [27]. The GWAS of clade C strains gave SNPs with significant scores mostly on chromosome I, but also on II and V. The 23 hits on chromosome I have the same or similar $-\log(\text{p-value})$ and form a cluster of SNPs adjacent to each other. This resembles a linkage group and might represent an example of hitchhiking, where one or a few SNPs which cause the phenotype are selected for and carry with them the linked SNPs by recombination [39]. Such a scenario would be expected for a trait that is passed between individuals by sexual reproduction. The observation that GWAS of strains from different clades identified different SNPs is again consistent with parallel evolution of the phenotype in clade C and clades A1/A2.

In an attempt to further investigate the evolution of the Htt phenotype, we measured allele frequencies for the GWAS hits. This showed that most of the identified SNPs were polymorphic in just a single clade, which is consistent with different loci being employed by individual *P. pacificus* clades to become high temperature tolerant. Alternatively, in theory an ancestral population that was polymorphic at all Htt loci could have evolved once. Subsequently, Htt could have been lost in individual clades by fixation or could have been regained by recombination across clades. This alternative scenario is consistent with the finding that for most significant SNPs, both

alleles are fixed in at least one other clade. However, even if this alternative scenario holds true for most Htt loci, we observe one clearly distinct and therefore independent pattern in clade C. All SNPs on chromosome I with significant association to Htt in the clade C specific GWAS are polymorphic only in clade C and only one of the alleles is present in all other clades. This indicates that clade C specific alleles in this region represent recently derived alleles that evolved *de novo* and therefore could not have been transferred by recombination across clades. Thus, we conclude that Htt evolved at least two times independently during the evolutionary history of *P. pacificus*.

Based on our findings and existing information about populations of *P. pacificus* on La Réunion, we can suggest a plausible series of events leading up to the current distribution of strains on the island. The common ancestor of clade C was most likely low temperature tolerant and, during diversification on the island, strains from that clade have gained the Htt phenotype at locations where temperatures are higher (SB, ES and CO, Fig. 4). The opposite seems to be the case for clade A, whose ancestral population had most likely already evolved Htt (Fig. 4 and sup. Fig. 3) making it ideally suited to fill the high temperature niche that is now SB, forming the hot sub-clade in clade A2. Strong selective pressure at this location meant that descendants of these strains kept the ability to survive at high temperatures. The ancestor of strain RSC028 was part of this Htt population at SB and was likely transported to GE (527 m) where temperatures are lower, was subjected to lower selective pressure or balancing selection, and then reverted to Ltt.

The fact that there are many SNPs associated with the phenotype suggests that the phenotype is polygenic with many loci influencing the phenotype. We therefore conclude that temperature tolerance is a complex trait, possibly a threshold trait. Threshold traits have an underlying continuous nature with a threshold above which the trait manifests itself [40]. The Htt phenotype fits this definition because each strain could have an upper temperature limit that falls onto a continuous scale and our definition of Htt involves a threshold of 30°C. Future work could confirm this by precisely measuring the upper temperature limit of strains which have upper temperature limits between those of Htt and Ltt strains. Adaptation to temperature has previously been reported for *Arabidopsis thaliana* [41] and *Drosophila melanogaster* [42]. There have also been several attempts to map loci responsible for

the temperature dependence of fertility related traits in *C. elegans* [43-46]. These studies could only identify large genomic regions containing many candidate genes because of the low marker densities used. Also, these studies utilized recombinant inbred lines made between lab adapted strains of *C. elegans*, which would mean that the ecological relevance of these phenotypes is limited [47].

We do not yet know the underlying cell biology mechanisms behind temperature adaptation. However, the ease by which the cell biology can be studied in these organisms suggests that it will be possible to directly relate adaptation to a temperature niche to the underlying cell biology mechanisms in the near future.

Methods

Collection of natural isolates of P. pacificus

Strains of *P. pacificus* had previously been collected by the Sommer lab (prefix RS), the Sternberg lab (prefix PS) and Félix lab (JU) from world-wide locations including many from La Réunion. To increase the number of strains for analysis, more strains were collected from La Réunion. See [16] for details of collection methods. Beetles were dissected along the midline and placed onto NGM plates seeded with *Escherichia coli* OP50. After three days of incubation, plates were inspected for gravid hermaphrodites using a dissecting microscope. The SSU gene was sequenced to confirm strains as *P. pacificus*.

Selection of sampling sites for temperature transect

Preliminary analysis set of 149 strains collected from La Réunion by the Sommer lab from 2008-2010 showed that one location had a high percentage of Htt strains. However, this dataset was lacking sufficient locations from 28-700m to properly test for a correlation between altitude and the occurrence of Htt strains. We identified locations around Plane d' Affourches (PD5-PD7) and Saint Phillippe (SP) where *Adoretus* sp. could be found. Sampling over the subsequent years (2011-2015) collected enough beetles to give nematodes from locations covering the full range of altitudes.

Strain maintenance

Worms were propagated on OP50/NGM plates according to standard methods for *C. elegans* [48]. In order to minimise epigenetic effects, all worms were kept at 20°C for at least two generations on abundant food before temperature experiments were performed. The incubators used for routine maintenance were Heraeus BK 6160 incubators (Thermo, accuracy $\pm 0.2^\circ\text{C}$).

High temperature tolerance assay

Carefully staged J3 worms from mixed stage plates incubated at 20°C were transferred singly to NGM plates, which were then shifted to 30°C. After seven days of incubation, the plates were inspected using a dissecting microscope and scored for

their ability of the founder worm to give rise to fertile offspring. A strain was designated as Htt if the founder worm gave rise to two generations of offspring (the F2). A strain was designated Ltt if the founder was: killed by heat; arrested during development; developed to adulthood but was sterile; or gave rise only to F1 that were infertile. Three to 18 worms were tested and the mean number of individuals tested was five. For large-scale application of the Htt assay, a custom-made 30°C room (stability $\pm 0.5^\circ\text{C}$) was used to incubate the strains at the test temperature. Any strain that did not give reproducible results was eliminated from the final dataset, giving a final dataset of 289 strains.

Statistical analysis

Statistical analysis was performed with RStudio software running R version 0.99.896 [49]. The data of the occurrence of Htt for all strains was fit with a generalised linear model (binomial distribution) using the glm command. The three variables for each strain were: the clade it belonged to; the altitude of its collections site; and the beetle species the strain was isolated from. See supplementary table 5 for the full data. Analysis of variance was performed using the anova command on the generalised linear model with the chi-square significance test. Bayesian Information Criterion analysis was performed using the bic command. The generalised linear model was fit to each variable independently and the output given as a weighted difference where the model that “best fits” the observed data has a value closest to 1. The results for the three models were 0.99 for altitude, 8.7×10^{-11} for clade and 3.0×10^{-6} for beetle.

GWAS

GWAS was performed on the easyGWAS cloud server [35], where genomic sequence data and phenotype data were uploaded. The site allows for gene annotation to be automatically linked to the sequence that was uploaded. The beetle species that each strain was isolated from was included as a covariate, transformed as a dummy variable. A minor allele frequency filter was set to 10% and the EMMAX algorithm selected. Detailed, interactive results are available at <https://easygwas.ethz.ch/data/public/datasets/>.

Data analysis for determining the altitudinal range for beetle species

For each sampling site visited, the number of beetles of each species collected was routinely noted for years 2010-2015. Also, the GPS coordinates and altitude of each site was noted. We define the altitudinal range for a beetle as being the range of altitudes where 95% of beetles were found and mean altitude of the collection site for all beetles of one species was also calculated.

Construction of a phylogenetic tree

SNP data was extracted using a custom pipeline implemented in Perl and Unix as previously described [27]. Whole genome data for the stains used in the study was already available [25]. Variant bases were identified and the genotype of all strains at all variant positions was extracted from the genome sequence covered by all the contigs in *P. pacificus* draft genome hybrid assembly 1 [50]. 873932 SNPs were selected and concatenated. This sequence was used to construct a maximum likelihood tree using IQ-Tree [31]. The substitution rate model GTR+ASC was applied (where ASC corresponds to use of a SNP ascertainment bias model) and the tree was bootstrapped 1000 times using the ultrafast bootstrapping method [32].

Inferred ancestral states

Bayesian analysis was performed using the ace (ancestral character estimation) command [51] implemented in the R phytools package [52]. A single rate model was applied and probabilities for either phenotype for each ancestor was calculated. The data was plotted with the R ggtree package with the probabilities at the internal nodes depicted as pie charts.

Calculation of timeframe for adaptation

One generation is estimated to be between 3 days, being the time to develop from egg to fertile adult at 25°C, to 3 months, being the maximum time a dauer can survive. The number of generations it took to generate the diversity in clade C is estimated to be 5.6×10^5 [37,38]. If one generation is 3 days, then 5.6×10^5 generations = $3 \times 5.6 \times 10^5$ days or 1.68×10^6 days. 365 days per year, then 1.68×10^6 days = 4602 years. A similar logic gives the upper limit at 140000 years for a generation time of 3 months.

514

515 **Acknowledgments**

516 We would like to thank: Jacques Rochat (Insectarium de Le Réunion), Matthias
 517 Hermann, Katie Morgan, Eduardo Moreno, and Jan Mayer for help sampling; Holger
 518 Brandl, Cameron Weadick, and Neel Prabh for help with statistical analysis; Waltraud
 519 Röseler and Andrea Zinke for strain maintenance; Heike Haussmann for freezing
 520 strains; Dominik Grimm for advice performing GWAS. M.L. was supported by an
 521 EMBO long-term fellowship (grant number ALTF:434- 2010).

522 **Author contributions**

523 ML and MK phenotyped strains. ML, AM and CR constructed the phylogenetic tree.
 524 ML and AM performed the GWAS. CR measured allele frequencies. ML, AM, CR,
 525 AAH and RJS conceived the study and wrote the paper.

526 **Competing interests**

527 The authors declare no competing interests

528 **Abbreviations**

529 Htt high temperature tolerant; GWAS genome wide association study; Anova analysis
 530 of variance; GE Grand Etang; SB Saint Benoit; SNPs single nucleotide
 531 polymorphisms

532

Figure 1. The occurrence of the Htt strains decreases with increasing altitude of their collection site.

The correlation between altitude and the occurrence of Htt strains. Strains were ranked by the altitude of their collection site then grouped into 10 bins. The percentage of high temperature tolerant strains in each bin was calculated and plotted against the average altitude of the sample site of all strains in that bin. The black line is a fit for the data between 0 and 1000 m. The red line is the altitude (867 m) where the percentage of Htt strains tends to zero as extrapolated from the fit.

Figure 2. The correlation between the percentage of Htt strains and altitude remains after accounting for the beetle host. Inset: the percentage of nematodes that are high temperature tolerant for strains isolated from each beetle species. Main panel, the percentage of Htt strains isolated from each beetle plotted against the mean altitude of their collection site. For nematodes isolated from *Adoretus sp.*, strains are grouped into bins and each bin plotted separately and fit with a straight line (dashed line). The solid line is the fit for the data as plotted in Fig.1.

Figure 3. The occurrence of Htt phenotype in strains that belong to different clades. Inset: the percentage of strains that are Htt belonging to each clade. Main panel: the percentage of Htt strains belonging to each clade is plotted against the mean altitude of their collection site. Mitochondrial clades A, B, C, D and strains of unknown clade were grouped into bins and each bin plotted separately.

Figure 4. Phylogenetic tree of *P. pacificus* strains shows one clade highly enriched with Htt strains. A maximum likelihood tree based on the sequence of 873932 concatenated SNPs across the whole genome for 212 strains. The phenotype of each strain is indicated by a circle at the terminal node of the tree (red = Htt, blue = Ltt). The sample site is indicated by a coloured square. For the nodes of the major clades, the degree of bootstrap support and the inferred phenotype is indicated by pie charts at the internal nodes.

References

1. Jones, F. C. et al. 2012 A Genome-wide SNP Genotyping Array Reveals Patterns of Global and Repeated Species-Pair Divergence in Sticklebacks. *Curr Biol* **22**, 83–90. (doi:10.1016/j.cub.2011.11.045)
2. Colosimo, P. F. et al. 2005 Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. *Science* **307**, 1928–1933. (doi:10.1126/science.1107239)
3. Elmer, K. R., Kusche, H., Lehtonen, T. K. & Meyer, A. 2010 Local variation and parallel evolution: morphological and genetic diversity across a species complex of neotropical crater lake cichlid fishes. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**, 1763–1782. (doi:10.1098/rstb.2009.0271)
4. Kocher, T. D., Conroy, J. A., McKaye, K. R. & Stauffer, J. R. 1993 Similar morphologies of cichlid fish in Lakes Tanganyika and Malawi are due to convergence. *Mol. Phylogenet. Evol.* **2**, 158–165. (doi:10.1006/mpev.1993.1016)
5. Schluter, D. 2001 Ecology and the origin of species. *Trends Ecol Evol* **16**, 372–380.
6. Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C. & Martin, P. R. 2008 Impacts of climate warming on terrestrial ectotherms across latitude. *Proc Natl Acad Sci USA* **105**, 6668–6672. (doi:10.1073/pnas.0709472105)
7. Huey, R. B. & Stevenson, R. D. 1979 Integrating thermal physiology and ecology of ectotherms: a discussion of approaches. *Amer. Zool.* **19**, 357–366. (doi:10.1007/BF00349191)
8. Hoffmann, A. A., Sørensen, J. G. & Loeschcke, V. 2003 Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology* **28**, 175–216. (doi:10.1016/S0306-4565(02)00057-8)
9. Alexander, L. A., Allen, S. K., Bindhoff, N. L., Bréon, F. M. & Church, J. A. 2013 *IPCC, 2013: Summary for Policymakers. In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.* Cambridge University Press.
10. Begasse, M. L., Leaver, M., Vazquez, F., Grill, S. W. & Hyman, A. A. 2015 Temperature Dependence of Cell Division Timing Accounts for a Shift in the Thermal Limits of *C. elegans* and *C. briggsae*. *CellReports* **10**, 647–653. (doi:10.1016/j.celrep.2015.01.006)
11. Petrella, L. N. 2014 Natural Variants of *C. elegans* Demonstrate Defects in Both Sperm Function and Oogenesis at Elevated Temperatures. *PLoS ONE* **9**,

- 601 e112377–13. (doi:10.1371/journal.pone.0112377)
- 602 12. Prasad, A., Croydon-Sugarman, M. J. F., Murray, R. L. & Cutter, A. D. 2010
603 Temperature-dependent fecundity associates with latitude in *Caenorhabditis*
604 *briggsae*. *Evolution* **65**, 52–63. (doi:10.1111/j.1558-5646.2010.01110.x)
- 605 13. Sommer, R. J. & McGaughran, A. 2013 The nematode *Pristionchus pacificus*
606 as a model system for integrative studies in evolutionary biology. *Mol Ecol* **22**,
607 2380–2393. (doi:10.1111/mec.12286)
- 608 14. Herrmann, M., Kienle, S., ROCHAT, J., Mayer, W. E. & Sommer, R. J. 2010
609 Haplotype diversity of the nematode *Pristionchus pacificus* on Réunion in the
610 Indian Ocean suggests multiple independent invasions. *Biological Journal of*
611 *the Linnean Society*, 170–179.
- 612 15. Ragsdale, E. J., Kanzaki, N. & Herrmann, M. 2015 Taxonomy and natural
613 history: the genus *Pristionchus*. In *Pristionchus pacificus A Nematode Model*
614 *for Comparative and Evolutionary Biology* (ed R. J. Sommer), Brill,
615 Leiden/Boston.
- 616 16. Herrmann, M., Mayer, W. E. & Sommer, R. J. 2006 Nematodes of the genus
617 *Pristionchus* are closely associated with scarab beetles and the Colorado potato
618 beetle in Western Europe. *Zoology (Jena)* **109**, 96–108.
619 (doi:10.1016/j.zool.2006.03.001)
- 620 17. Herrmann, M., Neuner, J., Voetsch, M., Wörner, J., Kanzaki, N. & Sommer, R.
621 J. 2015 *Pristionchus* Scratchpads--an online platform for taxonomy,
622 systematics and phylogeny. *Zootaxa* **3949**, 597–600.
623 (doi:10.11646/zootaxa.3949.4.10)
- 624 18. Morgan, K., McGaughran, A., VILLATE, L., Herrmann, M., Witte, H.,
625 BARTELMES, G., ROCHAT, J. & Sommer, R. J. 2012 Multi locus analysis of
626 *Pristionchus pacificus* on La Réunion Island reveals an evolutionary history
627 shaped by multiple introductions, constrained dispersal events and rare out-
628 crossing. *Mol Ecol* **21**, 250–266. (doi:10.1111/j.1365-294X.2011.05382.x)
- 629 19. McGaughran, A., Morgan, K. & Sommer, R. J. 2013 Unraveling the
630 evolutionary history of the nematode *Pristionchus pacificus*: from lineage
631 diversification to island colonization. *Ecol Evol* **3**, 667–675.
632 (doi:10.1002/ece3.495)
- 633 20. Rödelberger, C., Weller, A. M., Sommer, R. J., Witte, H. & Mayer, W. E.
634 2014 Characterization of Genetic Diversity in the Nematode *Pristionchus*
635 *pacificus* from Population-Scale Resequencing Data. *Genetics* **196**, 1153–1165.
636 (doi:10.1534/genetics.113.159855/-/DC1)
- 637 21. Meyer, J. M., Markov, G. V., Baskaran, P., Herrmann, M., Sommer, R. J. &
638 Rödelberger, C. 2016 Draft Genome of the Scarab Beetle *Oryctes borbonicus*
639 on La Réunion Island. *Genome Biol Evol* **8**, 2093–2105.
640 (doi:10.1093/gbe/evw133)
- 641 22. Ahrens, D. 2003 *Maladera affinis* (Blanchard, 1850) comb. n. (Coleoptera,

- 642 Scarabaeoidea, Sericini), an oriental faunal element in the Malagasy region.
643 *Deutsche Entomologische Zeitschrift* **50**, 133–142.
644 (doi:10.1002/mmnd.20030500113)
- 645 23. McGaughran, A. & Morgan, K. 2015 Population genetics and the La Réunion
646 case study. In *Pristionchus pacificus A Nematode Model for Comparative and*
647 *Evolutionary Biology* (ed R. J. Sommer), pp. 197–219.
- 648 24. Moreno, E., McGaughran, A., Rödelberger, C., Zimmer, M. & Sommer, R. J.
649 2016 Oxygen-induced social behaviours in *Pristionchus pacificus* have a
650 distinct evolutionary history and genetic regulation from *Caenorhabditis*
651 *elegans*. *Proc Biol Sci* **283**. (doi:10.1098/rspb.2015.2263)
- 652 25. McGaughran, A. et al. 2016 Genomic Profiles of Diversification and Genotype-
653 Phenotype Association in Island Nematode Lineages. *Molecular Biology and*
654 *Evolution* **33**, 2257–2272. (doi:10.1093/molbev/msw093)
- 655 26. Mayer, M. G., Rödelberger, C., Witte, H., Riebesell, M. & Sommer, R. J.
656 2015 The Orphan Gene dauerless Regulates Dauer Development and
657 Intraspecific Competition in Nematodes by Copy Number Variation. *PLoS*
658 *Genet* **11**, e1005146–20. (doi:10.1371/journal.pgen.1005146)
- 659 27. Leaver, M., Kienle, S., Begasse, M. L., Sommer, R. J. & Hyman, A. A. 2016 A
660 locus in *Pristionchus pacificus* that is responsible for the ability to give rise to
661 fertile offspring at higher temperatures. *Biology Open* **5**, 1111–1117.
662 (doi:10.1242/bio.018127)
- 663 28. Endler, J. A. 1986 *Natural Selection in the Wild*. Princeton University Press.
- 664 29. Mayr, E. 1942 *Systematics and the Origin of Species, from the Viewpoint of a*
665 *Zoologist*. Harvard University Press.
- 666 30. Burnham, K. P. 2004 Multimodel Inference: Understanding AIC and BIC in
667 Model Selection. *Sociological Methods & Research* **33**, 261–304.
668 (doi:10.1177/0049124104268644)
- 669 31. Nguyen, L.-T., Schmidt, H. A., Haeseler, von, A. & Minh, B. Q. 2015 IQ-
670 TREE: a fast and effective stochastic algorithm for estimating maximum-
671 likelihood phylogenies. *Molecular Biology and Evolution* **32**, 268–274.
672 (doi:10.1093/molbev/msu300)
- 673 32. Minh, B. Q., Nguyen, M. A. T. & Haeseler, von, A. 2013 Ultrafast
674 approximation for phylogenetic bootstrap. *Molecular Biology and Evolution*
675 **30**, 1188–1195. (doi:10.1093/molbev/mst024)
- 676 33. Baskaran, P. & Rödelberger, C. 2015 Microevolution of Duplications and
677 Deletions and Their Impact on Gene Expression in the Nematode *Pristionchus*
678 *pacificus*. *PLoS ONE* **10**, e0131136–18. (doi:10.1371/journal.pone.0131136)
- 679 34. Fournier-Level, A., Korte, A., Cooper, M. D., Nordborg, M., Schmitt, J. &
680 Wilczek, A. M. 2011 A Map of Local Adaptation in *Arabidopsis thaliana*.
681 *Science* **334**, 86–89. (doi:10.1126/science.1209271)

- 682 35. Grimm, D., Greshake, B., Kleeberger, S., Lippert, C., Stegle, O., Schölkopf, B.,
683 Weigel, D. & Borgwardt, K. 2012 easyGWAS: An integrated interspecies
684 platform for performing genome-wide association studies. *arXiv*. **q-bio.GN**.
- 685 36. Adeyemo, A. et al. 2009 A genome-wide association study of hypertension and
686 blood pressure in african americans. *PLoS Genet* **5**, e1000564–11.
687 (doi:10.1371/journal.pgen.1000564)
- 688 37. Molnar, R. I., BARTELMES, G., Dinkelacker, I., Witte, H. & Sommer, R. J.
689 2011 Mutation rates and intraspecific divergence of the mitochondrial genome
690 of *Pristionchus pacificus*. *Molecular Biology and Evolution* **28**, 2317–2326.
691 (doi:10.1093/molbev/msr057)
- 692 38. Weller, A. M., Rödelberger, C., Eberhardt, G., Molnar, R. I. & Sommer, R. J.
693 2014 Opposing forces of A/T-biased mutations and G/C-biased gene
694 conversions shape the genome of the nematode *Pristionchus pacificus*. *Genetics*
695 **196**, 1145–1152. (doi:10.1534/genetics.113.159863)
- 696 39. Smith, J. M. & Haigh, J. 2007 The hitch-hiking effect of a favourable gene.
697 *Genet. Res.* **89**, 391–403. (doi:10.1017/S0016672308009579)
- 698 40. Falconer, D. S. 1960 *Introduction to Quantitative Genetics*. The Ronald Press
699 Company, New York.
- 700 41. Hancock, A. M., Brachi, B., Faure, N., Horton, M. W., Jarymowycz, L. B.,
701 Sperone, F. G., Toomajian, C., Roux, F. & Bergelson, J. 2011 Adaptation to
702 climate across the *Arabidopsis thaliana* genome. *Science* **334**, 83–86.
703 (doi:10.1126/science.1209244)
- 704 42. Trotta, V., Calboli, F. C. F., Ziosi, M., Guerra, D., Pezzoli, M. C., David, J. R.
705 & Cavicchi, S. 2006 Thermal plasticity in *Drosophila melanogaster*: a
706 comparison of geographic populations. *BMC Evol Biol* **6**, 67.
707 (doi:10.1186/1471-2148-6-67)
- 708 43. Gutteling, E. W., Doroszuk, A., Riksen, J. A. G., Prokop, Z., Reszka, J. &
709 Kammenga, J. E. 2007 Environmental influence on the genetic correlations
710 between life-history traits in *Caenorhabditis elegans*. *Heredity* **98**, 206–213.
711 (doi:10.1038/sj.hdy.6800929)
- 712 44. Shook, D. R., Brooks, A. & Johnson, T. E. 1996 Mapping Quantitative Trait
713 Loci Affecting Life History Traits in the Nematode *Caenorhabditis elegans*.
714 *Genetics* **142**, 801–817.
- 715 45. Shook, D. R. & Johnson, T. E. 1999 Quantitative trait loci affecting survival
716 and fertility-related traits in *Caenorhabditis elegans* show genotype-
717 environment interactions, pleiotropy and epistasis. *Genetics* **153**, 1233–1243.
718 (doi:10.1007/BF01066638)
- 719 46. Harvey, S. C., Shorto, A. & Viney, M. E. 2008 Quantitative genetic analysis of
720 life-history traits of *Caenorhabditis elegans* in stressful environments. *BMC*
721 *Evol Biol* **8**, 15–16. (doi:10.1186/1471-2148-8-15)

- 722 47. Nadeau, N. J. & Jiggins, C. D. 2010 A golden age for evolutionary genetics?
723 Genomic studies of adaptation in natural populations. *Trends Genet* **26**, 484–
724 492. (doi:10.1016/j.tig.2010.08.004)
- 725 48. Brenner, S. 1974 The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71–94.
- 726 49. RStudio Team 2015 RStudio. (doi:http://www.rstudio.com/)
- 727 50. Borchert, N., Dieterich, C., Krug, K., Schutz, W., Jung, S., Nordheim, A.,
728 Sommer, R. J. & Macek, B. 2010 Proteogenomics of *Pristionchus pacificus*
729 reveals distinct proteome structure of nematode models. *Genome Res* **20**, 837–
730 846. (doi:10.1101/gr.103119.109)
- 731 51. Yang, Z., Kumar, S. & Nei, M. 1995 A new method of inference of ancestral
732 nucleotide and amino acid sequences. *Genetics* **141**, 1641–1650.
- 733 52. Revell, L. J. 2011 phytools: an R package for phylogenetic comparative
734 biology (and other things). *Methods in Ecology and Evolution* **3**, 217–223.
735 (doi:10.1111/j.2041-210X.2011.00169.x)

736

Figure 1. The occurrence of the Htt strains decreases with increasing altitude of their collection site.

Figure 2 - The correlation between the frequency of Htt strains and altitude remains after accounting for beetle species.

Figure 3 - The occurrence of Htt phenotype in strains belonging to different clades.

Figure 4 - Phylogenetic tree of *P. pacificus* strains shows one clade highly enriched with Htt strains.

Supplementary Figure 2 - The altitudinal range of beetle species from La Réunion.

The range of altitudes where beetle species are found. The vertical bar represents the maximum and minimum altitude each beetle species was found at. The point represents the mean altitude of the collection site for all beetles collected.

Supplementary Figure 3. Cladogram of clade A1 and A2 strains showing parallel evolution of the trait.

Cladogram based on clades A1 and A2 from the tree from figure 4. The phenotype of strains and the inferred ancestral phenotype are indicated by circles at the terminal nodes as in figure 4. Clade A1 is indicated by a yellow block and clade A2 by an orange block. The “hot” sub-clade is indicated by a black bar.

Supplementary table 1. The percentage of high temperature tolerant strains is higher at some low altitude collection sites. The number of strains collected for each location is shown as well as the number of strains able to give fertile offspring at 30°C (Htt strains) and the percentage of Htt strains per location (% Htt). Also shown are the total number of strains collected, the total number of Htt strains, and the percentage of Htt strains for the whole data set.

Code	Name	Altitude	Strains	Htt Strains	% Htt
ES	Etang Salé	17	3	1	33.3
SB	Saint Benoit	22	30	23	76.7
RG	Airport Roland Garros	26	1	0	0.0
PR	Petite Ravine	154	1	1	100.0
BB2	Bois Blanc 2	249	1	1	100.0
BB3	Bois Blanc 3	333	1	0	0.0
SP09	St Phillippe 9	339	34	11	32.4
SP10	St Phillippe 10	445	14	2	14.3
SP11	St Phillippe 11	511	24	8	33.3
GE	Grand Etang	527	11	0	0.0
SP12	St Phillippe 12	544	12	1	8.3
PD2	Plaine d'Affouches 2	691	12	5	41.7
CO	Colorado	694	41	7	17.1
PL	Plaines des Lianes	755	10	0	0.0
PD3	Plaine d'Affouches 3	809	6	0	0.0
TK	Takamaka	835	6	0	0.0
PD4	Plaine d'Affouches 4	886	9	2	22.2
PA0	Plaines des Palmiste 0	1199	1	0	0.0
SS	Sans Soucie	1353	10	0	0.0
TB	Trois Bassin	1413	30	0	0.0
PA1	Plaines des Palmiste 1	1466	2	0	0.0
PA3	Plaines des Palmiste 3	1627	3	0	0.0
NB	Nez de Bouef	2146	7	0	0.0
CK	Coteau Kerveguen	2150	10	0	0.0
CC	Cratere commerson	2327	10	0	0.0
		sum	289	62	21.5

Supplementary table 2. SNPs identified by GWAS.

Position	Major allele	Minor Allele	p-value	Location of SNP	GWAS
ChrI					
18417120	G	A	6.098x10 ⁻⁶	<i>hint-1</i>	All clades
ChrII					
16359384	C	A	2.947x10 ⁻⁶	intragenic between Contig145-SNAP2012.9 and ZK970.1	All clades
ChrIV					
13715610	A	C	2.065x10 ⁻⁶	Intragenic between Contig12-SNAP2012.347 and <i>nhr-213</i>	All clades
13634723	C	T	1.107x10 ⁻⁵	intragenic between <i>nhr-136</i> and <i>nhr-136</i>	All clades
23653301	G	C	2.296x10 ⁻⁵	<i>let-767</i>	All clades
ChrV					
19394757	T	A	2.631x10 ⁻⁶	<i>cfi-1</i>	All clades
6556437	A	C	9.989x10 ⁻⁶	between <i>gst-24</i> and <i>cyp-29A4</i>	All clades
6840753	T	A	1.689x10 ⁻⁵	<i>cuti-1</i>	All clades
6825501	T	G	1.689 x10 ⁻⁵	Contig25-SNAP2012.176 and <i>hpk-1</i>	All clades
ChrX					
1004401	C	G	6.098x10 ⁻⁶	<i>tag-53</i>	All clades
1004427	A	T	6.098x10 ⁻⁶	<i>tag-53</i>	All clades
1010844	A	T	6.098x10 ⁻⁶	between <i>tag-53</i> and <i>rab-35</i>	All clades
ChrV					
6556437	A	C	5.400x10 ⁻⁶	intragenic between <i>gst-24</i> and <i>cyp-29A4</i>	Clade A1+A2
ChrI					
19341896	A	T	2.211x10 ⁻⁶	<i>nhr-47</i>	Clade C
19355848	G	C	2.211x10 ⁻⁶	between <i>hpo-15</i> and Y41E3.7	Clade C
19355854	G	C	2.211x10 ⁻⁶	between <i>hpo-15</i> and Y41E3.7	Clade C
19355865	A	T	2.211x10 ⁻⁶	between <i>hpo-15</i> and Y41E3.7	Clade C
19368965	G	A	2.211x10 ⁻⁶	between C02F5.7 and Y41E3.7	Clade C
19371215	A	G	2.211x10 ⁻⁶	Y41E3.7	Clade C
19477595	C	A	2.211x10 ⁻⁶	ZC116.3	Clade C
21518470	G	A	2.211x10 ⁻⁶	between <i>eef-1G</i> and Contig14-SNAP2012.27	Clade C
21536930	A	T	2.211x10 ⁻⁶	Contig14-SNAP2012.30	Clade C
21550598	G	A	2.211x10 ⁻⁶	between Contig12-SNAP2012.32 and ZK856.8	Clade C
21602693	A	G	2.211x10 ⁻⁶	between <i>goa-1</i> and Contig14-SNAP2012.40	Clade C
21649980	T	A	2.211x10 ⁻⁶	<i>wars-1</i>	Clade C
21720251	C	G	2.211x10 ⁻⁶	<i>atg-18</i>	Clade C
21723671	G	T	2.211x10 ⁻⁶	ZC412.4	Clade C
21738474	G	A	2.211x10 ⁻⁶	between Y66A7A.7 and <i>fos-1</i>	Clade C

21788235	G	A	2.211×10^{-6}	Contig14-SNAP2012.73 and R08A2.7	Clade C
21793735	G	A	2.211×10^{-6}	between <i>rmd-6</i> and <i>rmd-2</i>	Clade C
21793884	G	A	2.211×10^{-6}	between <i>rmd-6</i> and <i>rmd-2</i>	Clade C
21821237	A	T	2.211×10^{-6}	K08F4.1	Clade C
22047574	A	G	6.579×10^{-6}	Contig14-SNAP2012.130 and C24G7.4	Clade C
22195492	T	A	2.211×10^{-6}	F45F2.10	Clade C
22250094	–	–	2.211×10^{-6}	<i>glr-3</i>	Clade C
22277854	G	A	6.579×10^{-6}	between <i>pgn-13</i> and Contig14-SNAP2012.17	Clade C
ChrIII					
1937583	A	C	2.211×10^{-6}	<i>ztf-12</i>	Clade C
ChrV					
1168890	T	A	1.573×10^{-5}	intragenic between <i>grh-1</i> and <i>lin-7</i>	Clade C
1201831	T	C	1.573×10^{-5}	<i>pad-1</i>	Clade C

Supplementary table 3. Allele frequencies of GWAS hits show some alleles are variable across clades while other alleles unique to one clade. For each position that was identified as being associated with the Htt phenotype, the allele frequency was calculated for all stains in each clade. SNPs that are variable in many clades are highlighted in yellow, those that are variable in only one clade are highlighted in orange, red and blue corresponding to clades A1, A2 and C respectively.

SNP position	GWAS	Allele frequencies in individual clades					
		A1	A2	A3	B	C	D
ChrI:18417120	All clades	G:0.79 A:0.21	A:1.00	G:1.00	A:1.00	G:1.00	G:1.00
ChrII:16359384	All clades	C:1.00	C:0.29 A:0.71	C:1.00	A:1.00	C:1.00	C:1.00
ChrIV:13634723	All clades	C:0.29 T:0.71	C:1.00	C:1.00	C:1.00	T:0.01 C:0.99	T:1.00
ChrIV:13715610	All clades	A:0.71 C:0.29	C:1.00	C:1.00	A:1.00	A:1.00	A:1.00
ChrIV:23653301	All clades	C:0.21 G:0.79	C:1.00	C:0.80 G:0.20	G:1.00	G:1.00	G:1.00
ChrV:19394757	All clades	A:0.64 T:0.36	T:0.68 A:0.32	T:1.00	A:1.00	T:1.00	T:1.00
ChrV:6825501	All clades	T:0.71 G:0.29	G:1.00	T:0.13 G:0.87	T:1.00	T:1.00	T:1.00
ChrV:6840753	All clades	T:0.71 A:0.29	A:1.00	T:0.13 A:0.87	T:1.00	T:1.00	T:1.00
ChrX:1004401	All clades	C:0.79 G:0.21	G:1.00	C:1.00	G:1.00	C:1.00	C:1.00
ChrX:1004427	All clades	T:0.21 A:0.79	T:1.00	A:1.00	T:1.00	A:1.00	A:1.00
ChrX:1010844	All clades	T:0.21 A:0.79	T:1.00	A:1.00	T:1.00	A:1.00	A:1.00
ChrV:6556437	Clade A1+A2	C:0.64 A:0.36	C:0.29 A:0.71	A:1.00	A:1.00	A:0.94 C:0.06	A:0.19 C:0.81
ChrI:19341896	Clade C	A:1.00	A:1.00	A:1.00	A:1.00	T:0.29 A:0.71	A:1.00
ChrI:19355848	Clade C	C:1.00	C:1.00	C:1.00	C:1.00	C:0.71 G:0.29	C:1.00
ChrI:19355854	Clade C	G:1.00	G:1.00	G:1.00	G:1.00	G:0.71 C:0.29	G:1.00
ChrI:19355865	Clade C	A:1.00	A:1.00	A:1.00	A:1.00	T:0.29 A:0.71	A:1.00
ChrI:19368965	Clade C	A:1.00	A:1.00	A:1.00	A:1.00	A:0.71 G:0.29	A:1.00
ChrI:19371215	Clade C	A:1.00	A:1.00	A:1.00	A:1.00	G:0.29 A:0.71	A:1.00
ChrI:19477595	Clade C	A:1.00	A:1.00	A:1.00	A:1.00	C:0.71 A:0.29	A:1.00
ChrI:21518470	Clade C	G:1.00	G:1.00	G:1.00	G:1.00	G:0.71 A:0.29	G:1.00
ChrI:21536930	Clade C	A:1.00	A:1.00	A:1.00	A:1.00	T:0.29 A:0.71	A:1.00
ChrI:21550598	Clade C	A:1.00	A:1.00	A:1.00	A:1.00	G:0.29 A:0.71	A:1.00
ChrI:21602693	Clade C	A:1.00	A:1.00	A:1.00	A:1.00	G:0.29 A:0.71	A:1.00
ChrI:21649980	Clade C	T:1.00	T:1.00	T:1.00	T:1.00	T:0.71 A:0.29	T:1.00

ChrI:21720251	Clade C	C:1.00	C:1.00	C:1.00	C:1.00	G:0.29 C:0.71	C:1.00
ChrI:21723671	Clade C	G:1.00	G:1.00	G:1.00	G:1.00	G:0.71 T:0.29	G:1.00
ChrI:21738474	Clade C	G:1.00	G:1.00	G:1.00	G:1.00	G:0.71 A:0.29	G:1.00
ChrI:21788235	Clade C	G:1.00	G:1.00	G:1.00	G:1.00	G:0.71 A:0.29	G:1.00
ChrI:21793735	Clade C	G:1.00	G:1.00	G:1.00	G:1.00	A:0.29 G:0.71	G:1.00
ChrI:21793884	Clade C	G:1.00	G:1.00	G:1.00	G:1.00	G:0.71 A:0.29	G:1.00
ChrI:21821237	Clade C	A:1.00	A:1.00	A:1.00	A:1.00	A:0.71 T:0.29	A:1.00
ChrI:22047574	Clade C	A:1.00	A:1.00	A:1.00	A:1.00	G:0.28 A:0.72	A:1.00
ChrI:22195492	Clade C	T:1.00	T:1.00	T:1.00	T:1.00	A:0.29 T:0.71	T:1.00
ChrI:22250094	Clade C	C:1.00	C:1.00	C:1.00	C:1.00	T:0.29 C:0.71	C:1.00
ChrI:22277854	Clade C	G:1.00	G:1.00	G:1.00	G:1.00	A:0.28 G:0.72	G:1.00
ChrIII:1937583	Clade C	C:1.00	C:1.00	C:1.00	C:1.00	C:0.29 A:0.71	C:1.00
ChrV:1168890	Clade C	T:1.00	A:0.14 T:0.86	T:1.00	T:1.00	T:0.81 A:0.19	T:0.95 A:0.05
ChrV:1201831	Clade C	T:1.00	T:0.86 C:0.14	T:1.00	T:1.00	T:0.81 C:0.19	T:0.95 C:0.05

Supplementary table 4. Genes containing or near to SNPs identified by GWAS. Predicted gene function based of protein family or closest homolog in *C. elegans*.

gene	protein	function	References
<i>atg-18</i>	Autophagy	essential for normal dauer morphogenesis and autophagy in daf-2 mutant animals	Wormbase
C24G7.4	ACD-2	sodium channel involved in mechanosensation	Wormbase
<i>cfi-1</i>	CEM Fate inhibitor	Cell fate determination of sensory neurons	Wormbase
<i>cuti-1</i>	cuticle and epithelial Integrity	cuticle development, embryo development, locomotion, the molting cycle, nematode larval development and reproduction	Wormbase
<i>cyp-29A4</i>	cytochrome P450	electron transport, xenobiotic detoxification, fatty acid synthesis	Wormbase
<i>eef-1G</i>	Eukaryotic translation Elongation Factor	involved in embryo development and reproduction. RNAi gives reduced broodsize, gonad defects and slow growth	Wormbase
F45F2.10	ankyrin repeat domain 36	involved in embryo development and reproduction: RNAi gives increased lifespan in dauers, embryonic lethal, maternal sterile, and sterile progeny	Wormbase
<i>fos-1</i>	B-Zip transcription factor	anchor cell invasion of the vulval epithelium and proper vulval and uterine development, fertility, and oogenesis	Wormbase
<i>goa-1</i>	G protein, O, Alpha subunit	regulation of locomotion, egg-laying, male mating, and olfactory-mediated behaviors; also required for asymmetric cell division in the early embryo	Wormbase
<i>glb-10</i>	globin	chemosensation	Wormbase
<i>grh-1</i>	Transcription factor	Cuticle synthesis and embryonic development	Wormbase
<i>glr-3</i>	GLutamate Receptor family		Wormbase
<i>gst-24</i>	glutathione S-transferase	Oxidative stress response	Wormbase
<i>hint-1</i>	histidine triad nucleotide binding protein 1		Wormbase
<i>hpk-1</i>	Homeodomain interacting Protein Kinase	localized to the nucleus and is expressed in cleavage stage embryos	Wormbase
<i>hpo-15</i>	Hypersensitive to PORE-forming toxin	predicted oxidoreductase activity	Wormbase
K08F4.1	chromosome transmission fidelity factor 18	cellular response to DNA damage stimulus	Wormbase
<i>let-767</i>	lethal	required for embryogenesis, molting, and female reproduction	Wormbase
<i>lin-7</i>	abnormal cell LINEage	Required for cell polarity in vulva precursor cells	Wormbase
M03B6.2	MCT-3	Solute carriers	Wormbase
<i>mua-3</i>	Vitrin	apoptotic process, cell-matrix adhesion, locomotion, nematode larval development and reproduction	Wormbase
<i>nhr-47</i>	nuclear hormone receptor	Expressed in response to estradiol exposure	Wormbase
<i>nhr-213</i>	nuclear hormone receptor	memory	Wormbase
<i>nhr-136</i>	nuclear hormone receptor	Starvation response	Wormbase
<i>pad-1</i>	PAtterning Defective	Required for embryonic development	Wormbase
<i>pgn-13</i>			
<i>rab-35</i>	Rab GTPase	intracellular vesicular trafficking and for regulation of endo- and exocytosis	Wormbase

<i>rmd-2</i>	Regulator of Microtubule Dynamics	involved in striated muscle myosin thick filament assembly	Wormbase
<i>rmd-6</i>	Regulator of Microtubule Dynamics	RNAi gives phenotypes in endosome recycling lysosome morphology defects	Wormbase
R08A2.7			
<i>tag-35</i>			
<i>wars-1</i>	tryptophanyl Amino-acyl tRNA Synthetase	affects fertility, embryonic viability, growth, and locomotion	Wormbase
Y66A7A.7			
<i>ztf-12</i>	SUP-37	involved in embryo development, embryonic digestive tract morphogenesis, nematode larval development and reproduction	Wormbase
ZC116.3	CUBILIN		Wormbase
ZC412.4			
ZK856.8	Chpf-1	involved in locomotion	Wormbase
Y41E3.7			
ZK970.1	NEP-26	A zinc metallopeptidases, found on the outer surface of animal cells that negatively regulate small signaling peptides	Wormbase

Supplementary Table 5. List of strains used in the study. The table lists for each strain its phenotype, its collection site, its beetle host and the mitochondrial clade to which it belongs. For strain that were not sequenced, no clade information is available, indicated by UK. See table 1 for the full name of the collection site. The altitude in meters for each site is also noted.

Strain	Phenotype	Location	Altitude	Beetle	Clade
RS5344	Ltt	ES	17	Mal	C
RS5345	Ltt	ES	17	Mal	D
RS5350	Htt	ES	17	Hop	C
RS5412	Htt	SB	22	Ado	A
RS5413	Htt	SB	22	Ado	A
RS5415	Htt	SB	22	Mal	C
RS5416	Htt	SB	22	Mal	A
RS5417	Htt	SB	22	Mal	A
RS5418	Ltt	SB	22	Mal	C
RS5419	Htt	SB	22	Mal	A
RS5420	Htt	SB	22	Mal	A
RS5421	Htt	SB	22	Mal	A
RS5422	Ltt	SB	22	Mal	A
RS5423	Htt	SB	22	Mal	A
RS5424	Htt	SB	22	Mal	A
RSB068	Htt	SB	22	Aph	A
RSB069	Htt	SB	22	Aph	A
RSB070	Htt	SB	22	Aph	A
RSB071	Htt	SB	22	Aph	A
RSB072	Ltt	SB	22	Aph	C
RSB073	Ltt	SB	22	Aph	C
RSB074	Ltt	SB	22	Aph	C
RSB077	Htt	SB	22	Aph	A
RSB078	Htt	SB	22	Aph	A
RSB079	Htt	SB	22	Aph	A
RSB080	Htt	SB	22	Aph	A
RSB081	Htt	SB	22	Mal	A
RSB082	Ltt	SB	22	Mal	A
RSB083	Htt	SB	22	Mal	A
RSB083	Htt	SB	22	Mal	A
RSB084	Ltt	SB	22	Mal	A
RSB085	Htt	SB	22	Mal	A
RSB086	Htt	SB	22	Aph	A
RS5343	Ltt	RG	26	Ali	C
RSA061	Htt	PR	154	Hop	C
RSE194	Htt	BB2	249	Mal	UK
RSE001	Ltt	BB3	333	Fig	D
RSF275	Ltt	SP09	339	Ado	D
RSF276	Ltt	SP09	339	Ado	D
RSF277	Htt	SP09	339	Ado	D
RSF278	Ltt	SP09	339	Ado	D
RSF279	Ltt	SP09	339	Ado	D

RSF280	Ltt	SP09	339	Ado	D
RSF281	Htt	SP09	339	Ado	D
RSF282	Ltt	SP09	339	Ado	D
RSF283	Htt	SP09	339	Ado	D
RSF284	Htt	SP09	339	Ado	D
RSF285	Htt	SP09	339	Ado	D
RSF286	Htt	SP09	339	Ado	D
RSF287	Htt	SP09	339	Ado	D
RSF288	Ltt	SP09	339	Ado	D
RSF289	Ltt	SP09	339	Ado	D
RSF290	Ltt	SP09	339	Ado	D
RSF291	Ltt	SP09	339	Ado	C
RSF292	Ltt	SP09	339	Ado	C
RSF293	Ltt	SP09	339	Ado	D
RSF294	Htt	SP09	339	Ado	D
RSF295	Htt	SP09	339	Ado	UK
RSF296	Htt	SP09	339	Ado	C
RSF297	Ltt	SP09	339	Ado	D
RSF298	Ltt	SP09	339	Ado	UK
RSF299	Ltt	SP09	339	Ado	D
RSF300	Ltt	SP09	339	Ado	C
RSF301	Ltt	SP09	339	Ado	C
RSF302	Ltt	SP09	339	Ado	C
RSF303	Ltt	SP09	339	Ado	C
RSF304	Ltt	SP09	339	Ado	C
RSF305	Ltt	SP09	339	Ado	UK
RSF306	Ltt	SP09	339	Ado	D
RSF307	Htt	SP09	339	Ado	D
RSF322	Ltt	SP09	339	Ado	UK
RSF231	Ltt	SP10	445	Ado	D
RSF232	Ltt	SP10	445	Ado	D
RSF233	Htt	SP10	445	Ado	D
RSF234	Htt	SP10	445	Ado	D
RSF235	Ltt	SP10	445	Ado	D
RSF236	Ltt	SP10	445	Ado	D
RSF237	Ltt	SP10	445	Ado	UK
RSF238	Ltt	SP10	445	Ado	D
RSF239	Ltt	SP10	445	Ado	D
RSF240	Ltt	SP10	445	Ado	D
RSF241	Ltt	SP10	445	Ado	C
RSF242	Ltt	SP10	445	Ado	C
RSF243	Ltt	SP10	445	Ado	C
RSF316	Ltt	SP10	445	Ado	D
RSF244	Ltt	SP11	511	Ado	D
RSF245	Ltt	SP11	511	Ado	D
RSF246	Ltt	SP11	511	Ado	C
RSF247	Ltt	SP11	511	Ado	C
RSF248	Ltt	SP11	511	Ado	D

RSF249	Htt	SP11	511	Ado	D
RSF250	Htt	SP11	511	Ado	D
RSF251	Ltt	SP11	511	Ado	D
RSF252	Ltt	SP11	511	Ado	D
RSF253	Ltt	SP11	511	Ado	D
RSF254	Htt	SP11	511	Ado	UK
RSF255	Ltt	SP11	511	Ado	D
RSF256	Ltt	SP11	511	Ado	D
RSF257	Ltt	SP11	511	Ado	D
RSF258	Htt	SP11	511	Ado	D
RSF259	Ltt	SP11	511	Ado	D
RSF260	Htt	SP11	511	Ado	UK
RSF261	Ltt	SP11	511	Ado	D
RSF262	Htt	SP11	511	Ado	D
RSF318	Htt	SP11	511	Ado	UK
RSF319	Htt	SP11	511	Ado	UK
RSF320	Ltt	SP11	511	Ado	D
RSF321	Ltt	SP11	511	Ado	D
SP11Ado20-2	Ltt	SP11	511	Ado	UK
RS5407	Ltt	GE	527	Ado	C
RS5408	Ltt	GE	527	Ado	A
RS5409	Ltt	GE	527	Ado	C
RS5410	Ltt	GE	527	Ado	C
RS5411	Ltt	GE	527	Ado	C
RSA046	Ltt	GE	527	Ado	C
RSA048	Ltt	GE	527	Ado	D
RSA049	Ltt	GE	527	Ado	D
RSA055	Ltt	GE	527	Ado	A
RSA056	Ltt	GE	527	Ado	D
RSA059	Ltt	GE	527	Ado	D
RSF263	Ltt	SP12	544	Ado	D
RSF264	Ltt	SP12	544	Ado	D
RSF265	ltt	SP12	544	Ado	D
RSF266	Ltt	SP12	544	Ado	D
RSF267	Ltt	SP12	544	Ado	D
RSF268	Ltt	SP12	544	Ado	D
RSF269	Ltt	SP12	544	Ado	D
RSF270	Htt	SP12	544	Ado	D
RSF271	Ltt	SP12	544	Ado	D
RSF272	Ltt	SP12	544	Ado	D
RSF273	Ltt	SP12	544	Ado	D
RSF274	Ltt	SP12	544	Ado	D
RSD033	Htt	PD2	691	Ado	C
RSD034	Ltt	PD2	691	Ado	C
RSD035	Ltt	PD2	691	Ado	C
RSD036	Ltt	PD2	691	Ado	C
RSD037	Ltt	PD2	691	Ado	C

RSD038	Htt	PD2	691	Ado	C
RSD039	Ltt	PD2	691	Ado	C
RSD041	Ltt	PD2	691	Ado	C
RSD042	Htt	PD2	691	Ado	C
RSD043	Ltt	PD2	691	Ado	C
RSD045	Htt	PD2	691	Ado	UK
RSD048	Htt	PD2	691	Ado	UK
RSB020	Ltt	COL	694	Ado	C
RSC014	Ltt	COL	694	Ado	C
RSC015	Ltt	COL	694	Ado	C
RSC016	Htt	COL	694	Ado	C
RSC017	Ltt	COL	694	Ado	C
RSC018	Ltt	COL	694	Ado	C
RSC019	Htt	COL	694	Ado	C
RSC020	Htt	COL	694	Ado	C
RSC021	Htt	COL	694	Ado	C
RSC022	Ltt	COL	694	Ado	C
RSF024	Ltt	COL	694	Ado	C
RSF025	Htt	COL	694	Ado	UK
RSF026	Htt	COL	694	Ado	UK
RSF027	Ltt	COL	694	Ado	UK
RSF028	Ltt	COL	694	Ado	C
RSF029	Ltt	COL	694	Ado	C
RSF030	Ltt	COL	694	Ado	UK
RSF031	Ltt	COL	694	Ado	C
RSF032	Ltt	COL	694	Ado	C
RSF033	Ltt	COL	694	Ado	C
RSF034	Ltt	COL	694	Ado	C
RSF035	Ltt	COL	694	Ado	C
RSF036	Ltt	COL	694	Ado	UK
RSF037	Ltt	COL	694	Ado	C
RSF038	Ltt	COL	694	Ado	C
RSF039	Ltt	COL	694	Ado	C
RSF040	Ltt	COL	694	Ado	C
RSF041	Ltt	COL	694	Ado	C
RSF042	Ltt	COL	694	Ado	UK
RSF043	Ltt	COL	694	Ado	UK
RSF044	Ltt	COL	694	Ado	UK
RSF045	Ltt	COL	694	Ado	UK
RSF046	Ltt	COL	694	Ado	UK
RSF047	Ltt	COL	694	Ado	UK
RSF048	Ltt	COL	694	Ado	UK
RSF049	Ltt	COL	694	Ado	UK
RSF050	ltt	COL	694	Ado	UK
RSF051	Htt	COL	694	Ado	UK
RSF052	Ltt	COL	694	Ado	UK
RSF053	Ltt	COL	694	Ado	UK
RSF309	Ltt	COL	694	Ado	UK

RSB059	Ltt	PL	755	Ado	D
RSB060	Ltt	PL	755	Ado	D
RSB062	Ltt	PL	755	Ado	D
RSB064	Ltt	PL	755	Ado	D
RSB065	Ltt	PL	755	Ado	D
RSB066	Ltt	PL	755	Ado	D
RSC051	Ltt	PL	755	Ado	D
RSC052	Ltt	PL	755	Ado	D
RSC053	Ltt	PL	755	Ado	C
RSC054	Ltt	PL	755	Ado	D
RSD049	Ltt	PD3	809	Ado	D
RSD050	Ltt	PD3	809	Ado	D
RSD051	Ltt	PD3	809	Ado	D
RSD053	Ltt	PD3	809	Ado	C
RSD055	Ltt	PD3	809	Ado	C
RSD057	Ltt	PD3	809	Ado	D
RSC094	Ltt	TK	835	Ado	UK
RSC095	Ltt	TK	835	Ado	UK
RSC096	Ltt	TK	835	Ado	UK
RSC097	Ltt	TK	835	Ado	UK
RSC098	Ltt	TK	835	Ado	UK
RSC099	Ltt	TK	835	Ado	UK
RSD059	Ltt	PD4	886	Ado	UK
RSD060	Ltt	PD4	886	Ado	UK
RSD062	Ltt	PD4	886	Ado	UK
RSD063	Htt	PD4	886	Ado	UK
RSD065	Htt	PD4	886	Ado	UK
RSD073	Ltt	PD4	886	Ado	UK
RSD075	Ltt	PD4	886	Ado	UK
RSD076	Ltt	PD4	886	Ado	UK
RSD077	Ltt	PD4	886	Ado	UK
RSC037	Ltt	PA0	1199	Ory	C
RSA096	Ltt	SS	1353	Ory	C
RSA097	Ltt	SS	1353	Ory	C
RSA098	Ltt	SS	1353	Ory	C
RSA100	Ltt	SS	1353	Ory	C
RSA102	Ltt	SS	1353	Ory	D
RSA104	Ltt	SS	1353	Ory	C
RSA106	Ltt	SS	1353	Ory	C
RSA108	Ltt	SS	1353	Ory	C
RSA110	Ltt	SS	1353	Ory	C
RSA111	Ltt	SS	1353	Ory	C
RS5334	Ltt	TB	1413	Ory	C
RS5336	Ltt	TB	1413	Ory	C
RS5337	Ltt	TB	1413	Ory	C
RS5347	Ltt	TB	1413	Ory	C
RS5351	Ltt	TB	1413	Ory	C
RS5385	Ltt	TB	1413	Ory	C

RS5397	Ltt	TB	1413	Ory	C
RS5399	Ltt	TB	1413	Ory	C
RS5402	Ltt	TB	1413	Hop	C
RS5403	Ltt	TB	1413	Hop	C
RS5404	Ltt	TB	1413	Hop	C
RS5405	Ltt	TB	1413	Hop	C
RSA062	Ltt	TB	1413	Hop	C
RSA064	Ltt	TB	1413	Hop	C
RSA065	Ltt	TB	1413	Hop	C
RSA066	Ltt	TB	1413	Hop	C
RSA069	Ltt	TB	1413	Hop	C
RSA071	Ltt	TB	1413	Hop	C
RSA072	Ltt	TB	1413	Hop	C
RSA073	Ltt	TB	1413	Hop	C
RSA089	Ltt	TB	1413	Hop	C
RSA090	Ltt	TB	1413	Hop	C
RSA091	Ltt	TB	1413	Hop	C
RSA092	Ltt	TB	1413	Hop	C
RSA113	Ltt	TB	1413	Ory	C
RSB087	Ltt	TB	1413	Hop	UK
RSB088	Ltt	TB	1413	Hop	C
RSB096	Ltt	TB	1413	Hop	C
RSB120	Ltt	TB	1413	Ory	C
RSC093	Ltt	TB	1413	Hop	C
RSC046	Ltt	PA1	1446	Ory	C
RSC047	Ltt	PA1	1446	Ory	C
RSC048	Ltt	PA3	1627	Ory	C
RSC049	Ltt	PA3	1627	Ory	C
RSC050	Ltt	PA3	1627	Ory	C
RSA076	Ltt	NB	2146	Amn	B
RSB033	Ltt	NB	2146	Amn	B
RSB034	Ltt	NB	2146	Amn	B
RSB035	Ltt	NB	2146	Amn	B
RSB037	Ltt	NB	2146	Amn	B
RSC035	Ltt	NB	2146	Amn	B
RSC036	Ltt	NB	2146	Amn	B
RSC007	Ltt	CK	2150	Amn	B
RSC008	Ltt	CK	2150	Amn	B
RSC009	Ltt	CK	2150	Amn	B
RSC010	Ltt	CK	2150	Amn	B
RSC011	Ltt	CK	2150	Amn	B
RSC012	Ltt	CK	2150	Amn	B
RSC013	Ltt	CK	2150	Amn	B
RSC100	Ltt	CK	2150	Amn	B
RSC172	Ltt	CK	2150	Amn	B
RSC173	Ltt	CK	2150	Amn	B
RSB001	Ltt	CC	2327	Amn	B
RSB005	Ltt	CC	2327	Amn	B

RSB008	Ltt	CC	2327	Amn	B
RSB013	Ltt	CC	2327	Amn	B
RSC001	Ltt	CC	2327	Amn	B
RSC002	Ltt	CC	2327	Amn	B
RSC003	Ltt	CC	2327	Amn	B
RSC004	Ltt	CC	2327	Amn	B
RSC005	Ltt	CC	2327	Amn	B
RSC006	Ltt	CC	2327	Amn	B