The ISME Journal (2018) 12, 77–86

www.nature.com/ismej

ORIGINAL ARTICLE

Evidence for a primate origin of zoonotic *Helicobacter suis* colonizing domesticated pigs

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Helicobacter suis **is the second most prevalent** ***Helicobacter*** **species in the stomach of humans suffering from gastric disease. This bacterium mainly inhabits the stomach of domesticated pigs, in which it causes gastric disease, but it appears to be absent in wild boars. Interestingly, it also colonizes the stomach of asymptomatic rhesus and cynomolgus monkeys. The origin of modern human-, pig- or non-human primate-associated** ***H. suis*** **strains in these respective host populations was hitherto unknown. Here we show that** ***H. suis*** **in pigs possibly originates from non-human primates. Our data suggest that a host jump from macaques to pigs happened between 100 000 and 15 000 years ago and that pig domestication has had a significant impact on the spread of** ***H. suis*** **in the pig population, from where this pathogen occasionally infects humans. Thus, in contrast to our expectations,** ***H. suis*** **appears to have evolved in its main host in a completely different way than its close relative** ***Helicobacter pylori*** **in humans.**
The ISME Journal (2018) **12,** 77–86; doi:10.1038/ismej.2017.145; published online 8 September 2017

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Received 30 December 2016; revised 11 May 2017; accepted 4 August 2017; published online 8 September 2017

Introduction

A number of *Helicobacter* species have been described to cause gastrointestinal disease in humans and animals. By far the best known and studied species is *Helicobacter pylori*, which can cause severe gastric disease in humans (Kusters *et al.*, 2006). Humans are the major reservoir for *H. pylori*, although *H. pylori* infection in captive socially housed rhesus macaques also seems to be ubiquitous (Solnick *et al.*, 2003). No evidence so far has been found on the presence of *H. pylori* in the stomach of

chimpanzees, our closest living relatives (Moodley *et al*., 2012).

Besides *H. pylori*, other mainly animal-associated gastric *Helicobacter* species have been found colonizing the stomach of human patients, albeit at a much lower prevalence (Haesebrouck *et al*., 2009; Blaecher *et al*., 2013). These include dog- and cat-associated *Helicobacter* species, as well as *H. suis*. The latter commonly inhabits the stomach of domestic pigs and several studies in various countries on different continents have reported prevalences in pig herds exceeding 50% (Grasso *et al*., 1996; Park *et al*., 2004; Hellemans *et al*., 2007; Haesebrouck *et al*., 2009; Foss *et al*., 2013). Humans occasionally acquire the infection, most likely through direct contact with pigs or consumption of contaminated meat, and develop a gastritis which in some cases evolves to gastric MALT lymphoma (Stolte *et al*., 1997; Van den Bulck *et al*., 2005; Haesebrouck *et al*., 2009; De Cooman *et al*., 2013; Joosten *et al*., 2013). Direct human-to-human transmission of *H. suis* has not been reported so far. A strong correlation between *H. suis* infection and gastritis has also been observed in pigs (Grasso *et al*., 1996; De Bruyne *et al*., 2012) and conflicting evidence on the role of this bacterium in the development of ulceration of the keratinized *pars oesophagea* of the stomach has been reported (Grasso *et al*., 1996; Queiroz *et al*., 1996; Szeredi *et al*., 2005; De Bruyne *et al*., 2012). Furthermore, several studies attempting to detect this *Helicobacter* species in wild boars were unsuccessful (Fabisiak *et al*., 2010; Bassi, 2013), raising the question on the origin of *H. suis* infections in domesticated pigs.

H. suis has also been described to colonize rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (crab-eating macaques; *Macaca fascicularis*) (O'Rourke *et al.,* 2004; Martin *et al*., 2013; Bosschem *et al*., 2017). In contrast to what has been described in humans and pigs, *H. suis* infection in macaques in general seems asymptomatic (Drevon-Gaillot *et al*., 2006), although mild gastritis has been described occasionally (Dubois *et al*., 1991). All reports on *H. suis* infection in macaques relate to captive animals and, very often, a large number of animals from the studied groups are infected (Dubois *et al*., 1991; O'Rourke *et al*., 2004; Drevon-Gaillot *et al*., 2006; Nakamura *et al*., 2007; Martin *et al*., 2013). However, no data are available concerning the prevalence of *H. suis* in wild macaque populations, raising the question of a possible anthropogenic origin of *H. suis* infection in these animals.

The population structure and phylogeography of *H. pylori* have been shown to reflect human demographic events, such as ancient migrations of anatomically modern humans from Africa to the rest of the world (Falush *et al*., 2003; Moodley *et al*., 2009, 2012). These studies support the hypothesis that *H. pylori* co-evolved with its host for at least 100 000 years. For *H. suis*, however, no information is available on the worldwide phylogeny and at present, the origin of modern human-, pig- or non-human primate-associated *Helicobacter suis* strains in these respective host populations is unknown.

Table 1 Summary of samples used for typing of *Helicobacter suis* strains

Host species	*Sample type*	*Country of origin*	*Sampling date*	*Number of samples*
Sus scrofa (hybrid)	Isolate	Belgium	04/2006–03/2013	35
Sus scrofa (hybrid)	Tissue	Belgium	2009–2011	17
Sus scrofa (hybrid)	Tissue	Belgium	2013	2
Sus scrofa	Tissue	Italy	2014	6
Sus scrofa	Tissue	Czech Republic	2013	11
Sus scrofa	Tissue	United States—Oklahoma	2007	3
Sus scrofa	Tissue	United States—Michigan	2009	7
Sus scrofa	Tissue	Brazil	2010	5
Sus scrofa (local mix)	Tissue	Nigeria	2014	1
Sus scrofa (Mong Cai)	Tissue	Vietnam	2013	6
Sus scrofa ('White')	Tissue	Vietnam	2013	10
Sus scrofa (Taihu)	Tissue	China	2014	1
Sus scrofa (Sutai)	Tissue	China	2014	1
Sus scrofa (Jinhua)	Tissue	China	2014	3
Sus scrofa (Luchuan)	Tissue	China	2014	1
Sus scrofa ('western')	Tissue	China	2014	4
Sus scrofa (wild boar)	Tissue	Belgium	2014	2
Homo sapiens	Tissue	Belgium	2009	1
Homo sapiens	Tissue	Australia	1998	2
Homo sapiens	Tissue	Japan	2003–2008	3
Macaca fascicularis	Tissue	Japan	1994	1
Macaca fascicularis	Tissue	Australia	1998	2
Macaca fascicularis	Isolate	Netherlands	2014–2015	6
Macaca mulatta	Isolate	Netherlands	2014–2015	7
Macaca mulatta	Tissue	Australia	1998	1
Macaca mulatta	Tissue	United States	2001	2
Macaca mulatta	Tissue	United States	2005	7
Mandrillus sphinx	Tissue	Australia	1998	2

In the present study, we aimed at resolving the structure of the *H. suis* population colonizing pigs, humans and non-human primates, in order to investigate the true origin of the infection in these hosts. We show that *H. suis* in pigs possibly originates from non-human primates. Our data suggest that a host jump from macaques to pigs happened between 100 000 and 15 000 years ago and that pig domestication has had a significant impact on the spread of *H. suis* in the pig population, from where this pathogen occasionally infects humans.

Materials and methods

Samples

A list of the samples used for *H. suis* strain typing and their specifications is shown in Table 1 and Supplementary Table S1. Human samples were collected for diagnostic purposes during routine upper gastrointestinal tract endoscopy (O'Rourke *et al.,* 2004; Joosten *et al.*, 2013; Matsui *et al.*, 2014). Stomachs from pigs (*Sus scrofa*) were collected in slaughterhouses or from wild boars that had been shot during authorized hunting. Gastric mucosal samples were collected as described previously (Liang *et al.*, 2013). Tissues from captive cynomolgus monkeys, rhesus monkeys and mandrills from the USA, Japan and Australia had been previously collected and used for other purposes (Dubois *et al.*, 1991; Lindén *et al.*, 2004; O'Rourke *et al.*, 2004; Nakamura *et al.*, 2007; Martin *et al.*, 2013).

In addition to tissues samples, seven and six *H. suis* strains were isolated using a previously described technique (Baele *et al.*, 2008; Liang *et al.*, 2015) from the stomach of socially housed rhesus monkeys (*M. mulatta*) and cynomolgus monkeys (*M. fascicularis*), respectively. These monkeys were bred and housed at the Biomedical Primate Research Centre (BPRC, Rijswijk, The Netherlands), accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). BPRC facilities comply with Dutch law on animal experiments and the EU Directive 63/2010, and have an Animal Welfare Assurance from NIH (A5539-01). Stomachs were collected at necropsy from healthy animals that were killed for reasons unrelated to this study.

All gastric tissue samples from pigs and humans, as well as *in vitro* isolated porcine strains described and used in the study by Liang *et al.* (2015) were included in the analyses as well.

Multilocus sequence typing

DNA from gastric biopsies as well as from pure *H. suis* strains was extracted using the Isolate Genomic DNA MiniKit (Bioline, London, UK) or DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. All DNA samples were screened for the presence of *H. suis* using a Taq polymerase-based species-specific PCR, as described by De Groote *et al.* (2000).

All *in vitro* isolated *H. suis* strains, as well as one tissue sample from every *H. suis*-positive animals and humans were subjected to multilocus sequence typing (MLST), as described previously (Liang *et al.*, 2013). This technique allows culture-independent strain typing of fastidious microorganisms such as *H. suis* and is based on partial nucleotide sequencing of the following seven *H. suis* housekeeping genes: *atpA, efp, ppa, mutY, trpC, ureAB* and *yphC*.

Analysis of H. suis population structure, recombination analysis and estimation of time of divergence

The Maximum Composite Likelihood nucleotide distance was calculated based on the concatenated MLST sequences (4084 bp) using MEGA6 software (Tamura *et al.*, 2004, 2013) and population structure and gene flow were inferred using Bayesian Analysis of the Population Structure (BAPS) 6 (Cheng *et al.*, 2013).

To ensure that possible recombination events did not bias the phylogenetic analysis, two distinct approaches were used to analyze the sequences. First, ClonalFrame v1.2 was used to infer the ancestral relationship between *H. suis* strains analyzed in this study (Didelot and Falush, 2007). This software uses MLST data to estimate the clonal relationship between strains, while also taking into account the influence of horizontal gene transfer (Didelot and Falush, 2007; Moodley *et al.*, 2012). The program was run two times with 500 000 Markov Chain Monte Carlo iterations with a thinning interval of 50 after an initial burn-in phase of 50 000 iterations and two times with 1 000 000 Markov Chain Monte Carlo iterations with a thinning interval of 100 after an initial burn-in phase of 100 000 iterations. A majority-rule consensus tree of the four independent ClonalFrame runs was computed and edited using the Interactive Tree of Life (iTOL) tool (Letunic and Bork, 2011). In a second approach, the method of Feil *et al.* (2003) was used to determine the pairwise nucleotide distances between strains as a function of MLST allele distances. Genes that have experienced recombination appeared as outliers in this analysis. The analysis was performed on the entire data set and for each BAPS cluster. After exclusion of genes affected by recombination, time of separation between the BAPS clusters HSU1 and HSU3 (see the Results section), showing a clear increasing linear tread in the analysis according to Feil and colleagues, was calculated using Bayesian Evolutionary Analysis by Sampling Trees (BEAST) (Drummond and Rambaut, 2007). A constant population size was assumed, and the HKY85 model of nucleotide substitution (Hasegawa *et al.*, 1985) was used with strict clock and gamma rate heterogeneity with six categories (Drummond and Rambaut, 2007). A log-normal prior with a mean of 1.0 and s.d. of 1.25 on the logarithmic scale was assumed for the

transition:tranversion ratio κ, and an exponential prior with a mean of 0.5 was utilized for the gamma shape parameter α. As prior, a normal distribution of the mutation rate ranging approximately between $1.8e^{-7}$ and $3.6e^{-7}$ (mean: $2.6e^{-7}$; s.d.: $8e^{-7}$; 95%, $1.28e^{-7}$–$3.91e^{-7}$) was assumed, which was based on the long-term population-based mutation rate in the closely related human pathogen *H. pylori* (Moodley *et al*., 2009; Morelli *et al*., 2010). Linear regression, calculated using IBM SPSS statistics 23, was used to assess the strength of the correlation between the coalescent units estimated by Clonalframe and the time to the most recent common ancestor (TMRCA) estimated by BEAST, and to extrapolate the time of separation between the other BAPS clusters.

Determination of porcine mitochondrial DNA haplotype

Porcine gastric tissue was used to classify pigs as having a European or Asian haplotype, according to a previously described classification based on mitochondrial DNA (Larson *et al*., 2010). Briefly, PCR was performed in 20 µl reaction volumes containing 5U GoTaq Flexi DNA polymerase (Promega), 1× GoTaq Flexi PCR buffer, 2.5 mM $MgCl_2$ (Promega), 200 µM dNTPs (Bioline), 0.25 µM of both primers (DloopL: 5′-ACTAACTCCGCCATCAGC AC-3′; DloopR: 5′-GTTTGGCAAGGCGTTATAG-3′) and 1 µl DNA. PCR reactions were run in a MasterCycler thermal cycler (Eppendorf) using the following cycling conditions: initial denaturation for 3 min at 94 °C, followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min and a final extension of 10 min at 72 °C. Alternatively, a newly designed primer pair was used to amplify and sequence part of the mitochondrial control region (D-loop; F: 5′-CTCGCTCCGGGCCCATAA-3′; R: 5′-TTTTTGGGGTTTGGCAA-3′). Based on the obtained sequences (Supplementary Figure S1), animals were classified as having a European or Asian haplotype, according to the classification described by Larson *et al.* (2005) (Supplementary Table S1).

The circos software package (Krzywinski *et al*., 2009) was used to visualize the distribution of the *H. suis* BAPS clusters among the different host species and genotypes (i.e. *Homo sapiens*,

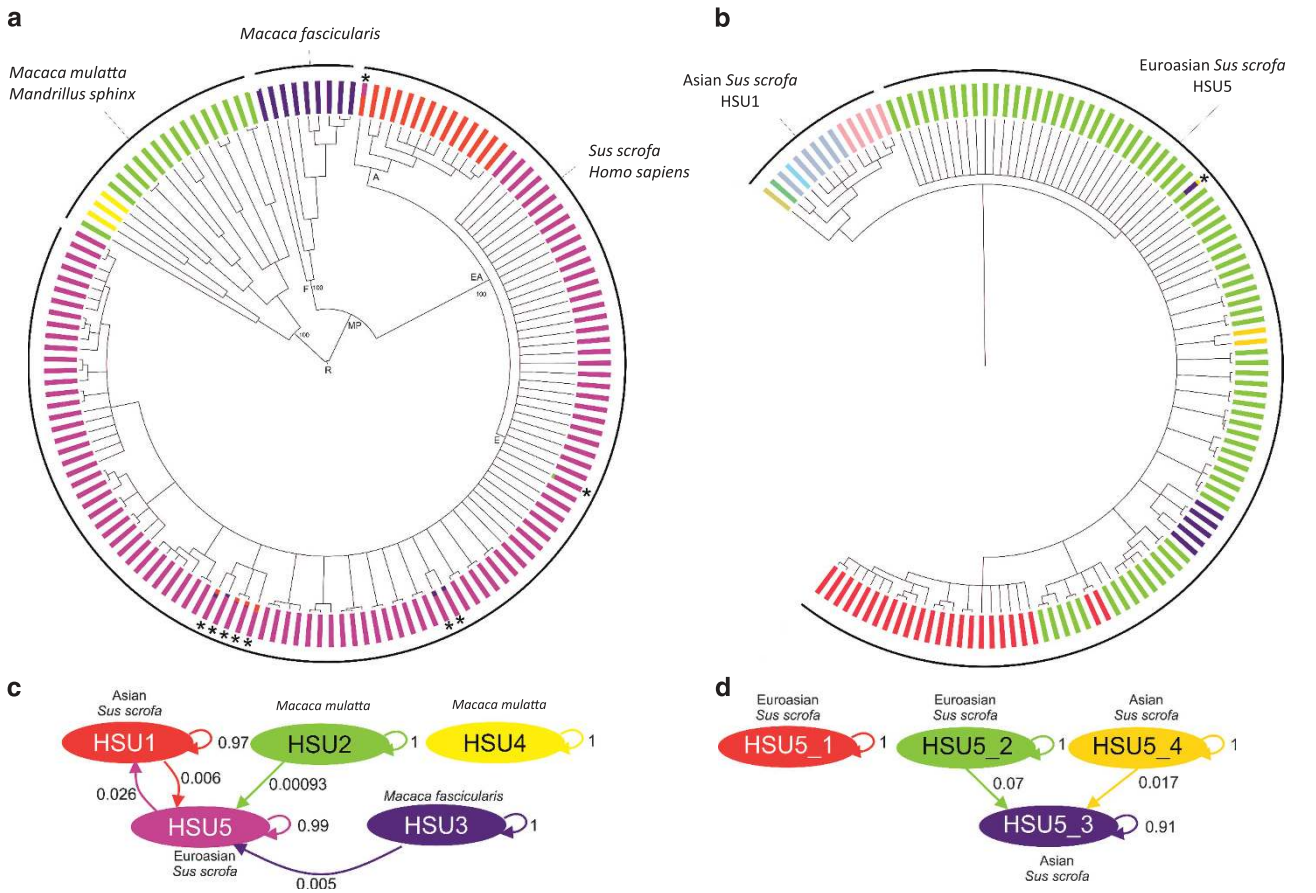

Figure 1 *Helicobacter suis* population structure. ClonalFrame and BAPS analysis showed that *H. suis* strains clearly belong to five distinct populations (HSU1–HSU5) (**a**). Population HSU5 was further divided into four subpopulations (HSU5_1–HSU5_4) (**b**). Gene flow between different *H. suis* BAPS populations (**c**) and HSU5 subpopulations (**d**) are indicated by arrows, accompanied by a number between 0 and 1, representing the proportion of ancestral DNA flow in this direction. Colors in Figures 1a and b correspond to the colors attributed to different BAPS clusters in Figures 1c and d, respectively. An * designates *H. suis* strains showing a mixed ancestry, as shown by mixed bar colors.

M. fascicularis, M. mulatta, Mandrillus sphinx, Asian *S. scrofa*, European *S. scrofa*).

Results

For 56 porcine samples out of a total of 80 for which a piece of gastric tissue was available, we were able to determine the mitochondrial DNA haplotype as being European or Asian (Supplementary Table S1 and Supplementary Figure S1). All Chinese and Vietnamese pigs were shown to have an Asian haplotype. Most pigs from Europe and the Americas revealed a European haplotype, although some of these animals revealed an Asian haplotype.

A total of 149 *H. suis*-positive samples from domestic pigs, wild boars, cynomolgus monkeys, rhesus monkeys and humans were collected and subjected to MLST. All obtained sequences are available at http://pubmlst.org/hsuis/. ClonalFrame and BAPS analysis revealed that *H. suis* strains clearly belong to five distinct populations (Figures 1a and 2 and Supplementary Table S1). Population HSU3 includes all *H. suis* strains isolated from or detected in cynomolgus monkeys. Populations HSU2 and HSU4 contain all strains colonizing the stomach of rhesus monkeys, as well as the two strains from the mandrills. The fact that no distinction could be made between *H. suis* strains from rhesus monkeys and mandrills, while both primate species originate from a different continent, suggests that the two mandrills harboring *H. suis* may have contracted the infection through direct or indirect contact with or the proximity of rhesus monkeys in the same Australian zoo.

Populations HSU1 and HSU5 comprise all the strains detected in or isolated from pigs and humans. More specifically, HSU1 (referred to as the 'Asian' pig-associated population) contains most, but not all, of the strains colonizing pigs with Asian mitochondrial DNA (mtDNA) haplotypes originating from Vietnam and China, while population HSU5 (referred to as the 'Euroasian' pig- and human-associated *H. suis* population) comprises all strains colonizing pigs with European haplotypes (originating from a European, American or African host), some strains colonizing pigs with Asian haplotypes (both originating from Europe and Asia) as well as all *H. suis* strains colonizing humans (Supplementary Table S1). This, along with the relatively low prevalence of *H. suis* infection in humans, suggests that *H. suis* bacteria occasionally jump from pigs to humans. Finally, population HSU5 was further divided (Figure 1b) into HSU5_1 and HSU5_2, harboring *H. suis* strains colonizing pigs with European or Asian haplotypes, and HSU5_3 and HSU5_4, comprising strains circulating only in pigs showing Asian haplotypes. Overall, a clear association was observed between the population structure of the host and that of *H. suis* (Figure 2).

Admixture analysis revealed gene flow between the different BAPS clusters, particularly from *H. suis* populations circulating in both macaque species (HSU2 and HSU3) to the pig- (and human)-associated Euroasian population (HSU5) and between the two *H. suis* populations circulating in pigs (HSU_1 and HSU_5) (Figure 1c). In addition, gene flow between Euroasian and Asian strains was also inferred within the HSU5 population (Figure 1d).

The common ancestor of the pig-associated *H. suis* population was shown to lie within the diversity of all macaque-associated *H. suis*, with the cynomolgus monkey-associated helicobacters being the sister clade of pig-associated *H. suis* strains (Figure 1a). Despite the higher number of pig- and human-associated *H. suis* strains analyzed in this study, the diversity of *H. suis* strains in non-human primates appeared to be much greater compared with the diversity of *H. suis* strains circulating in humans and pigs, which showed a star-like phylogeny characterized by long internal and short external tree branches. This strongly indicates a more ancient association between *H. suis* and non-human primates compared with that between *H. suis* and pigs, as well as a possible recent clonal expansion of the *H. suis* population colonizing domesticated pigs worldwide.

Nucleotide distance analysis revealed an identity of approximately 95% (94.4–95.15%) between the different *H. suis* populations from monkeys and pigs, whereas the highest distance (93% identity) was observed between the two groups harboring macaque-associated *H. suis* strains.

Using BEAST, we estimated the age of the association between *H. suis* and its hosts, based on the described population-based mutation rate for the closely related species *H. pylori* (Table 2). To minimize the effect of recombination in the estimation, the *yphC* gene was excluded from the analysis, since this was shown to be affected by pervasive recombination. In addition, we limited the BEAST analysis to populations HSU1 (*H. suis* strains associated with Asian pigs) and HSU3 (*H. suis* strains found in *M. fascicularis*). These two populations were selected since they showed a clear increasing linear tread in the analysis according to Feil *et al.* (2003), indicating little effect of recombination on the evolution of these lineages. Medians and the 95% highest posterior density interval of the estimation of the time to the most recent common ancestor (TMRCA) calculated by BEAST are summarized in Table 2. Furthermore, we attempted to infer the ages of the other nodes in the ClonalFrame tree by applying linear regression between the coalescent time calculated by Clonalframe and the $\log_{10}$ of the TMRCA inferred by BEAST. A strong correlation was observed ($R^2 = 0.942$) and the predicted ages of the other nodes are listed in Table 2. We estimated that a common ancestor for the *H. suis* population in pigs and monkeys

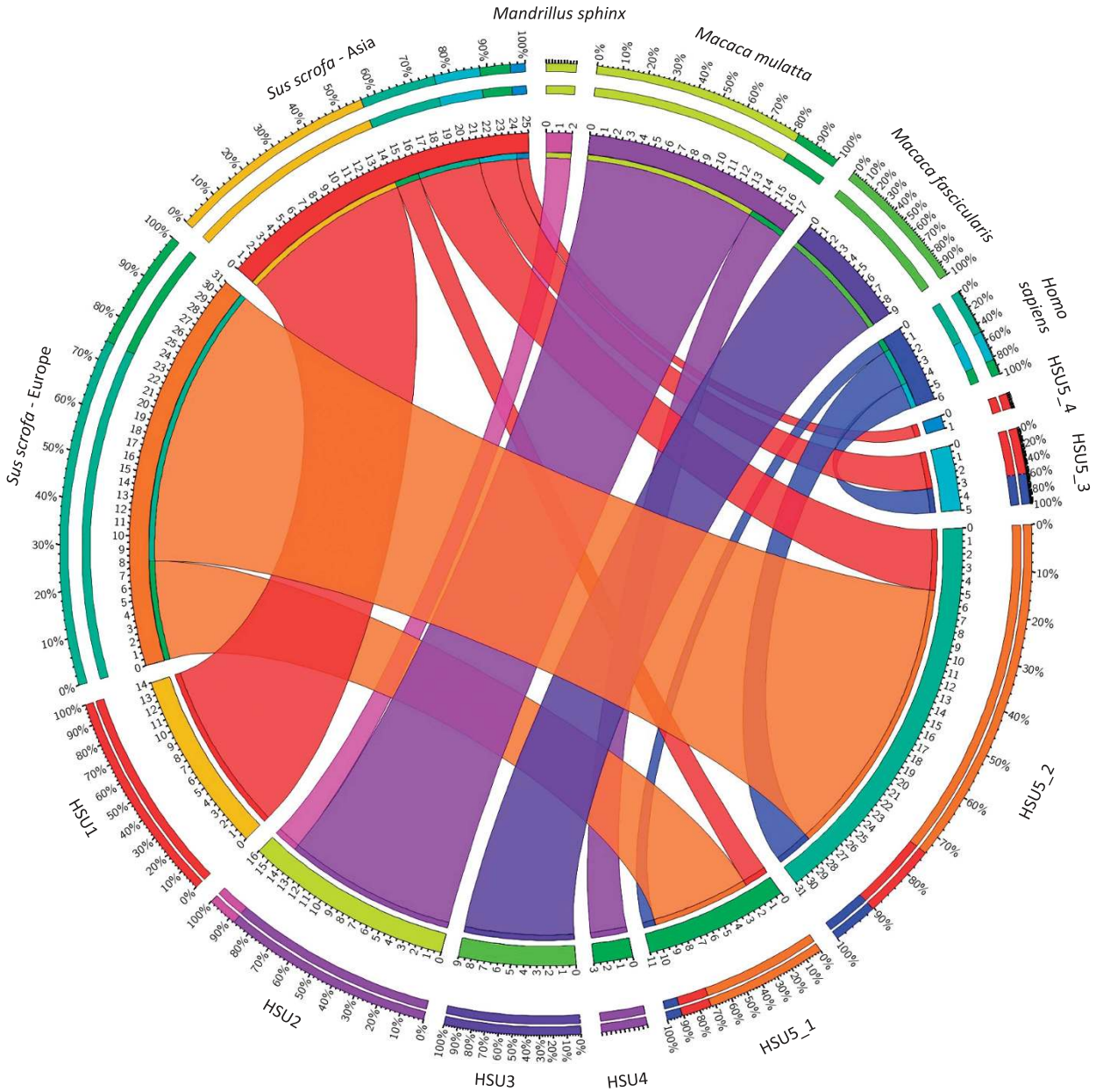

Figure 2 Association between the population structure of the host and that of *H. suis*. This figure shows the distribution of the *H. suis* strains from different BAPS clusters (HSU1–HSU5) among the different host species and genotypes (i.e. *Homo sapiens*, *Macaca fascicularis, Macaca mulatta, Mandrillus sphinx,* Asian *Sus scrofa*, European *Sus scrofa*).

existed until approximately 200 000 (193 118–197 704) years ago. In addition, we dated the split between monkey-associated and pig-associated *H. suis* populations approximately 100 000 (146 650–71 649) years ago. The *H. suis* population in pigs underwent a subsequent clonal expansion starting approximately 15 000 years ago. This, together with the limited diversity in the pig-associated *H. suis* population and the mixed ancestry (Figure 1a) observed for several pig-associated *H. suis* strains, strongly suggests that *H. suis* in pigs originates from non-human primates, a jump which seems to have taken place somewhere between 100 000 and 15 000 years ago.

Discussion

Numerous studies performed in Europe, the Americas and Asia have shown that *H. suis* infection in pigs is very common (Grasso *et al.*, 1996; Park *et al.*, 2004; Hellemans *et al.*, 2007; Foss *et al.*, 2013) with prevalences exceeding 50% and often being as high as 80–90%. These numbers resemble the high prevalence of *H. pylori* infection in humans worldwide, leading to the hypothesis that *H. suis* has evolved in pigs in a similar way to what has been described for *H. pylori* in humans. Several studies have suggested a long-standing coevolution of *H. pylori* and humans for at least 100 000 years,

Table 2 Age of the association between *H. suis* and its different hosts

Nodes		*BEAST (years BP)*			*Linear regression (years BP)*[a]		
		Median	*95% HPD lower*	*95% HPD upper*	*Prediction*	*95% lower*	*95% upper*
R[b]	Root	—	—	—	195 505	193 118	197 704
MP	*M. fascicularis*/pigs	106 580	71 649	146 650	93 399	92 408	94 319
F	*M. fascicularis*	52 972	36 279	73 270	60 348	59 765	60 893
EA	Euroasian/Asian	—	—	—	15 139	15 038	15 236
E	Euroasian	—	—	—	13 135	13 051	13 216
A	Asian	12 263	7358	17 547	11 620	11 549	11 689

Abbreviation: HPD, highest posterior density.
For nodes A, F and MP, as indicated in Figure 1a, the age of the association between *H. suis* and its hosts was determined using BEAST. For the other nodes in the ClonalFrame tree (Figure 1a), the ages were inferred by applying linear regression between the coalescent time calculated by Clonalframe and the $\log_{10}$ of the TMRCA inferred by BEAST.
[a] Years BP = 10^[3204 (Lower: 3197; Upper: 3210) × Coalescent Unit+3765 (Lower: 3763; Upper: 3767)]; R^2 = 0,942.
[b]As indicated in Figure 1.

resulting in a large genetic diversity and relatively strong phylogeographic signals (Achtman *et al.*, 1999; Falush *et al.*, 2003; Moodley *et al.*, 2009). In contrast, the results of the present study revealed that the *H. suis* population in pigs shows a fairly limited genetic diversity. In addition, the main pig-associated *H. suis* population (HSU5) showed a star-like phylogeny with relatively few structured geographic signals. The clearest distinction was found between approximately half of the *H. suis* strains colonizing Asian pigs and all other *H. suis* strains, associated with pigs in Europe, the Americas and Africa as well as infected humans from different continents. In contrast and somewhat surprisingly, the *H. suis* populations colonizing rhesus and cynomolgus monkeys could clearly be subdivided into two clades with a much greater genetic diversity. This very strongly suggests a more ancient association between *H. suis* and non-human primates than the association between *S. scrofa* and *H. suis*, and it appears to disprove the original idea of the swine origin of *H. suis*. In addition, for some pig-associated *H. suis* strains, a mixed ancestry was observed with the population colonizing cynomolgus monkeys indicating possible gene flow between these populations. This further indicates that *H. suis* infection in the pig population worldwide may very well originate from non-human primates. This could, for instance, have taken place through the consumption of monkey stomachs or their contents by pigs, which are indeed considered to be omnivorous. However, we cannot exclude other possible scenarios including the involvement of a so far unknown intermediate host species of *H. suis* or the (near-) extinction of the original pig-associated *H. suis* population due to a severe bottleneck as observed in other pathogens (Kapusinszky *et al.*, 2015). In our case, the domestication of *S. scrofa* and subsequent selection for specific traits may have caused a significant bottleneck effect on the diversity of pig-associated *H. suis* originally circulating in the ancestral wild boar population. This hypothesis implies the possibility that specific *H. suis* strains still colonize wild boars around the world. However, this theory is contradicted by several studies which unsuccessfully attempted to detect *H. suis* in these animals (Fabisiak *et al.*, 2010; Bassi, 2013).

In the present study, samples from Belgian and Chinese wild boars were also tested for the presence of *H. suis* DNA. The two Chinese animals were negative for *H. suis*, as well as 7/9 Belgian wild boars (data not shown). The two positive animals only revealed the presence of very low numbers of *H. suis* in the cardiac gland zone or the *pars oesophagea*, which have been described not to be the main colonization sites for this bacterium in pigs (Hellemans *et al.*, 2007; De Bruyne *et al.*, 2012). Both strains belong to the 'Euroasian' pig- and human-associated *H. suis* population, so detection of *H. suis* DNA in atypical stomach regions in these animals might be a consequence of recent contamination, for instance through contact with domesticated animals or their excretes. In general, our results are consistent with previous studies revealing that *H. suis* seems to be absent in the majority of the wild boar populations (Fabisiak *et al.*, 2010; Bassi, 2013). This further supports our theory of a fairly recent host jump of *H. suis* to pigs from macaques or other unknown hosts, although further research should be performed in additional wild boar populations around the globe to provide more insights. Recently, a novel *Helicobacter* species, *H. apri,* has been described in wild boars, but this is an enterohepatic *Helicobacter* species that does not produce urease (Zanoni *et al.*, 2016). It was, therefore, not included in our study.

An estimation of the divergence time between the different clades suggests that the host jump from macaques to pigs may have taken place somewhere between 100 000 years ago (ya), the inferred date of the split between monkey- and pig-associated populations, and 15 000 ya, which is the time to the most recent common ancestor of pig-associated *H. suis* populations. This would imply that pigs got infected

before the domestication of this animal species. Both zooarcheological and molecular studies have revealed at least two centers of pig domestication, which clearly happened independently in the Near East (Anatolia) and East Asia (Mekong valley) around 9000 ya (Larson *et al.*, 2005; Cucchi *et al.*, 2011; Frantz *et al.*, 2015). The first domestic pigs in Europe were most likely dispersed from Anatolia into Europe around 7500 ya, forming the basis for western Eurasian domestic pigs (Larson *et al.*, 2007). Nevertheless, recent data have revealed a more complex domestication process involving continuous post-domestication gene flow between domestic pig and wild boar populations, both in Asia and Europe, leading to the loss of Near Eastern mtDNA signatures in European domesticated pigs (Larson *et al.*, 2007; Frantz *et al.*, 2015). Our data showed that the host jump from *H. suis* was followed by a clonal expansion starting ~15 000 ya, suggesting that domestication of *S. scrofa* 7000–9000 ya and selection post-domestication for behavioral and morphological traits (Frantz *et al.*, 2015) may have had an significant impact on the spread of this bacterium in the modern pig population.

The results of the present study do not allow an exact calculation of the age of the association between *H. suis* and macaques. Possibly, the ancestor of *M. fascicularis* and *M. mulatta*, which have separated ~2.5 Mya, was infected with an ancestor of modern *H. suis*, or alternatively, one macaque species served as a source of infection for the other species, for instance during periods of interbreeding that have been described to have taken place after approximately 170 000 before the present in Indochina (Tosi *et al.*, 2002; Kanthaswamy *et al.*, 2008). As mentioned above, for some pig-associated *H. suis* strains a mixed ancestry was observed with the clade harboring all cynomolgus monkey-associated *H. suis* strains. The latter was also shown to be the sister clade of pig-associated *H. suis* strains, showing that cynomolgus monkeys are the most likely ancestral source of *H. suis* infection in pigs, rather than rhesus macaques, unless an unknown intermediate host species is involved as well. Bearing in mind the natural habitat of the animals (Kanthaswamy *et al.*, 2008), a potential host jump of *H. suis* from either macaque species to pigs most likely would have happened in East Asia. In turn, East Asian pigs may have been the source of *H. suis* infection in western domesticated pigs. Indeed, Asian pigs, possibly harboring *H. suis*, were introduced into Europe during the eighteenth and nineteenth centuries, as shown by historical records and recent investigations. These have revealed an Asian introgression of about 20% into the genome of European commercial pigs (Bosse *et al.*, 2014), as well as the presence of a minority of Asian mtDNA haplotypes in several European and American pig breeds (Giuffra *et al.*, 2000; Larson *et al.*, 2010), which was also demonstrated in the present study.

In our study, we clearly demonstrated differences between *H. suis* strains isolated from pigs and both macaque species. Similar reports have been made for other pathogens that have jumped between different hosts, such as *Staphylococcus aureus* (Smith *et al.*, 2014). It is of course important to realize that not only the pathogen but also the interaction between the host and pathogen determines the outcome of an infection. This has been nicely demonstrated for the closely related human pathogen *H. pylori*. African *H. pylori* ancestry, for instance, has been shown to be relatively benign in humans from African origin, whereas it is deleterious in individuals with substantial Amerindian ancestry, showing that the disruption of coevolution, as is the case for Amerindian humans infected with an African *H. pylori* strain, may result in more severe pathology (Kodaman *et al.*, 2014). Another example of disease severity associated with disrupted coevolution is that of bovine papillomavirus-1 (BPV-1), which causes relatively innocent warts in cows, its natural host. Recent research has revealed evidence of multiple, relatively recent host jumps of BPV-1 into horses, in which this virus can cause the development of invasive and aggressive sarcoids (Trewby *et al.*, 2014). In the present study, we revealed a more ancient association between *H. suis* and macaques, compared with that between *H. suis* and pigs. The seemingly more innocent character of *H. suis* infection in macaques fits with the examples described above for *H. pylori* and the bovine papillomavirus-1, showing that coevolution between the microbe and the host generally results in decreased pathogenicity. In contrast, the fairly recent host jump and expansion of a clonal population, especially predicted for the European domestic pig population, may explain the pathogenicity of *H. suis* strains infecting pigs.

This study provides the first insights into the phylogeny and population structure of *H. suis*. In contrast to our expectations, we showed that *H. suis* in pigs probably originates from non-human primates. After a possible host jump from macaques to pigs, pig domestication may have had a significant impact on the spread of *H. suis* in the pig population, from where this pathogen occasionally infects humans. This is a rare example showing that an important microbial pathogen in pigs and humans in fact seems to originate from a completely different host species, in which infection causes little or no harm. Future research should aim to answer the question whether other, hitherto unknown host species may have contributed to the evolution and worldwide spread of *H. suis.* In addition, it should be investigated which adaptations may have facilitated the remarkable jump of *H. suis* from non-human primates to pigs and the subsequent spread of the microorganism in its new host.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This study was funded by a grant from the Research Fund of Ghent University, Ghent, Belgium (grant BOF14/GOA/010) and the Research Foundation—Flanders (FWO; grant FWO14/PDO/067). JC was funded by the COIN centre of excellence, Academy of Finland grant 2551170. SKL was funded by grants from the Swedish Research Council (grant 521-2011-2370) and Formas (grant 221-2013-590).

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