



## Geographic and species association of hepatitis B virus genotypes in non-human primates

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### Abstract

Infection with hepatitis B virus (HBV) has been detected in human populations throughout the world, as well as in a number of ape species (*Pan troglodytes*, *Gorilla gorilla*, gibbons [*Nomascus* and *Hylobates* species] and *Pongo pygmaeus*). To investigate the distribution of naturally occurring HBV infection in these species and other African Old World monkey species (*Cercopithecidae*), we screened 137 plasma samples from mainly wild caught animals by polymerase chain reaction (PCR) using several of highly conserved primers from the HB surface (HBs) gene, and for HBs antigen (HBsAg) by ELISA. None of the 93 *Cercopithecidae* screened (6 species) showed PCR or serology evidence for HBV infection; in contrast 2 from 8 chimpanzees and 5 from 22 gibbons were PCR-positive with each set of primers.

Complete genome sequences from each of the positive apes were obtained and compared with all previously published complete and surface gene sequences. This extended phylogenetic analysis indicated that HBV variants from orangutans were interspersed by with HBV variants from southerly distributed gibbon species (*H. agilis* and *H. moloch*) occupying overlapping or adjacent habitat ranges with orangutans; in contrast, HBV variants from gibbon species in mainland Asia were phylogenetically distinct. A geographical rather than (sub)species association of HBV would account for the distribution of HBV variants in different subspecies of chimpanzees in Africa, and explain the inlier position of the previously described lowland gorilla sequence in the chimpanzee clade. These new findings have a number of implication for understanding the origins and epidemiology of HBV infection in non-human primates.

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### Introduction

Infection with hepatitis B virus (HBV) is a major global health problem, and is estimated to account for approximately one million deaths from chronic liver disease and hepatocellular carcinoma each year (Thomas and Jacyna, 1993). High frequencies of active infection, ranging from 8–15% are found in South and East Asia, sub-Saharan Africa and amongst indigenous peoples in Central and South America (Andre, 2000). In Asia this endemic pattern of HBV infection is primarily maintained through mother-to-child perinatal transmission and establishment of a highly infectious carrier state that transmits infection to the next

generation. In contrast, horizontal transmission is the predominant mechanism in Africa.

Although HBV (classified as a member of the *Hepadnaviridae*) contains a DNA genome, replication occurs through an RNA intermediate sequence analogous to the genomic RNA sequence of retroviruses. The copying of DNA from sequences from genomic DNA templates, and from the intermediate RNA transcript, is carried out by a virally encoded polymerase protein. The lack of proof-reading during viral transcription introduces a high frequency of mutations into the copied sequences (Hannoun et al., 2000). Indeed, HBV populations are characterized by a moderate degree of genetic diversity, with a total of 8 currently classified genotypes infecting human populations worldwide, differing from each other by nucleotide sequence distances of approximately 10–13%. Genotypes A, D and possibly G have global distributions,

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genotypes B and C are found predominantly in East and South East Asia, genotype E in West Africa, and genotypes F and H amongst various population groups, including indigenous peoples, in Central and South America (Norder et al., 1994; Arauz-Ruiz et al., 1997; Arauz-Ruiz et al., 2002).

Recently, we (MacDonald et al., 2000) and others (Takahashi et al., 2000; Hu et al., 2000; Vartanian et al., 2002; Hu et al., 2001) have documented the existence of HBV infection in chimpanzees in the wild, findings which add to other descriptions of frequent infection of gibbons and orangutans in South East Asia (Warren et al., 1999; Grethe et al., 2000; Verschoor et al., 2001; Noppornpanth et al., 2003). Nucleotide sequencing of HBV recovered from these ape species revealed the existence of new genotypes of HBV generally specific to each species, although with some exceptions, such as the detection of a human genotype E variant in a captive chimpanzee (Takahashi et al., 2000), the close relatedness of HBV recovered from a lowland gorilla to chimpanzee sequences (Grethe et al., 2000), and the detection of a gibbon-like HBV sequence in a captive chimpanzee (Grethe et al., 2000).

Apart from humans and non-human primates, far more divergent HBV-like viruses have been detected in New World rodents such as the woodchuck (*Marmota monax*), and squirrel species (*Spermophilus beecheyi*, *S. parryii*), and a range of bird species (ducks, geese, and grey heron). The evolutionary history of HBV in these various host species has been the subject of great debate over the past 5 years, principally fueled by the difficulty in reconciling the frequently interspersed genotype distributions of human and non-human primate sequences that seems to fit neither hypothesis for very recent or very ancient origins for HBV (Magnius and Norder, 1995; Norder et al., 1994; MacDonald et al., 2000) [reviewed in (Simmonds, 2001)]. In the current study, we have attempted to discover more about the distribution of HBV infection in apes and Old World monkey species (*Cercopithecidae*) using a large archive of stored serum and plasma samples from a wide range of different African and South East Asian primate species. Combining the new data obtained in this study with recently published sequences from gibbons, orangutans and chimpanzees (Vaudin et al., 1988; MacDonald et al., 2000; Takahashi et al., 2000; Hu et al., 2000; Hu et al., 2001; Warren et al., 1999; Grethe et al., 2000; Verschoor et al., 2001; Noppornpanth et al., 2003; Takahashi et al., 2001; Vartanian et al., 2002), we have been able to carry out comprehensive genetic comparisons of the expanded dataset of primate and human-derived HBV variants that indicates that geographical separation in primates explains genotype distributions of HBV better than species associations.

## Results

### *Detection of HBV infection in non-human primates*

To determine the frequency of active HBV infection in Apes and Old World primate species, we screened available plasma or serum samples collected from a range of species by PCR using previously described primers from the pre-S and S region (S1, S2). All samples had been screened for HBsAg by commercially available ELISAs. The pre-S and S primers were conserved between human genotypes A–G, the more divergent F and H genotypes, and all published HBV sequences recovered from non-human primates including the HBV variant obtained from New World woolly monkey species (Fig. 1A and B).

Results from the two sets of primers were concordant with each other and with the results of HBsAg screening with the exception of 1 HBsAg positive chimpanzee that was DNA negative. The frequency of detection of active HBV infection depended on the host species. Within the apes, we detected 2 HBV-positive samples from the 8 chimpanzees screened, 5 from the 22 gibbons species, and none from 14 orangutans (total prevalence of active infection in apes: 7/44 [15.9%]). In contrast, none of the 93 samples available from a total of 6 Old World Monkey species were positive in either PCR assay, nor confirmed positive for HBsAg. The observed restriction of HBV infection to apes and the frequency of active infection found in this study was consistent with previous surveys of HBV infection in primates (Table 1; see Discussion). Further evidence for a lack of HBV infection in these samples of Old World monkey species was provided by the lack of detectable antibodies to the HBV core protein (anti-HBc), used diagnostically as an indication of past resolved infection in samples negative for HBsAg.

Previous studies have demonstrated that non-human primates are generally infected with species-specific genotypes of HBV (Vaudin et al., 1988; MacDonald et al., 2000; Takahashi et al., 2000; Hu et al., 2000; Hu et al., 2001; Warren et al., 1999; Grethe et al., 2000; Verschoor et al., 2001; Noppornpanth et al., 2003; Takahashi et al., 2001; Vartanian et al., 2002). Therefore, one explanation for the apparent absence of HBV infection in Old World monkey species is that the putative variants of HBV perhaps present in these more evolutionarily distant species may be too divergent to be detectable by conventional HBV primers and hence too different antigenically to be detectable by conventional HBsAg or anti-HBc screening. To investigate this possibility, we developed a new set of primers (S3) from a highly conserved region of the surface gene that matched not only all human and non-human primate HBV variants, but also the sequences of each of the HBV-like viruses recovered from rodents (ground and arctic squirrels, woodchuck; Fig. 1C). The new primers showed equivalent sensitivity for human and primate HBV sequences as the S1 and S2 primers for human and primate genotypes of HBV

A) S1 (Pre-S region)

Sequence alignment for S1 (Pre-S region) showing primer sequences (Primer 41, 42, 43, 19) and alignments for human (H-A to H-H), chimpanzee (CH), gibbon (GN), orangutan (OT), woolly monkey (WM), and rodent species (GS, AGS, WC, OHVCGC).

B) S2 (S region)

Sequence alignment for S2 (S region) showing primer sequences (Primer S1, S3, S6, S5) and alignments for human (H-A to H-H), chimpanzee (CH), gibbon (GN), orangutan (OT), woolly monkey (WM), and rodent species (GS, AGS, WC, OHVCGC).

C) S3 (Highly conserved region)

Sequence alignment for S3 (Highly conserved region) showing primer sequences (Primer 21, 22, 23, 24) and alignments for human (H-A to H-H), chimpanzee (CH), gibbon (GN), orangutan (OT), woolly monkey (WM), and rodent species (GS, AGS, WC, OHVCGC).

Fig. 1. Sequence conservation of HBV and HBV-like virus sequences from primates and rodents in regions of the genome targeted by S1, S2 and S3 primer sets used for PCR. Primer sequences were compared with consensus sequences from the human HBV genotypes A–H (H–A to H–H), consensus sequences from chimpanzee, gibbon, orangutan (CH, GN, OT), and the woolly monkey sequence (WM). The lower panel for each alignment contains HBV-like virus sequences from North American rodent species, ground squirrel (GS), arctic ground squirrel (AGS) and woodchuck (WC, OHVCGC). For each alignment, differences from the consensus genotype A sequence was shown; primer sequences included degenerate bases (represented by the codes: M: A/C; Y: C/T; R: A/G; K: G/T) in variable regions. Alignment numberings refer to a minimal alignment of human and primate derived HBV sequences listed in the Methods section.

(data not shown). However, screening of this sample of Old World primate species failed to detect any additional cases of HBV infection (Table 1).

Species and sub-species identification of HBV-infected apes

Of the two chimpanzees positive for HBV DNA, one (Osang) originated from Central Africa and its subspecies identification as P. troglodytes vellerosus was determined by mitochondrial sequencing. The other infected chimpanzee (Louisa) originated from Cameroon and belonged to the troglodytes subspecies. The infected gibbons belonged to species H. lar (Tamang), H. agilis (Crazy Woman), N. concolor (Happy) and N. gabriellae (Wendy). This species

information was provided by the Welsh Mountain Zoo and the rescue centre respectively. It was not possible to obtain a species assignment for the final HBV-infected gibbon analyzed in the study (TB Black), although it was possible to identify it as belonging to the Hylobates genus and most likely belonging to the agilis or moloch subspecies as Mitochondrial 12S region sequences for these species were interspersed when compared phylogenetically (data not shown).

Sequence comparison of HBV sequences from non-human primates

Sequence relationships between the 7 (from 44) ape samples positive for HBV DNA by PCR with previously

Table 1  
Frequencies of detection of HBV DNA sequences, HBsAg and anti-HBc in current and previous studies of primates

Species	PCR*		Total		Active <sup>‡</sup> infection (%)	anti-HBc	Reference
	S1/S2	S3	PCR <sup>†</sup>	HBsAg			
<b>APES</b>							
<i>Gorilla gorilla</i> spp.	—	—	7/18	2/15	7/18 (39%)	—	(Grethe et al., 2000; Zuckerman et al., 1978; Linnermann, Jr. et al., 1984; Thornton et al., 2001; Worley and Stalis, 2002)
<i>Pan troglodytes</i> spp.	2/8	—	28/38	38/496	54/517 (10.5%)	2/6	This study, (Takahashi et al., 2000; MacDonald et al., 2000; Hu et al., 2000; Takahashi et al., 2001; Hu et al., 2001; Grethe et al., 2000; Zuckerman et al., 1978) (Vaudin et al., 1988) (Deinhardt, 1976; Ogata et al., 1993; Eichberg and Kalter, 1980)
<i>Hylobates</i> spp.	5/22	—	53/177	35/191	55/213 (25.8%)	48/130	This study, (Grethe et al., 2000; Norder et al., 1996; Lanford et al., 2000; Vaudin et al., 1988; Deinhardt, 1976; Thornton et al., 2001; Noppornpanth et al., 2003)
<i>Pongo pygmaeus</i> spp.	0/14	—	32/104	58/141	58/297 (19.5%)	1/14	This study, (Warren et al., 1999; Davis et al., 2000; Vaudin et al., 1988; Deinhardt, 1976)
<b>Total Apes</b>	<b>7/45</b>	<b>—</b>	<b>120/337</b>	<b>133/843</b>	<b>174/1045 (16.7%)</b>	<b>51/150</b>	
<b>OLD WORLD MONKEYS</b>							
<i>Papio</i> spp.	—	—	0/4	0/168	0	0/103	(Deinhardt, 1976; Michaels et al., 1996; Eichberg and Kalter, 1980)
<i>Cercopithecus aetiops</i>	—	—	—	0/19	0	—	(Eichberg and Kalter, 1980)
<i>Cercopithecus torquatus</i>	0/4	0/4	0/4	0/4	0	0/4	This study
<i>Cebus albifrons</i>	—	—	—	0/10	0	—	(Eichberg and Kalter, 1980)
<i>C. mona</i>	0/3	0/3	0/3	0/3	0	0/3	This study
<i>C. nictitans</i>	0/2	0/2	0/2	0/2	0	0/2	This study
<i>C. erythrotis</i>	0/3	0/3	0/3	0/3	0	0/3	This study
<i>Mandrillus leucophaeus</i>	0/78	0/78	0/78	0/78	0	0/78	This study
<i>M. sphinx</i>	0/2	0/2	0/2	0/2	0	0/2	This study
<b>Total OWM</b>	<b>0/102</b>	<b>0/102</b>	<b>0/96</b>	<b>0/289</b>	<b>0</b>	<b>0/195</b>	
<b>NEW WORLD MONKEYS</b>							
<i>Lagothrix lagotricha</i>	—	—	10/35	7/31	10/33	—	(Lanford et al., 1998)
<i>Saimiri sciureus</i>	—	—	—	0/20	0	—	(Eichberg and Kalter, 1980)
<i>Callithrix jacchus</i>	—	—	—	0/6	0	—	(Eichberg and Kalter, 1980)
<i>Saguinus oedipus</i>	—	—	—	0/12	0	—	(Eichberg and Kalter, 1980)
<b>Total NWM</b>	<b>—</b>	<b>—</b>	<b>10/58</b>	<b>7/69</b>	<b>10/33 (30%)</b>	<b>—</b>	

\* PCR results from pre-S and S gene primers (S1, S2), and from highly conserved primers (S3; Fig. 1C) from this study.

† Combined PCR results from current survey and from previous studies (cited in last column).

‡ Combined results for PCR-positivity and detection of HBsAg indicating active HBV infection.

described variants of HBV from human and non-human primates were investigated by determination of their complete genome sequences and phylogenetic analysis. HBV variants included in the analysis comprised all previously published full length HBV sequences from non-human primates and 10 representative sequences from each of the human genotypes A–H. The maximum likelihood tree of these data provided bootstrap support for each human genotype and primate-associated species, with the exception of gibbon and orangutan sequences (Fig. 2). However, other than the close relationships between genotypes D and E, and F and H, there was little support for any other inter-clade relationship. A separate comparison of S gene sequences was also carried out to enable several further partial genomic sequences from primates to be included in the analysis (Fig. 3; see below).

#### *Chimpanzee sequences*

Both complete genome and surface gene sequences from the two HBV-infected chimpanzees (Louisa, Osang) grouped consistently with other previously published sequences (Fig. 2 and 3A). This together with the handling and testing history is consistent with infection in the wild. Within the divergent clade of chimpanzee sequences, individual sequences fell into a variety of groups, with a strong association with subspecies and/or geographical origin (Fig. 4A). For example, clusters were observed corresponding to verus subspecies from West Africa (whole genome sequences AB032433, HPBVCG, AB032432, AB032433, AF222323 and Chimp 4), troglodytes from Central Africa (AF222322, Chimp 2 and Louisa), vellerous from Central-West Africa (Osang, AF305327), and a separate clade for

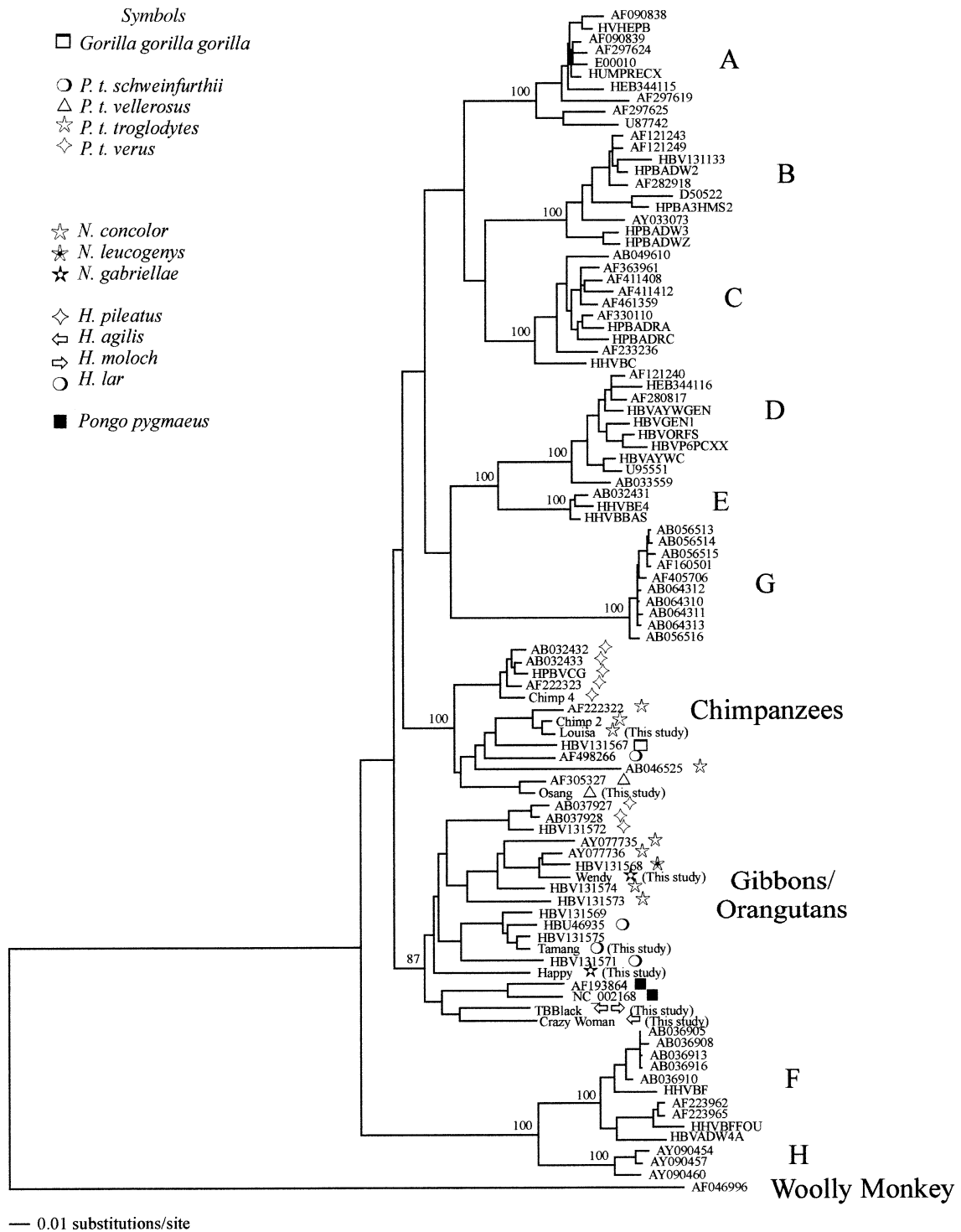


Fig. 2. Maximum likelihood phylogenetic tree for 99 complete HBV genomes (3342 bp) representing human genotypes A to H and all ape HBV sequences with the single woolly monkey HBV sequence as an outgroup. Neighbor-Joining bootstrap values (>75%) for major groupings are indicated on the relevant nodes. All horizontal branch lengths are drawn to scale.

the single Schweinfurthii (East African) HBV variant (AF498266). The only sequence that failed to group within its sub-species group was AB046525 obtained from a central African troglodytes species; this may be because it contains an unusual core gene sequence thought to have

originated through recombination with a highly divergent and hitherto undiscovered entirely separate genotype of HBV (Takahashi et al., 2001) (see below). Similar grouping of sequences into subspecies- or geographically-associated clades was found on analysis of partial S gene sequences.

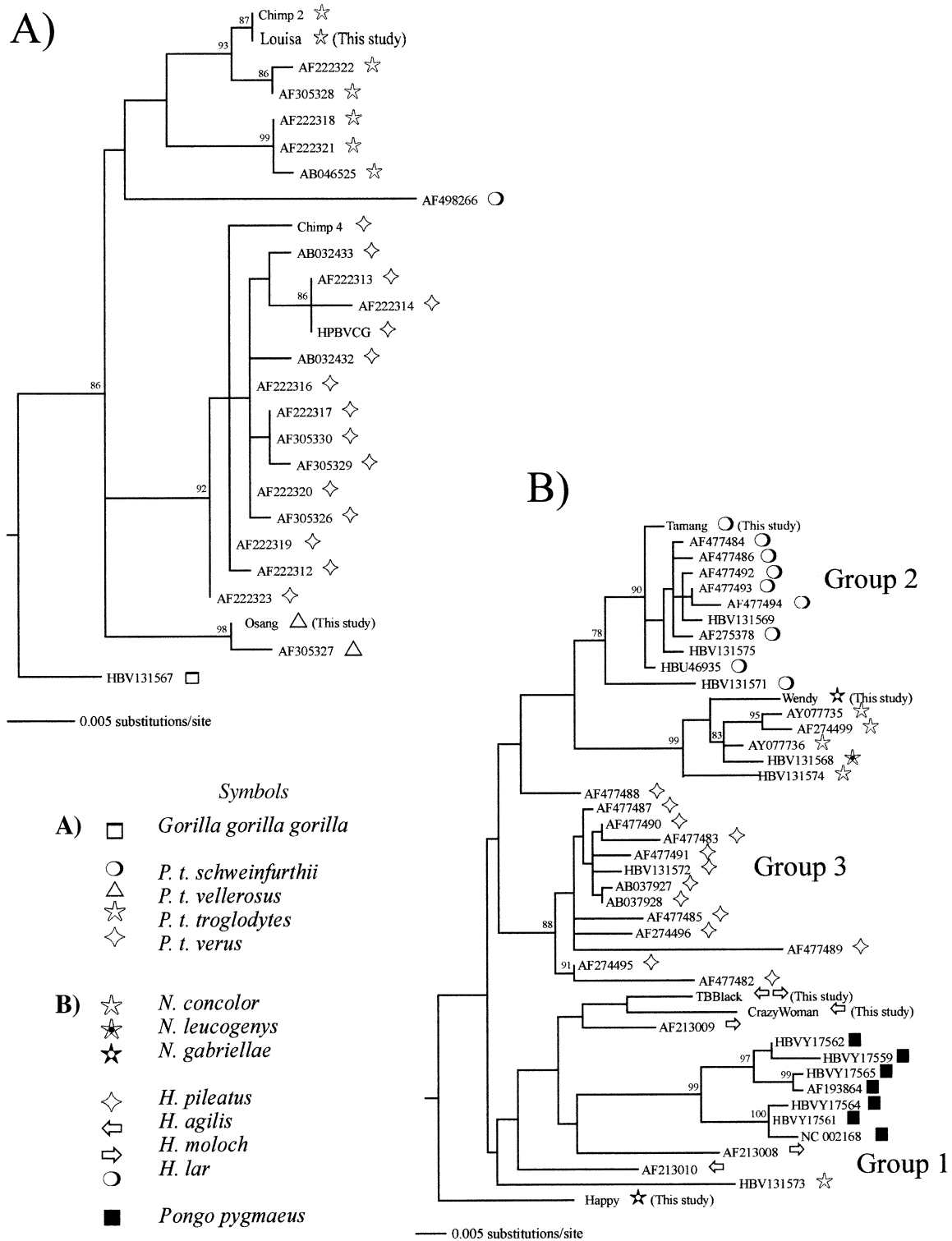
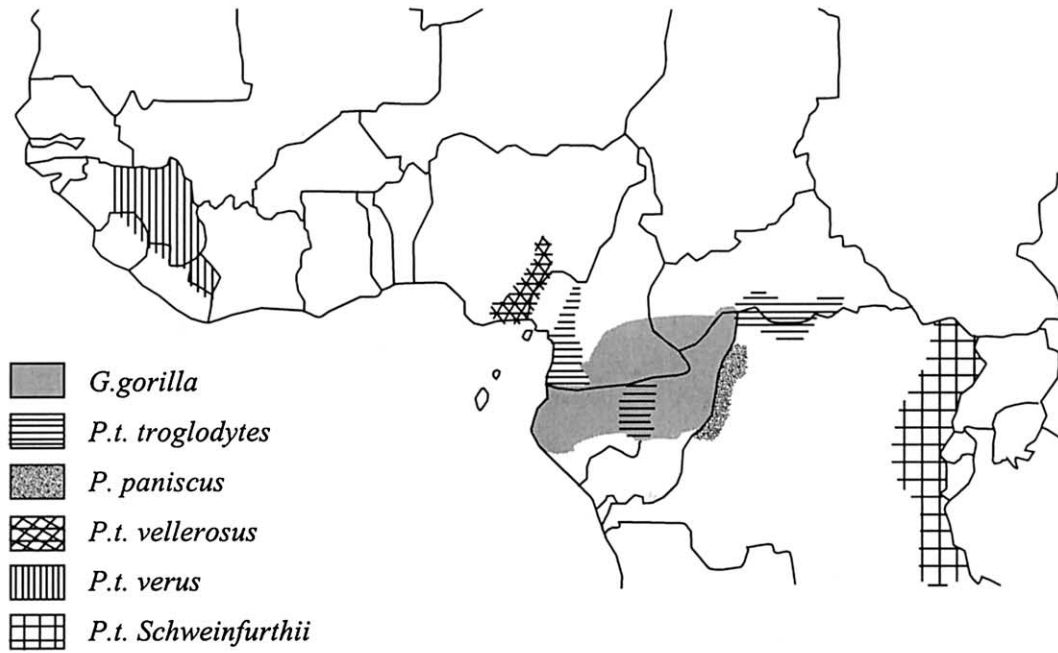


Fig. 3. Maximum likelihood phylogenetic trees of partial S gene sequences (689 bp) from (A) chimpanzees and (B) gibbons and orangutans. The chimpanzee tree is rooted using a single gibbon sequence (*Tamang*) and the gibbon/orangutan tree is rooted using a chimpanzee sequence (*Osang*). Neighbor-Joining bootstrap values (>75%) for major groupings are indicated on the relevant nodes. All horizontal branch lengths are drawn to scale.

Finally, the single available sequence from the gorilla (HBV131567) fell into its own unique clade within the chimpanzee genotype, an unexpected position if it is assumed that HBV variants are species (and sub-species)-specific, but not

inconsistent with the alternative hypothesis for a geographical basis for the observed sequence groups. As indicated (Fig. 4A), western lowland gorillas share their geographical range with the troglodytes subspecies of chimpanzee in Central Africa.

A)



B)

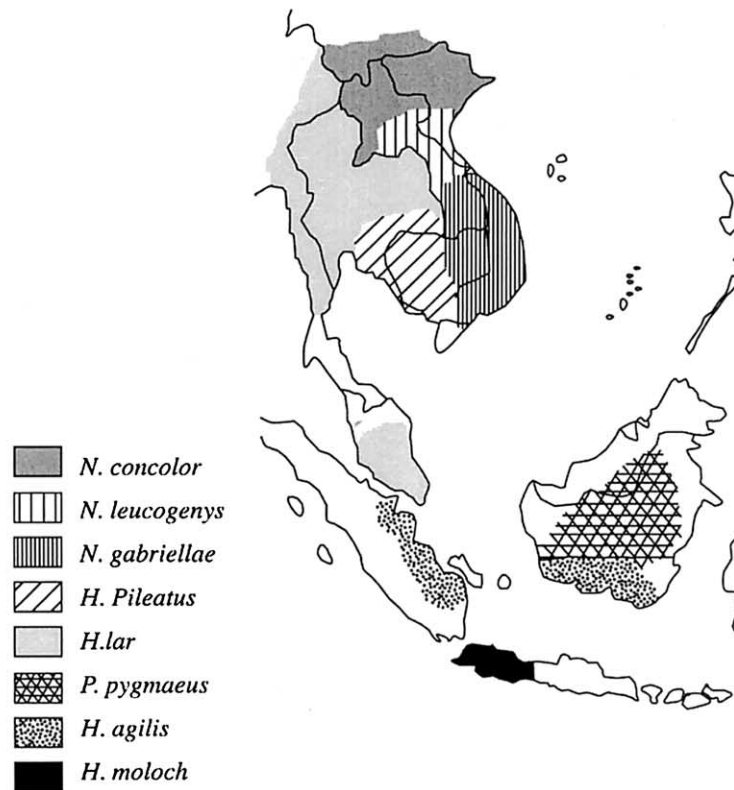


Fig. 4. Distribution of (A) chimpanzee subspecies and lowland gorillas in Africa, and (B) species of gibbons and orangutans in South East Asia.

Interestingly, the gorilla-derived sequence groups most closely with HBV sequences from this subspecies on comparison of complete genome sequences (Fig. 2), although this similarity does not lead to a bootstrap supported *G. gorilla/P.t.troglodytes* clade, nor is this similarity apparent upon sequence comparison of the surface gene fragment (Fig. 3A).

### Gibbon sequences

Complete genome sequences from 5 gibbons were obtained and compared with the 12 previously published HBV sequences from gibbon species and with those from two orangutans, and the single chimpanzee sequence (Fig. 2). As with the chimpanzee sequences, there was evidence for marked clustering of HBV sequences according to their host species, although the total sequence divergence within the clade was greater than found amongst the chimpanzee subspecies, and much greater than within human genotypes. There was also greater lineage differentiation within the gibbon sequence group, including well defined sequence clusters of sequences from *H. lar*, *nomascus* species (*N. concolor*, *leucogenys* and *gabriellae*) and from *H. pileatus* and a series of divergent single sequences, including those from Happy, TBBblack and CrazyWoman obtained in the current study. Lack of strict species-specificity of HBV variants was demonstrated in the S gene fragment by the highly divergent sequences obtained from *H. moloch* and *agilis* (CrazyWoman, AF213010, AF213008 and AF213009), the relation of an HBV variant from *N. concolor* (HBV131573) with these sequences and its failure to group with the main clade of *concolor* sequences (Fig. 3). Other anomalies include the outlier positions of the *H. pileatus* variant (AF477488) and the highly divergent sequence we obtained from Happy (*N. gabriellae*).

If a broader division of gibbon species into the *hylobates* (*moloch*, *agilis*, *pileatus* and *lar* species) and *nomascus* (*concolor*, *leucogenys* and *gabriellae* species) genera is made, there is still no association between host and HBV phylogeny. However, the greatest discordance between HBV grouping and species of origin is demonstrated by the phylogenetic position of the HBV sequences obtained from orangutans. The two complete genome sequences (NC\_002168 and AF193864), and the further 5 partial S gene sequences (HBVY17559–HBVY17565) grouped closely together, but they consistently fell within the gibbon clade, in an analogous manner to the position of the lowland gorilla sequence within chimpanzee sequences (see above). A better correlation was observed between geographical origin and/or host range of the HBV-infected species with the phylogenetic clustering of HBV variants recovered from them. The gibbon and orangutan sequences can be divided into three main groups, the first of which contains orangutan HBV variants and those infecting two of the *hylobates* species, which inhabit the Southern region of SE Asia, and overlap in range with orangutans. The second group contains sequences from northerly distributed *nomascus* species

of gibbons and *H. lar*, distributed predominantly in Central Thailand and Laos. HBV variants infecting *H. pileatus*, a gibbon species inhabiting Cambodia and Central Thailand made up the third clade of sequences.

The main inconsistency with the phylogeny of gibbon-derived HBV sequences is the anomalous position of the chimpanzee-derived HBV variant, HBV131575. This sequence groups with a number of HBV variants infecting *H. lar*, including HBV131571 from the same study (Grethe et al., 2000). This is the only case where an HBV variant infecting a non-Asian primate groups with gibbon and orangutan sequences, and further information is required on whether a cross-species transmission of HBV could have occurred in captivity.

## Discussion

### Host range and epidemiology of HBV in primates

Our PCR-based and serological survey of a wide range of Old World primate species provided further evidence for a substantial restriction of HBV infection to apes (Table 1), and is consistent with previous observations for the absence of detectable HBV infection in Old World monkey species (Deinhardt, 1976; Michaels et al., 1996; Eichberg and Kalter, 1980). Combining data from this and previous surveys, HBV infection appears to be common in all species of apes, with combined rates of PCR-positivity and/or HBsAg carriage of 16.7% (Table 1). This rate of active infection is remarkably similarly to that of human populations in areas of endemic infection, such as Central Africa and South East Asia.

Further parallels between the epidemiology of HBV infection in human and non-human primates is provided by the evidence for efficient mother-to-child transmission of HBV in captive primate species (Noppornpanth et al., 2003; Lanford et al., 2000). In humans, such perinatally acquired infections generally lead to lifelong HBV high infectivity carriage associated with partial immunotolerance and the production of the HBV “e” antigen (HBeAg). Persistent infection of females perpetuates the infection cycle to each succeeding generation. Observations of frequent vertical transmission in captive gibbons (Noppornpanth et al., 2003) supports the hypothesis that this may also be an important mechanism for the maintenance of HBV infection in gibbons and potentially other ape species in the wild.

One explanation for the failure to detect HBV infection in Old World monkey species is that they were infected with HBV variants so divergent in sequence from previously described HBV variants infecting humans and other primates that they would be refractory to amplification with conventional primers, and be serologically non-cross-reactive with reagents used in HBsAg and anti-HBc assays. Such viruses would therefore have to be more divergent than the outlier HBV variant found in a captive woolly



monkey (Lanford et al., 1998). To address this issue we re-screened the Old World monkey species by PCR using primers that matched even the highly divergent HBV-like viruses infecting North American rodents. Our failure to detect a single additional case of HBV infection in the 93 African monkey samples available provided further evidence for an absence of infection of hepadnaviruses in these species of the *Cercopithecoidea*, although it is possible that this picture may change with more extensive sampling in a greater range of species (see below).

Why HBV infection in the wild should be generally restricted to apes remains unclear at this stage. Given this highly selective distribution of HBV infection in Old World species, the existence of HBV infection in woolly monkeys (a New World primate species) becomes more difficult to account for. A total of 10 infected animals have been reported to be HBV-infected in a US zoo, although a previous serological survey of three other New World primate species (owl monkey, tamarin, and squirrel monkey) have failed to detect evidence of active or past HBV infection in this group [Table 1; (Eichberg and Kalter, 1980)]. Clearly further surveys would be required to determine whether HBV infection is present in wild caught monkeys in South America.

#### *HBV origins*

Reconstructing an evolutionary history of HBV using the data set of sequences of HBV variants infecting humans and other primates has proven to be both complex and difficult to reconcile with theories of either recent or remote times of origin (Bollyky et al., 1997; Magnius and Norder, 1995; Norder et al., 1994; MacDonald et al., 2000; Simmonds, 2001). The new sequence information obtained in this paper, and the combined analysis with the much larger dataset of HBV sequences now available from other studies, has provided strong evidence for a correlation between geographic origin of infection and sequence divergence that cuts across species barriers of non-human primates. Thus, HBV sequences from Central Africa group together, irrespective of their origin in chimpanzees or gorillas. A similar picture is observed in South Eastern Asian primates where gibbon and orangutan HBV sequences cluster together and their host species have overlapping geographic ranges.

Understanding the epidemiology of HBV infection in non-human primates is additionally complicated by the difficulty in constructing a time-scale for the divergence of the current distribution of variants found in non-human species. However, short-term rates of HBV sequence change have been determined for HBV infecting humans, with evidence for a higher rate of sequence change in individuals who mount an effective immune response to infection. Based on the HBeAg/anti-HBe status, mean frequencies of fixation of nucleotide substitution range from 2.1 to  $25 \times 10^{-5}$  nucleotide change per site per year (Hannoun et al., 2000). As HBeAg carriage (associated with a slower rate of sequence

change) is typically associated with early acquisition of HBV infection from vertical transmission, a mode of transmission that maintains HBV infection in human populations and potentially in primates (Noppornpanth et al., 2003), the sequence divergence of HBV variants infecting different subspecies of chimpanzee can be conservatively estimated as ranging at most from 1600 to 2200 years (mean sequence divergence 7%; rate of sequences change  $2.1 \times 10^{-5}$  nucleotide change per site per year). A similar time-scale for HBV evolution was inferred using non-overlapping regions of the HBV genome (Fares and Holmes, 2002).

The calculated time-scale of HBV evolution implied by measurement of rates of sequence change over short intervals indicates that the current wide distribution of HBV infection in apes must have arisen through several cross-species or subspecies transmissions in the relatively recent past. For example, HBV transmission following limited population contact between different ape species in South Eastern Asia (including between orangutans and gibbons), and between subspecies of chimpanzee and with gorillas with Africa may have provided the necessary conditions for both the introduction and subsequent differentiation of HBV genetic clusters into otherwise separate host populations.

In contrast to the co-speciation hypotheses discussed below, the recent emergence hypothesis for HBV infection in primates resolves many of the unexpected phylogenetic groupings evident from the comparison of the much larger datasets of ape-derived HBV sequences (Fig. 3). Despite the previous claim for the existence for an orangutan-specific genotype of HBV (Warren et al., 1999; Verschoor et al., 2001), the availability of a larger number of gibbon sequences, including those obtained in the current study, clearly places orangutan-derived variants deep into the gibbon clade of HBV sequences. Indeed, they show the closest genetic relationship with HBV variants infecting *Hylobates* species with geographically close or overlapping habitat ranges. In contrast, HBV variants infecting *H. lar* and the *Nomascus* gibbons from mainland Asia generally group separately.

The lack of sequence diversity of HBV sequences between separate infected orangutans further suggests that the introduction of HBV into this species may have been relatively recent. Indeed, the absence of HBV infection in our sample of 14 wild caught orangutans from Borneo suggests that HBV infection may not be so widely distributed geographically in this species as it evidently is in gibbons, and more consistent with recent introduction. Recent cross-species transmission events may also account for the close relationship between the single available gorilla-derived HBV variants with HBV infecting the troglodytes subspecies of chimpanzee whose ranges overlap in Central Africa (Fig. 2 and 3A), although more sequence information of HBV infecting other gorillas in the wild is required to substantiate this further possible example of cross-species transmission in the wild.

An alternative explanation of the distribution of non-

human HBV genotypes in Africa and South East Asia proposes that the distinct genotypes of HBV in different primate species arose during the evolution of different ape species over the past 20 million years. However, the implied very low rate of nucleotide substitution of HBV over these extended periods of host diversification is incompatible with extrapolations of the previously calculated rate of HBV sequence change over short observation periods. For example, while we estimate that the sequence divergence of HBV infecting different subspecies of chimpanzees arose  $\approx$ 1600–2200 years ago, the times of host species divergence range from 0.8 million years for the divergence between subspecies of chimpanzees from Central Africa (troglodytes, vellerosus and schweinfurthii subspecies) and an estimated 1.5 million years between these subspecies and *P.t. verus* from West Africa (Morin et al., 1994). Similarly, extrapolating from the above rates of sequence change, the radiation of gibbon- and orangutan-associated HBV variants can be timed as no more than 3500 years, a time-scale difficult to equate with the species and/or geographic differentiation of HBV variants in the wide range of primates infected with HBV.

In defence of the co-speciation hypothesis, it could be argued that constraints on sequence change, such as the unusual and extensive use of overlapping reading frames for protein coding in the HBV genome, as well as the role of RNA structures in transcription and translation (Mizokami et al., 1997) may prevent the simple and relatively unconstrained fixation of neutral mutations. For example, the absence of true synonymous sites in over 65% of the genome through the use overlapping reading frames, and the existence of *cis*-acting RNA structures required for HBV transcription and translation may lead to a rapid saturation of sequence substitutions and homoplasy in the (relatively few) phenotypically unconstrained sites, although low rates of divergence were also observed in non-overlapping regions of the HBV genome (Fares and Holmes, 2002). The loss of the variable, phylogenetically informative sites through homoplasy would therefore explain the instability of any sequence grouping of HBV variants below the level of (human) genotype or of non-human primate genetic groupings. More importantly, extensive homoplasy would prevent the extrapolation of the short term rate of sequence change of HBV (over 10 years) to the longer periods underlying the differentiation of HBV into separate genotypes.

However, no permutation of the co-speciation hypothesis can explain the inlier position of orangutan-derived HBV variants within the clade containing gibbon sequences (Fig. 2 and 3B), or the anomalous position of the lowland gorilla HBV sequence in the chimpanzee HBV phylogeny. Even more seriously, it fails to account for the substantial sequence diversity of HBV variants infecting humans. While it might be possible to compress human genotypes A to E and G into a single group that reproduces the diversity of HBV sequences found in gibbons (and orangutans), this approach cannot account for the outlier position of geno-

types F and H. Nor can the diversity of HBV variants infecting humans, with a maximum population age of 150,000 years (Stringer, 2002), be reconciled with the much more restricted sequence diversity of HBV variants infecting the several subspecies of chimpanzee which diverged over a time-scale 10 times greater, or different species of gibbon that diverged even longer ago.

Secondly, the hypothetical restrictions on sequence divergence can not explain how HBV-like viruses infecting rodents and birds have become so divergent from human and primate HBV variants over maximum chronological times of 100 to 300 million years. Even against the yardstick of these much longer periods for sequence diversification, greater sequence divergence and an outlier position for gibbon-derived HBV sequences would be expected to be resolvable over the 15 million years of ape diversification.

In summary, the proposed scenario for the relatively recent spread of HBV among African and South East Asian primates and cross-species transmission between animals in adjacent or overlapping ranges accounts much better for the geographical rather than the species association of HBV genotypes in non-human primates than previous co-speciation theories (Magnius and Norder, 1995; Norder et al., 1994; MacDonald et al., 2000). However, while the transmission networks of HBV in Africa and South East Asia are clearly separate, one difficult remaining question is how HBV could have spread between the African, Asian and potentially South American continents in the previous few thousand years. Resolving this question will require a better understanding of the relationship between human and non-human primate HBV variants, and the potential role of humans in disseminating HBV infection over these much larger geographical distances in the more remote past.

#### *HBV divergence*

Current and previous studies of HBV genotype distributions in apes have demonstrated the existence of HBV variants with relatively limited sequence diversity, manifested in particular by the grouping of these variants interspersed with the human genotypes A–E and G (Fig. 2). There is no evidence so far for HBV variants in apes as divergent as the human genotypes F and H, nor the HBV variant obtained from a captive woolly monkey.

However, the core gene sequence of the HBV variant, AB046525, recovered from a *P.t. troglodytes* subspecies in Central Africa (Takahashi et al., 2001) is highly divergent from equivalent regions in other HBV variants obtained from chimpanzees, other apes or humans, and indeed is as divergent from viruses in these latter species as the woolly monkey sequence (Lanford et al., 1998). Strangely the rest of the genome of AB046525 groups with HBV variants recovered from the troglodytes subspecies of chimpanzees (eg., in the S gene; Fig. 3B). As previously suggested, this virus is likely to be a recombinant between a troglodytes-associated HBV variant and another highly divergent HBV

variant of unknown origin. Combined with the woolly monkey variant (again of obscure origin), these findings hint towards the existence of much more divergent HBV variants in nature that are refractory to current primate surveillance methods. Further work to discover the original species distribution of such variants will provide considerable insights into the ultimate origins and evolution of HBV in primates, and may help to resolve the many conflicting theories for the origin and past epidemiology of HBV infection in human populations.

## Materials and methods

### Primate samples

A total of 137 primate serum and plasma samples were available for screening. From Africa, samples from 71 drills (*Mandrillus leucophaeus*) were available from a rescue centre in Nigeria; in the study population, 48 were wild-born and 23 captive-born; all samples were negative for HBV surface antigen (HBsAg). A total of 14 samples were available from 8 drills, 2 mandrills (*Mandrillus sphinx*), 4 cherry-capped mangabeys (*Cercopithecus torquatus*) kept at the Limbe Wildlife Center in Cameroon, all of which were wild-born. From the same centre, a total of 7 samples from chimpanzees were also available, of which 2 were HBsAg positive, 3 were negative and 2 were unknown. Finally, one sample from a known HBsAg positive chimpanzee and 3 HBsAg-negative samples from mona monkeys (*Cercopithecus mona*), 2 samples from putty-nosed monkeys (*C. nictitans*) and 3 samples from red-eared monkeys (*C. erythrotis*) were provided for the study from a rescue centre in Nigeria.

From South East Asia, 20 gibbon samples from various wild-born gibbon species (*Hylobates spp.*; 4 HBsAg positive and 16 negative), and 14 samples from wild-born Bornean orangutan (*Pongo pygmaeus pygmaeus*; all HBsAg negative), were provided by the Pingtung Rescue Center in Taiwan. One male and one female lar gibbon (*H. lar*) from the Welsh Mountain Zoo were also included. They were HBsAg tested at the City Hospital, Edinburgh and the female was found to be positive whereas the male was negative.

All samples were stored at 4°C prior to shipping to the UK according to CITES regulations, and then stored at –25°C prior to analysis.

### Source of infection in HBV-positive primates

Louisa (*P. troglodytes*, subspecies *troglodytes*) was confiscated in Southwest Cameroon from local people, after which she was housed in the Limbe Wildlife Centre. Since confiscation she has had no contact with any other primate species. The exact details of how she was kept prior to confiscation are unknown. The second chimpanzee was

Osang (*P. troglodytes*, subspecies *vellerosus*), and was donated to a conservation project in South-Eastern Nigeria in 1996, at which time he was positive for HBsAg. Osang was also kept by local people after he was captured, and was subsequently kept with other chimpanzees at the conservation centre.

Of the 5 HBV-infected gibbons, 4 were wild-caught and one was captive-born. The captive-born was a female, Tamang (*Hylobates lar*) from a captive-born father (Paignton Zoo) with known wild-caught parents from Malaysia and Thailand, and a captive-born mother (Stuttgart Zoo) with unknown parental origins. At the Welsh Zoo she is kept with a male, Jake (*H. lar*) who was born at the Zoo, who was both HBsAg and HBV PCR negative. The remaining 4 positive gibbons consist of Crazy Woman (*H. agilis*) confiscated in Taiwan in 1993, TB Black (species not identified) confiscated in Taiwan in 1996, Happy (*H. concolor gabriellae*) confiscated in Taiwan in 1996, and Wendy (*H. concolor gabriellae*) confiscated in Taiwan in 1999. Their histories prior to confiscation are unknown, as are their origins. For the latter 4, all were HBsAg positive on first testing after confiscation. Tamang likely acquired infection from her mother peri- or post-natally. The subgenus and species identifications of the HBV-infected gibbons were provided by the sanctuary.

### PCR screening and sequencing of HBV DNA

HBV sequences were amplified by PCR as previously described (MacDonald et al., 2000) from 100 µl volumes of serum or plasma using pre-S (S1) and S (S2) gene primers (Fig. 1A and B). Nucleotide sequencing was carried out directly on second round amplification products using either the Sequenase version 2.0 kit (United States Biologicals), or ABI PRISM d-rhodamine or Big Dye kits (Applied Biosystems). Positive primate samples were sequenced using overlapping primers covering the entire genome as described previously (MacDonald et al., 2000) using primers S5, S3, 4, 22, 25, 26, 30, 33, 42 and the new primers 44 (5'-GTCCTTGWTTGGGRTTGAAGTCCCAATC-3'; positions 2959 to 2985), 45 (GGTCACMWTAYTCYTGGGAA-CAAGA; positions 2824 to 2848), 46 (ACCAWTTTATG-CYRCAGCCTCCTA; positions 1778 to 1801) and 47 (GGACGTCCTTTGKTACGTCCC; positions 1415 to 1442).

For investigation of infection with possibly more divergent HBV-like viruses in Old World primates, a further PCR assay was developed using highly conserved primers (set S3) in the surface gene that matched the following additional sequences recovered from HBV-like viruses recovered from North American rodent species: K02715 (ground squirrel), NC004107 and M19183 (woodchuck) and U29144 (arctic ground squirrel). Samples were amplified using the assay and cycling conditions as for the S1 and S2 gene primers. Nucleotide sequences obtained in this study have been submitted to GenBank and have been

assigned the following accession numbers: Ay330911 to Ay330917

#### *Sequencing of the 12S mitochondrial region of primate samples*

Primate samples were also amplified and sequenced using primers specific for the 12S region of the mitochondria to confirm the species and distinguish between subspecies and individual animals to allow comparisons of the primate and HBV phylogenies. 2  $\mu$ l nucleic acid was added to PCR mix as described before and amplified using a single round PCR reaction. Primers 12S-S (5'-CCATAAACAMAYAG-GYTTGGTCC-3'; positions 641 to 664) and 12S-AS (CAGGGTTTGCTGAAGATGGCGGTATATA; positions 1270 to 1298) were used. PCR conditions were 40 cycles of 94°C for 60 sec, 55°C for 60 sec and 72°C for 60 sec, followed by a final extension at 72°C for 6 min. Amplified DNA was directly sequenced using the ABI PRISM Big Dye kit (Applied Biosystems).

Mitochondrial sequencing was used to determine the sub-species of the 2 HBV-infected chimpanzees, from which the following assignments were made: Osang: *P. troglodytes vellerosus*, Louisa: *P. troglodytes troglodytes*. Mitochondrial sequencing was unable to determine the species for gibbon TB Black because sequences for subspecies *agilis* and *moloch* were interspersed, and published sequences for gibbons were mainly determined in a different region of the mitochondrial genome. Mitochondrial sequencing was also performed on samples from the previously described HBV-infected chimpanzees from whom complete genome sequences were published [Chimp 2, Chimp 4; (MacDonald et al., 2000)]. Although both were originally classified as being versus subspecies on the basis of morphology, mitochondrial sequencing indicated that Chimp 2 was the *troglydytes* subspecies; this designation has subsequently been confirmed by the sanctuary.

#### *Sequence analysis*

Sequence data obtained using the ABI PRISM kits was viewed using the CHROMAS sequence viewer and directly imported into the SIMMONICS sequence alignment program and edited. HBV sequences were aligned with up to 10 representative sequences of each human genotype: Genotype A—HUMPRECX, AF090839, AF297624, E00010, HVHEPB, HEB344115, AF090838, U87742, AF297625, AF297619; Genotype B—AY033073, HPBA3HMS2, D50522, HPBADW3, HPBADWZ, AF282918, HBV131133, HPBADW2, AF121249, AF121243; Genotype C—AF233236, HHVBC, AB049610, AF411412, HPBADRA, AF330110, AF461359, HPBADRC, AF411408, AF363964; Genotype D—HEB344116, AB033559, AF280817, AF121240, U95551, HBVAYWC, HBVAYWGEN, HBVGEN1, HBVORFS, HBVP6PCXX; Genotype E—HHVBE4, HHVB-

BAS; Genotype F—HBVADW4A, HHVBFFOU, AF223965, AF223962, HHVBF, AB036910, AB036908, AB036916, AB036905, AB036913; Genotype G—AB056515, AF405706, AB056516, AB064312, AB064310, AB064311, AB056514, AF160501, AB056513, AB064313 and Genotype H—AY090454, AY090457, AY090460. Sequences were also compared with every available complete genome sequence obtained from primates: Chimpanzee—AB032431, HBV131575, AF242585, AF222322, AF498266, AB046525, AF305327, AF222323, AF242586, AB032432, HPBVCG, AB032433; Gorilla—HBV131567; Gibbon—AY077736, AY077735, HBV131568, HBV131574, HBV131571, HBV131569, HBU46935, HBV131572, ABO37927, AB037928; Orangutan—AF193864, NC\_002168 and woolly monkey—AF046996.

Sequence comparisons in the S-gene included the following additional further partial sequences from primates: Chimpanzees—AF305328, AF222318, AF222321, AF222312, AF222319, AF222313, AF222314, AF305326, AF222316, AF222320, AF305329, AF222317, AF305330; Gibbons—AF213009, AF213008, AF21210, AF274495, AF274496, AF274499, AF477482, AF477483, AF477484, AF477485, AF477486, AF477487, AF477488, AF477489, AF477490, AF477491, AF477492, AF477493, AF477494; Orangutans—HBVY17565, HBVY17562, HBVY17559, HBVY17564, HBVY17561.

Phylogenetic trees for these data sets were estimated using a maximum likelihood (ML) method. To undertake as robust an analysis as possible, we employed the most complex GTR+I+ $\Gamma$  model of nucleotide substitution available; this allowed each type of nucleotide change to occur at a different rate (the general time-reversible substitution model, GTR), a proportion of nucleotide sites to be invariant (I) and a gamma ( $\gamma$ ) distribution of among-site rate variation with the  $\alpha$  shape parameter (with 4 categories) estimated from the empirical data. The maximum likelihood base composition was also estimated from the data. All parameter values are available from the authors on request. An heuristic search procedure was used to find the ML tree using successive rounds of TBR branch-swapping, optimizing the ML substitution parameters at each stage. To determine the support for key nodes on the tree we conducted a bootstrap resampling analysis using 1000 replicate neighbor-joining trees constructed under the substitution model as defined above. All these analyses were performed using the PAUP\* package (Swofford, 1998). In all cases the single woolly monkey sequence (AF046996) was used as an outgroup to root the phylogenetic trees.

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