these contacts is consistent with the observed biophysical and biochemical characteristics of the capsid[7]. The virus particles are more stable at pH 5 than at pH 8. It is possible to dissociate the virus and reassemble the subunits without denaturation, reflecting the polar nature of individual subunits. Trypsin treatment of intact particles leads to truncation of the amino-terminal segment by about 70 residues. The remaining protein domain remains soluble and folded, and assembles into $T = 1$ (ref. 8) particles containing 60 subunits. In contrast to the dense packing of protein subunits within the capsid, virus particles do not make extensive contacts in the crystals. Therefore, no statistical analysis of these contacts is presented.

The method presented here of representing the shape of a three-dimensional object as a binary map and using this map to simplify the evaluation of contacts between protein molecules, might find application in other analyses of protein structure and architecture such as complementarity of surface features and analysis of van der Waals volumes. A similar approach has been used for protein-ligand docking[9]. This method is also useful for excluding spurious solutions that lead to penetration of neighbouring molecules in molecular replacement[10], a technique for protein structure determination based on homology to a previously known structure.

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Application of molecular sexing to free-ranging Asian elephant (*Elephas maximus*) populations in southern India*

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Selective poaching of Asian elephant (*Elephas maximus*) males for ivory has resulted in highly female-biased adult sex ratios, necessitating regular monitoring of population structure and demography. We demonstrate that molecular sexing from dung-extracted DNA, based on ZFX–ZFY fragment amplification and ZFY-specific *Bam*HI site restriction, can be applied to estimate sex ratios of free-ranging Asian elephants, in addition to or instead of field demographic methods. The adult sex ratios using molecular sexing in Nagarahole and Mudumalai–Bandipur reserves during May 2001 were 1 : 3.1, matching the demography-based sex ratio for the same month, and 1 : 9.4, respectively.

MOLECULAR sexing is the process of sexing individuals based on variation in DNA between sexes. Methods include amplification of Y-specific fragments, usually based on the *SRY* gene[1], and amplification of homologous fragments (the amelogenin gene or *ZFX–ZFY* genes in mammals/CHD-CHD-W in non-ratite birds) on both X and Y chromosomes, using length polymorphism[2] or restriction fragment length polymorphism (RFLP)[3] to differentiate between the sexes. Molecular sexing has been widely used to sex foetuses in humans, other primates and livestock[2,4,5], and to a lesser extent in sexing birds[3,6,7], whales[1,8,9], seals[10] and fish[11], which are difficult/impossible to sex visually.

Embryonic fluid, blood or tissue samples are generally used as a source of DNA in these instances. Since most large terrestrial mammals are sexually dimorphic, there have been only a few field studies of molecular sexing in such species, for example apes[12] and bears[13]. However, molecular sexing can be a useful tool to sex juveniles, which lack dimorphism, or to estimate population sex ratios by carrying out noninvasive sampling. Here, we demonstrate the applicability of molecular sexing to free-ranging populations of the Asian elephant (*Elephas maximus*).

Poaching for ivory in the Asian elephant began assuming threatening proportions during the 1970s, the average number of elephants poached over the last decade in

India being approximately 87 per year (data compiled by the Asian Elephant Research and Conservation Centre, Bangalore, and Wildlife Trust of India, New Delhi). The animals poached are sub-adult and adult males that carry tusks, and occasionally females that bear tushes. Thus, despite a population of 24,000–28,500 elephants in India[14–16], the estimated number of tusked adult male elephants in the country is only approximately 1500 and is further decreasing[17]. The increasingly female-biased adult sex ratios, particularly in southern India, could affect populations seriously, by lowering the effective population size, lowering birth rates, and decreasing genetic viability due to inbreeding[18]. It is thus imperative that sex-ratios are monitored on a regular basis in addition to population sizes.

Sex ratios have been traditionally estimated using demographic data from field observation. This is feasible as Asian elephants show sexual dimorphism, the chief difference being that females do not carry tusks while males may carry tusks, and differences in behaviour. However, sampling dung instead of the elephants themselves and using a molecular sexing method can be an addition or an alternative to the demographic method. The molecular method may be advantageous in places where elephant density is low, sex ratios are extremely skewed and/or visibility is poor, making direct sightings of animals very rare. The molecular method may also circumvent any bias associated with ageing/sexing animals in the field. In the present study RFLP in ZFX–ZFY fragments was used to molecular sex individuals. The restriction site within the ZFY fragment in the Asian elephant, identified by Fernando and Melnick[19], was used. Sampling was carried out in two areas in the Nilgiri Biosphere Reserve in southern India: the Kabini backwater area (about 25 km^2) of the Nagarahole National Park (henceforth Kabini) and the tourism area of the Mudumalai Wildlife Sanctuary and a part of the Bandipur National Park adjoining it (Mudumalai–Bandipur, approximately 140 km^2) (Figure 1). The habitat type in the sampled areas included open areas with short grass, and a lesser extent of dry deciduous forest in Kabini, and dry deciduous and dry thorn forest in Mudumalai–Bandipur. Demographic data were collected during May 2001 at Kabini and annual demographic data for Mudumalai were available for 1999 and 2000. Elephants were sexed visually and aged from their height and morphological characters[20]. All the herds that could be classified with respect to at least the presence or absence of an adult (> 15 years old) male, and for which total or approximate group size was known, were used to compute the adult sex ratio.

Dung samples less than a day old were collected by walking 0.5 km transects in the study sites in May 2001. Bolus diameter of dung was used as an indicator of age as these were known to be positively correlated[21]. Based on this and the distributions of dung diameter from adult males and females (Figure 2), we used 12 cm as the minimum cutoff for adult females and 14 cm for adult males. These corresponded to 93% of the distribution, while diameters lower than these overlapped substantially with those of sub-adult animals. Hence, all samples over 12 cm diameter were collected in the field and those below 14 cm that were molecular sexed as males were subsequently discarded.

The outermost layer of dung that is rich in endothelial cells was collected in 95% ethanol. DNA was extracted from dung following Fernando et al.[22], by digesting 0.5 g of the dung sample with SDS/Proteinase K, followed by extraction with phenol/chloroform/isoamyl alcohol, and purification using a QIAGEN gel purification kit. PCR using the primers P1-5EZ: 5′-ATAATCACATGGAGA-GCCACAAGCT-3′ and P2-3EZ: 5′-GCACTTCTTTGG-TATCTGAGAAAGT-3′[4] was carried out to amplify a ~ 300 bp segment of ZFX–ZFY. PCR reaction volumes were 25 µl, using 2 µl DNA, 0.5 µl each of P1-5EZ and P2-3EZ (Operon Technologies Inc. 10 pM), 0.2 µl of Taq DNA polymerase (MBI Fermentas 5 U/µl), 0.982 µl of 10 mM dNTP mix, 0.325 µl of 100 mg/ml BSA, 0.038 µl of 1 M MgCl$_2$, 0.308 µl of 4 M KCl, 0.245 µl of 1 M Tris pH 8.4 and 19.902 µl of water. PCR reactions were carried out following 40 cycles of denaturation at 92°C, annealing at 51°C, and extension at 72°C, for a minute each. PCR products were restriction-digested with 5 µl of BamHI (MBI Fermentas 5 U/µl), 5.5 µl of 10X buffer with BSA, 20 µl of PCR product, and 19.5 µl of water at 37°C for 2 h. Upon electrophoresis on a 2% 1 agarose : 1
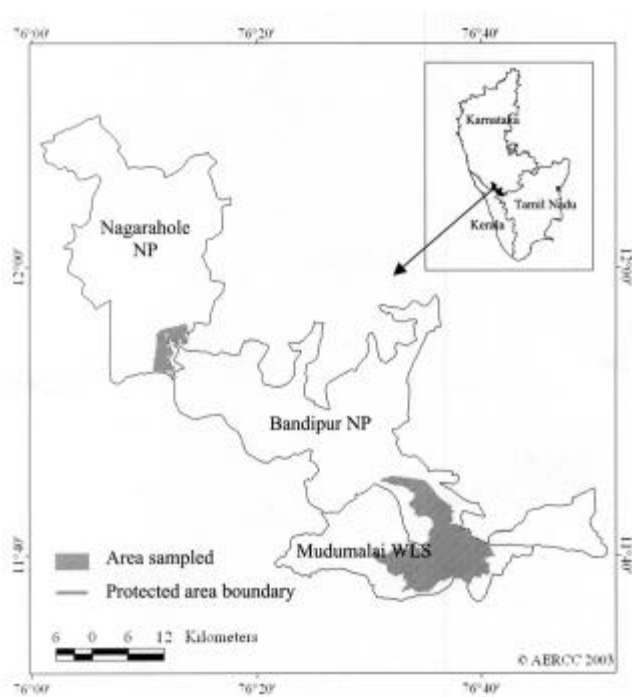

Figure 1. Areas sampled in Nagarahole National Park, Bandipur National Park, and Mudumalai Wildlife Sanctuary. Inset: Southern India showing three southern states and the location of the above protected areas.

low melt agar gel, females showed a single band due to the undigested ZFX fragment, and males showed three bands due to the presence of the undigested ZFX and the digested ZFY fragments (Figure 3). The procedure was
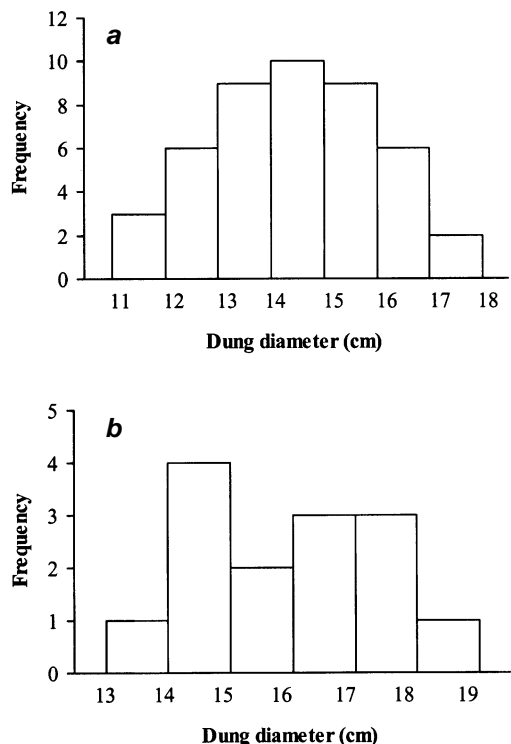
Figure 2. Frequency distributions of the long bolus diameter of dung from adult (*a*) females and (*b*) males.
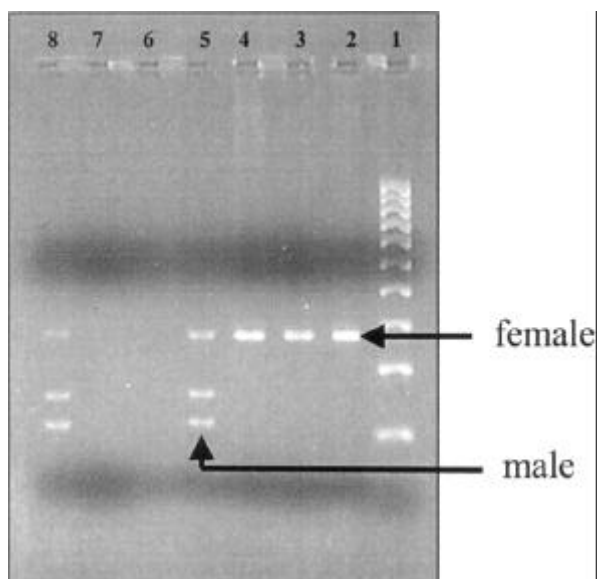

Figure 3. Restriction digests of ZFX–ZFY PCR products with *Bam*HI electrophoresed on a 2% agarose gel. Arrows point to lanes with a female and a male, the band of the female (intact ZFX fragment) corresponding to about 280 bp. A 100-bp ladder is shown in lane 1, and negative and positive controls in lanes 7 and 8 respectively.

standardized with blood and dung samples from captive animals. PCRs were always carried out with a negative control and the restriction digestions with a positive control, to minimize experimental error.

Over 93% of the 129 samples collected could be successfully amplified using this molecular technique. Defecation rates between the sexes were assumed to be similar (based on unpublished data of Surendra Varma, on the defecation rates of 25 adult female and 28 adult male captive elephants). The sex ratios calculated were 1 : 3.1 ($n = 10 \male$, $31 \female$; 95% CI = 1 : 2.78 to 1 : 3.48) and 1 : 9.4 ($n = 7 \male$, $66 \female$; 95% CI = 1 : 8.79 to 1 : 10.19) for Kabini and Mudumalai–Bandipur respectively. This sex ratio for Kabini was not significantly different from 1 : 2.9 ($n = 7 \male$, $19 \female$) calculated using field demographic data collected during May 2001 (2×2 *G*-test of independence[23], $G_{adj} = 0.1665$, $P = 0.68$) showing that the molecular method reflected demographic data very well. We did not have sufficient demographic data during May 2001 to calculate sex ratios for Mudumalai–Bandipur and could not directly compare the molecular method with the demographic data for the same month. Sex ratios calculated from annual demographic data for Mudumalai were 1 : 15.1 ($n = 57 \male$, $858 \female$), 1 : 15.7 ($n = 20 \male$, $313 \female$) and 1 : 29 ($n = 8 \male$, $240 \female$) for the years 1999, 2000 and 2001 respectively. These are much more skewed compared to 1 : 9.4, but wide monthly fluctuations are known (Figure 4) depending on the movement of adult males in and out of the area. With both demographic and molecular methods, one would have to consistently sample round the year to arrive at sex ratios representative of the area.

This is the first application of molecular sexing to free ranging elephants in India. Since the method is reflective of demographic data, as seen in Kabini, it can be applied to areas where sex ratios are unknown. It would also be useful in areas with highly skewed sex ratios such as the Periyar Tiger Reserve with an estimated adult sex ratio of
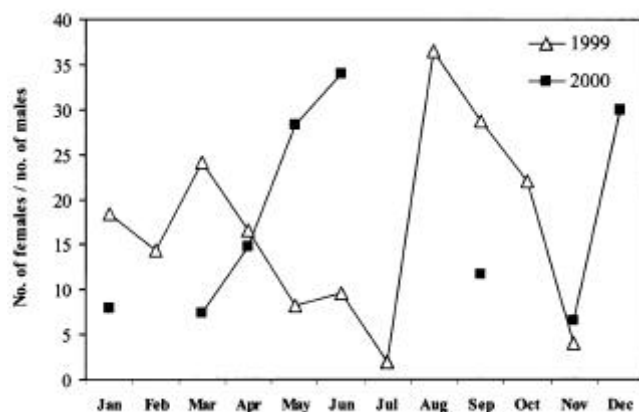
Figure 4. Adult sex ratios calculated for each month during 1999 and 2000. There were insufficient data to carry this out for 2001. Some points are not connected in the 2000 series because the intervening points were at infinity, no adult males having been sighted in those months.

about 1 male : 100 females[18], as direct sightings of males would be difficult. Sub-adult and juvenile sex ratios, which may otherwise be biased due to misidentification of *makhnas* or tuskless males, can also be calculated. The technique is feasible as samples can be collected rapidly with little training of manpower, and involves little subjectivity in ageing individuals. Molecular sexing can be used in novel situations like sexing crop-raiders and problem animals from dung left behind in the field, and for genetic tracking of animals[8], using the ZFX–ZFY locus in addition to microsatellite loci. As the *ZFX–ZFY* genes are present in other mammals[24] and the restriction site, which is conserved within species, is easily identifiable[19], this technique can also be applied to other mammals, and will be useful in monitoring the population structure of elusive species.

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