The Use of iDNA in Detecting Elusive Mammals: A Case Study on the Indonesian Island of Seram

A Rapid Assessment of the Status of the Seram Bandicoot *Rhynchomeles prattorum* and the Development of iDNA as a Survey Tool for Remote Locations

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Abstract

More than a fifth of all mammalian species on the planet are categorised as threatened. A significant proportion of these live in remote tropical forest and are difficult to survey hence their actual numbers cannot be determined. Distribution and abundance data for most tropical mammal species is data-deficient and therefore the development of cheap and reliable surveying methods is crucial to successfully assess these species. The use of carrion-feeding flies as vectors of mammalian DNA (referred to here as iDNA) and their application as a cost-effective tool for the assessment of mammalian biodiversity was first shown in 2013; however iDNA technology remains constrained by the persistence period of amplifiable iDNA in fly guts and the limitations of DNA preservation in remote locations.

Fieldwork was carried out on the island of Seram, Indonesia. Archive data and interviews with indigenous forest users were used to optimise locations for the detection of *Rhynchomeles prattorum*, an endemic marsupial recorded from Seram in 1920.

Carrion feeding flies were collected in montane forest, initially in the vicinity of a butchering site of Cuscus (Phalangeridae) (n = 99), and subsequently at remote sites in pristine forest (n = 50). At all locations camera trapping was conducted to provide independent evidence of mammal species present. Flies were individually dissected in the field and gut contents smeared onto FTA cards to preserve iDNA, or, if too small to facilitate dissection (n = 57), were placed individually into 95% ethanol at ambient temperature.

Following extraction, a PCR using pan-mammalian 12S mitochondrial DNA (mtDNA) primers yielded bands of the expected size. These bands were purified and sequenced, and a BLAST search confirmed the presence of Cuscus DNA, as well as other mammal species from the island. A lower recovery of amplifiable iDNA from flies collected in remote locations correlated with a longer processing time for flies post capture, potentially indicating a limitation of current iDNA methodologies.

In order to extend the time interval required to remove flies from traps for successful iDNA amplification, a new fly trap incorporating Propylene glycol as a fixative was designed and its capture efficacy evaluated. Through a laboratory feeding experiment using newly pupated blow flies *Calliphora vomitoria* the persistence period of amplifiable iDNA in the new trap was evaluated in comparison to a conventional dry fly trap design. After exposure to beef liver, flies were subsequently trapped in conventional dry traps and in the new fly trap. At 24, 48, 72, 96, 120 and 144 hours post-trapping flies were removed and their guts dissected onto FTA cards. Following extraction, a PCR using Beef Specific Primer (BSP) yielded bands of the expected size. An average higher intensity of band derived from flies caught in the new trap indicates its partial success in extending the persistence period of amplifiable iDNA.

Lay Summary

Recent estimates suggest that more than a fifth of mammal species are threatened with extinction. A major obstacle in conservation is to determine how many animals of a species are left. For rare and shy species, for example animals living in tropical forests, surveying is difficult. Recently scientists described using DNA extracted from guts of carrion-feeding flies to identify the mammals the flies had fed upon. These flies cover large areas of forest while feeding on faeces and dead animals. By removing the guts of trapped flies and sequencing the DNA present in their guts it is possible to identify which animal species they fed on. This technique was used on the Indonesian island of Seram to search for the Seram long-nosed bandicoot, a small marsupial known only from seven specimens collected in 1920. Flies attracted by the remains of Cuscus, an animal commonly hunted and eaten on the island, were trapped and the DNA of Cuscus and other local mammals was successfully sequenced from the guts of these flies. Using archive data and information collected from local hunters, the remote area presumed to be where the long-nosed bandicoot was originally collected was located and flies were trapped there. DNA from several mammal species, not including the long-nosed bandicoot, was successfully sequenced from the guts of these flies but there was a lower success rate than with freshly collected flies.

In order to overcome this limitation, a trap was designed to catch flies in media which preserves DNA for later extraction. The new trap was tested for its ability to catch flies and then an experiment was conducted to compare how long DNA persisted in flies caught in the new trap compared to those caught in conventional ‘dry’ traps.

The new trap was found to be partially successful in overcoming current limitations whereby flies have to be retrieved from traps within 24 hours to avoid DNA degradation and as such it has a practical application for scientists wishing to survey mammal species in remote or difficult to access terrain.

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Declaration

I, Andrew James Bagnall, do hereby declare that the work carried out in this thesis is original, was carried out by myself or with due acknowledgement, and has not been presented for award or degree at any other university.

Andrew James Bagnall

1 Introduction

Earth is currently facing a decline in biodiversity at a rate estimated to be 1000 times the natural extinction rate of species (Pimm et al. 2014). This accelerated decline results primarily from anthropogenic causes such as habitat change and hunting, attributable mainly to increased use of land for agriculture, over-exploitation of biological resources, introductions of non-native species and climate change (Daleszczyk et al. 2016).

1.1 Threats Facing Mammals

In the Class Mammalia, 25% of all species are threatened with extinction (Schipper et al. 2008; Hoffmann et al. 2011). Species occupying tropical forest habitats and restricted range species, many of which are concentrated in tropical mountain forest systems, are especially vulnerable to extinction (Schipper et al. 2008; Pimm et al. 1995) and this may be exacerbated with the increasing effects of climate change (Wright et al. 2009). South East Asia has been highlighted as one of the regions facing the greatest threat of biodiversity depletion across all taxa (Hoffmann et al. 2011; Laurance 1999; Sodhi & Brook 2008) and among terrestrial mammals, threatened species are concentrated in South and South East Asia (Schipper et al. 2008). Overall the assessment for mammalian species occupying forests in South East Asia is exceedingly bleak (Brooks et al. 1999) and constitutes a high priority for conservation.

Prioritising areas for protection and managing threatened species is dependent on quality data such as a species' range size, population density and temporal trends in population (Hoffmann et al. 2011). Globally, 15% of all mammalian species are classed as Data Deficient (Hoffmann et al. 2011), and for terrestrial mammals, the greatest concentration of data deficient species occurs in tropical forests (Schipper et al. 2008). Biodiversity Hotspots, regions containing exceptionally high concentrations of endemic species and frequently correlated with the greatest levels of threat, have been widely described (Mittermeier et al. 1998; Myers et al. 2000; Myers 1988). Of 25 Hotspots described by Myers *et al* in 2000, four are in South East Asia: Indo-Burma, the Philippines, Sundaland, and Wallacea. The island of Seram, the focus of fieldwork for this study, is the second largest landmass in Wallacea (Figure 1).

Map of Seram Island and also Ambon Island and Lease Islands / Lencer / Wikimedia Commons / http://creativecommons.org/licenses/by-sa/3.0

Figure 1: Map of Seram and its location within Indonesia

1.1.1 The Mammals of Seram

Seram is the second largest land mass, after the island of Halmahera, in the eastern Indonesian province of Maluku (Edwards et al. 1993). The island covers an area of approximately 17 100 km² and is characterised by a forested, mountainous interior with a central spine of mountains rising to a height of just over 3000 metres above sea level (asl) (a detailed description of Seram is given in Appendix A). For an island of its size, with a tropical environment, Seram has a relatively low level of mammalian diversity (Wikramanayake et al. 2002). The described wild-living mammal fauna is comprised of just 46 species, of which 26 are species of bat Chiroptera (Appendix B). Of the 20 non-volant species, at least 10 occur as a result of either deliberate or accidental human introductions. Two species of rat; *Rattus rattus* and *R. exulans*, together with the Asian house shrew, *Suncus murinus*, probably arrived as unintended stowaways on the ships of early traders (Kitchener et al. 1993). Although these species are frequently recorded as human commensals (Laurie & Hill 1954) on Seram they have become feral, possibly through expansion into unoccupied ecological niches (Kitchener et al. 1993). Ellen (Ellen 1993) suggests that dogs *Canis familiaris* have been present on Seram alongside humans for at least 3000 years and it is likely that cats *Felis catus* are similarly well established. Feral living populations of both cat and dog are reported by local inhabitants. The presence of pig *Sus domesticus* and of deer *Rusa timorensis* on Seram are as a result of historic introductions of these species as prey items (Macdonald et al. 1993). According to Groves

(1981), pigs living wild in the forests of Seram are a form resultant from the hybridisation of *S. scrofa* and *S. celebensis*. The Malay civet *Viverra tangalunga* and the Palm civet *Paradoxurus hermaphroditus* occur on Seram (Laurie & Hill 1954; Kitchener et al. 1993), both as a result of historical human introductions likely to date from around the 17th century, when civets were traded for the musk produced by their perineal glands which was used as a fixative in perfumes (Gijsels 1871; Kitchener et al. 1993; Ellen 1993).

Two species of cuscus (Order Marsupialia, Family Phalangeridae) occur on Seram (Laurie & Hill 1954; Macdonald et al. 1993; Flannery 1995); the spotted cuscus *Spilocuscus maculatus* and the Grey or Northern common cuscus *Phalanger orientalis*. The origin of both species is uncertain; Flannery (1995) suggests that both species arrived as a result of deliberate human introduction, however, Helgen (2003) cites the widespread natural distribution of both genera on islands in the vicinity of New Guinea as evidence that their occurrence on Seram may be natural. The remaining seven species is comprised of six species of rat (Order Rodentia, Family Muridae); five of which are endemic and the other is an endemic subspecies (*Melomys rufescens paveli* Helgen 2003), and an endemic bandicoot *Rhynchomeles prattorum* (Order Marsupialia, Family Peramelidae) (Thomas 1920) (Table 1). Collectively, all seven endemic mammal taxa are known from just 34 museum specimens (Thomas 1920; Helgen 2003). Knowledge of these species' distribution, habitat preferences and altitudinal range is limited to data associated with the specimens' collection and no further ecological data are known for these species.

Table 1: Endemic non-volant Mammals of Seram

Species	Number of Museum specimens (Occasions collected)	Altitude collected (metres a.s.l)	IUCN status
Rhynchomeles prattorum	7 (1)	1800*	Endangered
Nesoromys ceramicus	4 (2)	1500 - 1800	Endangered
Rattus feliceus	8 (3)	sea level - 1800	Near Threatened
Melomys aerosus	10 (3)	650 -1800	Endangered
Melomys fulgens	2 (1)	sea level	Data Deficient
Melomys fraterculus	2 (1)	1800	Critically Endangered
Melomys rufescens paveli	1 (1)	400	(Subspecies not assessed)

1.1.2 Mapea – The Seram Bandicoot

The Seram bandicoot *Rhynchomeles prattorum*, known locally as *Mapea*, was described from a series of seven specimens collected in 1920 (Thomas 1920). The series was collected at an altitude of approximately 1800 metres asl, in terrain recorded on a collector's label as 'very precipitous jungle clad limestone country. Since *Rhynchomeles'* initial description, no further conclusive information concerning its existence has been published, however Flannery (1995) notes that 'the trapping effort of recent expeditions (*sic*) has been in the wrong habitat and at too low an altitude and for that reason *Rhynchomeles* should not be regarded as extinct'. A detailed description of *Rhynchomeles* is given in Appendix C.

1.1.3 The Possibility of Unrecorded Mammals on Seram

Anecdotal evidence exists for the occurrence of additional mammal species on Seram. Based on descriptions passed on by Messrs Pratt from their local hunters, Thomas (1920) suggests that 'it seems probable that a species of *Dactylopsila* is also found on Seram'. Local inhabitants of Seram have described a small kangaroo like animal, occasionally encountered in forest inland from the North coast (Janes Augustyn,, personal communication 15th July 2014; Jemi Sohaly personal communication 14th July 2015). Pademelons (*Thylogale* spp.) occur both on New Guinea, less than 250 km off the East coast, and on the Aru Islands to Seram's south east. On the island of Halmahera to the north, fossil evidence of *Dorcopsis* has shown that this small macropodid previously had a larger range than its current distribution on New Guinea and islands in its immediate vicinity (Flannery et al. 1995). Both of these taxa could be potential candidates for the species described on Seram.

Although two species of cuscus are described on Seram, it has been reported that a greater number of distinct species are recognised locally (Macdonald et al. 1993; Jemi Sohaly personal communication 14th July 2015). Whilst sexual dimorphism, individual variation and pelage changes associated with development may account for the difference in species number, several specimens in museum collections show morphological differences from recognised forms of either species (Helgen 2003; Alastair A. Macdonald personal communication 14th January 2016). Helgen (2003) describes a trophy skin in the *Museum Zoologicum Bogoriensis* (MZB 14710), of an unidentified cuscus collected in Keloa, Seram, with 'an entirely dark chocolate brown dorsum and white venter, similar in appearance to *Phalanger sericeus* from New Guinea'. Helgen (2003) emphasises that additional trapping and collecting efforts are necessary, especially at higher altitudes, to complete the inventory of the mammal fauna of Seram.

Seram's montane area has the greatest number of endemic mammals of any island in the Maluku region (Flannery 1995). This high level of endemism together with extremely limited knowledge of its indigenous mammals, and the potential for unrecorded species, underlines the requirement for additional survey efforts, especially of the remote higher altitudes (Helgen 2003; Flannery 1995; Leary et al. 2008).

1.2 Survey Methods for Mammals

Although improvements in the capabilities of remote sensing have opened up its potential as a tool for collecting biological data (Gross et al. 2009; Vanden Borre et al. 2011), for many variables, such as invasive species or the occurrence of rare species, on-the-ground surveys remain essential to collecting accurate biological data (Wiens et al. 2009). Such data are, however, dependent on robust and reliable survey methodologies that can be deployed in the field. Currently the two principal methods for surveying mammals are trapping specimens and camera trapping (Hance 2011; Barnett & Dutton 1995; De Bondi et al. 2010).

1.2.1 Trapping Mammals

Trapping small mammals is done on either a lethal or a live-catch and release basis (e.g. Nichols & Pollock 1983). In areas where taxa are poorly known or those containing closely related species that are difficult to assign to species level, the collection of voucher specimens may be favoured to facilitate accurate identification of the species present (Flannery 1995; Richards 2007). This requires ethical consideration in terms of animal welfare and the effect that trapping may have on the population of species of conservation interest (Putman 1995). The action of lethal trapping may also have an impact on public attitudes towards survey activity, indeed, in protected areas such as National Parks, the use of lethal survey methods may not be permissible or may be discouraged (Balai Taman Nasional Manusela 2014). Mammal traps that capture the specimen alive are an alternative to lethal trapping although these are generally limited to smaller bodied species. Live capture traps tend to be bulky and, due to welfare considerations, standard procedures recommend they are checked at a regular interval. For surveys in remote areas without vehicular access and where it is preferable to deploy traps for an extended time period, live-trapping has significant limitations.

1.2.2 Camera Trapping

The use of remote photography has gained widespread favour with wildlife biologists since the early 1990s with the emergence of commercially available automatically triggered cameras (Cutler & Swann 1999; Hance 2011; Meek et al. 2014). Modern trail cameras typically consist of a digital camera coupled with an inbuilt infra-red or motion sensor that triggers the camera when an animal passes within range of the sensor. Current models with battery lives exceeding one month, and capable of recording in High Definition format, are now widely available for between £100 to £200 per unit (Rovero et al. 2013). Using trail cameras, researchers have documented the occurrence and conservation status of elusive and threatened mammals (Tobler et al. 2008), used individually distinguishable features to estimate population density (Silveira et al. 2010) and studied patterns of activity and resource partitioning (Tobler et al. 2009). The use of trail cameras is lauded as a non-invasive and cost-effective survey tool that facilitates the standardization of survey methods across sites and enables the rapid assessment of biodiversity in remote areas (Dobson & Nowak 2010). An incidental benefit of using camera traps is the capture of images of rare or elusive species which can then be used to generate awareness of, or support for, conservation efforts (Hance 2011; Dobson & Nowak 2010). In comparison to conventional live-trapping, camera trapping has been shown to perform favourably in terms of biological data collected, and to be considerably more cost effective (De Bondi et al. 2010). Despite the advantages of camera traps, their relatively high initial cost is a limitation to their widespread use in developing countries or areas where human interference is a likelihood (Silveira et al. 2009; Hance 2011). In remote areas that cannot be accessed by vehicle, the weight of cameras, typically 0.4 – 1 Kilograms per unit, limits the deployment of large numbers. Camera traps may also have inherent biases in data collected; Gompper *et al* (2006) reported that Coyote *Canis latrans* actively avoided camera traps. Identification of species is also reliant on expert knowledge and may be unreliable for certain taxa (Meek et al. 2013).

1.3 DNA as a Survey Tool

Recent advances in the application of DNA technologies have shown the potential for surveying mammals by means of sequencing their DNA from indirect sources. The recovery of mammal DNA may come from indirect field survey methods, such as scat sampling (Stenglein et al. 2010; Shehzad et al. 2012; Piggott & Taylor 2003; Ruibal et al. 2009; Bohmann et al. 2014) or hair trapping (Piggott & Taylor 2003; Bremner-Harrison et al. 2006; Jun et al. 2011; Ruibal et al. 2010). Increasingly, samples of environmental material such as soil (Andersen et al. 2012), freshwater (Thomsen & Willerslev 2015; Goldberg et al. 2015; Ficetola et al. 2008), ancient sediment (Boessenkool et al. 2012) or even air (Folloni et al. 2012) are being utilised as a source of DNA. Environmental samples as a source of DNA, termed eDNA (Ogram et al. 1987), were initially used only to study microbial communities (Taberlet et al. 2012) and their use as a tool to survey higher order taxa has only been developed within the last decade (Thomsen & Willerslev 2015; Bohmann et al. 2014). A more focussed source of mammalian DNA that has shown potential as an effective survey tool has been the utilisation of invertebrates such as haematophagus mosquitoes, tsetse flies and leeches as vectors of their host's DNA (Kent 2009; Townzen et al. 2008; Schnell et al. 2015; Adams et al. 2006). The term 'iDNA' has been introduced to collectively describe all invertebrate-derived mammalian DNA (Calvignac-Spencer, Leendertz, et al. 2013).

1.3.1 iDNA as an Emerging Survey Tool

The sequencing of mammalian DNA from insect vectors is not a recent occurrence. Five years after the forensic application of genetic profiling was first described (Gill et al. 1985), Coulson and colleagues (1990) demonstrated that the technology could be used to identify human DNA derived from a mosquito blood-meal. A practical application of this; the ability to identify individual human hosts from DNA sequenced from mosquito blood-meals, was reported shortly after (Gokool et al. 1993). Around the same time as these developments, there was a recognition in forensic science that developmental stages of blow fly (Diptera; Calliphoridae) could be used to estimate post-mortem interval (Greenberg 1991). Following this discovery, genetic profiling was used to first positively identify the species of fly larva feeding on corpses (Sperling et al. 1994) and then further developed to identify and match individual fly larva with the corpse they had fed on (Campobasso et al. 2005; Wells et al. 2001). Concurrent to these developments in the field of forensics, DNA analyses of the blood-meals of haematophagous arthropods were being undertaken to understand their role as vectors of blood-borne human pathogens (e.g. Kent 2009; Townzen et al. 2008).

The first use of iDNA as a tool for biodiversity assessment was published by Schnell and colleagues (2012) who recorded the presence of rare and cryptic mammalian species from the blood meals of a series of terrestrial *Haemadipsa* spp. leeches caught in a tropical Vietnamese rainforest. Furthermore, the group demonstrated that amplifiable mammalian DNA is recoverable from *Hirudo* spp. leeches at least four months post feeding, leading to the suggestion that most wild caught adult leeches will contain DNA traces of their last blood meal (Schnell et al. 2012). The viability of iDNA from carrion flies (a term used to encompass blow flies Calliphoridae and flesh flies Sarcophagidae), as a tool for assessing mammalian diversity, was shown the following year by Calvignac-Spencer, Merkel and colleagues (2013). The group screened 201 carrion flies collected in West African and Madagascan forests for mammal DNA. Using multiple Polymerase Chain Reaction (PCR) systems, they were able to retrieve DNA sequences of twenty six species of mammal, representing species of diverse size and from a range of forest strata (Calvignac-Spencer, Merkel, et al. 2013). Recently, the targeted detection of specific mammal species from carrion fly iDNA, and the potential capability of iDNA to facilitate the identification of individual mammals were reported (Schubert et al. 2015). Using species-specific PCR assays, Schubert and colleagues increased detection of target species by up to threefold in comparison to non-specific PCR assays (Schubert et al. 2015). The capability to identify individual mammals using iDNA was subsequently confirmed by Martinez-De la Puente and colleagues (2015) by identification of an individual Iberian lynx *Lynx pardinus* using iDNA collected from an *Anopheles* mosquito. Through a laboratory feeding experiment, Lee et al. (2015) determined the persistence period of amplifiable mitochondrial DNA (mtDNA) in blow fly guts. Their results showed that whilst it was possible to amplify mtDNA sequences up to 96 hours post feeding, this was achievable in only 22% of flies tested. At 24 hours post feeding, retrieval of amplifiable mtDNA was successful in 100% of flies; they therefore recommended a standardised trapping protocol with flies retrieved at no more than 24 hour intervals. The recommended trapping protocol of 24 hour intervals is problematic for surveys in remote territory where traps may typically be placed and left for extended periods.

1.3.2 Current Limitations of iDNA

The last three years have seen the introduction and development of iDNA as a cost effective and viable tool for surveying mammal diversity. However, a significant obstacle to the use of iDNA for surveying mammals in remote areas remains the relatively short persistence period of amplifiable DNA within the fly's gut. The recommended 24 hour maximum interval for retrieving flies (Lee et al. 2015) compares poorly to camera trap studies where researches may check cameras at intervals of up to two weeks (Silver et al. 2004; Wang & Macdonald 2009) or even longer. In a survey of wolverines *Gulo gulo* in Alberta, Canada, cameras set in very remote locations were visited by helicopter at intervals of one month (Fisher 2004). The same study analysed DNA from hair samples retrieved from hair traps at one to two month intervals. Surveys utilising iDNA to date have had rapid access to deep freeze storage to preserve iDNA; a requirement which further limits the potential use of iDNA as a survey tool in remote areas.

Although results collected so far indicate that iDNA has potential as a comprehensive and cost-effective tool for surveying mammals, its efficacy has so far been demonstrated in tropical forests only and limited to sites where traps are easily accessible and facilities are close at hand for the rapid preservation of collected flies. In order to extend the potential of iDNA to a wider variety of habitats and to field sites in remote areas, methods of trapping and processing flies in the field over a longer timescale are required.

1.4 The Development of iDNA for Remote, Montane Habitats

In this study iDNA was developed for use in remote mountainous terrain utilising an extremely data deficient species as a model. Optimum sites for the Seram bandicoot were identified through a search of archive data and by conducting interviews with forest users. Flies with known exposure to local mammals were collected at a remote site in montane forest and processed in the field, using field-appropriate techniques to preserve DNA for subsequent iDNA analysis. A fly trap suitable for montane environments was developed and deployed alongside camera traps in order to compare the success of iDNA to a conventional survey method in a range of undisturbed montane habitats. In a controlled experiment, the ability of a newly designed trap to preserve iDNA, and thus extend its persistence period, was evaluated against a conventional fly trap design.

This study discusses the applicability of iDNA as a method for surveying mammals in remote montane habitats and presents a method for field preservation of iDNA and a cost-effective and lightweight trap suitable for fly trapping in montane habitat. A newly designed fly trap with the potential to extend the persistence period of iDNA is presented.

2 Materials and Methods

2.1 Study Sites

The relevant permits to conduct research on Seram were obtained (Appendix E). Study sites were selected by applying information on the capture locality of *Rhynchomeles* obtained from archive materials at the Natural History Museum (London) to relief map data, and were subject to modification from anecdotal information collected during interviews on Seram (Table 1). Selection was done to maximise the likelihood of collecting evidence of *Rhynchomeles*. A discussion of the interpretation of details within archive documents is given in Appendix C.

Table 2: Study Sites

Study site	**Location**	**Altitude (metres asl)**	**Dates**	**Rationale for inclusion**
Gunung Manusela	S3° 13.5' E129° 37.1'	1839	14.7.14 - 17.7.14	Approximate location attributed as the 1920 collection locality of *Rhynchomeles* (Thomas 1920). Spotted cuscus (*Spilocuscus maculatus*) butchered next to the camp site allowed for the collection of flies that had been observed feeding on a known species.
Sepi Nahu	S3° 14.1' E129° 36.6'	1097	12.7.14 - 14.7.14	Enclave of montane forest with a low level of disturbance by hunters. This was the site where Rhynchomeles was reported first hand to have been trapped in 199?.
Ramatiti	S3° 10.7' E129° 33.3'	1290 - 2432	15.7.15 - 22.7.15	Area of undisturbed habitat including montane forest and alpine scrub above the treeline. Access to upper zone limited to day trek from staging camp: lack of water to establish a higher camp
Gunung Loa Loa	S3° 01.7' E129° 11.8'	1603	15.7.16 - 19.7.16	Montane forest at northern end of contiguous central mountainous spine with low levels of disturbance. Reported as containing several unrecorded species of mammal

The sites selected provided an opportunity to evaluate trapping methods in montane forest and to process flies with known exposure to mammal species occurring in that habitat. The selected sites enabled remote processing of flies and preservation of DNA to be trialled in the field and provided an opportunity to assess the efficacy of iDNA as a survey tool in comparison to camera trapping in undisturbed montane habitats and to evaluate the success of fly traps deployed over multiple days. A map showing location of study sites is shown in Appendix D.

2.2 Field Methods

Two principal survey methods were used in the field: Camera trapping and iDNA. Interviews of local forest users were also conducted to gain a rapid awareness of local knowledge of wild species of mammals and to inform study site selection.

2.2.1 Interviews of Forest Users

Anecdotal information on *Rhynchomeles* was sought from indigenous inhabitants during informal interviews. Interviews were conducted with multiple informants and all informants were made aware that responses were being recorded for research purposes and that the research pertained to the fauna of Seram. Free-listing (Weller & Romney 1988) was employed to collect data on the terrestrial vertebrate fauna of Seram, utilising the accompanying guide from Ambon as a translator. The structured question used was: *What kinds of animals live wild on Seram*? Four supplementary interviewing techniques were incorporated to maximise the output from the interviews (Brewer 2002; Harper 2010): Non-specific prompting, reading back to an informant the information given, using free-listed items as semantic clues and photo-elicitation. The techniques were applied in the sequence listed; the first three techniques following Brewer (2002). For photo-elicitation, three A4 sheets containing colour images of 14 mammal species were presented to the informant (see appendix). The species illustrated included species known to occur on Seram (n = 9) and selected species from elsewhere (n = 5). The non-Seram species selected were four potential candidates for unrecorded mammals on Seram (Thomas 1920; Helgen 2003) and a neotropical species known not to occur in the region (
Table 3). Informants may report the presence of all species shown if this is presumed to be the response sought by the interviewer (Padmanaba et al. 2013) so the neotropical species was included as a control for respondent reliability. Images were arranged to be correctly proportional to each other within each sheet and two sheets containing smaller sized species included a tape measure as scale; the tape was taken to Seram and used to illustrate size of species during interviews. A recording template was used to collate interview data (Appendix F).

Table 3: Mammal Species on Photo-elicitation sheets & Reason for Inclusion

Image	Common name	Species	Reason for inclusion
1	Seram bandicoot	*Rhynchomeles prattorum*	Seram endemic; poorly known
2	Spiny Seram island rat	*Rattus feliceus*	Seram endemic; poorly known
3	Seram mountain rat	*Nesoromys ceramicus*	Seram endemic; poorly known
4	Seram Long-tailed melomys	*Melomys fulgens*	Seram endemic; poorly known
5	Dusky Mosaic-tailed rat	*Melomys aerosus*	Seram endemic; poorly known
6	Manusela Mosaic-tailed rat	*Melomys fraterculus*	Seram endemic; poorly known
7	Striped possum	*Dactylopsila trivigata*	Possible candidate for descriptions given in 1920
8	Long-fingered triok	*Dactylopsila palpator*	Possible candidate for descriptions given in 1920
9	Masked ringtail possum	*Pseudochirulus larvatus*	Possible candidate for descriptions given in 1920
10	Common spotted cuscus	*Spilocuscus maculatus*	Common wild mammal on Seram
11	Silky cuscus	*Phalanger sericeus*	Similar species to skin collected on Seram in 1987
12	Asian Palm civet	*Paradoxurus hermaphroditus*	Introduced species recorded from Seram
13	South American coati	*Nasua nasua*	Does not occur in Asia: included to assess respondent reliability
14	Malay civet	*Viverra tangalunga*	Introduced species recorded from Seram

2.2.2 iDNA Survey

Fly trapping or collecting was carried out at each of the four study sites (Table 4). At Gunung Manusela eight fly traps were hung from vegetation at heights of 30 – 100 cm, at approximately 80 metre intervals along a broad ridge line transect. At Sepi Nahu eight fly traps were placed at random trapping stations within 550 metres of a temporary camp site.

Table 4: Fly Trapping Conducted

Study site	Dates	Nights	Fly traps set	Trap nights
Sepi Nahu	12.7.14 - 14.7.14	2	8	16
Gunung Manusela	14.7.14 - 17.7.14	3	8	24
Ramatiti	15.7.15 - 22.7.15	7	24	136
Gunung Loa Loa	15.7.16 - 19.7.16	4	1	4

Fly traps at both sites were of a modified bottle trap design, previously used to capture carrion feeding flies (Calvignac-Spencer, Merkel, et al. 2013) (Figure 2). Traps were baited with entrails of Cuscus (*Spilocuscus maculatus*) (Gunung Manusela) or rotting fish (Sepi Nahu). At Gunung Manusela, flies were also actively netted at a site where two cuscus *Spilocuscus maculatus* caught by local hunters had been butchered. All captured flies were transferred to 10ml sample tubes and anaesthetised by holding this tube end to end with a tube containing tissue soaked in acetone solution (Avione nail polish remover; Avion-Kosmetic, Bandung, Indonesia).

Figure 2: Bottle Fly Trap Used at Gunung Manusela and Sepi Nahu

At Ramatiti 24 sticky tube traps were wired to woody vegetation in a horizontal orientation at heights ranging from ground level to 90 cm height at approximately 200 metre intervals along a 5 km long irregular transect with an altitudinal range of 1142 metres. The traps were made from 1.5 litre plastic bottles with the funnel-shaped tops removed to form a tube with a closed end. A rolled adhesive fly-paper sheet (Sticky fly catcher: PestTrappa, Chesterfield, UK) was inserted and secured inside the open end of the tube with two paperclips. The cut-off base from a 500ml plastic bottle, containing a synthetic bait (removed from a Fly Bag: AgriSense-BCS, Glamorgan, UK) and netted over to prevent entry by flies, was placed at the closed end of the tube (Figure 3). Captured flies were removed from the fly-paper with fine forceps. At Gunung Loa Loa a single fly trap with a reservoir of Propylene glycol as a preservative medium was deployed over four nights (Figure 4). The trap was placed on the forest floor at an altitude of 1603 metres asl.

Figure 3: Open-tube Fly Trap Used at Ramatiti

For dissection, flies were placed on compact plastic foam matting on their dorsal side and insect pins inserted on the lateral side of the mesothorax. Dissection followed Boonsriwong *et al* (2011) and was performed with fine forceps and a 10a scalpel blade using a 6X Linen Tester Magnifier (Maplin Electronics Ltd., Rotherham, UK). Specimens were dissected ventrally by removal of abdominal sclerites in a posterior to anterior direction. Once all of the sclerites had been removed, the alimentary canal was severed anterior to the junction of the mid gut and the hind gut by probing into the muscular thorax. The hind gut together with the retained chains of malpighian tubules were then removed by forceps and smeared onto the sample area of an FTA™ Classic Card (GE Healthcare Life Sciences, Whatman™, Buckinghamshire, UK) as per manufacturer's instructions.

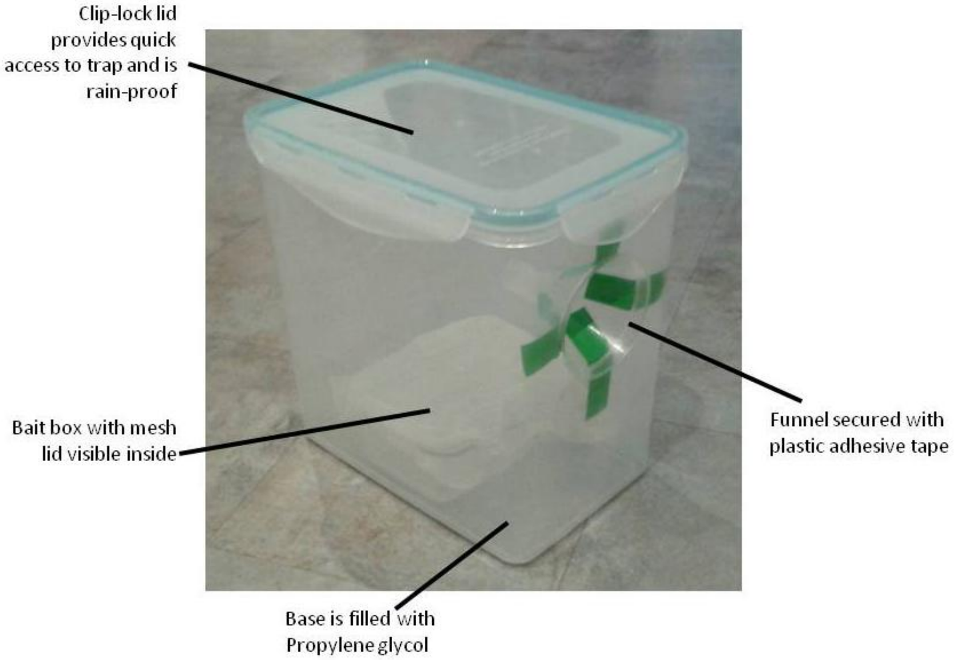

Figure 4: New Fly Trap Deployed at Gunung Loa Loa

2.2.3 Camera Trapping – Calibration of iDNA

Trail cameras were deployed in the vicinity of fly traps at each field site (Table 5). Cameras utilised were Cuddeback Capture (Cuddeback, WI 54307 USA) and Bushnell NatureView CamHD (Bushnell Outdoor Products, 92150 Suresnes, France). At Sepi Nahu and Gunung Manusela, only Cuddeback Capture cameras were used, whilst as Ramatiti, cameras were mostly Bushnell NatureView (n = 12), with four Cuddebacks set at the lower end of the transect. Bushnell cameras were used exclusively at Gunung Loa Loa. Cameras were set in a vertical orientation at between 20 and 90 cm height, strapped to woody vegetation or to stakes pushed into the ground. Camera traps were set in the vicinity of fly traps and following the same deployment pattern; e.g. at Gunung Manusela; approximately equally distributed along a transect. Where present, suspected mammal trails and containment features such as fallen logs were utilised when placing cameras to give the greatest chance of image capture. Cameras were baited with peanut butter, a known olfactory attractant to *Isoodon* and *Perameles* bandicoots in Australia (Paull et al. 2011), supplemented with small amounts of fish and cuscus entrails when available. Cameras were checked daily at Sepi Nahu and Gunung Manusela and on removal at day four at Gunung Loa Loa and on day four or seven at Ramatiti. Details of the settings for all adjustable parameters are given in Appendix G.

Table 5: Camera Traps Deployed

Study site	Dates	Nights	Camera stations	Trap nights
Sepi Nahu	12.7.14 - 14.7.14	2	19	38
Gunung Manusela	14.7.14 - 17.7.14	3	19	57
Ramatiti	15.7.15 - 22.7.15	7	16	112
Gunung Loa Loa	15.7.16 - 19.7.16	4	5	20

2.3 Trap Design to Extend the Persistence of iDNA

A new fly trap, designed to preserve iDNA through the inclusion of a well of Propylene glycol, was developed. For DNA preservation, Propylene glycol has been shown to perform well in comparison to commonly used preservatives such as 95% ethanol, dimethyl sulfoxide (DMSO) and RNAlater® (Ambion Inc., Austin, TX, USA) (Moreau et al. 2013). Cooled Propylene glycol has recently been demonstrated as a suitable preservative for high quality DNA extraction from remote field-collected Diptera (Patrick et al. 2016).

2.3.1 The New Fly Trap Design

The trap was manufactured from a clear plastic box, 20 x 13 x 22 cm high with a transparent snap-top lid. Round holes were cut in the upper half of the two shorter ends of the box, into which were fitted 7 cm diameter plastic funnels, held in place with plastic adhesive tape (Figure 1). The use of entry funnels on the vertical sides rather than the upper surface was designed to prevent rain entering the trap. A smaller plastic box, 11 x 8 x 6 cm high, designed to hold an olfactory bait, was placed in the base of the larger box. The bait box was covered with taut nylon mesh, secured with an elastic band, to prevent contact between flies and the bait. Food grade Propylene glycol (ClassiKool Ltd; Leigh-on-Sea, Essex, UK) was poured into the base of the trap, around the bait box, to a depth of 2 cm. In order to ascertain the trap's efficacy at trapping flies, two of the traps, baited with fish (*Sprattus* spp.) were placed in outdoor service areas adjacent to animal enclosures (Banteng *Bos javanicus* and

Gelada *Theropithecus gelada*) in Edinburgh Zoo. The traps were left in place for 26 days and the number of trapped flies recorded at weekly intervals.

2.3.2 A Comparison of the New Fly Trap with a Conventional Trap

The new fly trap's ability to preserve mammalian DNA in the guts of blow flies was evaluated in comparison to conventional fly traps without preservative media in a controlled experiment. Approximately two hundred pupae of the Bluebottle fly *Calliphora vomitoria* were obtained (Blades Biological Ltd., Edenbridge, UK) and maintained within a thermostatically controlled room at a temperature of 20°C (+/- 4°C) with a photoperiod of 12 hours light / 12 hours dark. Pupae were divided into six equal sized groups, housed in clear plastic boxes measuring 30 x 30 x 35 cm high (Figure 5). Opaque tights were stretched over the open top of the box and secured in place with adhesive tape. To facilitate access inside the box, the end of one leg of the tights was cut off and secured with a klippit fastening (WeLoc – Weland M. AB., Sweden). Eclosion of the pupae commenced immediately on arrival and continued for two days resulting in the emergence of approximately 150 flies. After five days no further flies had emerged and the remaining pupae were removed and discarded. Newly emerged flies were provided with granulated white sugar and water *ad libitum*.

At the commencement of the experiment, sugar was removed from all containers for 24 hours to standardise fly appetite. Post-fasting, all groups were given access to food for four hours (1800 – 2200 hours): four groups were provided with beef liver *ad libitum* whilst provision of sugar was reinstated to the remaining two groups. All flies were observed to feed at this time. A sample of beef liver was frozen for use as a positive control. At the end of the feeding phase, food and water was removed from all containers and Propylene glycol at ambient temperature was added to two of the containers to a depth of 2 cm (T = 0 hours) (Figure 6).

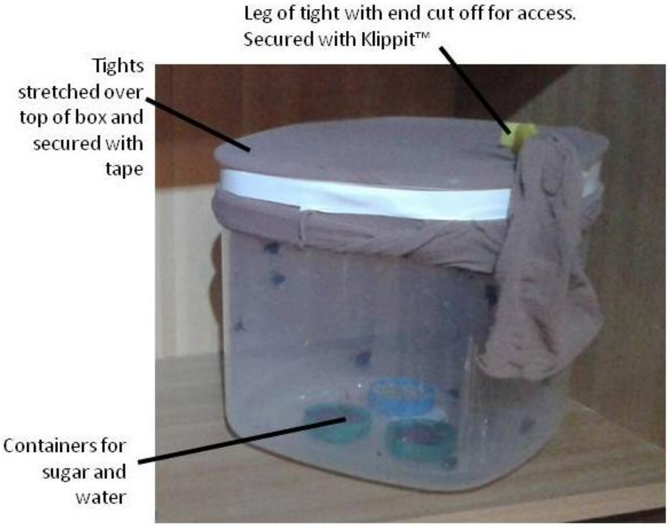

Figure 5: Box Housing Blow Flies

Four flies were removed from each box at 24 hour intervals at 24, 48, 72, 96, 120, and 144 hours. Upon removal, flies were pinned on their dorsal side, and their guts dissection onto FTA cards as described above. Separate pinning mats and forceps were used for each treatment; these were cleaned in between each fly with DNAZap™ solutions (Thermo Fisher Scientific Inc., Waltham, MA, USA) as per manufacturer's instructions. Separate pins and scalpel blades were used for each fly. FTA cards for each group and time point were stored in separate paper envelopes at room temperature.

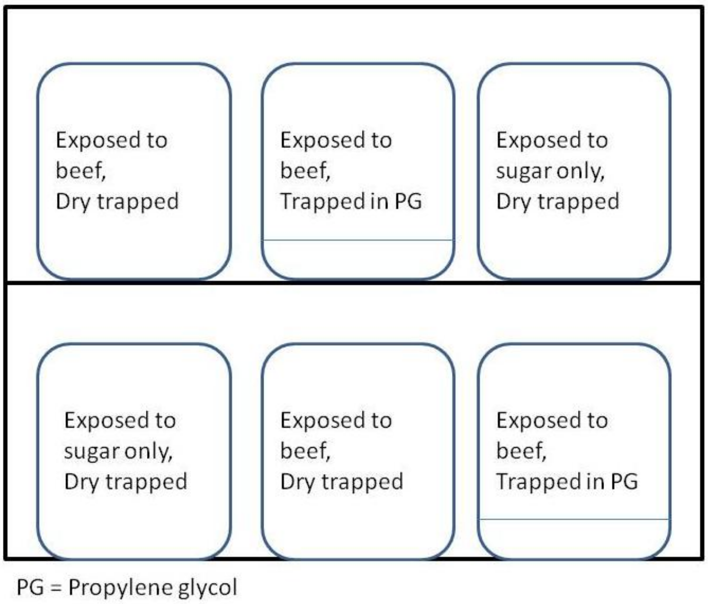

Figure 6: Layout of Fly Containers on Two Shelves

2.4 Laboratory Processing of Samples

DNA extraction and PCR of all field collected samples (Seram samples) was carried out at the RZSS WildGenes laboratory, Edinburgh Zoo, Edinburgh, UK. Samples pertaining to the new fly trap trial (UK samples) were processed at The Roslin Institute, The University of Edinburgh, Easter Bush, UK. Procedures for processing samples are described below.

2.4.1 Extraction of DNA

For each sample stored on an FTA card, three disks of 3 mm diameter were removed from the card's sample area using a Harris Uni-Core™ punch and cutting mat (GE LifeSciences) and placed together in a labelled 1.5ml Eppendorf. Between samples the Uni-Core™ punch and cutting mat were thoroughly cleaned using paper roll and Distel High Level Laboratory Disinfectant solution (Tristel Solutions Ltd., Snailwell, Cambs., UK) at a dilution of 1:100.

Extraction of Seram samples was carried out using the QuickGene DNA tissue kit S (Fujifilm Corporation, Minato, Tokyo, Japan) following manufacturer's instructions. In brief, cell lysis was performed by the addition of 25 µl of Proteinase K and 180 µl of MDT lysis buffer to the sample, reagents were mixed by brief vortexing and centrifugation before incubating on a thermoblock at 55°C for 60 minutes. Two hundred µl LDT buffer was added and mixed by brief vortexing and centrifugation before incubation on a thermoblock at 72°C for 60 seconds. Two hundred µl of 100% ethanol was then added and mixed by immediate vortexing followed by centrifugation at 3000 rpm for 3 minutes to produce an ‘extraction supernatant’. Elution of Seram samples was carried out using a Quickgene Mini80 personal extraction system (Fujifilm Corporation, Minato, Tokyo, Japan) following the manufacturer’s instructions. The extraction supernatant was pipetted into a cartridge placed above a collection tube and blown through for one cycle (approximately 1 minute). Seven hundred and fifty µl of wash buffer was added into the cartridge and blown through for one cycle. This step was repeated twice to give three rinses in total. The bases of each cartridge were cleaned with a separate clean cotton bud before being placed over clean, labelled 1.5 ml micro-centrifuge tubes. Samples were eluted by adding 30 µl of elution buffer, blown through for one cycle followed by a further 25 µl elution buffer, also blown through for one cycle.

For UK samples, extraction was carried out using the DNeasy® Blood & Tissue Kit (Qiagen Ltd., Manchester, UK) following manufacturer’s instructions. Briefly, cell lysis was performed by the addition of 20 µl of Proteinase K and 180 µl of lysis buffer ATL to the sample, reagents were mixed by brief vortexing and centrifugation before incubating on a thermoblock at 56°C for 60 minutes. Two hundred µl buffer AL was added and mixed by brief vortexing and centrifugation. Two hundred µl of 100% ethanol was then added and mixed by immediate vortexing. Elution was done by DNeasy Mini Spin column (Qiagen Ltd., Manchester, UK) following manufacturer’s instructions. The extraction supernatant was pipette into the Mini spin column, placed in a 2 ml collection tube and centrifugation at 8000 rpm applied for 1 minute. The collection tube containing the flow through was then discarded and the Mini spin column placed in a new collection tube. Five hundred µl of Buffer AW1 was added and centrifugation repeated as before. The collection tube and flow-through was discarded and replaced with a new tube. Five hundred µl of Buffer AW2 was added and forced through the spin column’s membrane by centrifugation at 14000 rpm for 3 minutes. Spin columns were then placed in 1.5 ml micro-centrifuge tubes and 100 µl Buffer AE pipetted directly onto the membrane. After incubation at ambient temperature for 1 minute, samples were eluted by centrifugation at 8000 rpm for 1 minute. Eluted samples were stored at -20°C when not in use.

2.4.1.1 DNA Quantification

For all samples stored on FTA cards, DNA quantity and purity was determined using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), following the manufacturer’s guidelines for quantifying nucleic acid quality and concentration. DNA has a maximum absorption at 260nm so DNA concentration of each sample was determined by measuring the optical density at that wavelength (A260). Every 1.0 unit of density equates to 50 µg/ml of DNA. Absorption was also measured at 280nm and 230nm. The A260/A280 ratio is an indication of the level of protein contamination in the sample. A A260/A280 ration of 1.8 is considered ‘pure’ for DNA (Wilfinger 1997). A high peak at A230 can indicate other contaminants such as carbohydrate carryover or phenol, when used for extraction. The ideal A260/A230 ration is greater than 1.5, ideally close to 1.8.

Prior to quantifying samples, calibration was done as *per* instructions using 1.5 µl of nuclease free water followed by 1.5 µl of elution buffer. Once calibrated, 1.5 µl of each sample was pipette directly onto the measurement pedestal of the NanoDrop™ 1000 Spectrophotometer. A column of the eluted sample was then drawn between the ends of two optical fibres to create a measurement path. Absorbance was measured and recorded using the pre-set programme for DNA quantification.

2.4.2 Amplification of Mammalian Sequences Using PCR

Post-extraction and assessment of DNA quality and quantity, PCR was used to amplify targeted mammal sequences contained within the samples elutes.

2.4.2.1 Primer Selection

For Seram samples the DNA fragment amplified by primers must facilitate identification at a species level but ideally avoid high levels of intra-specific variation. In order to do this it must of adequate length to display inter-specific variation but must also be short enough to allow potentially degraded DNA to be amplified. A trial of four prospective pan-mammalian primer pairs was carried out (Table 6).

For UK samples, a beef specific primer pair; BSPF and BSPR (Tanabe et al. 2007) was used as beef was the only mammalian food source available to these flies and to avoid mis-amplification of human DNA contaminants. This primer set amplified a 119 bp region of the bovine Cytochrome b (Cyt b) gene. Use of this set aimed to facilitate comparison with results from a controlled experiment by Lee et al. (2015), using the same primers to determine the persistence of mammalian DNA within blow flies post-feeding. For all primers, stock primers, at 100 pmol/µl concentration were stored at -20°C and aliquots taken from this and diluted to a concentration of 10µM for use in PCR procedures.

Table 6: Primers Tested and Used

Forward primer	**F primer 5'-3' sequence**	**Reverse primer**	**R primer 5'-3' sequence**	**Author**
BSP F	CCCGATTCTTCGCTTTCCAT	BSP R	CTACGTCTGAGGAAATTCCTGTTG	Tanabe et al 2007
BRCA1.126F	GTTTCAAACTTGCATGTGGAGCC	BRCA1.3012R	GTTGGAAGCAGGGAAGCTCTTCATC	Teeling et al 2000
RAG1.2204F	GCTTCTGGCTCWGTCTACATYTGTAC	RAG1.2749R	AAACGCTGTGARTTGAAACT	Amrine-Madsen et al 2003
12Sa.490F	CTG GGA TTA GAA CCC CAC TAT	12So.614R	GTCGATTATAGGACAGGTTCCTCTA	Kuch et al 2002
		12Sm.680R	GAGGATGGCGGTATATAGGCTG	

2.4.2.2 *Polymerase Chain Reaction*

PCR of Seram samples was carried out using a Bio-Rad PTC-200 thermal cycler (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts. UK). UK samples were amplified using a G-Storm GS4822 thermal cycler (G-Storm, Somerton, Somerset, UK). All reactions incorporated positive and negative controls. Negative controls were included to indicate the occurrence of contaminants or non-specific amplification whilst positive controls allowed verification of the PCR reaction. For Seram samples, the negative control consisted of a reaction mix with the sample substituted for nuclease free water, whilst as a positive control a DNA sample from European beaver *Castor fiber* was used. For UK samples, DNA extracted from flies that had not been exposed to beef was used as a negative control and a DNA sample extracted directly from the beef liver was used as a positive control. HotStarTaq Master Mix kit and HotStar Taq Polymerase kit (both Qiagen Ltd., Manchester, UK) were used for Seram samples and UK samples respectively. For each reaction, a master mix (**Table** 7) was prepared and then aliquots mixed with individual samples. Thermal cycle conditions are given below (Table 8).

Table 7: PCR Reaction Mix

Reagent	**Volume (µl)**	
	Seram samples	**UK samples**
Hot StarTaq Master Mix	14	
HotStar Taq Polymerase		0.2
dNTP mix (10mM)		0.8
10x PCR Buffer		2
Nuclease free water		11
Forward primer (10pmol/µl)	2	2
Reverse primer (10pmol/µl)	2	2
DNA template	2	2
Total reaction volume	**20**	**20**

Table 8: PCR Thermal Cycle Conditions

Stage of cycle	**Number of cycles**	**Temperature (°C)**	**Time (minutes)**
Enzyme initiation	1	95	5
Denaturing	40	95	0.5
Annealing	40	50	0.5
Extension	40	72	1
Final extension	1	72	10
Hold	1	10	∞

2.4.2.3 Gel Electrophoresis

Post-PCR DNA samples were analysed using gel electrophoresis. Agarose gels were made by dissolving UltraPure™ agarose (Invitrogen Corporation, Carlsbad, California, USA) in TAE buffer using a microwave. Seram samples were run on 1.5% gels whilst UK samples, with smaller DNA fragments amplified, were run on 3% gels. Once the gel had cooled to 50 – 60°C, GelRed™ Nucleic Acid Gel Stain (Biotium Inc., Fremont, California, USA) was added at a concentration of 1 µl to 20 ml (Seram samples) or 1 µl to 10 ml (UK samples) of gel. The solution was poured into a gel casting tray with gel combs in place and allowed to set. All samples were mixed with a loading dye; BlueJuice™ Loading Buffer (Invitrogen Corporation, Carlsbad, California, USA) for Seram samples, Blue/Orange Loading Dye (Promega UK Ltd., Southampton, UK) for UK samples, as per manufacturer's instructions. The gel was submerged in TAE buffer and the samples, together with a 20 bp DNA ladder

(Promega UK Ltd.) were loaded into the wells. Gels were connected to a power supply; the positive electrode furthest from the samples as DNA is negatively charged, and run at 65 – 120 V for 20 – 45 minutes respectively. Completed gels were visualised under UV and images captured using a CCD digital camera. Seram samples were visualised on a D1-HD light box (Cleaver Scientific, Rugby, UK) mounted on a MUV21-312 Transilluminator (Cleaver Scientific), whilst a Molecular Imager®GelDoc™XR Imaging System (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts. UK) was used for UK samples.

2.4.2.4 Sequencing

Sequencing was done by an external provider; Edinburgh Genomics (Ashworth Laboratories, The King's Buildings, Edinburgh, UK) using the BigDye® Terminator v3.1 sequencing reaction (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA). The reaction, based on the Sanger sequencing technique, utilises dideoxynucleotides that terminate chain elongation after their incorporation into the DNA strand.

For Seram samples, all post-PCR samples showing bands in the correct location on gels were sequenced in both directions. An initial clean-up reaction utilising Exo I and FastAP (Thermo Fisher Scientific Inc., Waltham, MA, USA) was carried out following manufacturer's protocols. In brief; 0.5 µl of each of the aforementioned reagents was added directly to 5 µl of post-PCR sample and incubated at 37°C for 45 minutes, followed by 85°C for 15 minutes. Once purified, cycle sequencing was performed using BigDye® Terminator v3.1, as per manufacturer's instructions. Per sample, 0.5 µl of BigDye® Terminator v3.1, 1.75 µl of BigDye buffer, 1.0 µl of primer and 4.75 µl of nuclease free water were mixed and added to 2 µl of purified sample. The resulting mixture was incubated at 37°C for 45 minutes then 85°C for 15 minutes before final sequencing at Edinburgh Genomics. A sub-set of UK samples was selected for sequencing. The sub-sample, consisting of one sample from the two trap conditions at each of the six time points, together with a positive and a negative sample, was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega UK, Southampton, UK) following the manufacturer's instructions for DNA purification by centrifugation. All samples had only single bands observed on gels and so were purified directly. For direct purification of PCR products, an equal volume of membrane binding solution was added to the PCR reaction mix. The solution was transferred to a SV Minicolumn™, placed in a collection tube, and incubated at room temperature for 1 minute before centrifugation at 14,000 rpm for 1 minute. Two subsequent washes of the Minicolumn™ were performed by adding 700 µl and 500 µl of previously diluted membrane wash solution and centrifugation at 14,000 rpm for 1 and 5 minutes respectively. Flow-through was discarded following each wash. The Minicolumn™ was removed and left for 1 minute with the lid off to allow evaporation of residual ethanol. The SV Minicolumn™ was then transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of nuclease free water was pipette onto the centre of the membrane. This was incubated at ambient temperature for 1 minute before centrifugation at 14,000 rpm for 1 minute. The eluted DNA was assessed for quantity and quality using the NanoDrop™ 1000 Spectrophotometer, as detailed above. Purified samples were then stored at -20°C prior to sequencing.

2.4.3 Assigning Species Identities

Sequences returned for Seram samples were quality controlled, aligned and then assigned species identities against reference sequences on the NCBI curated database 'GenBank' (Benson et al. 2013) as detailed below.

2.4.3.1 Quality Assurance of Sequences

Post-sequencing, the returned chromatograms (Figure 7) were viewed to assess quality using MEGA6.06 genetic analysis software (Tamura et al. 2013). Ambiguous sequences typically occur at the ends of the traces and these were removed prior to further quality checks. The overall intensity of the nucleotide peaks, the spacing between peaks and the level of background noise was then visually assessed on chromatograms. Traces were rejected if the intensity was too low to allow clear peaks to be identified or if there was no discernible difference between intensity of peaks and background noise. Following this step, traces and text sequences were scanned to identify heterozygous peaks (i.e. base pair positions with more than one clear peak). IUPAC degenerate codes were substituted for assigned bases to account for such situations.

2.4.3.2 Alignment of Sequences

Quality controlled sequences were added to the alignment explorer in MEGA6.06. Sequences generated with reverse primers were reverse complemented and concatenated with the corresponding forward primer sample sequence. The consensus sequences were aligned using the Muscle alignment tool 06 (Edgar et al. 2004) contained within MEGA6.06. The GenBank database was mined for 12S sequences of wild living mammal species recorded from Seram. Reference samples of these species, together with additional reference sequences for several species of interest were added to the alignment. Once concatenated and aligned, sequences that were 100 % homologous were reduced to a single representative sequence. A search was performed for each representative sequence using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) to identify the sequences in

GenBank with the greatest similarity to it. The first search result returned was recorded and accepted as the sequence identify if the identity value was $\geq 97\%$. To further verify the assigned species identities, a Phylogenetic tree was constructed using the inbuilt phylogeny construction tools in MEGA 6.06. Following instructions in Hall (2013) the sequence alignment was exported as a MEGA file (.meg) and then opened from the Phylogeny menu in the main window of MEGA. A *Neighbour Joining* tree was selected,

and in the Analysis Preferences window, the *Partial deletion* option was selected as this retains greater information than the default; *Complete deletion*, especially for alignments with multiple gaps in columns. In the *Test of Phylogeny* section, the *Bootstrap method* was selected and set at 500 replications prior to computing the tree.

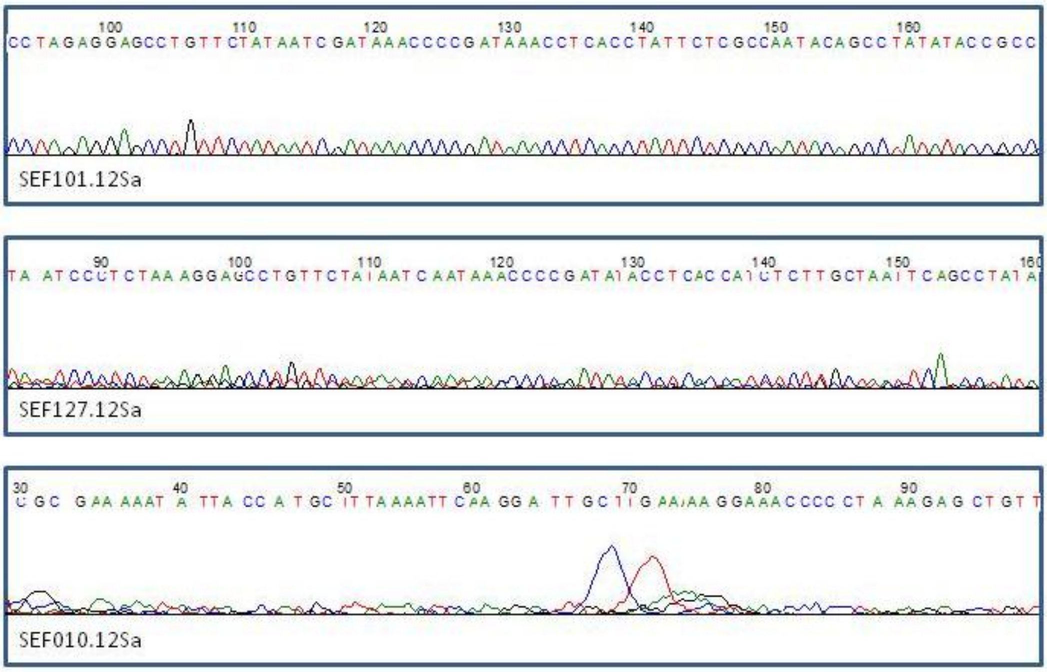

Figure 7: Examples of Chromatograms Showing Acceptable (Top) and Unacceptable (Middle and Bottom) Sequence Traces

2.4.4 Assessing the Persistence of iDNA in the New Fly Trap

Images of gels produced in the New Fly Trap experiment (see 2.3 above) were visually assessed to determine the relative brightness of bands as this is indicative of the concentration of DNA present (Lee et al. 2015). To further assess band intensity, images were analysed using ImageJ software (Schneider et al. 2012). Using the option *Analyze>Gels>Select First Lane* a selection rectangle was placed over the first band. Using the option *Analyze>Gels>Select Next Lane* additional rectangles were set over all subsequent lanes. The option *Analyze>Gels>Plot Lanes* was selected to draw a profile plot of each lane. Using the **Straight line** selection tool from the ImageJ toolbar, the area of each peak was isolated by adding vertical lines (Figure 8). Using the **Wand** tool from the toolbar, the internal area of each peak was selected and recorded. The area of the profile plots represents the relative density of the contents of each band. Using this technique, relative intensities of separate bands on the same gel image can compared. In the new fly trap trial, a direct comparison of each condition at the same time point was made. To make comparisons over successive time points, shown on different gels, the positive control band on each gel was utilised as a standard sample against which to normalise all other bands on the same gel. Once normalised, values were compared across all time points.

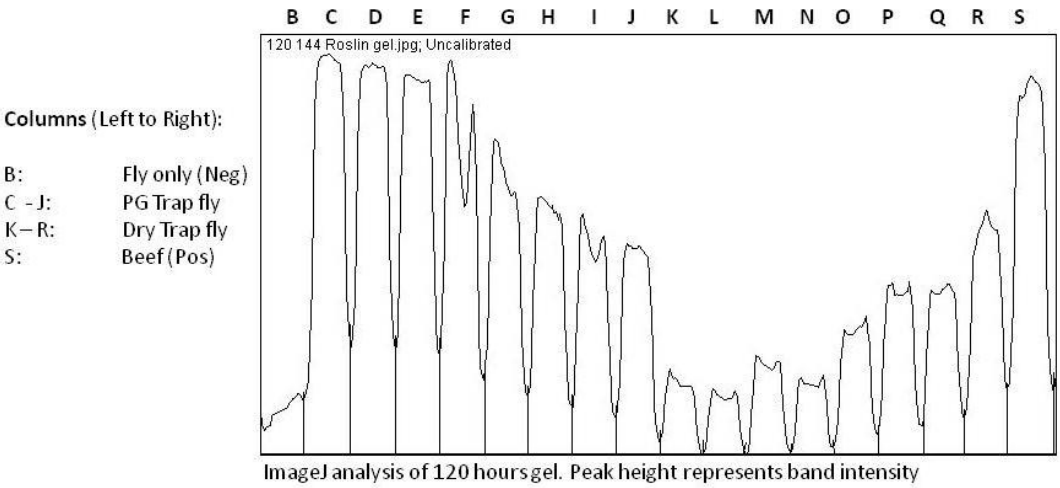

Figure 8: ImageJ Analysis of Bands: Peak Height Represents Band Intensity

3 Results

3.1 Interviews of local forest users

Nine interviews were conducted with forest users (**Table** 9). Interviews were conducted in forest camps (n = 3) and in villages (n = 6). Despite the intention to conduct individual interviews, within the environment it was not possible to prevent other individuals from participating and so data was recorded for multiple informants. Unstructured responses given by informants were recorded as accurately as possible in a notebook as interviews took place.

Table 9: Interviews of Local Forest Users Conducted

Date	Location	Location number	Location type	Number of informants
11.07.2014	S3° 14.978' E129° 36.316'	1	Camp at Gua Dua	8
15.07.2014	S3° 13.517' E129° 37.081'	2	Gunung Manusela	4
17.07.2014	S3° 10.233' E129° 35.253'	3	Maraina village	7
21.07.2014	S2° 57.891' E129° 12.082'	4	Masihulan village	2
11.07.2015	S3° 00.106' E129° 40.207'	5	Kaloa village	4
12.07.2015	S3° 05.932' E129° 38.584'	6	Hato'Olo village	5
14.07.2015	S3° 10.233' E129° 35.253'	7	Maraina village	4
16.07.2015	S3° 10.692' E129° 33.302'	8	Camp at Ramatiti	6
27.07.2015	S2° 57.483' E129° 10.742'	9	Sawai village	1

During interviews all terrestrial wild-living mammals recorded for Seram, not including resolution of different Murid rodents, were named and described (Table 10). During all interviews rodent species were referred to collectively as *Tikus*, the only variation to this was the description by two informants in one interview of a giant rat, said to live near the tops of the mountains. Two informants also described a small kangaroo; one relatively detailed description of multiple first-hand sightings, the other as a second-hand account, but given independently. Potential candidates for a small macropodid were not included on photo-elicitation sheets, which were constructed prior to its reported presence. Anecdotal evidence volunteered spontaneously out-with an interview framework was also recorded. In total the Seram bandicoot, *Mapea*, was referred to in one first-hand account, three second-hand accounts of named individuals who had seen or killed one, and three generalised reports of knowledge of the species existence. Of the four first and second-hand reports; the first-hand report was of the capture and release of a single *Mapea*, reported to have occurred in the vicinity of Sepi Nahu (1097 metres asl) in 1998. The three second-hand reports included a recent sighting (approx. 2013) on the treeless summit area of Gunung Binaiya (approx. 2800 – 3000 metres asl) and two reports of *Mapea* killed in hunts; one in 2013 – 2015 at approximately 1800 metres asl near Gunung Hoale, and one at approx. 1850 metres asl on the South Eastern side of Gunung Murkele Kercil.

Table 10: Interview Results

Type of animal	**Local name given**	**Frequency listed**	**Notes**
Cuscus (all types)	kuskus	1.00	Up to six forms described. Inconsistent data given; see additional note
Pig	babi	1.00	No differentiation between pig types given
Deer	rusa	1.00	
Malay civet	tingalunga	1.00	
Asian palm civet	musang	1.00	
Rat	tikus	1.00	No differentiation between rat species
Dog (feral)	anjing	0.89	Described as having originated from village dogs but can be found living wild in the forest
Cat (feral)	kucing hutan	0.78	Described as having originated from village cats but now living wild in the forest
Bandicoot	mapea	0.78	One first hand sighting; trapped in 1998, two second hand reports
Small kangaroo	(none given)	0.22	One first hand sighting, one second hand sighting; both described from forest on northern central area of island
Giant rat	tikus besar	0.11	One (multiple informant) description; said to live on top of mountains, looks like normal rat but much bigger
Small civet	lau lau	0.11	One informant; described as smaller than musang, brown body with white neck, hunts cuscus in trees.

In response to images on photo-elicitation sheets, *Rhynchomeles* was identified twice as a *Tikus* present on Seram, but not identified as *Mapea* by any respondent, including the individual reporting the first-hand sighting and three individuals reporting second-hand sightings. *Dactylopsila, Pseudochirulus* and *Nasua* were stated as not occurring on Seram by 100% of respondents (Table 11). *Phalanger sericeus*; the silky cuscus from New Guinea, was reported as occurring on Seram by 100% of respondents.

Table 11: Photo-Elicitation Results

Image	**Common name**	**Species**	**Frequency listed**
1	Seram bandicoot	*Rhynchomeles prattorum*	**0.22**
2	Spiny Seram island rat	*Rattus feliceus*	**0.78**
3	Seram mountain rat	*Nesoromys ceramicus*	**0.78**
4	Seram Long-tailed melomys	*Melomys fulgens*	**0.78**
5	Dusky Mosaic-tailed rat	*Melomys aerosus*	**0.78**
6	Manusela Mosaic-tailed rat	*Melomys fraterculus*	**0.78**
7	Striped possum	*Dactylopsila trivigata*	**0.00**
8	Long-fingered triok	*Dactylopsila palpator*	**0.00**
9	Masked ringtail possum	*Pseudochirulus larvatus*	**0.00**
10	Common spotted cuscus	*Spilocuscus maculatus*	**1.00**
11	Silky cuscus	*Phalanger sericeus*	**1.00**
12	Asian Palm civet	*Paradoxurus hermaphroditus*	**1.00**
13	South American coati	*Nasua nasua*	**0.00**
14	Malay civet	*Viverra tangalunga*	**1.00**

3.2 Camera Trap Survey

As cameras were deployed for relatively short periods and battery exhaustion was not of concern, adjustable parameters were set to take the maximum number of pictures. Photograph interval was set to 20 seconds on the Cuddeback Capture cameras, whilst the Bushnell NatureView cameras were set to take three photographs each time they were triggered with an interval of just 3 seconds. In order to process results, images were categorised into events. An image of a species is recorded as a distinct event if there is an independence interval of ≥60 minutes between other images of that species (P. D. Meek et al. 2014). In total, camera traps captured 30 events over 227 trap nights; a success rate of 13% (Table 12).

Table 12: Camera Traps Capturing Images

Study site	Camera stations	Trap nights	Number of cameras successful	Percentage of cameras Successful
Sepi Nahu	19	38	7	37
Gunung Manusela	19	57	2	11
Ramatiti	16	112	7	44
Gunung Loa Loa	5	20	2	40
All Study sites	59	227	18	31

Camera traps captured images of four mammals identifiable to species level, and images identified as Murid rodents for which species identity could not be resolved (Table 13). Rodents comprised 87% of all events captured. Two events were recorded for Malay civet *Viverra tangalunga* and one each for Palm civet *Paradoxurus hermaphroditus* and deer *Rusa timorensis*; an adult male. The latter three species were recorded individually at altitudes previously recorded for these species (Kitchener et al. 1993; Macdonald et al. 1993; Choudhury 2013).

Table 13: Species Captured by Camera Traps

	Species (number of events*)				
Study site	**Rat *Nesoromys & Rattus spp.***	**Malay civet *Viverra tangalunga***	**Palm civet *Paradoxurus hermaphroditus***	**Deer *Rusa timorensis***	**Trapping success rate (%)**
Sepi Nahu	14	1			39
Gunung Manusela	2				4
Ramatiti	10	1			10
Gunung Loa Loa			1	1	10
All Study sites	26	2	1	1	13

*An event is defined as an image capture with no other capture of that species within a 60 minute interval

3.3 iDNA Survey

3.3.1 Fly Trapping

In total 149 flies were collected in the field. Thirty three of these were trapped and 116 were caught by hand in nets (Table 14). Initial fieldwork utilised a bottle fly trap design which had successfully trapped flies in lowland tropical forest elsewhere (Calvignac-Spencer, Merkel, et al. 2013). In montane habitat on Seram the traps were unsuccessful. The traps were used at both Sepi Nahu and Gunung Manusela for a total of 40 trap nights with only a single fly captured. In subsequent fieldwork, a sticky tube trap was used at comparable altitude at Ramatiti and resulted in 32 flies trapped during 136 trap nights (a trap night refers to a 24 hour period). During the final fieldwork phase, a new fly trap containing Propylene glycol was placed on the forest floor at 1603 metres asl for four nights. During this time heavy rain occurred almost constantly during daylight, conditions which prevent blow flies from flying, and no flies were trapped.

Table 14: Flies Collected at Study Sites

Study site	Trap nights	Number of traps successful	Number of Flies trapped	Number of flies netted by hand	Total number of flies collected
Sepi Nahu	16	0	0	0	0
Gunung Manusela	24	1	1	98	99
Ramatiti	136	18	32	18	50
Gunung Loa Loa	4	0	0	0	0
All Sites	180	19	33	116	149

3.3.2 DNA Extracted from Collected Flies

In the field only larger bodied flies were dissected onto FTA cards. Of the 149 flies collected, 92 were dissected onto FTA cards whilst the smaller remaining 57 were transferred to 2 ml collection tubes with 95% ethanol. DNA was extracted from all flies dissected onto FTA cards and from 16 of the whole flies collected. Mean total DNA extracted was highly variable with a higher mean value for samples from Ramatiti (mean = 55.8 ng) than those collected at Gunung Manusela (mean = 29.3) (Table 15). This is despite there being a potentially longer interval between capture in traps and dissection onto card at Ramatiti; up to seven days compared to less than 24 hours at Gunung Manusela. Many of the flies collected at Gunung Manusela were attracted to the site by cuscus entrails and are presumed to have fed from these, an assumption supported by sequencing results (Chapter 3.3.3). However, the large standard deviations indicate the huge variability in samples from both sites (Table 15). It should also be noted that an unknown but potentially significant proportion of the total DNA measured at this pre-PCR stage will be from the fly itself.

Table 15: Total DNA Extracted from FTA cards for Seram Samples

Site	Mean Total DNA Extracted (ng)	Standard Deviation
Gunung Manusela	29.3	54.2
Ramatiti	55.8	75.3
All Seram sites	36.2	61.1

3.3.3 PCR and Sequencing of Samples

The first PCR of Seram samples was a pilot to test four mammal-specific primer sets. The four primer pairs were added to mixes with five Seram samples, together with a sample of

Beaver *Castor fiber* DNA and pure fly DNA as positive and negative controls respectively. PCR products were run out on a gel and visualised under UV light (Figure 9). BRCA1 amplified DNA from one Seram sample but failed to amplify the positive control. RAG1 amplified one sample, but different to the one Seram sample positive for BRC1, as well as the positive control. The two 12S pairs, which target overlapping regions of 12S mitochondrial DNA, showed strong amplification of the positive control and also amplified sequences from each of the Seram samples. All the primer pairs were negative for the pure fly DNA. Following the trial, the primer pair 12Sa.490F / 12Sm.680R, designed to amplify a 205 base bp region of the mitochondrial 12S gene, was selected (Kuch et al. 2002). Although the performance of both the 12S pairs was similar, the longer fragment was selected as the slightly longer sequence may provide greater detail for species' identification and it had a greater area of overlap with the single sequence published for *Rhynchomeles*.

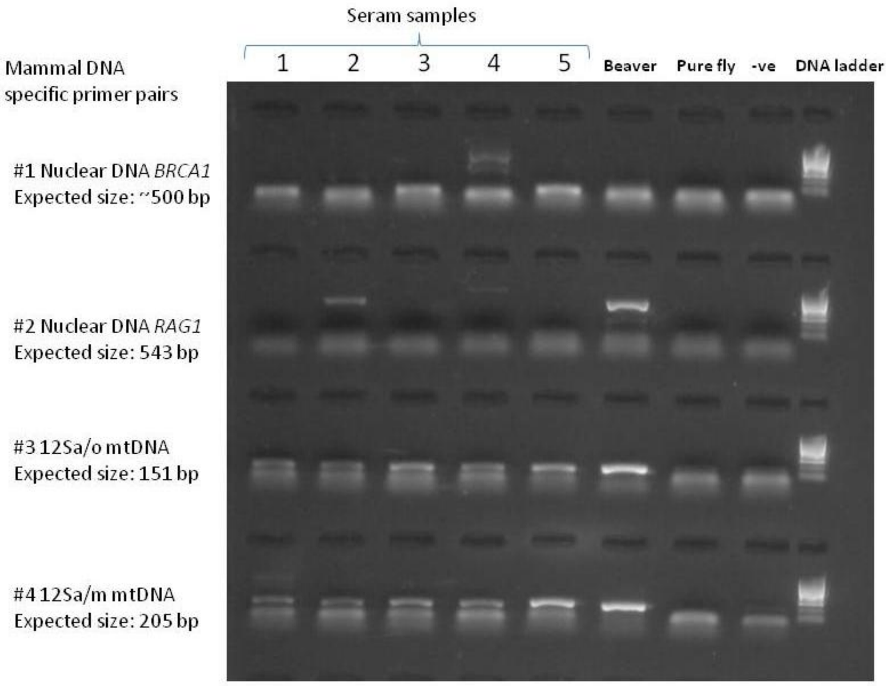

Figure 9: Gel Image Showing Results From Test of Four Mammal-specific Primer Sets

A PCR using the 12Sa / 12Sm primer pair was carried out for all extracted samples as detailed above. Of 108 extracted samples, 82 had visible bands on gels and were sequenced in both directions (Figure 10).

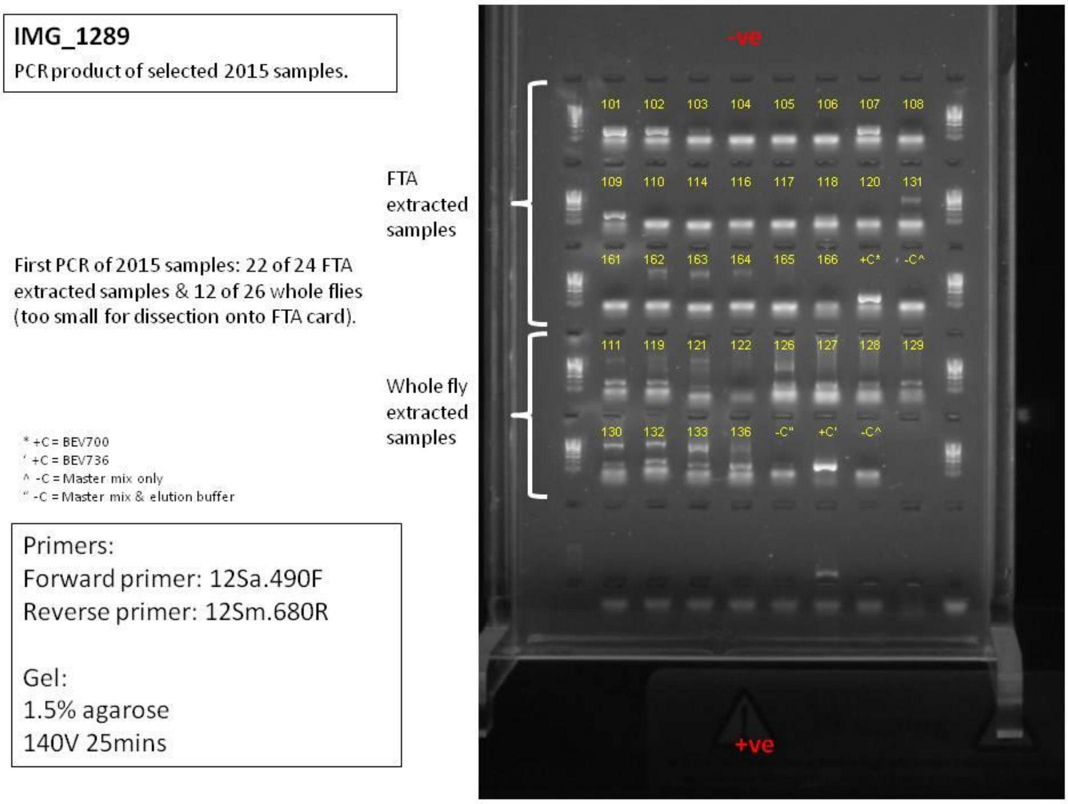

Figure 10: Gel Image of Samples Collected at Ramatiti. Presence of a Band e.g. # 109 Indicates the Amplification of Mammalian DNA

3.3.4 Assigning Identities to the Samples

The 164 sequences (forward and reverse sequences for each of the 82 samples) were subject to the quality assurance process previously described (Chapter 2.4.3.1). From the 164, 80 (49%) were assessed as of acceptable quality and added to an alignment with reference sequences from GenBank (Appendix H). The 80 sequences added to the alignment were derived from 55 samples. Of these 55 samples, 30 were represented by either a forward (n = 6) or a reverse (n = 24) sequence only. Twenty five samples were represented by both forward and reverse sequences so to obtain consensus sequences, reverse sequences were reverse complemented and concatenated with the forward sequence. This resulted in a 55 sequences, each corresponding to a single sample. The 55 sequences were aligned using Muscle and 100% homologous sequences reduced to a single representative sequence. This process resulted in six distinct sequences which were labelled SEF Sequence A – F (Appendix I). An example of a portion of the alignment for *Rhynchomeles* and cuscus species is shown below (Figure 11).

```
Species/Abbrv                        Group *** **** *****  * *      *   *** ***  *  ****************** ** **
1. Rhynchomeles prattorum  JF694112.1      GCTTAGCCCTAAACCTAAATTATT-ATTTAACAAAATAACTCGCCAGAGAACTACTAGCAAATGC
2. Spilocuscus maculatus KJ868160.1        GCTTAGCCCTAAACCGAGATAATTTATTTAACGAAACTATTCGCCAGAGAACTACTAGCCAACGC
3. Phalanger maculatus AF108220.1           GCTTAGCCCTAAACCGAGATAATTAATTTAACGAAATTATTCGCCAGAGAACTACTAGCCAACGC
4. Phalanger maculatus AF108222             GCTTAGCCCTAAACCGAGATAATTAATTTAACGAAATTATTCGCCAGAGAACTACTAGCCAACGC
5. SEF Sequence A                          GCTTAGCCCTAAACCGAGATAATTAATTTAACGAAATTATTCGCCAGAGAACTACTAGCCAACGC
6. Phalanger vestitus AB241057.1            GCTCAGCCATAAACTAAGATAGTCCAAACAACAAAACTATCCGCCAGAGAACTACTAGCTAACGC
7. Phalanger carmelitae U21182.1           GCTCAGCCATAAACCAAGATAATTCAAACAACAAAATTATCCGCCAGAGAACTACTAGCCAACGC
8. Phalanger lullulae AF108219.1            GCTCAGCCTTAAACCAAGATAATTAAAATAACAAAACTATCCGCCAGAGAACTACTAGCCAACGC
9. SEF Sequence B                          GCTCAGCCATAAACTAAGATAGCTCAAACAACAAAACTATCCGCCAGAGAACTACTAGCCAATGC
10. Phalanger orientalis U33496.1          GCTCAGCCCTAAACCAAGACAATTAAAATAACAAAATTATCCGCCAGAGAACTACTAGCCAACGC
```

Figure 11: Portion of the Alignment for Bandicoot and Cuscus

The six sequences were labelled SEF Sequence A – F. A blast search returned identities for the six sequences (Table 16). Two of the sequences represent haplotypes of human, whilst three of the other identities returned are species known to live wild on Seram: spotted cuscus, pig and cat. The first match of SEF Sequence B was *Phalanger vestitus* (Stein's cuscus) with an identity of 95%. Further support for the identity of each sequence was through the construction of a Neighbour Joining Tree (Figure 12). Sequences clustered as expected with Sequence B forming a branch basal to the cuscus grouping.

Table 16: First Result Returned by BLAST Search of Seram Consensus Sequences A - F

SEF Sequence	**Description**	**Max score**	**Total score**	**Query cover (%)**	**E value**	**Identity (%)**	**Accession**
A	*Phalanger maculatus*	375	375	98	2.00E-100	99	gi|4324664|AF108220.1
B	*Phalanger vestitus*	323	323	98	8.00E-85	95	gi|94481215|AB241057.1
C	*Homo sapiens*	367	367	98	4.00E-98	99	gi|1064842824|KX146835.1
D	*Homo sapiens*	367	367	98	4.00E-98	99	gi|1062950603|KX821323.1
E	*Felis catus*	371	371	98	3.00E-99	99	gi|1098537|U20754.1
F	*Sus scrofa*	362	362	99	1.00E-96	98	gi|1049741382|KU556691.1

The description table provided by BLAST includes measures to describe the similarity of the returned sequences to the input sequence. Max score describes the highest alignment score of a set of aligned segments from the same database sequence. The score is calculated from the sum of the match rewards and the mismatch, gap open and extend penalties independently for each segment. The Total score is the sum of alignment scores of all segments from the same subject sequence. Query cover is the percentage of the query length that is included in the aligned segments. E values describe the number of hits that could be expected by chance when searching a database of a particular size. The lower the E-value, the more significant the match to a database sequence is. Identity (%) describes the highest percentage identity for a set of aligned segments to the same subject sequence

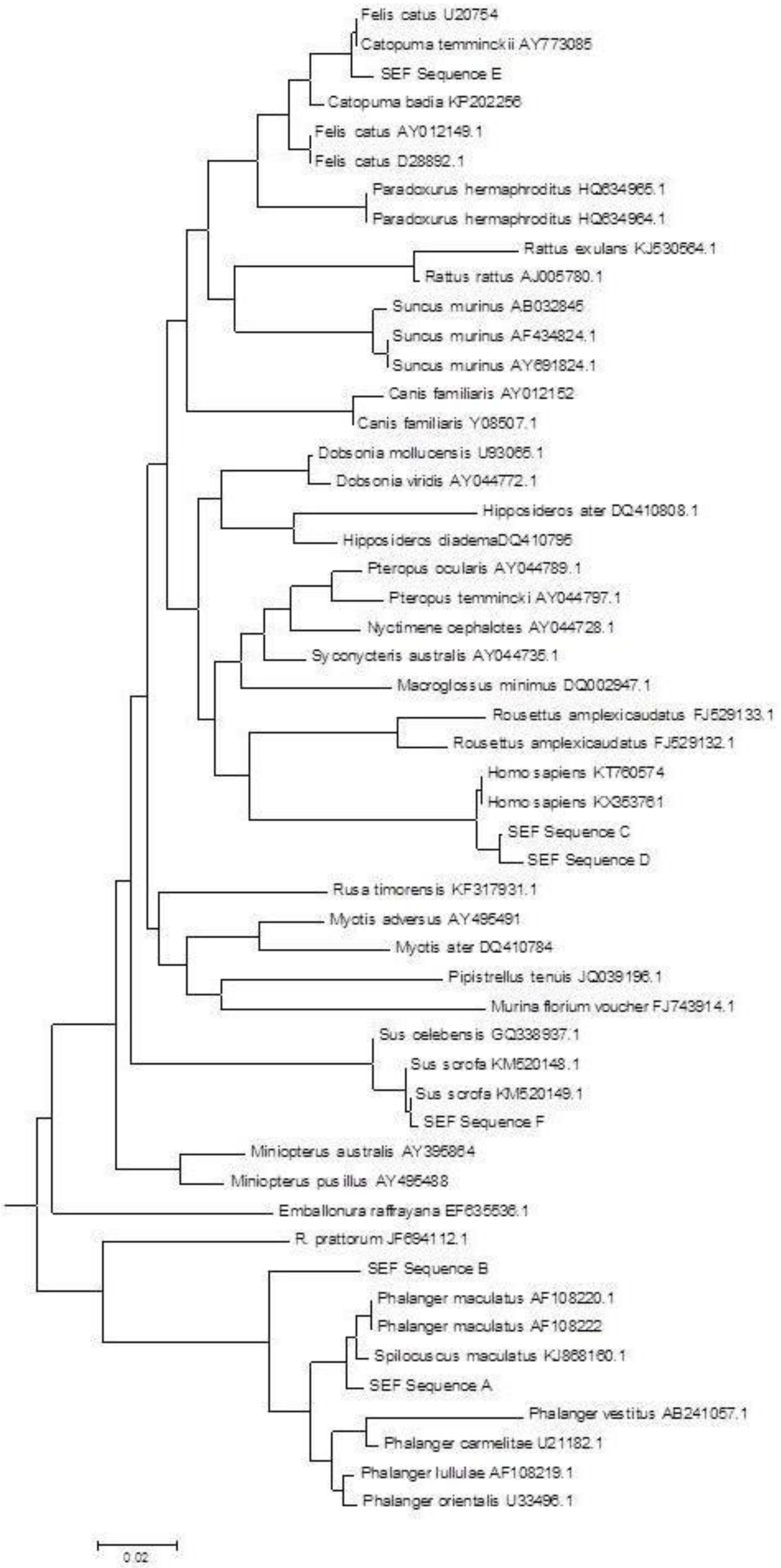

Figure 12: Neighbour-joining Tree Showing Affiliations of Seram Sequences A - F

3.4 Trap Design to Extend the Persistence of iDNA

The trap's efficacy at catching flies was assessed by placing two traps in off-exhibit outdoor areas at Edinburgh Zoo for 26 days in late summer. Flies were collected and counted at approximately weekly intervals. In total 89 flies were caught during the period (Table 17).

Table 17: Flies Caught in Two New Design Traps at Edinburgh Zoo

	Number of Flies Removed from Trap			
Trap location	**Day 7**	**Day 13**	**Day 20**	**Day 26**
Banteng Stand-off	8	13	0 (trap knocked over)	7
Gelada Service Yard	16	9	14	22

Assessment of the trap's ability to preserve DNA was assessed through a controlled experiment. Flies that had fed on beef were trapped in Propylene glycol (PG) and in conventional dry traps. Eight flies were removed from each treatment and dissected onto FTA cards at 24 hour intervals up to 144 hours. DNA was extracted from samples and quantity and quality of DNA assessed as previously described (Chapter 2.4.1). Total DNA extracted is shown for each treatment at each time point (Table 18).Samples were amplified with a beef specific primer pair and the samples for each condition were run alongside each other on a gel (Figure 13). Brighter bands indicate a higher concentration of DNA (Lee et al. 2015).

Table 18: Total DNA Extracted from Flies by Trap Type Over Time

	Total DNA extracted (ng)			
Hours	**Propylene glycol: Mean**	**Propylene glycol: Standard Deviation**	**Dry trap: Mean**	**Dry trap: Standard Deviation**
24	6956.63	7470.75	18840.00	10697.42
48	4727.50	3123.00	10405.00	9830.08
72	2540.00	1158.88	11878.75	10249.20
96	3356.25	1752.67	11642.50	14097.16
120	2130.00	1329.50	3702.50	4608.60
144	2512.50	2992.28	1207.50	445.28

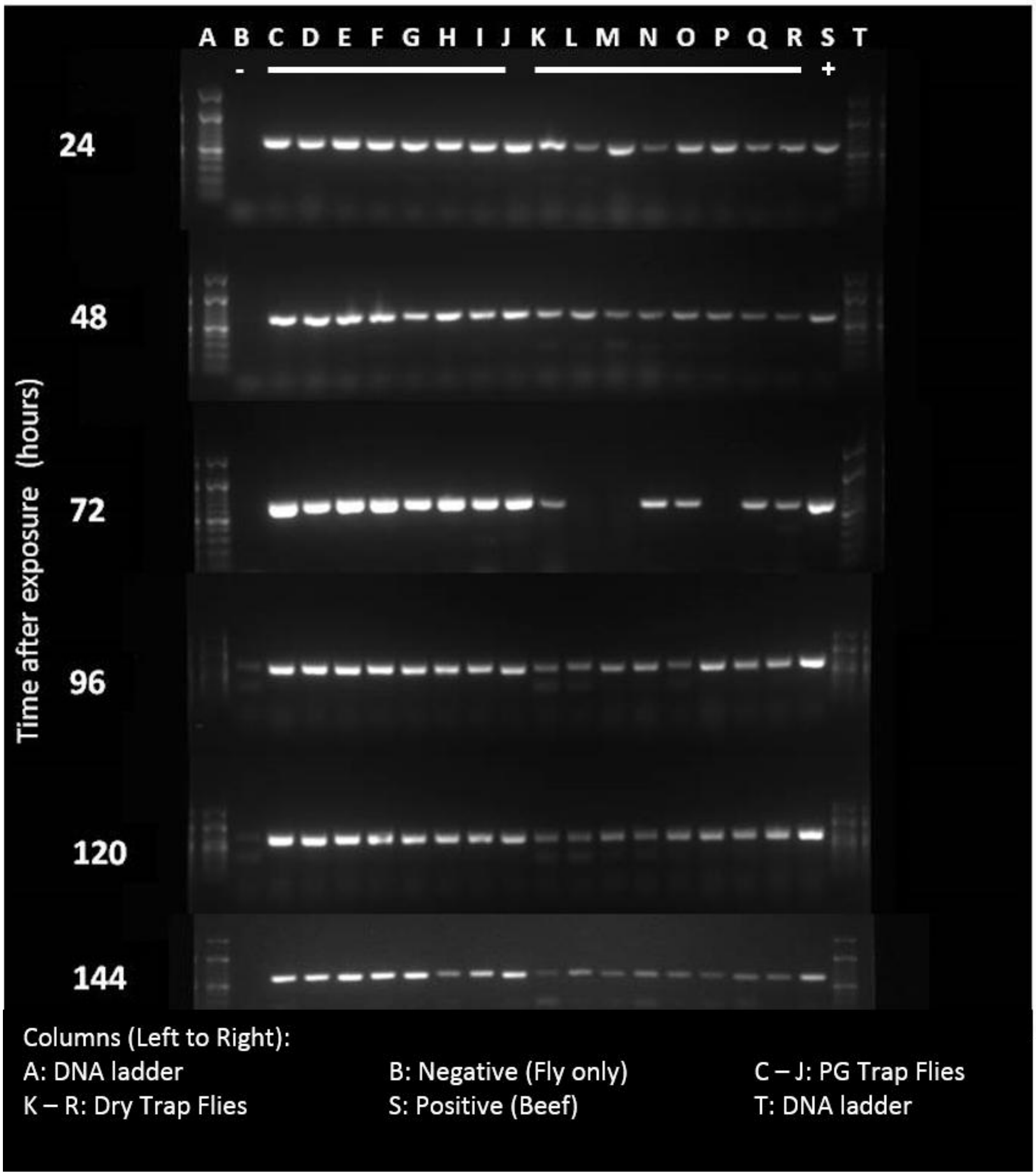

Figure 13: PCR Product from New Trap and Conventional Trap at 24 Hour Intervals

Band intensity was assessed using ImageJ software as described above. Mean values were calculated for band intensity at each time point (Appendix J for raw data). Using the intensity of the positive controls at each time point, mean values were normalised and the relative density of each treatment was calculated across the six time points (Figure 14). The Propylene glycol traps produced bands with greater intensity for all time points as well as consistently producing bands for all flies. There appeared to be no deterioration in the concentration of DNA for the first 48 hours in the Propylene glycol traps, whilst a decrease in concentration already occurred at this stage in the dry traps. The DNA bands produced after 72 hours in the Propylene glycol traps were of equivalent intensity as the dry trap at 24 hours. A subset of UK samples was sequenced to verify the identity of bands. One sample from each condition at each time point, together with a positive and a negative sample were sequenced. Beef *Bos taurus* was the only identity returned from a BLAST search of sequences. However, despite visible bands on gels, sequence quality was poor and eight samples did not produce a sequence. Sequencing of samples needs to be repeated but it was not possible for this to be done in the available time.

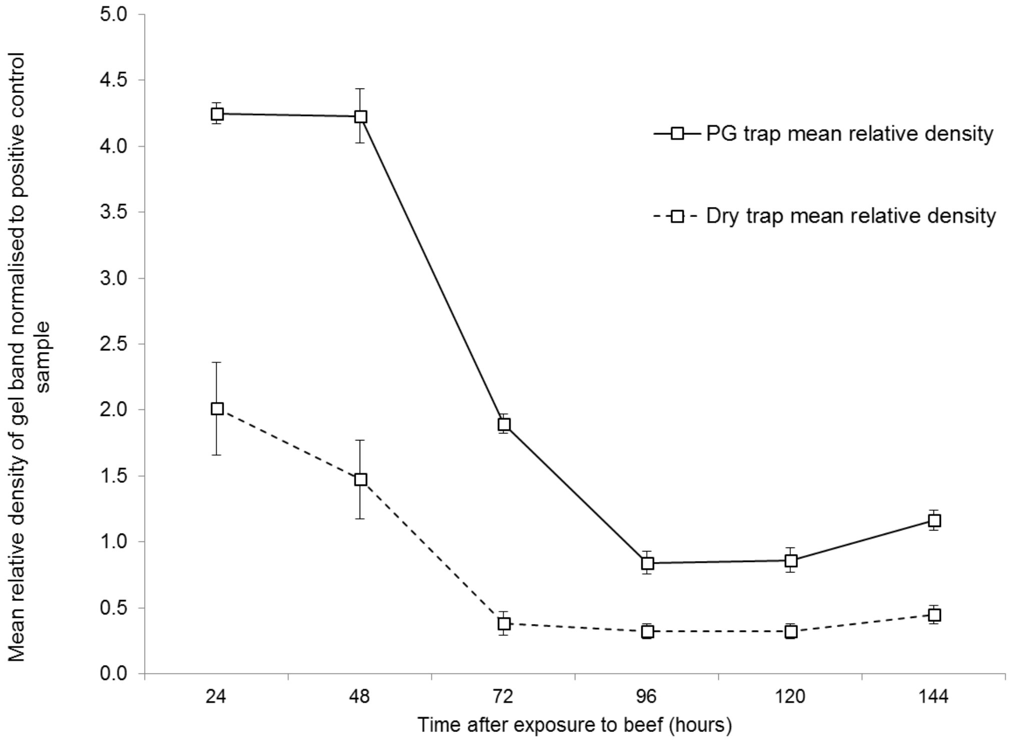

Figure 14: Graph Showing Relative Intensity of Bands by Trap Type Over Time. Error bars show Standard Error of the Mean (+/- SEM)

4 Discussion

4.1 Fieldwork sites

The study sites selected on Seram provided an authentic test of survey methodology in remote, mountainous areas. The interior of Seram is infrequently visited by people other than the indigenous population and the higher altitudes at Ramatiti had reportedly not previously experienced human visitation.

4.2 Information from Interviews

Interviews were conducted to rapidly access local knowledge of wild species of mammals on Seram and to direct the selection of study sites. Of particular interest was evidence of the status of the Seram bandicoot. Anecdotal evidence for the continued existence of the Seram bandicoot was obtained from seven interviews. All recounted information on the bandicoot was consistent with it being a species limited to undisturbed montane forest, a requirement concordant with the hypothesis of Kitchener et al. (1993). This information validated selection of higher altitude study sites and justified the effort to access the site at Ramatiti which had minimal human disturbance. Only one direct modification was made to study sites as a result of information given in interviews: the site at Sepi Nahu was a route diversion due to a single first hand report of *Rhynchomeles* trapped in the vicinity of this site.

Despite a widespread cultural familiarity with *Rhynchomeles* amongst the indigenous population and a reported first hand sighting, its image on the photo-elicitation sheet was not recognised by anyone questioned. The absence of recognition by the informant reporting a sighting casts doubt on the reliability of his account, whilst the widespread lack of recognition may represent shifting baseline knowledge of a species that may have undergone recent extinction (Turvey et al. 2010). The use of anecdotal evidence to assess the presence of elusive species has been cautioned (McKelvey et al. 2008), however for some species, abundance estimates derived from interviews have been shown to correlate closely with data from conventional survey techniques. (Anadón et al. 2009). With a single exception, species illustrated on the photo-elicitation sheets that were not from Seram were categorically stated as such by all respondents, adding support to the reliability of positive recognition of other species. The exception to this was the universal recognition of *Phalanger sericeus.* However, it should be noted that the related Grey cuscus *Phalanger orientalis* was not illustrated and the positive responses may represent general recognition of *P. sericeus* as a cuscus (Andrew Kitchener personal communication 5th August 2016). Interviews were found to be useful tool to rapidly gather data on local mammals, although data gained in this way must be reported as anecdotal evidence only. Future use of photo-elicitation should include a comprehensive rather than representative set of the recorded mammal species.

4.3 Camera trapping in Comparison to iDNA

Camera traps captured 30 events over 227 trap nights; a success rate of 13%. Comparisons with camera trapping studies elsewhere are difficult to make due to local ecological variables and a lack of standardised methods for reporting camera trap results (Meek et al. 2014). In this study camera trapping was included as an attempt to calibrate iDNA against a widely used conventional survey tool (Schnell et al. 2015). Camera trapping identified a similar number of taxa but an entirely different set of species to those identified by iDNA. This result is consistent with recent evaluations by Lee et al. (2016) of the performance of iDNA in comparison to ‘traditional’ ecological surveying methodologies in two forest areas in Malaysia. Camera trap placement on Seram limited the range of species likely to be detected, especially in consideration of the largely arboreal cuscuses, a significant component of the mammal assemblage on Seram. Results from the two methods used on Seram support the conclusion that surveys using multiple approaches are likely to achieve a more accurate representation of mammal fauna (Lee et al. 2016; De Bondi et al. 2010; Catling et al. 1997). In this study camera traps captured images of three taxa that could be identified to species level; two civet Malay and Palm civet and Rusa deer. The majority of images captured by the cameras were of rodents that could not be positively identified. Although captured images of rodents were of poor quality, it is likely that even high quality images would prove problematic to identify due to a lack of published reference material and limited expertise. In comparison, sequences produced by iDNA provide a powerful tool for assigning species identities (Wilson et al. 2016) that can be processed with minimal specialist training.

4.4 Application of iDNA

Successful use of iDNA from blow flies is dependent on a series of steps. Flies must be collected, their DNA must be preserved and a suitable fragment must be identified and sequenced to facilitate identification of the species present. In a lowland tropical environment with easy access to both traps in the field and laboratory resources such as liquid nitrogen, collecting flies and preserving their DNA presents few problems. In remote areas and in mountainous habitats, these steps may present significant challenges to overcome.

4.4.1 Trapping flies

In montane forest on Seram, a fly trap design that had previously been successful in lowland tropical forest failed to catch flies, despite observation of blow fly activity in the area. A 'sticky tube' fly trap subsequently proved effective at trapping flies in comparable habitat at Ramatiti. However, flies removed from these traps contained few mammalian sequences able to be amplified. At Gunung Manusela trapping duration (the interval between potential first capture or actual capture of a fly and subsequent preservation of iDNA) was ≤ 24 hours. The success rate for samples preserved on FTA cards and subsequently positive for iDNA (73%) compares favourably to other studies (Table 19). Samples from Ramatiti, where traps were *in situ* for 4 – 6 days and flies recovered at the end of this period, had a much lower percentage of samples positive for iDNA (11%). This decline in success is attributed to a decrease in amplifiable DNA over time (Lee et al. 2015). For surveys in remote areas where recommended standardised trapping protocols (Lee et al. 2015) cannot be achieved, and for long term trapping, this is a limitation. This conclusion emphasises the importance of developing trapping methods that extend the persistence of iDNA for remote sites where traps cannot be accessed frequently.

Table 19: Samples Positive for iDNA from Different Sites

Researcher	Location	Trapping duration (hours)	Number of flies tested	Region targetted	Fragment length (bp)	Total number of sequences obtained	Samples positive for iDNA (%)
Bagnall	Gunung Manusela	< 24	67	12S	205	49	73
Bagnall	Ramatiti	(up to) 72	36	12S	205	4	11
Calvignac-Spencer et al (2013)	Tai forest, Ivory Coast	< 24	86	12S & 16S	both <170	61	71
Calvignac-Spencer et al (2013)	Tai forest, Ivory Coast	< 24	75	12S & 16S	both <170	29	39
Calvignac-Spencer et al (2013)	Kirindy, Madagascar	< 24	40	12S & 16S	both <170	17	43

On Seram, persistent heavy rain at two sites, coupled with a reduction in temperature at higher altitude, reduced blow fly activity. This supports a requirement for long term fly trapping to mtigate short term variations in weather. A trap able to be deployed in the field for long intervals would increase efficiency in such circumstances.

However, for conclusion drawn from field based evidence, it should be borne in mind that a proportion of flies may have either not recently fed or fed on non-mammalian sources, and as such have no potential to yield mammal sequences. Ecological variable such as feeding rates and preferences are poorly known for wild living blow flies (Norris 1965) and further research on these and other factors, such as dispersal, may aid the development of iDNA as a survey tool.

4.4.2 DNA preservation

Collection of samples from remote locations for subsequent iDNA analysis is dependent on robust methods for preservation of DNA in the field. In this study, Mammal DNA was successfully sequenced from flies dissected onto FTA cards in the field. This provides a proof of concept that FTA cards can preserve amplifiable iDNA. Once on FTA cards, large numbers of samples can be carried with little weight and no urgency for processing. For samples collected in this study, extraction from cards was up to six months after dissection. However, it is worth noting that dissection of individual flies is time consuming and the development of a trap containing a preservative may facilitate a simpler procedure in which samples are simply stored in the trap for removal from the field.

In this study Propylene glycol was evaluated as an in-trap preservative for flies. Previous comparison of Propylene glycol against desiccation, DMSO, RNAlater and different types and concentrations of alcohol has shown it to be an effective preservative for DNA in insects (Moreau et al. 2013). The inert property of Propylene glycol makes it a safe liquid in which to transport samples (Patrick et al. 2016) and prevents it from evaporating rapidly from traps. In contrast to alcohols, Propylene glycol is odourless and does not produce vapours that may interfere with olfactory baits in fly traps. Results presented here from the evaluation of iDNA extracted from flies trapped in conventional dry traps and traps containing Propylene glycol indicate that such traps have the potential to overcome the survey constraints imposed by the relatively short persistence period of iDNA in fly guts.

4.4.3 Which site to target?

For flies collected on Seram, only a single PCR assay was conducted for each sample. It is possible that greater concentrations of commoner species, or greater concentrations of their DNA, may have masked the presence of DNA from other species (Schubert et al. 2015). For surveys targeting specific taxa, the use of species specific assays in comparison to non-specific assays can increase the detection of target species up to threefold (Schubert et al. 2015). However, Lee et al. (2015) found no difference between amplification success when using a beef specific primer and a newly designed pan-mammalian primer pair. Advances in Next Generation Sequencing (NGS) may provide a powerful tool for rapidly sequencing multiple taxa from pooled samples.

The choice of target site is an important one. For surveying biodiversity, such as the samples collected on Seram, the optimum size of fragment is around 200 bp (Lee et al. 2015). This length allows amplification from degraded samples, such as iDNA, while still retaining the ability to distinguish species. A further benefit is that it is within the maximum read length for high-throughput sequencing (Tillmar et al. 2013; Lee et al. 2015). For iDNA surveys, mtDNA has several advantages over nuclear DNA. A single somatic cell typically contains between 10 - 1000 mitochondria, each carrying 2 - 10 mtDNA copies, in comparison nuclear DNA has just two copies per cell. This is particularly significant when using highly degraded sources. Additionally, mtDNA has a relatively high and constant mutation rate making it useful to trace evolution and resolve taxa to species level. Following evaluation of four primer pairs, a 12S primer set targeting a 205bp was used in this study to identify mammals on Seram. Although the Cytochrome C oxidase 1 gene (CO1) has been promoted as the ideal gene for barcoding animal species (Ratnasingham & Hebert 2007), analysis of cuscus sequences in GenBank supports the selection of 12S (Table 20).

A BLAST search conducted for SEF sequence B returned *Phalanger vestitus* as the closest match, although this species is not recorded on Seram. The identity value of 95% is below the threshold stated previously for species identity to be assigned. Despite 12S having the highest number of mtDNA sequences for cuscus species on GenBank, there are only single 12S sequences available for four *Phalanger* species. This includes *P. orientalis*; one of the two species of cuscus described from Seram. The BLAST search result for SEF sequence B may be attributable to only a single reference sequence for *P. orientalis*; a widely distributed species with a complex taxonomy (Menzies et al. 1986), or may be indicative of a genetically distinct form on Seram. The result highlights the complexity of interpreting

BLAST search results and cautions discarding sequences from alignments based on simple thresholds.

Table 20: Sequences in GenBank for Selected Species of Marsupial

	Sequences on GenBank						
Species	**Total number of records**	**12S**	**16S**	**COI**	**ND2**	**unverified sequences**	**Nuclear DNA**
Rhynchomeles prattorum	1	1					
Spilocuscus maculatus	9	1				1	7
Phalanger maculatus	4	2					2
Phalanger orientalis	9	1	1		1		6
Phalanger sericeus	1	1			1		
Phalanger carmelitae	2	1			1		
Phalanger lullulae	4	1					1

Note: *Spilocuscus maculatus* is synonym of *Phalanger maculatus* (Helgen & Flannery 2004).

4.4.4 The Future of iDNA

An increasing number of studies have demonstrated the potential of iDNA as a cost effective alternative to conventional survey methodologies. Although genetic analysis methodologies are often perceived as expensive, the increasing availability of low cost sequencing, which can be as little as US$8 per sample (Wilson et al. 2014), or even less using Next Generation Sequencing (NGS) (Meier et al. 2016), negates some of these perceptions. Further research is required to quantify the success of traps designed to preserve iDNA and also to optimise trap design to specific environments. The exponential increase in sequences available in publicly accessible databases such as GenBank will greatly aid the identification of poorly known taxa.

It is envisaged that iDNA surveys in remote areas, including montane habitat, will be possible through the longer term deployment of fly traps containing a preservative. Preserved samples will be transported from field sites within the trap and sequenced as a pooled sample using NGS.

4.5 Conclusions

The development of iDNA is a valuable addition to the survey tools available in conservation and has potential use in important biomes including montane habitats where it had not previously been utilised.

Dissection onto FTA cards is a viable method for preservation of iDNA in remote locations but may be superseded by removal of whole traps containing preservative and sequencing of pooled samples.

A trapping method enabling longer intervals between trap visits is required to overcome limitations to the use of iDNA in remote areas. A design for such a trap has been presented. Propylene glycol may be appropriate as a medium for such a trap but further evaluation is required.

5 References

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Appendices

A. Description of Seram

The area in which Seram resides is geologically extremely complex (Villeneuve et al. 2010; Audley-Charles 1981) being at the intersection of two major tectonic plates; the Eurasian and Australian plates, and one minor tectonic plate; the Philippine plate. Seram originated as a Gondwanan fragment, most likely separated from present day West Papua by slip strike action around 20 – 14 million years ago (Villeneuve et al. 2010). Prior to the late Miocene, Seram is thought to have been entirely submarine (Audley-Charles 1981) and so has never been joined to any other large land mass since it emerged from the sea. However, some 25000 – 15000 years ago, when ice caps were at their greatest extent, sea levels were approximately 160 metres lower than present levels. At this time Seram was joined to the smaller islands of Ambon, Haruku, Saparua and several smaller islets to form a 'paleolandmass' (Flannery 1995). Aside from these smaller, near neighbour islands, Seram is separated from other landmasses by open sea. The next significant island closest to Seram is Buru, approximately 70 km to the West. To the north, the Islands of Misool and Obi are 100 and 120 km away respectively with the largest Molluccan landmass, Halmahera, a distance of 215 km from Seram. To the south of Seram lies the Banda Sea and the diminutive Banda Islands before the Aru Islands, 400 km away and beyond them, the northern coast of Australia approximately 900 km distance. To the East of Seram, approximately 140 km between the closest points, lies West Papua (the Indonesian portion of New Guinea; formerly called Irian Jaya). From an ecological colonisation perspective, non-volant species of mammal have therefore either arrived as deliberate or incidental introductions by humans, or by sweepstake dispersals across the sea (Edwards et al. 1993; Flannery 1995; Helgen 2003). Seram's state of isolation is a formidable barrier to colonisation, which is reflected by the high level of endemism amongst its native mammals and is likely to have also been a significant factor in determining the paucity of mammal species on the island relative to its land mass.

Seram is located at the Eastern extremity of Melanesia; a subdivision of Oceania. The term Melanesia; the etymology meaning the 'Black Islands' was first used in the early nineteenth century to delineate a group of islands from those of Polynesia an Micronesia. Early European scholars identified the people of Melanesia as constituting a distinct racial group, a view supported by more recent genetic studies (Friedlaender et al. 2008). Ellen (1993) places the earliest evidence of human settlement of Seram; flake and blade industries reported from a site on the south coast of Seram (Bellwood 1985), at some time after 8000 years BP. It has been speculated, despite conclusive evidence such as radiocarbon dated excavations, that humans have been present on Seram as early as 40 – 60 000 years BP (Ellen 1993).

Seram is also a part of the biogeographic region of Wallacea. Named after Alfred Russel Wallace, who noted the geographic affinities of the area's fauna, Wallacea encompasses the area east of the Malay Peninsula and the major islands of Sumatra, Java and Borneo (collectively termed Sundaland) and west of New Guinea. Significant islands included in the area include Sulawesi, Lombok, Sumba, Flores, Halmahera and Buru, as well as Seram. The region's fauna, particularly the order Mammalia, is characterised by influences of both Asian and Australasian origin.

Approximately 19% of Seram's land area is protected within Manusela National Park (Balai Taman Nasional Manusela 2014). The park was formed following a recommendation to amalgamate and extend two previously designated conservation areas; Way Mual and Way Nua Nature Reserves, both formed in December 1972 (Wind & Amir 1978). The formation of the National park occurred by legislation enacted in October 1982 and the name 'Manusela National Park' came into first usage at this point. Further legislation in 1997 cemented the status of the area as a single protected area (Balai Taman Nasional Manusela 2014).

All of Seram's main biotopes, ranging from coastal mangrove swamp to treeless southern alpine grassland, are represented within the National Park. In April 2013, the National Park Authority introduced a zoning system within the park. The stated aim of this is to delineate areas within the park for specific functions including the development of nature tourism and recreation, and a traditional zone for utilization of natural resources and ecosystems by local communities in a sustainable manner. The National Park is inhabited by an indigenous population centred upon four villages situated in an enclave within the park. Three of the villages; Manusela, Maraena and Solemena are sited within a broad valley, drained by the Wai Isal (River Isal), running approximately East to West to the north of the main line of mountains. The fourth village, Kanikeh, is sited further west on a different watershed. At present, the indigenous inhabitants appear to have little awareness of the National Park authority's zoning system and seem largely unaffected by it (Personal observation July 2014, July 2015).

B. Wild-living Mammal Species on Seram

Order	Species	notes
Marsupialia	Rhynchomeles prattorum	
	Spilocuscus maculatus	Formerly Phalanger maculatus. Possibly introduced
	Phalanger orientalis	Possibly introduced
Insectivora	Suncus murinus	Introduced
Chiroptera	Rousettus amplexicaudatus	
	Pteropus chrysoproctus	
	Pteropus melanopogon	
	Pteropus ocularis	
	Pteropus temmincki	
	Dobsonia moluccense	
	Dobsonia viridis	
	Nyctimene cephalotes	
	Macroglossus minimus	
	Syconycteris australis	
(Microchiroptera)	Emballonura nigrescens	
	Emballonura raffrayana	
	Rhinolophus euryotis	
	Rhinolophus megaphyllus	
	Hipposideros ater	
	Hipposideros diadema	
	Hipposideros macrobullatus	
	Aselliscus tricuspidatus	
	Myotis adversus	
	Myotis ater	
	Pipistrellus tenuis	
	Miniopterus australis	
	Miniopterus pusillus	
	Murina florium	
	Tadarida jobensis	
	Mormopterus beccarii	
Carnivora	Canis familiaris	Introduced as human commensal. Some now feral
	Viverra tangalunga	Introduced c.17th Century
	Paradoxurus hermaphroditus	Introduced c.17th Centuy
	Felis catus	Introduced as human commensal. Some now feral
Artiodactyla	Sus spp.	S. scrofa x S. celebensis (according to Groves)
	Rusa timorensis	Cervus timorensis in Macdonald et al 1993
Rodentia	Nesoromys ceramicus	Rattus ceramicus in Macdonald et al 1993
	Rattus exulans	Introduced
	Rattus feliceus	
	Rattus nitidus	Introduced
	Rattus argentiventer	Introduced
	Rattus rattus	Introduced
	Melomys aerosus	
	Melomys fraterculus	
	Melomys fulgens	
	Melomys rufescens paveli	Described by Helgen (2003) from single specimen

C. Details of the Seram Bandicoot

The Seram bandicoot *Rhynchomeles prattorum,* was described in 1920 by Oldfield Thomas from a series of seven specimens received at the British Museum (Natural History). No further specimens of *Rhynchomeles* have been collected since. The Skulls and study skins of six of the specimens remain at the Natural History Museum, London (formerly the British Museum (Natural History), whilst the seventh specimen (AMM 29415; formerly BMNH 20.7.26.33) has resided in the Australian Museum, Sydney, since it was part of an inter-museum exchange in 1993. Specimens range in size from male BMNH 20.7.26.35 with an overall length of 458 mm from tip of snout to tip of tail, to female BMNH 20.7.26.36 at just 252 mm long. *Rhynchomeles* has a slender, elongated snout, which is hairless for the anterior 11 – 22 mm. The tail, which accounts for 20 -28% of the overall length, appears hairless, although under magnification, small, sparse hairs can be observed. The feet are naked and bear five toes. On the forefeet, the first and fifth digits are reduced and do not bear nails whilst the second and third digits are of approximately equal length and bear elongated nails, up to 12 mm in length. The fourth digit is intermediate in size and bears a small nail. On the hind feet, nails are present on all toes but are shorter than on the forefeet. The third and fourth toes are fused by soft tissue for the majority of their length and are reduced in thickness. This condition is apomorphic amongst bandicoots (Archer & Clayton 1984). The undersides of the nails are concave and appear adapted for digging: a suggestion supported by the observation of traces of soil on the underside of nails on three of the specimens. Other than the naked parts mentioned the rest of the body is covered in firm, non-spineous hair: dark brown over the back and flanks and a lighter shade underneath with a patch of cream-coloured hair on the chest of variable size. The hair is longest on the back, up to 15 mm, and shortest on the ventral side: at most 9.5 mm long. Males have a pronounced scrotum at the posterior ventral position, whilst on the females the marsupium is ventral, posterior and inguinal. It is not possible to ascertain conclusively the direction of opening of the pouch but it would appear to be towards the rear as with other Peramelidae bandicoots (Tate 1948). On all specimens, traces of a white, powdery material are present at the base of a small posterior ventral area of fur, these may be indicative of anal glands. *Rhynchomeles prattorum* is the sole member of its genus. Recent phylogenetic studies have placed *Rhynchomeles* allied to *Echimypera* and *Microperoryctes* within the subfamily Echymiperinae (Westerman et al. 2012), largely concurring with previous classification based on morphology (Thomas 1920; Tate 1948). *Rhynchomeles* is classified as Endangered by the IUCN (Leary et al. 2008) and it is one of the Zoological Society of London's EDGE (Evolutionary Distinct, Globally Endangered) mammal species of interest (Isaac et al. 2007).

Archer and Clayton (1984) and Westerman and colleagues (2012) speculate that Rhynchomeles may have undergone extinction, although neither provided evidence to support their assertion other than the lack of sightings or specimens since its original capture. Flannery (1995) notes that the trapping effort of recent expeditions has been in the wrong habitat and at too low an altitude and for that reason *Rhynchomeles* should not be regarded as extinct.

The seven specimens received at the British Museum (Natural History) in 1920 were part of a consignment of thirty-seven mammals dispatched from Seram by Charles, Joseph and Felix Pratt: three English brothers who were mainly concerned with collecting Lepidoptera. A letter sent by the Pratt brothers in January 1920 to Thomas states: 'We have also a peculiar animal with a long snout + rather short tail like that of a rat. The natives say it grunts like a pig + certainly has pig like eyes. It is very rare indeed + is confined to the highest altitudes in those mountains'. The letter, and details on the collectors' labels, indicates that the Bandicoot was familiar to the local hunters employed by Messrs Pratt, although not a species frequently encountered by them. A note on one of the specimen labels (BMNH 20.7.26.37) records the native name for the animal as 'Mabaya' although more recent discussions with locals has given the name 'Mapea' as the accepted variation (Macdonald et al. 1993)(However, see Ellen (1972) for alternative local names).

The Pratt's specimen labels give the locality that *Rhynchomeles* was collected at as Mt. Manusela, Central Ceram, at an altitude of 6000 feet. Talbot (1920), in reference to the Lepidoptera that were the principle reason for the Pratts' expedition, reported that 'after much difficulty the three brothers established a camp on the Manusela range at 6000 ft and were able to start collecting in October '. In a letter dated 24th March 1920 to Thomas, Messrs Pratt reported that they had remained at that altitude for only three weeks before they were forced to move camp due to the activity of the local rats. In reference to *Rhynchomele*s, the letter also states that the local trappers employed by them 'were fortunate in finding a locality for it, so that in just over a month they caught seven of them'. Dates on the specimen labels indicate that the series of *Rhynchomeles* was collected during January and February, with the initial specimen referenced by the Messrs Pratt in a letter sent from their camp at 4600 feet, on 10th January 1920. It seems apparent that the specimens of *Rhynchomeles* were brought to Messrs Pratt whilst they were based at a lower altitude and consequently, the 6000 feet altitude given as the collection locality should be regarded as an approximation. It cannot be ascertained how far the hunters employed by the Pratts travelled to collect the seven specimens, however the statements '+ is confined to the highest altitudes in those mountains' and 'our trappers were fortunate in finding a locality for it' suggest that the collection locality may have been at some distance to their camp. The Manusela range contains several peaks above 2500 metres and an area of approximately 95 km² above 2000 meters (= 6562 feet) altitude. Altitudinal ranges for selected closely related species are given in Westerman et al. (2012) (Table 1); these are frequently above the 6000 feet (1829 metres) reported for *Rhynchomeles*. It is notable that Seram supports several endemic species of bird restricted to montane habitat such as the Blue-Eared Lory *Eos semilarvata* and the Spectacled Honeyeater *Lichmera monticola* and the endemic subspecies of the Island Thrush *Turdus poliocephalus deningeri*; the latter limited to above 1800 metres (Bowler & Taylor 1993).

Table 21: Altitudinal Ranges for Selected Bandicoots on New Guinea

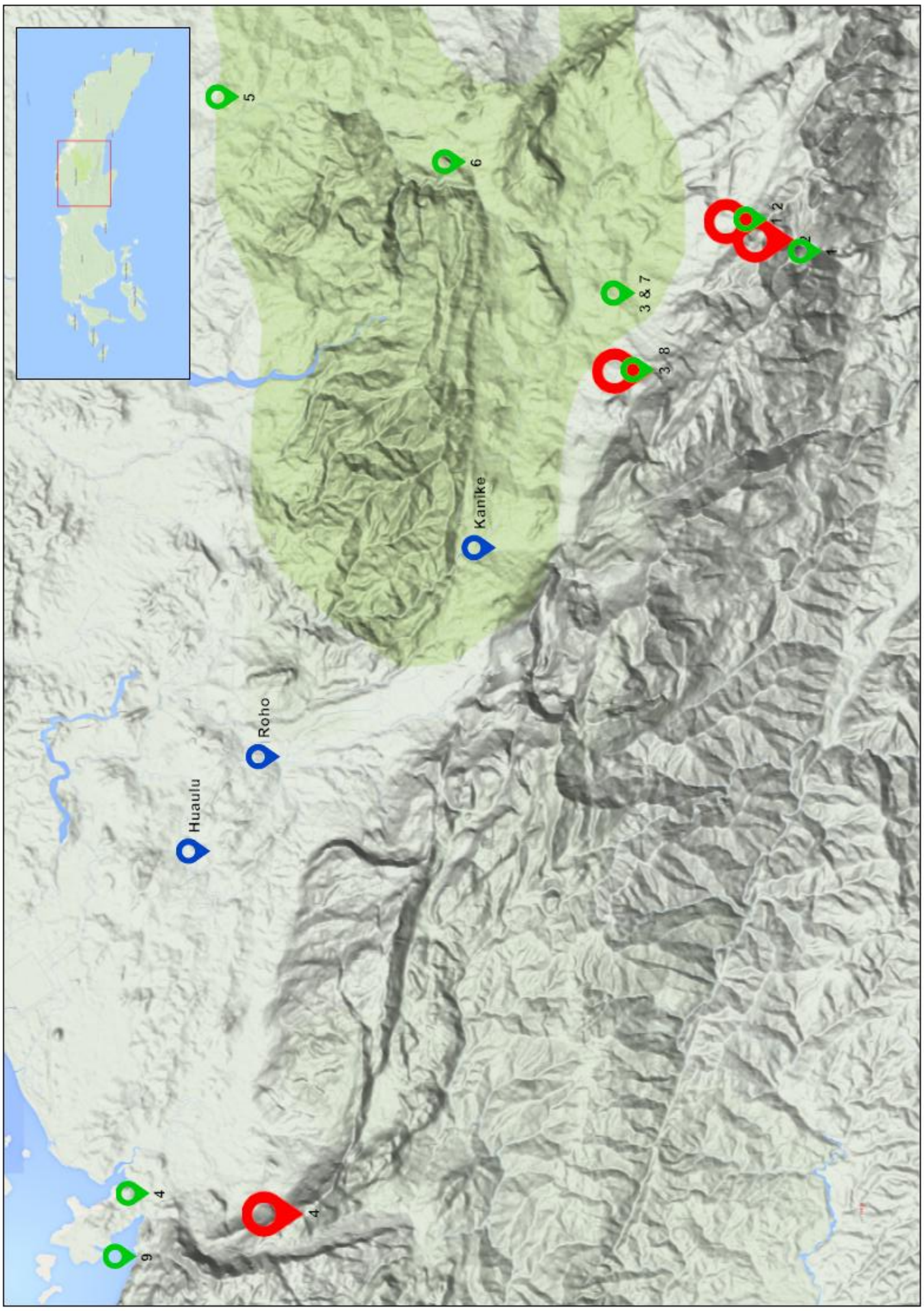
Species	Altitude (metres asl)
Microperoryctes longicaudata	1900 – 2200
M. murina	2500
M. aplini	1900 – 2200
M. ornata	1000 – 3600
M. papuensis	1200 – 2650

(Adapted from Westerman et al. 2012)

Since *Rhynchomeles* initial description, no further conclusive information concerning its existence has been published, however, several authors have presented anecdotal evidence of its continued existence. Drawing on extensive fieldwork among the Nuaulu people of Seram's interior, Ellen (1972) states 'other marsupials occur on Seram and are reasonably familiar to the Nuaulu: the taxa *lau* and *kuha* which appear to be two varieties of the Seram Island bandicoot (*Rhynchomeles* spp)'. Reporting on the Operation Raleigh Expedition to Seram, Macdonald and colleagues (1993) reported that despite failing to trap *Rhynchomeles*, the local mountain people knew of its presence by its distinctive droppings which were reportedly seen in montane forest during 1987. Kitchener and colleagues (1993) reported that despite 80 trap nights in montane forest on Gunung Binaiya, Seram, targeting mammals of the appropriate size range, no bandicoots were caught and no evidence of bandicoot activity was detected at their study sites despite intensive searches. The authors presented three hypotheses to explain the absence of Rhynchomeles: I. Complete or local extinction by introduced predators, II. Competition from other introduced mammals, and III. Habitat disturbance by human activity. The authors assert that due to the historic nature of predator introductions such as civet species *Viverra tangalunga*, *Paradoxurus hermaphrodites*, Cat *Felis catus* and dog *Canis familiaris* any extinction attributable to predation would have occurred possibly hundreds of years ago, and as such they dismiss this hypothesis as a likely cause of *Rhynchomeles*' absence. They suggest that the introduction of possible candidates for ecological competitors, such as the Asian house shrew *Suncus murinus* and the Polynesian rat *Rattus exulans*, filled vacant niches on Seram, rather than forcing out endemic mammals, and for this reason they dismiss hypothesis II. The authors report that dialogue with local informants living on and around Gunung Binaiya suggests the persistence of *Rhynchomeles* in undisturbed montane forest to the West of their study site. The authors conclude that habitat disturbance by human activities may account for their lack of records through local exclusion of Rhynchomeles (hypothesis III).

D. Map of Study Sites and Interview Locations

Red pins represent study sites: Gunung Manusela (1), Sepi Nahu (2), Ramatiti (3) and Gunung Loa Loa (4). Green pins are interview locations. See Table 9 for numbering. The villages of Kanike, Roho and Huaulu are also shown.



E. Permits to Conduct Research on Seram

Permit to conduct research in Indonesia (front).

KEMENTERIAN DALAM NEGERI REPUBLIK INDONESIA
DIREKTORAT JENDERAL POLITIK DAN PEMERINTAHAN UMUM
Jalan Medan Merdeka Utara Nomor 7 Telp. (021) 3450038 Jakarta 10110

SURAT PEMBERITAHUAN PENELITIAN
letter of research notification

NOMOR : 070/2795/Polpum

MEMBACA : Surat Kementerian Riset Teknologi dan Pendidikan Tinggi/Sekretariat Perizinan Peneliti Asing Nomor : **1651**/FRP/E5/Dit. KI/VII/2016 tanggal 11 Juli 2016

MENGINGAT :
1. Peraturan Pemerintah Nomor : 41 Tahun 2006 tentang Izin Penelitian Bagi Perguruan Tinggi Asing, Lembaga Penelitian Asing, Badan Usaha Asing dan Orang Asing;
2. Peraturan Menteri Dalam Negeri Nomor : 49 Tahun 2010 tentang Pedoman Pemantauan Orang Asing dan Organisasi Masyarakat Asing di Daerah.
3. Peraturan Menteri Dalam Negeri Nomor 43 Tahun 2015, tentang Organisasi dan Tata Kerja Kementerian Dalam Negeri

MEMPERHATIKAN :
1. Surat Izin Penelitian Kementerian Riset Teknologi dan Pendidikan Tinggi/Sekretariat Perizinan Peneliti Asing Nomor: **232**/SIP/FRP/ E5/Dit.KI/VII/2016 tanggal 11 Juli 2016
2. SKJ POLRI Nomor Pol.: SKJ/Subbid ORAS – **8191**/VII/2016/Baintelkam, berlaku mulai tanggal 11 Juli 2016 s/d 09 Agustus 2016

NAMA : **Mr. ANDREW JAMES BAGNALL**
ALAMAT : 32 Howe Park, Edinburgh EH 10 7HF
PEKERJAAN : Peneliti (Postgraduate Master's Degree)
KEBANGSAAN : Inggris
NOMOR PASPOR : 528803095

PENGIKUT : -
KEBANGSAAN : -
NOMOR PASPOR : -

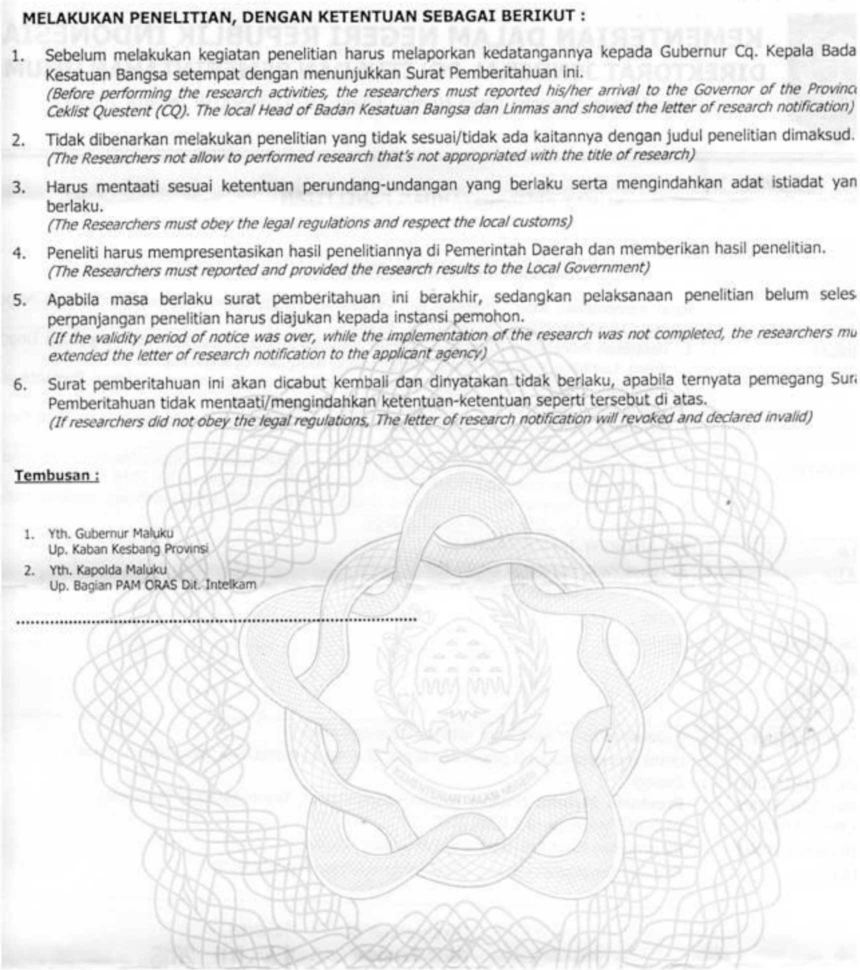
JUDUL PENELITIAN : "*Identification of food items used by small mammals*"
TUJUAN : Untuk mengetahui jenis pakan dan kebiasaan makan mamalia di Pulau Seram, Maluku
BIDANG PENELITIAN : Zoologi
DAERAH PENELITIAN : **Provinsi : Maluku** (Maluku Tengah – Seram Utara, Taman Nasional Manusela)
LAMA PENELITIAN : 01 (satu) bulan, mulai 11 Juli 2016
PENANGGUNG JAWAB : Kementerian Riset Teknologi dan Pendidikan Tinggi.
MITRA KERJA : Fakultas Kedokteran Hewan, IPB (Professor Srihadi Agungpriyono)

Dikeluarkan di : Jakarta
Pada Tanggal : 14 JUL 2016

A.n. DIREKTUR JENDERAL
POLITIK DAN PEMERINTAHAN UMUM
SEKRETARIS DITJEN

BUDI PRASETYO, SH, MM
Pembina Utama Madya (IV/d)
NIP. 19570108 198703 1 001

Permit to conduct research in (back).



MELAKUKAN PENELITIAN, DENGAN KETENTUAN SEBAGAI BERIKUT :

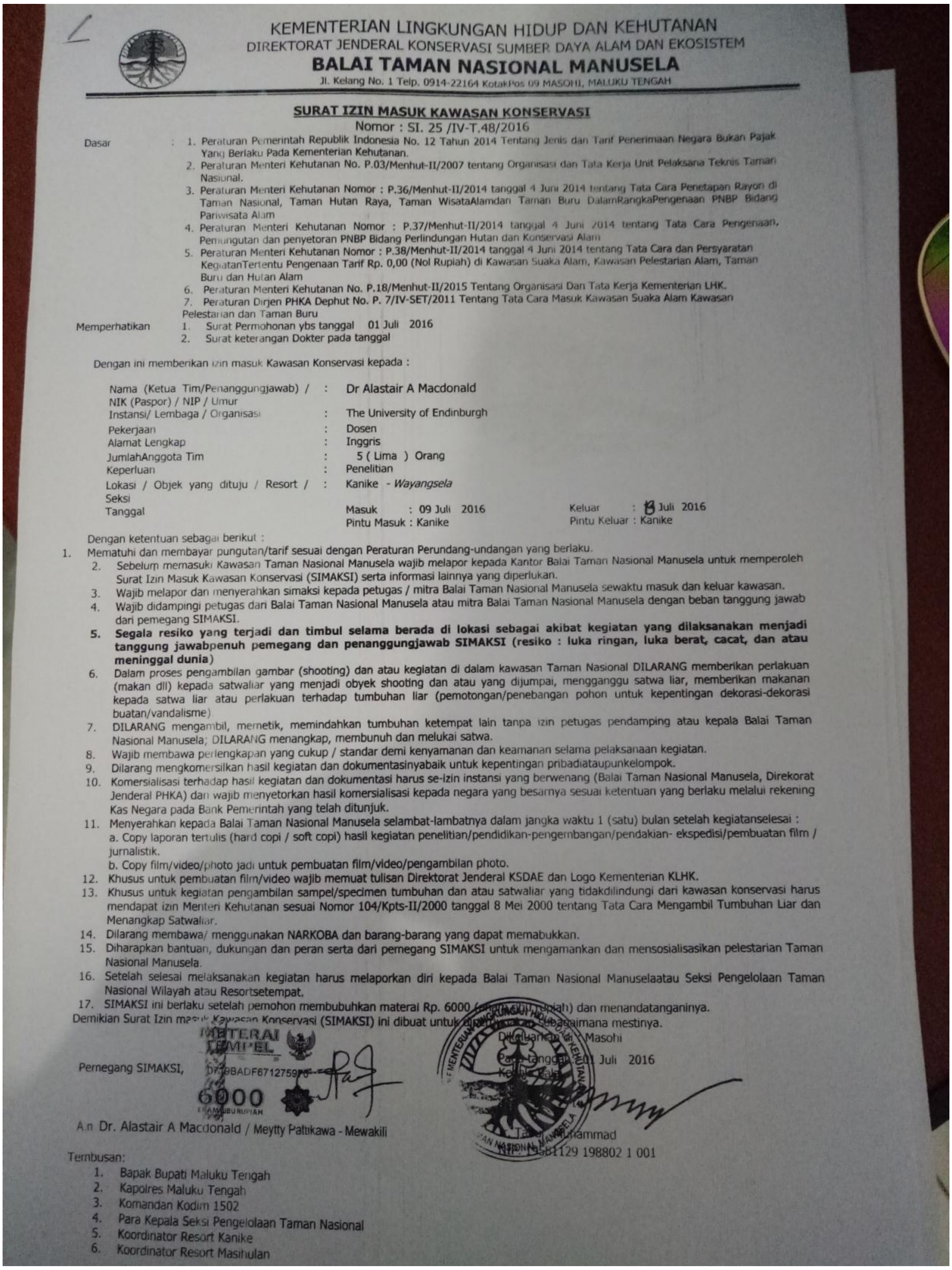
1. Sebelum melakukan kegiatan penelitian harus melaporkan kedatangannya kepada Gubernur Cq. Kepala Bada Kesatuan Bangsa setempat dengan menunjukkan Surat Pemberitahuan ini.
   (Before performing the research activities, the researchers must reported his/her arrival to the Governor of the Provinc Ceklist Questent (CQ). The local Head of Badan Kesatuan Bangsa dan Linmas and showed the letter of research notification)
2. Tidak dibenarkan melakukan penelitian yang tidak sesuai/tidak ada kaitannya dengan judul penelitian dimaksud.
   (The Researchers not allow to performed research that's not appropriated with the title of research)
3. Harus mentaati sesuai ketentuan perundang-undangan yang berlaku serta mengindahkan adat istiadat yan berlaku.
   (The Researchers must obey the legal regulations and respect the local customs)
4. Peneliti harus mempresentasikan hasil penelitiannya di Pemerintah Daerah dan memberikan hasil penelitian.
   (The Researchers must reported and provided the research results to the Local Government)
5. Apabila masa berlaku surat pemberitahuan ini berakhir, sedangkan pelaksanaan penelitian belum seles perpanjangan penelitian harus diajukan kepada instansi pemohon.
   (If the validity period of notice was over, while the implementation of the research was not completed, the researchers mu extended the letter of research notification to the applicant agency)
6. Surat pemberitahuan ini akan dicabut kembali dan dinyatakan tidak berlaku, apabila ternyata pemegang Sur Pemberitahuan tidak mentaati/mengindahkan ketentuan-ketentuan seperti tersebut di atas.
   (If researchers did not obey the legal regulations, The letter of research notification will revoked and declared invalid)

Tembusan :

1. Yth. Gubernur Maluku
   Up. Kaban Kesbang Provinsi
2. Yth. Kapolda Maluku
   Up. Bagian PAM ORAS Dit. Intelkam

……………………………………………………………………

Permit from Forestry Department to conduct research in a National Park (page 1).



KEMENTERIAN LINGKUNGAN HIDUP DAN KEHUTANAN
DIREKTORAT JENDERAL KONSERVASI SUMBER DAYA ALAM DAN EKOSISTEM
BALAI TAMAN NASIONAL MANUSELA
Jl. Kelang No. 1 Telp. 0914-22164 KotakPos 09 MASOHI, MALUKU TENGAH

SURAT IZIN MASUK KAWASAN KONSERVASI
Nomor : SI. 25 /IV-T.48/2016

Dasar :
1. Peraturan Pemerintah Republik Indonesia No. 12 Tahun 2014 Tentang Jenis dan Tarif Penerimaan Negara Bukan Pajak Yang Berlaku Pada Kementerian Kehutanan.
2. Peraturan Menteri Kehutanan No. P.03/Menhut-II/2007 tentang Organisasi dan Tata Kerja Unit Pelaksana Teknis Taman Nasional.
3. Peraturan Menteri Kehutanan Nomor : P.36/Menhut-II/2014 tanggal 4 Juni 2014 tentang Tata Cara Penetapan Rayon di Taman Nasional, Taman Hutan Raya, Taman WisataAlamdan Taman Buru DalamRangkaPengenaan PNBP Bidang Pariwisata Alam
4. Peraturan Menteri Kehutanan Nomor : P.37/Menhut-II/2014 tanggal 4 Juni 2014 tentang Tata Cara Pengenaan, Pemungutan dan penyetoran PNBP Bidang Perlindungan Hutan dan Konservasi Alam
5. Peraturan Menteri Kehutanan Nomor : P.38/Menhut-II/2014 tanggal 4 Juni 2014 tentang Tata Cara dan Persyaratan KegiatanTertentu Pengenaan Tarif Rp. 0,00 (Nol Rupiah) di Kawasan Suaka Alam, Kawasan Pelestarian Alam, Taman Buru dan Hutan Alam
6. Peraturan Menteri Kehutanan No. P.18/Menhut-II/2015 Tentang Organisasi Dan Tata Kerja Kementerian LHK.
7. Peraturan Dirjen PHKA Dephut No. P. 7/IV-SET/2011 Tentang Tata Cara Masuk Kawasan Suaka Alam Kawasan Pelestarian dan Taman Buru

Memperhatikan
1. Surat Permohonan ybs tanggal 01 Juli 2016
2. Surat keterangan Dokter pada tanggal

Dengan ini memberikan izin masuk Kawasan Konservasi kepada :

Nama (Ketua Tim/Penanggungjawab) / NIK (Paspor) / NIP / Umur : Dr Alastair A Macdonald
Instansi/ Lembaga / Organisasi : The University of Endinburgh
Pekerjaan : Dosen
Alamat Lengkap : Inggris
JumlahAnggota Tim : 5 (Lima) Orang
Keperluan : Penelitian
Lokasi / Objek yang dituju / Resort / Seksi : Kanike - *Wayangsela*
Tanggal
Masuk : 09 Juli 2016
Pintu Masuk : Kanike
Keluar : 18 Juli 2016
Pintu Keluar : Kanike

Dengan ketentuan sebagai berikut :

1. Mematuhi dan membayar pungutan/tarif sesuai dengan Peraturan Perundang-undangan yang berlaku.
2. Sebelum memasuki Kawasan Taman Nasional Manusela wajib melapor kepada Kantor Balai Taman Nasional Manusela untuk memperoleh Surat Izin Masuk Kawasan Konservasi (SIMAKSI) serta informasi lainnya yang diperlukan.
3. Wajib melapor dan menyerahkan simaksi kepada petugas / mitra Balai Taman Nasional Manusela sewaktu masuk dan keluar kawasan.
4. Wajib didampingi petugas dari Balai Taman Nasional Manusela atau mitra Balai Taman Nasional Manusela dengan beban tanggung jawab dari pemegang SIMAKSI.
5. **Segala resiko yang terjadi dan timbul selama berada di lokasi sebagai akibat kegiatan yang dilaksanakan menjadi tanggung jawabpenuh pemegang dan penanggungjawab SIMAKSI (resiko : luka ringan, luka berat, cacat, dan atau meninggal dunia)**
6. Dalam proses pengambilan gambar (shooting) dan atau kegiatan di dalam kawasan Taman Nasional DILARANG memberikan perlakuan (makan dll) kepada satwaliar yang menjadi obyek shooting dan atau yang dijumpai, mengganggu satwa liar, memberikan makanan kepada satwa liar atau perlakuan terhadap tumbuhan liar (pemotongan/penebangan pohon untuk kepentingan dekorasi-dekorasi buatan/vandalisme)
7. DILARANG mengambil, memetik, memindahkan tumbuhan ketempat lain tanpa izin petugas pendamping atau kepala Balai Taman Nasional Manusela; DILARANG menangkap, membunuh dan melukai satwa.
8. Wajib membawa perlengkapan yang cukup / standar demi kenyamanan dan keamanan selama pelaksanaan kegiatan.
9. Dilarang mengkomersilkan hasil kegiatan dan dokumentasinyabaik untuk kepentingan pribadiataupunkelompok.
10. Komersialisasi terhadap hasil kegiatan dan dokumentasi harus se-izin instansi yang berwenang (Balai Taman Nasional Manusela, Direkorat Jenderal PHKA) dan wajib menyetorkan hasil komersialisasi kepada negara yang besarnya sesuai ketentuan yang berlaku melalui rekening Kas Negara pada Bank Pemerintah yang telah ditunjuk.
11. Menyerahkan kepada Balai Taman Nasional Manusela selambat-lambatnya dalam jangka waktu 1 (satu) bulan setelah kegiatanselesai :
    - a. Copy laporan tertulis (hard copi / soft copi) hasil kegiatan penelitian/pendidikan-pengembangan/pendakian- ekspedisi/pembuatan film / jurnalistik.
    - b. Copy film/video/photo jadi untuk pembuatan film/video/pengambilan photo.
12. Khusus untuk pembuatan film/video wajib memuat tulisan Direktorat Jenderal KSDAE dan Logo Kementerian KLHK.
13. Khusus untuk kegiatan pengambilan sampel/specimen tumbuhan dan atau satwaliar yang tidakdilindungi dari kawasan konservasi harus mendapat izin Menteri Kehutanan sesuai Nomor 104/Kpts-II/2000 tanggal 8 Mei 2000 tentang Tata Cara Mengambil Tumbuhan Liar dan Menangkap Satwaliar.
14. Dilarang membawa/ menggunakan NARKOBA dan barang-barang yang dapat memabukkan.
15. Diharapkan bantuan, dukungan dan peran serta dari pemegang SIMAKSI untuk mengamankan dan mensosialisasikan pelestarian Taman Nasional Manusela.
16. Setelah selesai melaksanakan kegiatan harus melaporkan diri kepada Balai Taman Nasional Manuselaatau Seksi Pengelolaan Taman Nasional Wilayah atau Resortsetempat.
17. SIMAKSI ini berlaku setelah pemohon membubuhkan materai Rp. 6000 ([illegible] rupiah) dan menandatanganinya.

Demikian Surat Izin masuk Kawasan Konservasi (SIMAKSI) ini dibuat untuk [illegible] sebagaimana mestinya.

Dikeluarkan di : Masohi
Pada tanggal : 01 Juli 2016
Kepala Balai

Pemegang SIMAKSI,

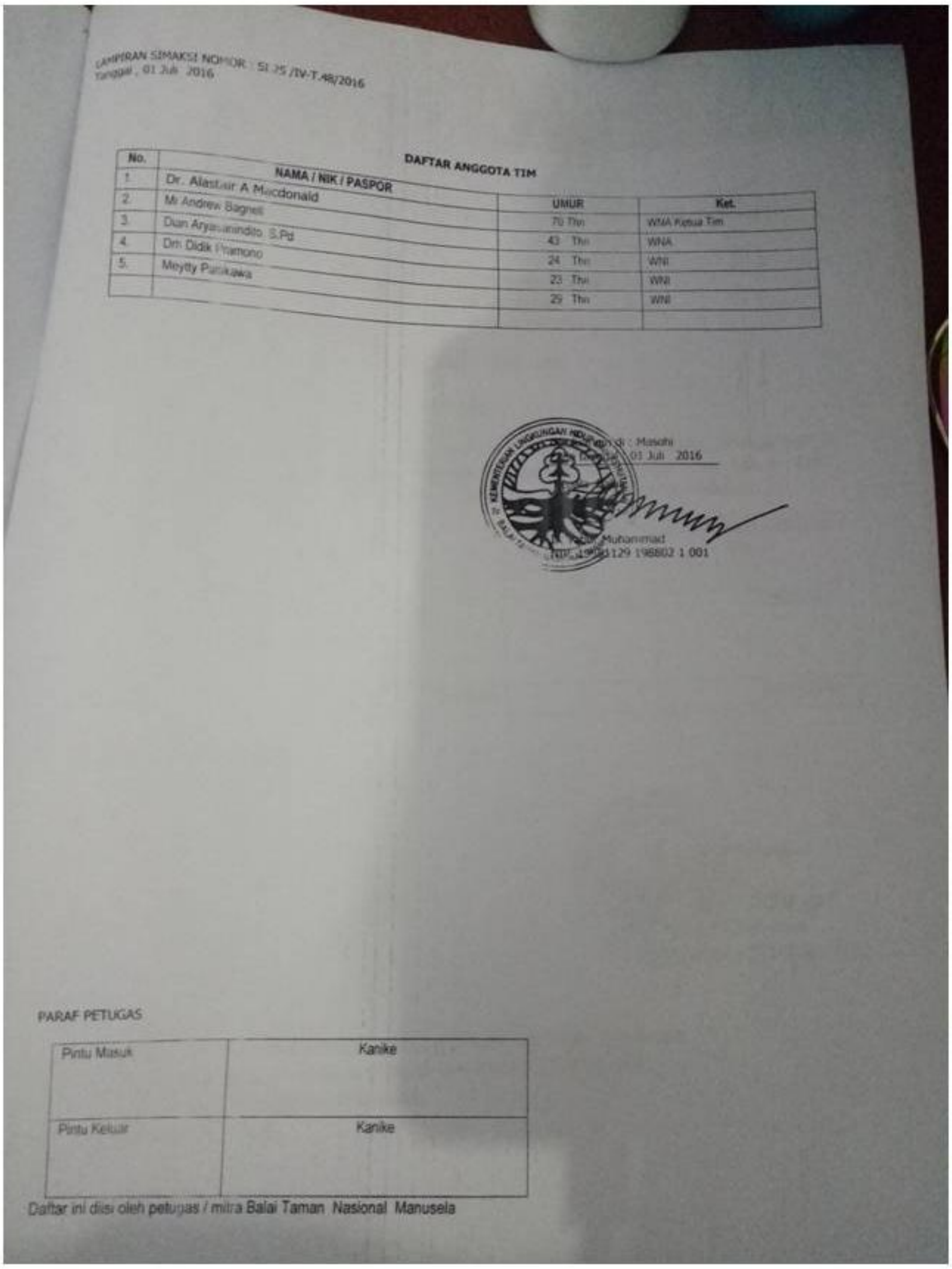
METERAI TEMPEL
6000
ENAM RIBU RUPIAH

A.n Dr. Alastair A Macdonald / Meytty Pattikawa - Mewakili

[illegible] Muhammad
NIP. 19581129 198802 1 001

Tembusan:
1. Bapak Bupati Maluku Tengah
2. Kapolres Maluku Tengah
3. Komandan Kodim 1502
4. Para Kepala Seksi Pengelolaan Taman Nasional
5. Koordinator Resort Kanike
6. Koordinator Resort Masihulan

Permit from Forestry Department to conduct research in a National Park (page 2).



LAMPIRAN SIMAKSI NOMOR : SI.25 /IV-T.48/2016
Tanggal , 01 Juli 2016

DAFTAR ANGGOTA TIM

No.	NAMA / NIK / PASPOR	UMUR	Ket.
1.	Dr. Alastair A Macdonald	70 Thn	WNA Ketua Tim
2.	Mr Andrew Bagnell	43 Thn	WNA
3.	Duan Aryasunindito. S.Pd	24 Thn	WNI
4.	Drn Didik Pramono	23 Thn	WNI
5.	Meytty Purikawa	29 Thn	WNI

[illegible] di : Masohi
[illegible] 01 Juli 2016

[illegible] Muhammad
NIP. 19[illegible]1129 198802 1 001

PARAF PETUGAS

Pintu Masuk	Kanike
Pintu Keluar	Kanike

Daftar ini diisi oleh petugas / mitra Balai Taman Nasional Manusela

Foreign Researcher Permit (Front and back).

No. 232

KEMENTERIAN RISET, TEKNOLOGI, DAN
PENDIDIKAN TINGGI
Jl. M.H. Thamrin No. 8, Jakarta 10340, Gedung II BPPT Lantai 20
Tel. 021-3169777 Fax. 021-39836180, e-mail: frp@ristekdikti.go.id

KARTU IZIN PENELITI ASING

Nama : **Mr. Andrew James Bagnall**
No. Pasport : **528803095**
Warganegara : **Inggris**
Berlaku : **11-7-2016 s.d 10-8-2016**
No. Surat Izin : **232/SIP/FRP/E5/Dit.KI/VII/2016**

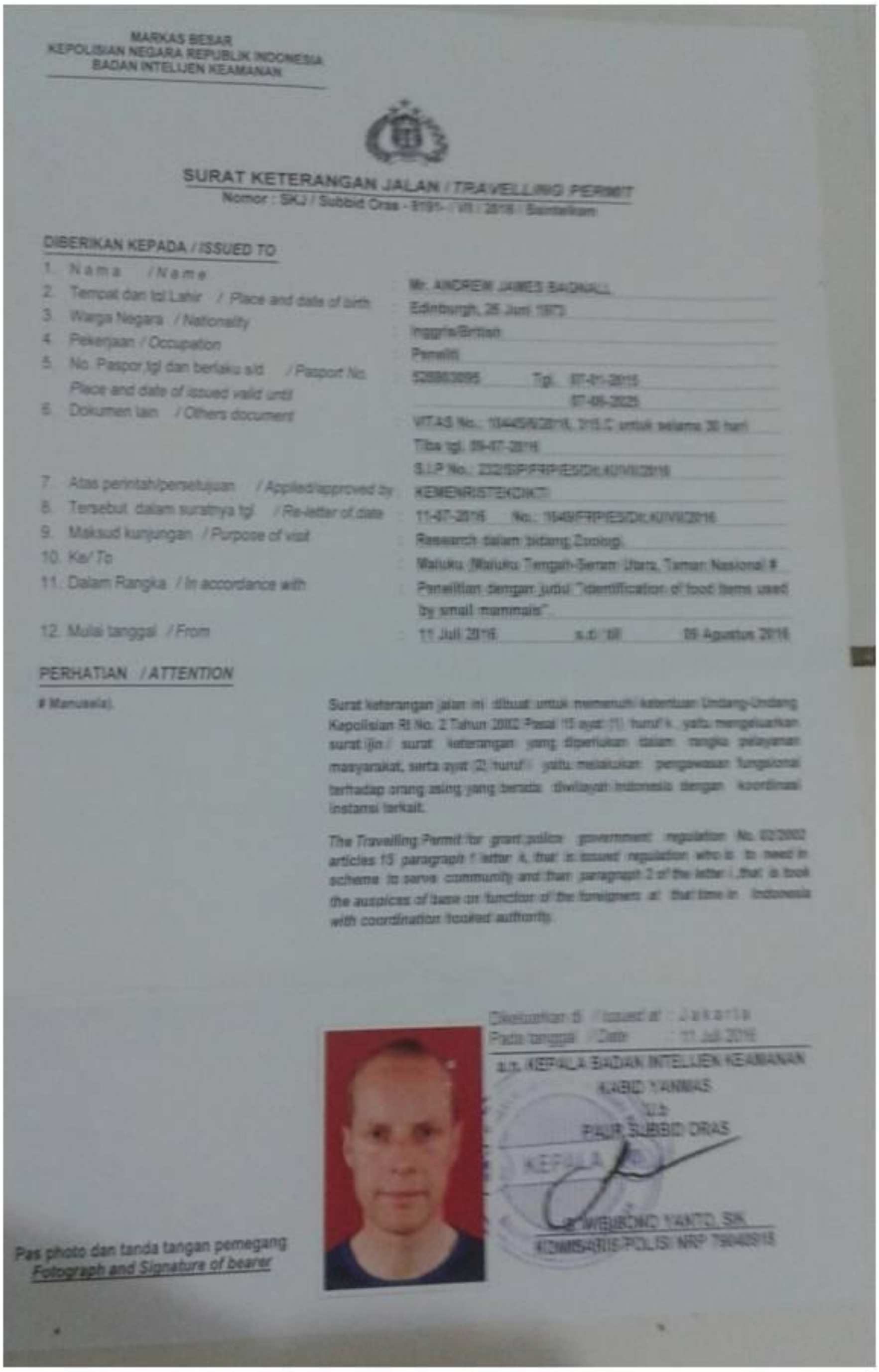
Sesuai dengan Peraturan Pemerintah Republik Indonesia Nomor 41 Tahun 2006, Kartu Izin Peneliti Asing diberikan kepada yang bersangkutan untuk dipergunakan seperlunya. Kami mohon dengan hormat kiranya instansi-instansi Pemerintah / Swasta maupun perorangan yang dihubungi untuk memberikan bantuan kepada yang bersangkutan sesuai dengan peraturan yang berlaku.

Jakarta, 11 Juli 2016

KEMENTERIAN RISET, TEKNOLOGI, DAN
PENDIDIKAN TINGGI
a.n. Ketua Tim Koordinasi Pemberian Izin Penelitian Asing
Direktur Pengelolaan Kekayaan Intelektual/selaku
Sekretaris TKPIPA

Dr. Sadjuga, M.Sc

Permit from the National Police Department to travel within Indonesia in fulfilment of research.



MARKAS BESAR
KEPOLISIAN NEGARA REPUBLIK INDONESIA
BADAN INTELIJEN KEAMANAN

SURAT KETERANGAN JALAN / *TRAVELLING PERMIT*
Nomor : SKJ / Subbid Oras - 9191- / VII / 2016 / Baintelkam

DIBERIKAN KEPADA / *ISSUED TO*

1. Nama / *Name* : Mr. ANDREW JAMES BAGNALL
2. Tempat dan tgl Lahir / *Place and date of birth* : Edinburgh, 26 Juni 1973
3. Warga Negara / *Nationality* : Inggris/British
4. Pekerjaan / *Occupation* : Peneliti
5. No. Paspor,tgl dan berlaku s/d / *Pasport No. Place and date of issued valid until* : [illegible] Tgl. 07-01-2015 07-06-2025
6. Dokumen lain / *Others document* : VITAS No.: [illegible], 315.C untuk selama 30 hari Tiba tgl. 09-07-2016 S.I.P No.: [illegible]
7. Atas perintah/persetujuan / *Applied/approved by* : KEMENRISTEKDIKTI
8. Tersebut dalam suratnya tgl / *Re-letter of date* : 11-07-2016 No.: [illegible]
9. Maksud kunjungan / *Purpose of visit* : Research dalam bidang Zoologi.
10. Ke/ *To* : Maluku (Maluku Tengah-Seram Utara, Taman Nasional #
11. Dalam Rangka / *In accordance with* : Penelitian dengan judul "Identification of food items used by small mammals".
12. Mulai tanggal / *From* : 11 Juli 2016 s.d. *till* 09 Agustus 2016

PERHATIAN / *ATTENTION*

Manusela).

Surat keterangan jalan ini dibuat untuk memenuhi ketentuan Undang-Undang Kepolisian RI No. 2 Tahun 2002 Pasal 15 ayat (1) huruf k, yaitu mengeluarkan surat ijin / surat keterangan yang diperlukan dalam rangka pelayanan masyarakat, serta ayat (2) huruf i yaitu melakukan pengawasan fungsional terhadap orang asing yang berada diwilayah Indonesia dengan koordinasi instansi terkait.

The Travelling Permit for grant police government regulation No. 02/2002 articles 15 paragraph 1 letter k, that is issued regulation who is to need in scheme to serve community and than paragraph 2 of the letter i, that is took the auspices of base on function of the foreigners at that time in Indonesia with coordination hooked authority.

Dikeluarkan di / *Issued at* : Jakarta
Pada tanggal / *Date* : 11 Juli 2016

a.n. KEPALA BADAN INTELIJEN KEAMANAN
KABID YANMAS
u.b.
PAUR SUBBID ORAS

[illegible] YANTO, SIK
KOMISARIS POLISI NRP [illegible]

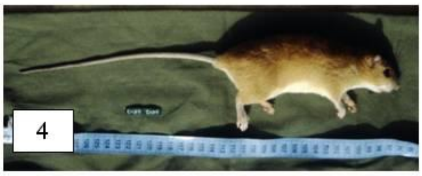
Pas photo dan tanda tangan pemegang
Fotograph and Signature of bearer

F. Photo-elicitation and Interview Recording Sheets

Photo-elicitation sheet 1


1


3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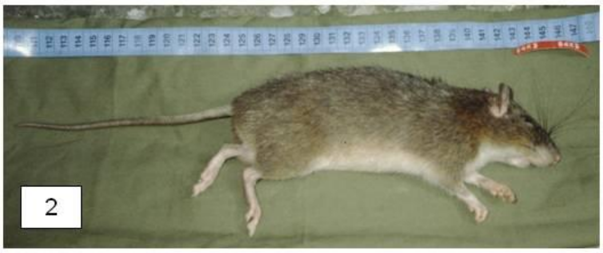
5

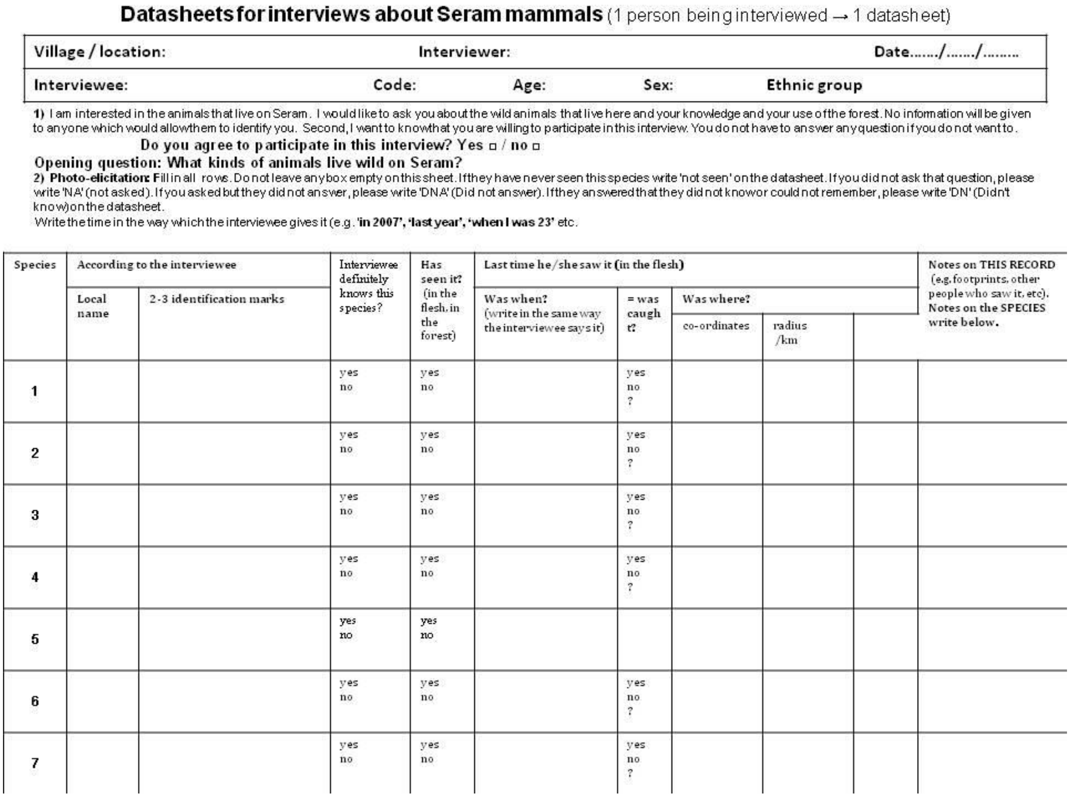

2


6

Photo-elicitation sheet 2

Photo-elicitation sheet 3

Recording sheet for interviews & photo-elicitation (front)



Datasheets for interviews about Seram mammals (1 person being interviewed → 1 datasheet)

Village / location:	**Interviewer:**	**Date……/……/………**

Interviewee: **Code:** **Age:** **Sex:** **Ethnic group**

1) I am interested in the animals that live on Seram. I would like to ask you about the wild animals that live here and your knowledge and your use of the forest. No information will be given to anyone which would allow them to identify you. Second, I want to know that you are willing to participate in this interview. You do not have to answer any question if you do not want to.

Do you agree to participate in this interview? Yes ☐ / no ☐

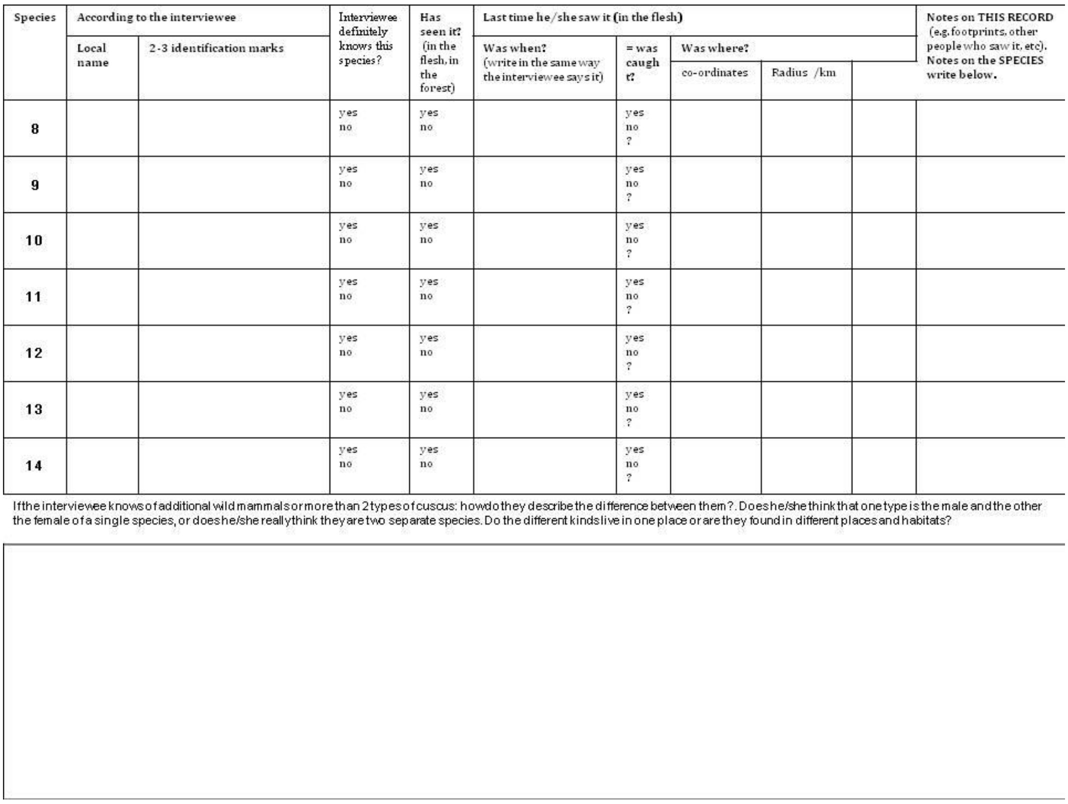
Opening question: What kinds of animals live wild on Seram?

2) Photo-elicitation: Fill in all rows. Do not leave any box empty on this sheet. If they have never seen this species write 'not seen' on the datasheet. If you did not ask that question, please write 'NA' (not asked). If you asked but they did not answer, please write 'DNA' (Did not answer). If they answered that they did not know or could not remember, please write 'DN' (Didn't know) on the datasheet.

Write the time in the way which the interviewee gives it (e.g. **'in 2007', 'last year', 'when I was 23'** etc.

Species	According to the interviewee		Interviewee definitely knows this species?	Has seen it? (in the flesh, in the forest)	Last time he/she saw it (in the flesh)					Notes on THIS RECORD (e.g. footprints, other people who saw it, etc). Notes on the SPECIES write below.
	Local name	2-3 identification marks			Was when? (write in the same way the interviewee says it)	= was caught?	Was where?			
							co-ordinates	radius /km		
1			yes no	yes no		yes no ?				
2			yes no	yes no		yes no ?				
3			yes no	yes no		yes no ?				
4			yes no	yes no		yes no ?				
5			yes no	yes no						
6			yes no	yes no		yes no ?				
7			yes no	yes no		yes no ?				

Recording sheet for interviews & photo-elicitation (back)

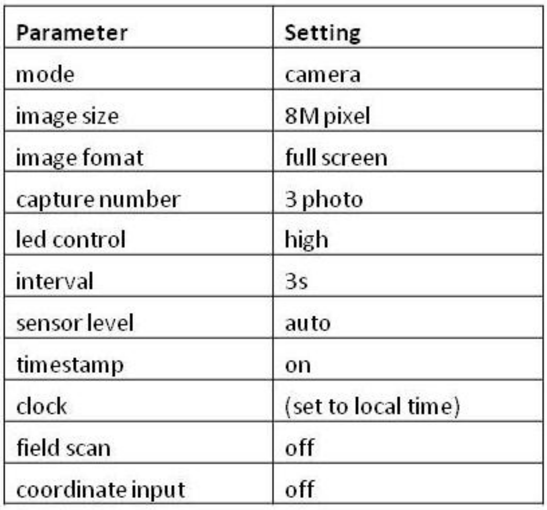


Species	According to the interviewee		Interviewee definitely knows this species?	Has seen it? (in the flesh, in the forest)	Last time he/she saw it (in the flesh)					Notes on THIS RECORD (e.g. footprints, other people who saw it, etc). Notes on the SPECIES write below.
	Local name	2-3 identification marks			Was when? (write in the same way the interviewee says it)	= was caught?	Was where?			
							co-ordinates	Radius /km		
8			yes no	yes no		yes no ?				
9			yes no	yes no		yes no ?				
10			yes no	yes no		yes no ?				
11			yes no	yes no		yes no ?				
12			yes no	yes no		yes no ?				
13			yes no	yes no		yes no ?				
14			yes no	yes no		yes no ?				

If the interviewee knows of additional wild mammals or more than 2 types of cuscus: how do they describe the difference between them?. Does he/she think that one type is the male and the other the female of a single species, or does he/she really think they are two separate species. Do the different kinds live in one place or are they found in different places and habitats?

G. Camera Trap Settings for all Adjustable Parameters

Bushnell NatureViewHD Parameter settings



Parameter	Setting
mode	camera
image size	8M pixel
image fomat	full screen
capture number	3 photo
led control	high
interval	3s
sensor level	auto
timestamp	on
clock	(set to local time)
field scan	off
coordinate input	off

H. FASTA Files for Sequences in Alignments

Seram Bandicoot
>Rhynchomeles prattorum JF694112.1
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CTAAACCCATCTAGAGGAGCCTGTTCTATAA-
TCGATAAACCCCGATATACCTCACCCTCTCTTGCCAATACAGTCTATATACCGCCATCGTC

Cuscus
>Spilocuscus maculatus KJ868160.1
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>Phalanger vestitus AB241057.1
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>Phalanger carmelitae U21182.1
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>Phalanger lullulae AF108219.1
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Asian House Shrew
>Suncus murinus AB032845
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Bats (Chiroptera)
>Rousettus amplexicaudatus FJ529133.1
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>Rousettus amplexicaudatus FJ529132.1
C--
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>Pteropus temmincki AY044797.1
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>Dobsonia mollucensis U93065.1
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>Dobsonia viridis AY044772.1

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>Nyctimene cephalotes  AY044728.1
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>Emballonura raffrayana  EF635536.1
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>Hipposideros ater DQ410808.1
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>Pipistrellus tenuis JQ039196.1
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>Miniopterus australis AY395864
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>Murina florium voucher FJ743914.1
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Human

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Carnivora (Dogs, Civets & Cats)

```
>Canis familiaris AY012152
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>Canis familiaris Y08507.1
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>Paradoxurus hermaphroditus HQ634965.1
```

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>Catopuma temminckii AY773085
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>Catopuma badia KP202256
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TCGATAAACCCCGATATACCTCACCATCTCTTGCTAATTCAGCCTATATACCGCCATCCTC

Pigs
>Sus celebensis GQ338937.1
AACTGGGATTAGATACCCCACTATGCCTAGCCCTAAAC-CCAAATAGTCA--
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>Sus scrofa KM520148.1
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>SEF Sequence F
T-CTGGGATTAGA-ACCCCACTATGCCTAGCCCTAAAC-CCAAATAGTTA--
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CACATCCACCTAGAGGAGCCTGTTCTATAA-
TCSATAAACCCCGATAGACCTTACCAACCCTTGSCAATTCAGCCTATATACCGCC------

Deer
>Rusa timorensis KF317931.1
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GTAAACAAAACTATTCGCCAGAGTACTACCGGCAATAGCTTAAAACTCAAAGGACTTGGCGGTGCT
TTATACCCTTCTAGAGGAGCCTGTTCTATAA-
TCGATAAACCCCGATAAACCTCACCATTCCTTGCTAATACAGTCTATATACCGCCATCTTC

Rats
>Rattus exulans KJ530564.1
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>Rattus rattus AJ005780.1
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TATATCCATCTAGAGGAGCCTGTTCTATAA-
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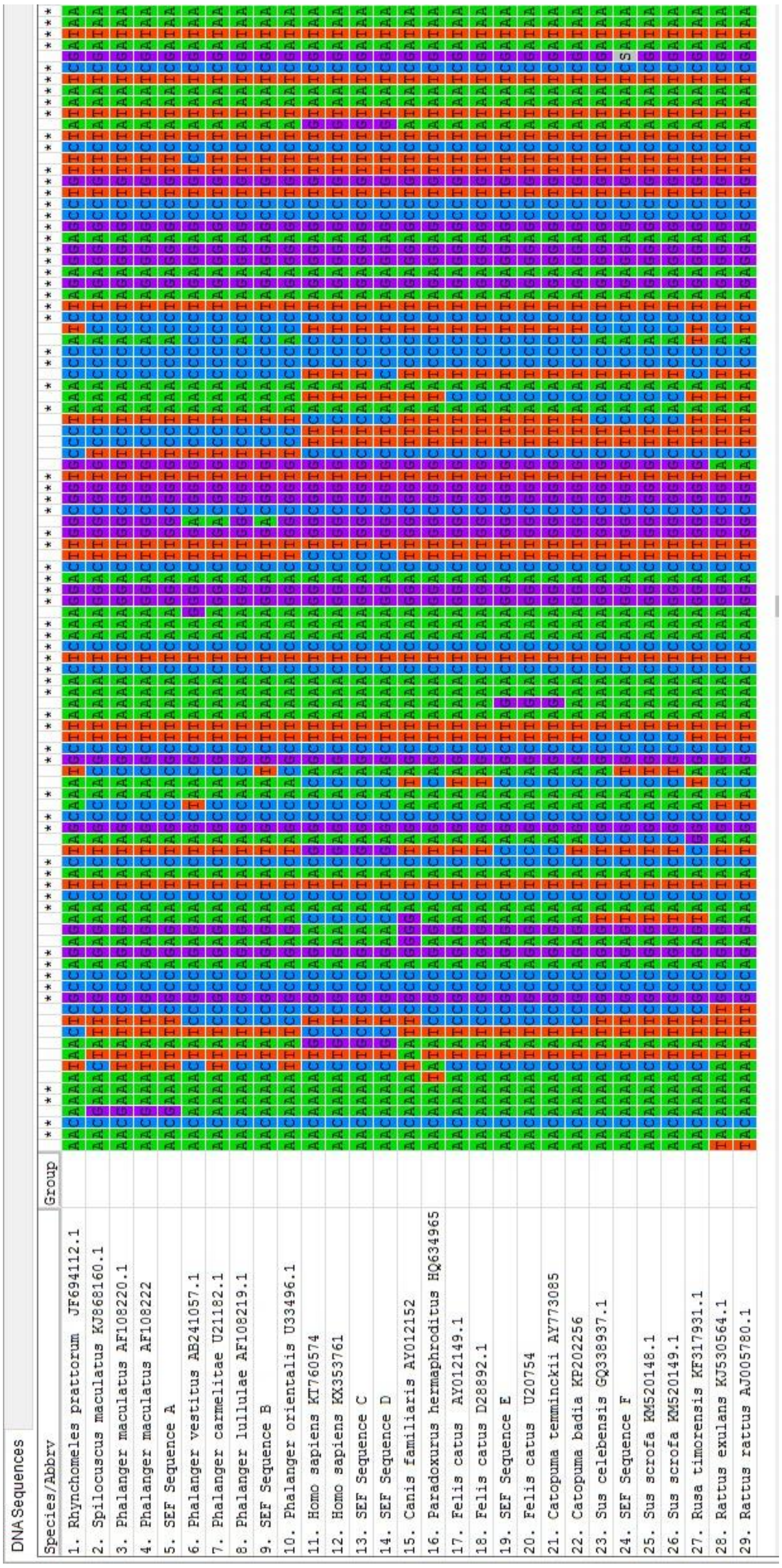
I. Seram Sequences Alignment: Sites 55 – 155



DNA Sequences

1. Rhynchomeles prattorum JF694112.1
2. Spilocuscus maculatus KJ868160.1
3. Phalanger maculatus AF108220.1
4. Phalanger maculatus AF108222
5. SEF Sequence A
6. Phalanger vestitus AB241057.1
7. Phalanger carmelitae U21182.1
8. Phalanger lullulae AF108219.1
9. SEF Sequence B
10. Phalanger orientalis U33496.1
11. Homo sapiens KT760574
12. Homo sapiens KX353761
13. SEF Sequence C
14. SEF Sequence D
15. Canis familiaris AY012152
16. Paradoxurus hermaphroditus HQ634965
17. Felis catus AY012149.1
18. Felis catus D28892.1
19. SEF Sequence E
20. Felis catus U20754
21. Catopuma temminckii AY773085
22. Catopuma badia KP202256
23. Sus celebensis GQ338937.1
24. SEF Sequence F
25. Sus scrofa KM520148.1
26. Sus scrofa KM520149.1
27. Rusa timorensis KF317931.1
28. Rattus exulans KJ530564.1
29. Rattus rattus AJ005780.1

J. Raw Data for Gel Band Intensities Assessed Using ImageJ

Timepoint (hours):		24	24	48	48	72	72	96	96	120	120	144	144
column		Area	relative density										
	1	9297.87	4.03	9658.28	4.76	10507.70	2.12	9878.04	1.10	10049.58	1.14	7923.58	1.14
	2	9296.87	4.03	9764.75	4.81	9289.87	1.87	9909.87	1.11	9946.34	1.13	8755.28	1.26
	3	10648.04	4.61	9734.87	4.79	10586.16	2.13	9531.75	1.06	9828.34	1.12	9783.58	1.41
	4	10025.04	4.34	9339.58	4.60	10421.75	2.10	8432.58	0.94	8467.58	0.96	9214.58	1.33
	5	9269.87	4.02	6731.04	3.31	8750.46	1.76	6960.28	0.78	6857.16	0.78	8687.58	1.25
	6	9643.46	4.18	8262.46	4.07	9524.58	1.92	5914.04	0.66	5929.46	0.67	5156.87	0.74
	7	9857.58	4.27	7378.58	3.63	7907.92	1.59	5096.87	0.57	5011.58	0.57	6804.16	0.98
	8	10402.75	4.51	7848.58	3.86	8278.28	1.67	4686.75	0.52	4511.75	0.51	8274.99	1.19
PG trap mean		9805.18	4.25	8589.77	4.23	9408.34	1.90	7551.27	0.84	7575.22	0.86	8075.08	1.16
	9	8560.16	3.71	5511.87	2.71	2981.75	0.60	1704.80	0.19	1742.80	0.20	3095.80	0.45
	10	4152.21	1.80	5279.87	2.60	893.26	0.18	1397.75	0.16	1462.92	0.17	5228.58	0.75
	11	7323.41	3.17	3301.63	1.63	749.19	0.15	2057.04	0.23	1904.34	0.22	4060.75	0.59
	12	2776.04	1.20	2764.87	1.36	3753.04	0.76	1667.21	0.19	1596.51	0.18	4250.16	0.61
	13	4860.04	2.11	2955.28	1.46	2445.63	0.49	2922.16	0.33	2808.46	0.32	3043.87	0.44
	14	4784.87	2.07	2203.16	1.08	71.78	0.01	3870.87	0.43	3777.34	0.43	1839.46	0.27
	15	2303.58	1.00	1173.04	0.58	2392.75	0.48	3983.58	0.44	3951.46	0.45	1979.63	0.29
	16	2380.87	1.03	734.63	0.36	1954.09	0.39	5456.16	0.61	5508.16	0.63	1309.63	0.19
Dry trap mean		4642.65	2.01	2990.54	1.47	1905.19	0.38	2882.45	0.32	2844.00	0.32	3100.98	0.45
Positive		2307.46	1.00	2031.04	1.00	4961.28	1.00	8967.99	1.00	8809.58	1.00	6932.04	1.00