Mus musculus populations in Western Australia lack VKORC1 mutations conferring resistance to first generation anticoagulant rodenticides: Implications for conservation and biosecurity

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Short title
Anticoagulant rodenticide resistance in *Mus musculus* of Western Australia

Abstract

Background

Humans routinely attempt to manage pest rodent populations with anticoagulant rodenticides (ARs). We require information on resistance to ARs within rodent populations to have effective eradication programs that minimise exposure in non-target species. Mutations to the VKORC1 gene have been shown to confer resistance in rodents with high proportions of resistance in mice found in all European populations tested. We screened mutations in *Mus musculus* within Western Australia, by sampling populations from the capital city (Perth) and a remote island (Browse Island). These are the first Australian mouse populations screened for resistance using this method. Additionally, the mitochondrial D-loop of house mice was sequenced to explore population genetic structure, identify the origin of Western Australian mice, and to elucidate whether resistance was linked to certain haplotypes.

Results

No resistance-related VKORC1 mutations were detected in either house mouse population. A genetic introgression in the intronic sequence of the VKORC1 gene of Browse Island house mouse was detected which is thought to have originated through hybridisation with the Algerian mouse (*Mus spretus*). Analysis of the mitochondrial D-loop reported two haplotypes in the house mouse population of Perth, and two haplotypes in the population of Browse Island.

Conclusions

Both house mouse populations exhibited no genetic resistance to ARs, in spite of free use of ARs in Western Australia. Therefore weaker anticoagulant rodenticides can be employed in pest control and eradication attempts, which will result in reduced negative impacts on non-target species. Biosecurity measures must be in place to avoid introduction of resistant house mice, and new house mouse subspecies to Western Australia.

Introduction

Commensal rodents are resident on all land masses (except Antarctica)[1,2] and are expanding their geographical distributions, which is indicative of a high adaptability to challenging environmental conditions and ability to exploit resources provided by humans.[3] These rodents are considered pests, and poisons are the most commonly used method for control and eradication programs.[4] Due to their efficacy, anticoagulant rodenticides (ARs) have been used to carry out eradications on over 300 islands with a 90% success rate.[5,6] Anticoagulant rodenticides can be subdivided into first generation (FGARs) or second generation (SGARs), according to when they were first synthesised and commercialised.[7] In 1958, the first evidence of resistance to ARs was discovered in *Mus musculus* populations, which were resistant to warfarin and diphacinone.[8] Second generation compounds with greater acute toxicities than FGARs were developed in response, resulting in mortality after a single feed at a lower concentration.[7,9,10]

Resistance-linked mutations within rodent species can arise independently and be positively selected in populations that are exposed to ARs, or can be acquired via

58 introgression with other species, for example, by hybridisation between *M. musculus* and
59 the Algerian mouse (*Mus spretus*).[11–13] Resistance to ARs in *M. musculus* populations has
60 been linked to single nucleotide polymorphisms in subunit 1 of the *Vitamin K epOxide*
61 *Reductase Complex* (VKORC1*)*, which is involved in the synthesis of vitamin K and is
62 important in blot clotting[14] and bone formation.[15] However, mutations in the VKORC1
63 gene should be selected against in the absence of exposure to rodenticides due to negative
64 pleiotropic effects.[14–17] Extensive testing of resistance to SGARs in *M. musculus* in
65 Europe, Canada and some in Australia via feeding trials, blood clotting response tests,
66 and/or genetic screening has uncovered high prevalence of SGAR resistance.[11,18,19] For
67 example, every European population tested (100 from 6 countries) had levels of VKORC1-
68 related resistance ranging from 70 to 100%.[11,13,19–21]
69
70 It is vital to understand levels of resistance in rodent populations so that appropriate
71 chemicals can be applied at a suitable rate for management purposes, as chemical residues
72 of ARs have been detected in a wide variety of non-target species, in both terrestrial and
73 aquatic environments. The presence of these chemicals outside of their target species can
74 result directly from the rodenticide, or indirectly via consumption of poisoned rodents as
75 persistence of rodenticides[22–24] results in liver retention and ultimately biomagnification
76 and bioaccumulation in food webs.[9]
77
78 There is scarce information regarding the presence and distribution of resistance in
79 rodent populations of Australia, and a complete absence of information for *M. musculus*
80 populations on the Australian mainland.[25,26] In Western Australia, neither rats nor mice
81 have been tested to quantify the levels of resistance to ARs despite the pervasive use of

82 poisons by government agencies and the public, whose use of both FGARs and SGARs is
83 unmonitored and unregulated.[9]
84
85 As ARs are the most commonly used class of chemicals to control both mainland and
86 island rodent populations in Australia, it is critical to evaluate the levels of resistance in
87 rodent populations in order to reduce the impact on non-target species.[9] For this reason,
88 the aim of this study was to investigate whether VKORC1 mutations that have been linked
89 to AR resistance are present within populations of *M. musculus* in the urban area of Perth
90 (the largest city in Western Australia). As a comparison, the same investigation was
91 undertaken for a population of south-east Asian house mouse (*M. musculus castaneus*)
92 from Browse Island off the coast of Western Australia where ARs have not been used. For
93 both populations, analyses of the mtDNA D-loop region were conducted to investigate
94 whether resistance (if present) is correlated with genetic lineages within these species.
95

96 Materials and methods

97 Sample collection and preparation

98 Western house mice (*Mus musculus domesticus*) from the Perth metropolitan area
99 (Western Australia) were collected by use of traps or other methods not involving ARs
100 (n=34); confirmation that death was not due to ARs was derived from the absence of
101 internal haemorrhaging noted during dissection. Mice were stored at -20°C at Edith Cowan
102 University until liver extraction, and livers were then stored in 100% ethanol at -8°C until
103 DNA extraction. Liver samples of south-east Asian house mouse (*Mus musculus castaneus*)
104 that had been collected using snap traps from Browse Island were donated by the

105 Department of Biodiversity, Conservation and Attractions of Western Australia (n=15). Liver
106 samples from these mice were preserved in salt saturated dimethyl sulfoxide and stored at -
107 8°C at Edith Cowan University until DNA extraction.
108

109 Sensitivity analysis

110 We carried out a sensitivity analysis to ascertain the likelihood of detecting
111 mutations in our sample of 34 mice under different proportions of mutation in the Perth
112 population. Using a binomial distribution, we tested proportions of resistant individuals in
113 the population ranging from 70%[11] (the lowest percentage of resistance detected in the
114 literature) down to 10%. The probability of not collecting any mice with resistance in our
115 sample size of 34 was well below 0.000001 at 70% and 0.028 at 10% in the population
116 (Table 1). In fact, even at the lowest resistance rate tested, we could have expected to
117 collect at least 3 resistant mice, and for a population with 70% resistance that number rises
118 to 24 mice.
119

120 **Table 1. Binomial sensitivity analysis.**

	Proportion of resistant mice in population (%)			
	10	30	70	95
Probability of finding no resistant mice	0.028	<0.0001	<0.000001	<0.000001
Mode (resistant mice detected)	3	10	24	33

121 Finding a resistant mouse was taken as a success. The analysis was based on the Perth
122 population with a sample size of 34.
123

124

125 ## DNA extraction and PCR

126 Prior to DNA extraction, the liver was washed in Milli-Q water for 5 minutes on a
127 rocking platform at room temperature, then the water was drained and replaced, and the
128 process repeated under the same conditions for 10 minutes. Genomic DNA was extracted
129 from the liver using a DNeasy Blood & Tissue kit following the manufacturer's instructions
130 (Qiagen). Concentration and purity of the DNA were measured using a NanoDrop (Thermo
131 Fisher Scientific), and then concentration of DNA in all samples was standardised to 10
132 ng/µL by dilution with Milli-Q water unless it was below 10 ng/µL.

133

134 Polymerase chain reaction was used to amplify the three exons of the VKORC1 gene
135 region[21] and the complete mitochondrial D-loop and flanking regions[32] using primer sets
136 from the sources indicated. Reactions included 20 ng of DNA template, 12.5 µL of Platinum
137 II HotStart (VKORC1) or Platinum HotStart (D-loop) master mix (Thermo Fisher Scientific),
138 0.5 µL of both forward and reverse primers made up to 25 µL with DNA-free H_2O. Cycling
139 conditions for PCR are shown in Table 2. Successfully amplified PCR products were purified
140 and sequenced at the Australian Genome Research Facility in Perth, Western Australia.

141

142 **Table 2. Adapted PCR parameters for VKORC1 and D-loop amplification.**[11]

Gene region	Master Mix	Initial denaturation	Denaturation	Annealing	Extension	Final extension
VKORC1	Platinum HotStart	2 min @ 94°C	30 sec @ 94°C	30 sec @ 58°C	110 sec @ 72°C	10 min @ 72°C

	Platinum II HotStart	2 min @ 94°C	30 sec @ 94°C	30 sec @ 60°C	30 sec @ 72°C	10 min @ 72°C
D-loop	Platinum HotStart	2 min @ 94°C	30 sec @ 94°C	30 sec @ 55°C	1 min @ 72°C	10 min @ 72°C

All conditions included 40 cycles.

Sequence construction

Sequence chromatograms were manually checked and edited using FinchTV (Geospiza) and aligned in Geneious Prime 2019.0.4 with multiple reference *Mus musculus* and *Mus spretus* sequences downloaded from GenBank to confirm (sub)species identification, identify haplotypes, and detect VKORC1 SNPs. Reference sequences for the VKORC1 gene included wild type (susceptible to ARs) *M. m. domesticus*, AR resistant *M. m. domesticus*, the Algerian mouse *M. spretus*, and the hybridised (and resistant to ARs) *M. m. domesticus*/*M.spretus*.

Haplotype network construction

The D-loop sequences of Perth metropolitan mice and Browse Island specimens were aligned in Geneious with 578 *M. m. domesticus* D-loop sequences and 246 *M. m. castaneus* D-loop sequences, respectively. Non-unique sequences from the same country were filtered out from both datasets, and sequence length was standardised. Haplotype networks were created from the aligned datasets using the minimum spanning network method in PopART (Population Analysis with Reticulate Trees).[27]

Ethical approval

All protocols were approved by the Edith Cowan University Animal Ethics Committee (permit number 21776).

Results

VKORC1 mutations, insertions and deletions

VKORC1 amplification covered three exons. Exons 1 and 2 were amplified using one primer set and resulted in complete sequences for 31 mice and incomplete sequences in 21 mice. Exon 3 resulted in 26 complete sequences and one incomplete. In total, 15 mice from Browse Island and 34 mice from Perth were sequenced, in approximately equal sex ratio.

Sequences from *M. m. castaneus* presented two noteworthy features: all mice carried two silent single nucleotide polymorphisms in exon 1 at amino acid positions 10 and 37, and all mice had a 68 base pair insertion at nucleotide position 1454, located in the intron following exon 2. When compared to published sequences it was found that the insertion (12 mice) was 100% homologous to a section of DNA in *M. spretus* as well as certain *M. m. domesticus* that hybridised with the Algerian mouse. The insertion was not identical in all individuals sequenced from the island: the remaining three individuals differed from the published sequence by a single identical point mutation. None of the mutations linked to the *M. spretus* resistant allele were detected in any of the Browse Island mice, despite all displaying the intragenic sequence. Furthermore, all 15 Browse Island samples reported an eight bp deletion at position 1143 that is shared with the published sequences previously mentioned. One mouse from Browse Island displayed a seven bp and

184 an 11 bp insertion at nucleotide position 939 and 1266 respectively, which were not
185 exhibited in any of the published VKORC1 sequences. The VKORC1 insertion discovered on
186 Browse Island is here defined as an introgression in agreement with previous studies that
187 have used simulation and statistical methods to reach this conclusion.[28–30]
188
189 In contrast, no mutations (associated with AR resistance or not) were detected in the
190 coding regions of the VKORC1 gene in western house mice (*Mus musculus domesticus*) from
191 the Perth metropolitan area.
192

193 *Mus musculus* D-loop haplotypes in Western Australia

194 Mitochondrial D-loop sequences were obtained for all house mice in this study
195 (n=49), with sequenced PCR products between 900 and 1100 base pairs. The Perth mouse
196 population had two haplotypes, with the most common being 100% homologous to
197 published sequences of *M. m. domesticus* from Australia. One mouse from the metropolitan
198 area displayed a different haplotype, which did not match any published D-loop sequences;
199 this mouse was most closely related to haplotypes from Australia and The Netherlands, but
200 differed from them by a single base pair change at position 718. The haplotype network (Fig
201 1) groups Perth mice with haplotypes from West Europe, New Zealand, and the British Isles,
202 as well as Southeast Asia, Africa and Oceanic Islands. The western house mouse from Perth
203 with a unique haplotype was most closely related to *M. m. domesticus* from the Middle
204 East, Cyprus, and Europe.
205 **Figure 1 Minimum spanning haplotype network from *Mus musculus domesticus* D-loop**
206 **data.** The numbers in the legend are representative of the number of unique haplotypes

207 included in the analysis. The size of each circle indicates the frequencies of the haplotype,
208 and each colour represents the geographical origin of the sequences. The numbered nodes
209 in the haplotype network correspond to the numbered inset boxes. A black cross in legend
210 indicates that M. m. domesticus in that area has not been tested for anticoagulant
211 rodenticide (AR) resistance.[31] A white square indicates that M. m. domesticus has been
212 tested for Vkorc1 mutations related to AR resistance and was found to be negative (this
213 study). A red triangle indicates that M. m. domesticus has been tested for resistance
214 through a lethal feeding period test and was found to be resistant to ARs.[5,8,20,25,26] A
215 white triangle indicates that M. m. domesticus has displayed Vkorc1 mutations related to
216 AR resistance.[20] A black triangle indicates that a blood clotting response test has been
217 performed and M. m. domesticus was found to be resistant to ARs.[26] A blue triangle
218 indicates that an assay of the VKOR enzyme has been carried out and resistance to ARs was
219 determined.[5]

220 The D-loop sequences of 15 mice from Browse Island were identical to published *M.*
221 *m. castaneus* D-loop sequences from Asia and Kenya. One mouse differed from the other
222 haplotype by one base pair at position 101 and was 100% homologous to published *M. m.*
223 *castaneus* sequences from Asia, Russia and Kenya. On the haplotype network (Fig 2),
224 mitochondrial D-loop sequences of *M. m. castaneus* from Browse Island were grouped with
225 sequences from China, Bangladesh, Japan, Indonesia and Kenya, as well as other Asian
226 countries.

227 **Fig 2 Minimum spanning haplotype network from mitochondrial D-loop data of *Mus***
228 ***musculus castaneus*.** The numbers in the legend are representative of the number of unique
229 haplotypes included in the analysis. The size of each circle indicates the frequencies of the
230 haplotype, and each colour represents the geographical origin of the sequences. The

numbered node in the haplotype network corresponds to the numbered inset box. Haplotype diversity resulting from deletions and insertions is not represented in PopART (1.7) networks, therefore all Browse Island samples appear in the same haplotype node.

Discussion

Mus musculus domesticus of Perth lack VKORC1 mutations

Resistance to ARs is usually present in populations of house mice and rats that have been exposed to toxicants, especially when exposure is repeated and involves intensive use of FGARs such as warfarin.[32] ARs have been intensively used by the public and professionals for conservation outcomes, or in agricultural, residential and commercial settings in Western Australia,[9] and professional pest managers have anecdotally observed resistance to the ARs warfarin, coumatetralyl (FGARs) and bromadiolone (SGAR) in Perth house mice (Piggott D, 2019, pers. comm.). Where mouse populations carry resistance, larger quantities or stronger ARs tend to be used, increasing the likelihood of non-target animals being affected directly through feeding on baits and indirectly through feeding on resistant mice that have ingested higher doses of poison (in addition to consuming dead susceptible mice).

Prior to this study, the resistance to warfarin in Australian *Mus musculus* to ARs had been recorded only using no-choice feeding tests on animals from Lord Howe Island (700 km off the coast of New South Wales), and Sydney but not in Western Australian populations.[25,33] In Australia, the *Vkroc1* gene has not previously been sequenced in

253 mice, and therefore the results of this study are the only available information currently on
254 genetically-based resistance to ARs of mice in Australia.
255
256 No mutations correlated with resistance to ARs were detected in the VKORC1 gene
257 of *M. m. domesticus* from the Perth metropolitan area in this study. Previous VKORC1
258 screening in Europe has recorded high frequencies of mutations related to AR resistance
259 ranging 70-100% (Table 3).[11,19,21,28,34] All published research at the time of preparing
260 this manuscript reported resistance-related mutations, therefore the absence of VKORC1
261 sequence variants in the Perth region is a unique result and poses challenges and
262 opportunities for pest management and biosecurity.
263
264 **Table 3. Anticoagulant resistance conferred by mutations in the VKORC1 gene in *Mus***
265 ***musculus* populations worldwide.**

Location	Number of mice	Resistance (%)	Reference
France (65 locations)	266	70	[11]
United Kingdom (5 counties)	53	89	[20]
Germany (25 locations)	352	90	[19]
Ireland	50	84	[21]
Switzerland (3 locations)	48	100	[19]
Spain	29	93	[30]

266
267
268 We consider six scenarios as likely explanations for the lack of genetic resistance in
269 Perth mice (Table 4). The simplest scenario is a founder effect: the mutations in the VKORC1

270 gene were never present in the Western Australian mice population. As *M. m. domesticus*
271 was probably introduced in Australia by European settlers in the late 18th Century, the
272 absence of mutations in the exons of VKORC1 might because they were absent from these
273 founder animals.[35,36] The mitochondrial D-loop data supports this, as the low haplotype
274 diversity indicates rapid expansion from a small founding population.[37] ARs were invented
275 in the 1940s, and the first evidence of resistance in rodents dates to 1958.[18] Therefore,
276 the highly conserved coding regions of VKORC1 have been maintained free of mutations
277 because the initial rodent population that colonised Western Australia did not exhibit any.
278
279 **Table 4. Possible scenarios that could have led to the absence of SNPs in the VKORC1 gene**
280 **of *Mus musculus domesticus* of the Perth metropolitan area, Western Australia.**

Scenario	Description	Likelihood
Founder effect	Mutations have never been in Western Australia because the founder population did not carry VKORC1 mutations.	Possible
SGARs	The use of strong SGARs removed any warfarin resistance (the most common kind of resistance inferred from VKORC1 mutations) from the Western Australian house mouse population.	Very likely
Pleiotropy	Defective VKOR enzyme activity posed a negative selection pressure on VKORC1 mutations related to rodenticide resistance.	Not likely

Scenario	Description	Likelihood
Other factors	The arid Australian environment selects for natural tolerance to vitamin K depleted conditions; other unidentified genes (such as cytochrome P450) confer resistance to ARs in Western Australian mice.	Possible
Detection failure	As only a subset of the Western Australian house mouse population was tested, the sample could have missed individuals from the population carrying VKORC1 mutations.	Not likely

281

282

283 Alternatively, western house mice of Western Australia may have lost mutations
284 conferring AR resistance. In this scenario, it may be that heavy use of SGARs has removed
285 them from the population. In Australia, eight ARs are commercially available for rodent
286 control, which are intensively used, and readily available to the public without
287 restrictions.[9] While there is no information available regarding the volume of use of
288 rodenticides in Australia, evidence of secondary poisoning and AR residues in non-target
289 species indicates heavy use of rodenticides in Western Australia, both by professionals and
290 the public.[9] As the most common resistance is exhibited towards the anticoagulant
291 warfarin, the use of stronger SGARs may have removed any individuals carrying mutations
292 related to warfarin resistance from Western Australian populations.

293

294 Another explanation could be that a large pleiotropic has removed the alleles.
295 Dysfunctional VKORC1 activity has a cost, as blood clotting requires the enzyme to perform
296 well.[14] Furthermore, vitamin K is essential during foetal bone formation and therefore

297 females with normal VKOR activity are better breeders.[15] VKORC1 mutations are
298 therefore selected against in conditions where rodenticides are not used and rodent
299 populations are not exposed to the poisons;[14–17] however, continuous exposure of house
300 mice to ARs in the Perth Metropolitan area poses a selection pressure to maintain resistant
301 mutations, and therefore it is unlikely that mutations in the VKORC1 gene are absent due to
302 negative pleiotropic effects, and resistant rodents might be consuming exogenous vitamin K
303 to overcome the defective enzyme activity.[17]
304
305 It is also possible that Perth mice have AR resistance through other mechanisms (i.e.
306 through mutations in other genes conferring resistance), which would result in there being
307 no selection in favour of VKORC1 mutations (and they may be actively selected against as
308 per the pleiotropy scenario). The absence of resistance-related alleles in the VKORC1 gene
309 does not rule out the presence of resistance in Western Australia as this gene is not the only
310 known mechanism of resistance to anticoagulant rodenticides. Cytochrome P450 2C9
311 (CYP2C9) metabolises anticoagulant rodenticides, and genetic variants are associated with
312 the need for higher dosages.[26,38] As Cytochrome P450 also detoxifies other pollutants,
313 such as pesticides, the presence of environmental pollutants in general could lead to an
314 increase of cytochrome P450 activity and render rodents pre-adapted to rodenticides.[39]
315 Other unidentified genes could be involved in rodenticide resistance as well, and resistance
316 to certain ARs, such as difenacoum, has been shown to be polygenic.[40,41]
317 Rodents adapted to living in arid environments are tolerant to anticoagulant
318 rodenticides, an adaptation to a scarcity of available vitamin K. This could be an alternative
319 cause of AR resistance in western house mice of Western Australia, where the climate
320 ranges from Mediterranean in the south (where Perth is located) and transitions through

321 semi-arid to desert further inland.[38,42,43] Until resistance to anticoagulant rodenticides
322 is tested in *M. m. domesticus* of Western Australia through laboratory tests such as feeding
323 trials and blood clot response tests, it cannot be said whether the mice of this region are
324 resistant to ARs even though this study has shown that they do not carry resistance-linked
325 alleles in the VKORC1 gene. Pest managers have anecdotally reported resistance in the
326 Perth region, and therefore it is possible that house mice of Western Australia are resistant.
327
328 A final possibility is that mutations in the VKORC1 gene of Perth house mice are
329 present but were not detected. Sample size (34 mice) was adequate to detect resistant mice
330 at levels of resistance in the population to as low as 10% (Table 1). Measures were taken to
331 avoid sample collection bias according to sex (equal number of male and female samples
332 were used), location (a geographical range spanning through the entire Perth metropolitan
333 area was preferred), and sample collection was designed not to underestimate the
334 frequency of VKORC1 mutations (i.e. no mice killed with rodenticides were collected).
335 Furthermore, western house mouse populations in Europe reported high percentages of
336 resistance (between 70% to 100%) with much smaller sample sizes than our study (as few as
337 four mice per location)[11] while all other tests performed on house mouse populations
338 worldwide detected alleles associated with resistance.[11,19–21,30] We therefore hold the
339 possibility of a false negative not to be a concern.
340
341 Combinations of the scenarios discussed could be active (potentially in different
342 places and at different times); for example, if the initial colonising population did not have
343 mutations in the VKORC1 gene (scenario founder effect), any mutations arising

344 independently could have been quickly removed from the population by the intensive use of
345 strong anticoagulant rodenticides that killed those individuals (scenario SGARs).
346

347 VKORC1 introgression between *Mus* species

348 *M. m. castaneus* inhabiting Browse Island had an intragenic insertion in the VKORC1
349 gene, which was 100% homologous with the GenBank *Mus spretus* reference sequence, as
350 well as some *M. m. domesticus* that have hybridised with *M. spretus*.[30] There is evidence
351 of an ongoing process of hybridisation between the *M. musculus* subspecies, which,
352 combined with the retention of ancestral polymorphism and introgression in the wild,
353 renders it difficult to distinguish between subspecies.[44]
354
355 The most researched *M. musculus* subspecies, *M. m. musculus* and *M. m.*
356 *domesticus*[45] present evidence for introgression in at least 10% of their nuclear genomes,
357 as well as multiple mitochondrial restriction sites.[46,47] When compared to *M. m.*
358 *musculus* and *M. m. domesticus*, *M. m. castaneus* is more polymorphic, and its retention of
359 ancestral polymorphisms might be the reason behind the complexity of defining its lineage
360 and identifying introgression,[45] making it difficult to interpret the history of the Browse
361 Island mice.
362
363 The intragenic insertion detected in this study in the VKORC1 gene has been
364 previously found to have transferred from *M. spretus* to *M. m. domesticus* in areas of
365 sympatry in Spain and allopatry in Germany, where hybridisation and introgression between
366 allopatric species might have been caused by human interaction that increased the

367 geographic distribution of the *Mus* species.[30] Although there is a selective disadvantage
368 from hybridisation between *M. musculus* and *M. spretus*, including sterile male offspring
369 and the possibility of sterile female offspring depending on the direction of the
370 backcross,[48] the introgression of the *M. spretus* haplotype between distant populations
371 has been possible because it involved an adaptive gene such as VKORC1 and its spread has
372 been driven by the selection pressure posed by the common use of ARs.[29]
373
374 Previous studies identified three distinct introgression events between *M. m.*
375 *domesticus* and *M. spretus*, including an ancient event that occurred more than 2000 years
376 ago prior to the colonisation of Europe by *M. musculus*.[28] The other more recent
377 introgression events are dated around 50 to 60 years ago, since the introduction of ARs.[30]
378 Introgression in the VKORC1 gene was present but in low frequencies and it was therefore
379 considered far from becoming fixed and the details were not reported.[29] The 68 bp
380 insertion detected in the present study was present in all Browse Island mice so it can be
381 concluded that the introgression event was likely to be fixed in this small population due to
382 founder effect and genetic bottlenecks. Therefore the region of the VKORC1 gene from *M.*
383 *spretus* that has been detected in the *M. m. castaneus* sample from Browse Island is
384 considered a comet allele[47] that has migrated from one evolutionary lineage to another
385 and its frequency has increased to reach fixation.
386
387 The D-loop places the mice on Browse Island most closely to mice from China,
388 Indonesia, and other Asian countries, but as no other studies have sequenced the VKORC1
389 gene region in this subspecies or attempted to identify introgression in different *Mus*
390 species from Asian countries, we cannot make inferences on the process and location of the

391 hybridisation between the two *Mus* species. Less is known about hybridisation involving *M.*
392 *m. castaneus* than the European subspecies;[49] additionally, introgression in the VKORC1
393 gene between *M. spretus* and *M. m. castaneus* has not been described previously, possibly
394 because the two taxa are allopatric.
395
396 The length of the insertion in the VKORC1 gene (68bp), could indicate an ancient
397 introgression event (>2000 years ago) characterised by continued backcrossing, drift and
398 recombination;[28] however the insertion is not fragmented, and is identical across most
399 individuals from Browse Island and the reference sequences from GenBank. The fact that
400 the region is highly conserved is evidence of its adaptive value as selection would maintain it
401 over time, but this affects the length-based method used to determine the age of the
402 hybridisation. The location of the insertion on the gene could influence the way it is
403 conserved. Noncoding intron regions are usually conserved when the exons are alternatively
404 spliced.[50] In humans, the VKORC1 gene is alternatively spliced, and three isoforms have
405 been described, while the VKORC1 of *M. musculus* has a single described isoform and two
406 potential isoforms that have been computationally mapped.[51] If the VKORC1 gene of *Mus*
407 *musculus* is indeed alternatively spliced, the possibility of the intron being conserved is
408 much higher, as this phenomenon is observed in 77% of the conserved exons.[50]
409
410 The VKORC1 gene is highly conserved across organisms and no south-east Asian
411 house mouse from Browse Island carried mutations in the coding sequence that are linked
412 to the *M. spretus* allele. The absence of these adaptive mutations is consistent with the
413 absence of exposure to ARs.[13]
414

Low genetic diversity of *Mus musculus* in Western Australia

Mus musculus domesticus of the Perth metropolitan area belongs to two closely related haplotypes that differ from each other by a single base pair change, and are most closely related to mice from European countries, such as The Netherlands and United Kingdom. A phylogeographic study of mice in Australia detected 12 haplotypes, with Western Australian house mice belonging to two major clades.[36] The clade occurring geographically closest to Perth is the most widespread clade in Australia, and this haplotype group is also the most common in the United Kingdom, which suggests Western Australian *M. m. domesticus* likely originated from the British Isles.[36]

Apart from mice from Madeira, which were grouped closely in the haplotype network, no countries were characterised by pockets of diversity. The resulting network was highly reticulate, indicating western house mice are closely related worldwide (Fig 1). The genotypic analysis of the mitochondrial D-loop of house mice from the Perth metropolitan area indicated the presence of a single, large panmictic population. Our network places Iranian mice in a central position (Fig 1), indicating Iran is an old (or ancestral) population[52] and potentially a centre of origin for the subspecies. This concurs with the assumed origin of the commensal relationship between mice and humans that was established in the Middle East around 10,000 BC when humans first formed large settlements in the Fertile Crescent.[53] The expansion of *M. m. domesticus* would have been aided by human transport and trade into the Mediterranean Sea during the Iron Age, and then to colonies like Australia by European settlers.[54–56]

438 A previous phylogeographic study of *M. musculus* in Australia documented the
439 presence of *domesticus* as the predominant and only subspecies of house mouse in
440 Australia[36], however mitochondrial D-loop data from mice of Browse Island from our
441 study confirm previous records based on morphology suggesting that the *castaneus*
442 subspecies of house mouse is present in Australia. However, unlike the European origins of
443 the house mouse in Australia, the haplotype network of *M. m. castaneus* suggests that the
444 origin of these Browse Island mice is China and Indonesia. Previous studies have also
445 identified the Indonesian islands of Rote and Timor as the source of Australian *M. m.*
446 *castaneus*.[57]
447

448 Implications for pest management

449 When planning pest eradications or population control, it is vital to gather
450 information on the resistance of the population in order to choose a control method that
451 can successfully eliminate most of the population,[70] ideally 99% of the population.[31]
452 However, eradication attempts and rodent control programs in Western Australia have
453 been carried out with no knowledge of the level of resistance in rodent populations.
454
455 As resistance-conferring VKORC1 mutations were not detected in the Perth
456 metropolitan area, nor on Browse Island, house mouse populations in these areas can be
457 managed with the use of a) FGARs with fewer negative impacts on non-target species or b)
458 weaker SGARs when the natural tolerance exhibited by mice poses restrictions on the use of
459 first generation compounds. Even so, regular testing should be used to detect developing
460 resistance which can then be eliminated by a temporary switch to a SGAR,[33] or methods

461 with low risk of bioaccumulation and secondary toxicity such as lethal trapping or a non-
462 anticoagulant rodenticide containing actives such as cholecalciferol or corn gluten
463 meal.[9,58]
464
465 As Perth is close to a major commercial port,[59] the risk of resistant mice invading
466 the area through international shipping is high. Despite a reported genetic resilience of
467 established mouse populations to new migrants,[60] in the event that resistant mice reach
468 Perth these new individuals would be at a highly advantageous position due to the intense
469 use of ARs.[14] Although our data indicate that, so far this has not occurred, it is an ongoing
470 risk. A biosecurity and quarantine system has been developed to defend Western Australia
471 and all other States and Territories on the Australia continent against incursions of pests,
472 and checkpoints are in place at the State borders and all airports[61,62] and stringent
473 biosecurity protocols need to be established for all ports also.[63] Vessels should be
474 certified free of rodents and provide evidence that no re-infestation occurred since the
475 certificate was issued.[63] Furthermore, resistance to ARs is not always concomitant in rat
476 and mouse species, and if rats and mice of Perth exhibit different levels of resistance, more
477 complex control programs are needed.[33]
478
479 The presence of a different mouse subspecies on Browse Island poses a biosecurity
480 risk to mainland Australia.[64] Fortunately, the introgression between Browse Island mice
481 and *M. spretus* was limited to intronic sequences in the VKORC1 gene, and resistance-
482 related mutations were not carried by any individual from Browse Island that has been
483 sequenced. Since the south-east Asian house mouse population on Browse Island carries
484 silent VKORC1 mutations that do not impair rodenticide activity, the use of ARs to achieve

pest eradication is possible, reducing the biosecurity risk of invasion of mainland Australia and will promote breeding of indigenous species by removing the risk of egg predation of both seabirds and green turtles (*Chelonia midas*) that nest on the island[1],[57]. The use of anticoagulant rodenticides on all islands should be carefully planned to reduce the impact of toxicants to endemic fauna of the island and coastal waters, since broadcast applications of rodenticides on islands are linked to exposure of non-target wildlife in coastal environments.[65] The waters around Browse Island are part of the Australian Fishing Zone, and are often visited by Indonesian fishermen who harvest marine organisms for consumption; as this biota might be exposed to ARs, the use of toxicants threatens human health through possible secondary exposure.[65,66]

Conclusion

The order Rodentia is characterised by a high number of successful invaders, which humans have attempted to manage with the use of anticoagulant rodenticides (ARs) since the 1940s.[4,7] However, this has selected for resistance in the rodents and negatively impacted non-target species.[7,9,22,24] VKORC1 screening in multiple European countries has detected high frequencies of mutations that confer resistance to ARs.[11,18,19,39] In Australia, strong ARs can be purchased from supermarket and retail outlets and are routinely and intensively used in residential and commercial settings.[9] These factors led to the hypothesis that *Mus musculus* of Perth have a high probability of carrying resistance-related mutations in the VKORC1 gene. The aim of this study was to provide the first genetic data on AR resistance levels in two contrasting house mouse populations, and to make suggestions for conservation and biosecurity based on the evidence gathered.

508 Concomitantly this is the first genetic data available for resistance in mice on the Australian
509 continent. The absence of resistance-conferring VKORC1 mutations in both Perth
510 metropolitan area and Browse Island house mouse populations contrasts starkly with all
511 other mouse populations screened thus far. Our results indicate that FGARs can be
512 employed to control and/or eradicate mice effectively, minimising potential harm to non-
513 target species, promoting biodiversity on the island[57] and reducing the biosecurity threat
514 to mainland Australia posed by *Mus musculus castaneus*.
515
516 A general lack of data regarding the levels of AR resistance in commensal rodents in
517 Australia and the impact of rodenticides on non-target biota should motivate future
518 research to investigate resistance in mouse and rat populations throughout Australia.
519 Investigation of genetic resistance should also include native rodents targeted for control in
520 agricultural settings like *Rattus sordidus* and *Melomys burtoni*.[67] Anticoagulant
521 rodenticides are likely to continue to be effective in controlling house mouse populations (at
522 least in the short-term) and there should be a focus on: preventing the introduction of
523 potentially resistant house mice to Western Australia through the port of Fremantle and
524 other routes; genetic screening of other mainland Australian mouse and also rat
525 populations; screening populations prior to eradication programs on islands to minimise the
526 risk of coastal water contamination; and, generally reducing the threat of secondary
527 poisoning to non-target species in both environments by careful planning and application of
528 ARs underpinned by a thorough understanding of resistance in the target populations.
529

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536

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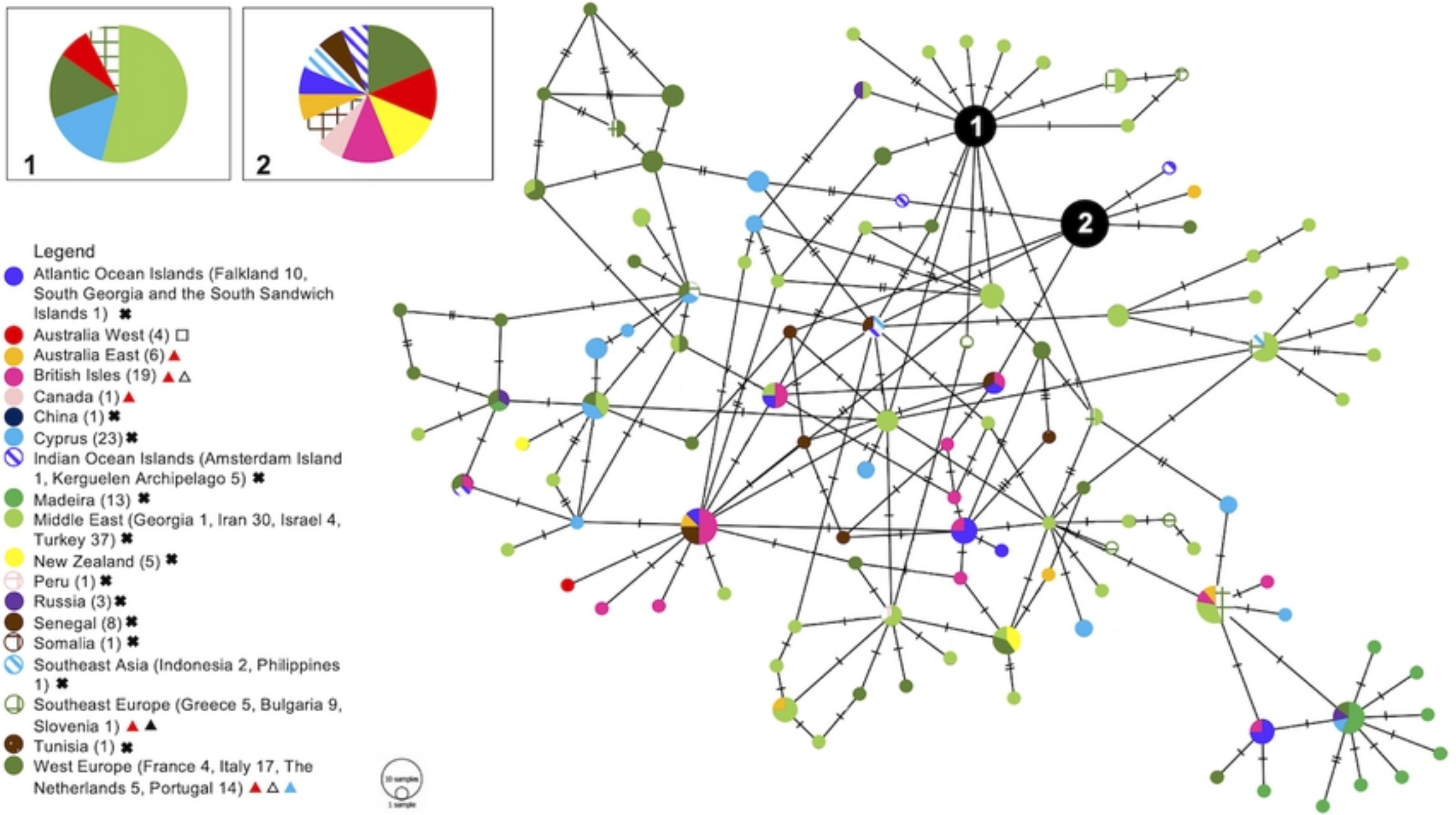
1
2
Legend
Atlantic Ocean Islands (Falkland 10, South Georgia and the South Sandwich Islands 1)
Australia West (4)
Australia East (6)
British Isles (19)
Canada (1)
China (1)
Cyprus (23)
Indian Ocean Islands (Amsterdam Island 1, Kerguelen Archipelago 5)
Madeira (13)
Middle East (Georgia 1, Iran 30, Israel 4, Turkey 37)
New Zealand (5)
Peru (1)
Russia (3)
Senegal (8)
Somalia (1)
Southeast Asia (Indonesia 2, Philippines 1)
Southeast Europe (Greece 5, Bulgaria 9, Slovenia 1)
Tunisia (1)
West Europe (France 4, Italy 17, The Netherlands 5, Portugal 14)

Fig1

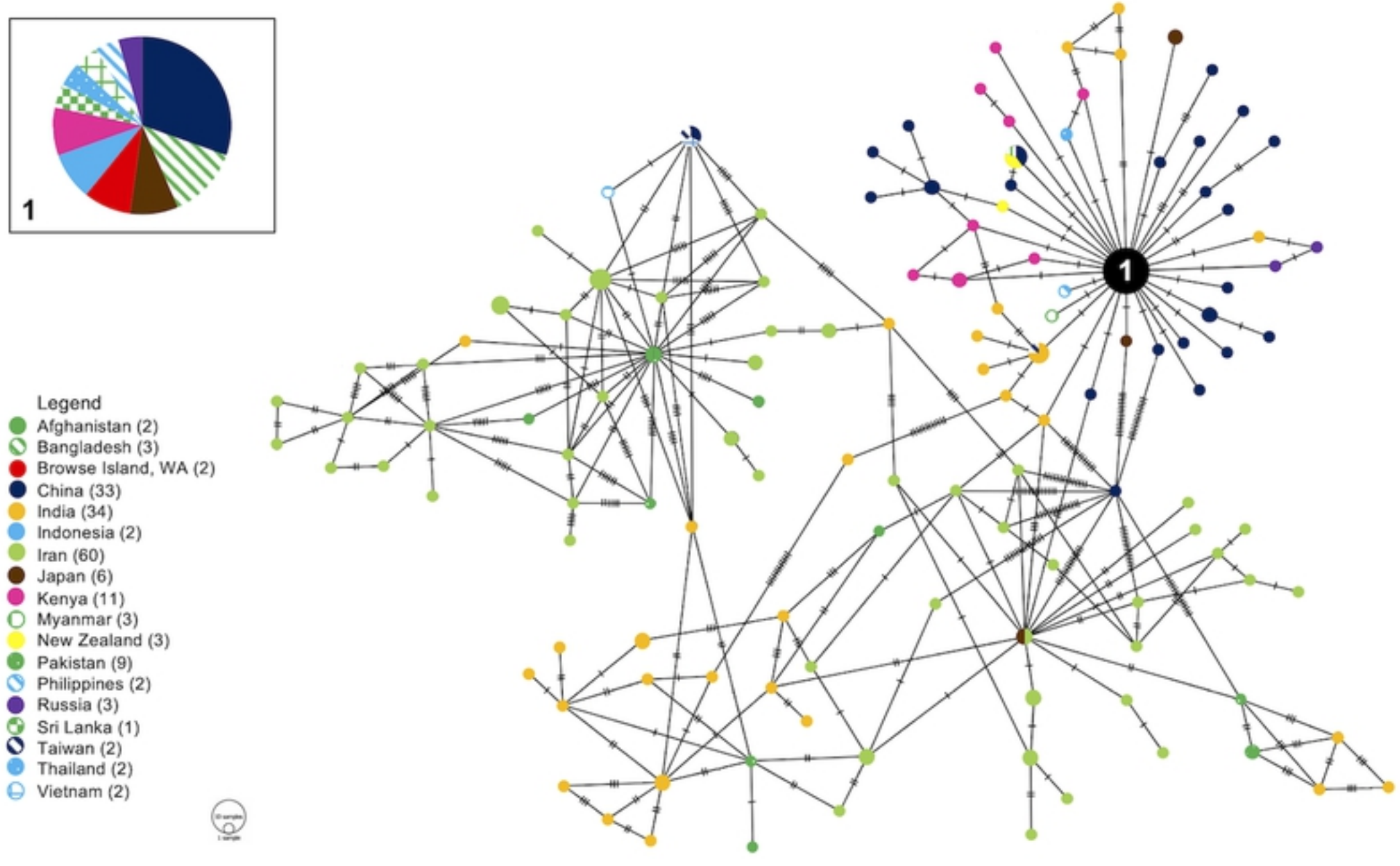
1
1
Legend
Afghanistan (2)
Bangladesh (3)
Browse Island, WA (2)
China (33)
India (34)
Indonesia (2)
Iran (60)
Japan (6)
Kenya (11)
Myanmar (3)
New Zealand (3)
Pakistan (9)
Philippines (2)
Russia (3)
Sri Lanka (1)
Taiwan (2)
Thailand (2)
Vietnam (2)

Fig2