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Choice of molecular assay determines ranavirus detection probability and inferences about prevalence and occurrence

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Choice of molecular assay determines ranavirus detection probability and inferences about incidence and impact

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Abstract

Infectious diseases are emerging at an increasing rate and reliable diagnostic strategies are essential for effective surveillance and research. Ranavirus is an emerging pathogen that can cause morbidity, mortality and population declines in ectothermic hosts. Despite the economic and conservation importance of these viruses, there is no standardized approach to diagnostics, with many molecular assays currently in use. Here, we compared the inter-assay variation and intra-assay precision among two commonly used quantitative and two conventional polymerase chain reaction assays, using laboratory propagated virus and field-collected amphibian and reptile samples. The assays varied in their diagnostic and analytical sensitivity and diagnostic specificity as well as the sequence similarity of oligonucleotides to a set of published ranavirus isolates. Some assays exhibited poor diagnostic sensitivity resulting in a high proportion of false negative results whilst false positive results were also generated. Amplicon sequences from the two conventional PCRs were used to confirm

results but were also shown to be useful for inferring viral lineage. Inaccurate results bias estimates of the distribution and impacts of ranaviruses and together these findings emphasize that molecular assays should be chosen carefully in the context of study aims and ideally combined with other lines of evidence when diagnosing infection and disease.

Key words

Diagnostic methods, nested PCR, qPCR, ranavirus, sensitivity, specificity

1. INTRODUCTION

Disease emergence is occurring at an unprecedented rate (Jones et al. 2008) requiring surveillance systems which incorporate sensitive and reliable diagnostic assays that limit bias (Kuiken et al. 2005, Hyatt et al. 2007, Lachish et al. 2012, DiRenzo et al. 2017). Molecular methods – predominantly nucleic acid-based techniques such as conventional polymerase chain reaction (PCR), quantitative (q)PCR and sequencing – do not usually provide explicit information about pathology but are increasingly used for pathogen detection due to their perceived ease of use, reducing costs and rapid turnaround times (Boyle et al. 2004, Hyatt et al. 2007, Black et al. 2017). They also often have broader applications, greater sensitivity and specificity and require less training and skill than traditional techniques, such as histology (Hyatt et al. 2007, Lorch et al. 2010, Skerratt et al. 2011).

Ranaviruses (family *Iridoviridae*) have caused incidents of mortality and morbidity in amphibians, reptiles and bony fish around the world (Gray et al. 2009, Price et al. 2017). The distribution of *Ranavirus* and the drivers of outbreaks are often unclear. Ranaviruses have been suggested to be ubiquitous (Warne et al. 2016, Campbell et al. 2018) but severe disease outbreaks are patchy in distribution and many studies have linked disease emergence to human behaviors including translocations of infectious materials (Price et al. 2014, 2016, Rijks et al. 2016). Ranaviruses have been associated with population declines of a frog species in the United Kingdom and entire amphibian assemblages in Iberia (Teacher et al. 2010, Price et al. 2014, Rosa et al. 2017), underlining them as important pathogens of potentially significant conservation concern. Reliable, sensitive and specific diagnostic methods are essential for detecting low intensity infections, which will aid a more complete understanding of ranavirus disease ecology and epidemiology.

53 The majority of laboratories use a single molecular assay when screening samples for
54 ranavirus infection, but approaches are not standardized despite the availability of published
55 guidelines from the World Organisation for Animal Health (OIE 2017, Black et al. 2017).
56 Eleven different PCR methods for ranavirus detection were used in the published literature
57 between 2009-2014, but information about the sensitivity or specificity of these assays was
58 usually lacking (Black et al. 2017). Therefore, results may not be reproducible, low intensity
59 infections may be missed, prevalence estimations may be biased, and pathogens may be
60 misidentified. Sequencing of PCR amplicons can be used to confirm the specificity of an
61 assay and the sequences can also be used to investigate a pathogen's taxonomy and genetics
62 (OIE 2017). Nevertheless, even when the primary goal is presence-absence detection,
63 laboratories may favor qPCR and have frequently utilized unpublished methods (Black et al.
64 2017). Additionally, there appears to be both uncertainty and inconsistency about how to
65 define a positive result from qPCR assays (e.g. Miller et al. 2015, Black et al. 2017), which
66 can reduce the comparability and accuracy of results. This study compared the inter-assay
67 variation and intra-assay precision of four molecular methods used in ranavirus detection.
68 The Mao *et al* (1997) assay is one of the most commonly PCR methods used for ranavirus
69 diagnostics (Black et al. 1997). Meng *et al.*(2013) is a nested method based on Mao *et al*
70 (1997) which might therefore offer greater specificity and sensitivity. The Brunner *et*
71 *al.*(2004) assay is one of the most commonly used qPCR methods in the literature and Leung
72 *et al.* (2017) is a new qPCR assay which is likely to be attractive to users since it is paired
73 with a host assay enabling precise quantification of viral loads.

74 **2. MATERIALS AND METHODS**

75 Four molecular assays were compared for their analytical and diagnostic (see Saah & Hoover
76 1997 for definitions) sensitivity and diagnostic specificity, including two conventional PCRs
77 (from Mao *et al.*, 1997 and Meng *et al.*, 2013; Table 1) and two probe-based qPCRs

78 (Brunner *et al.*, 2004 and Leung *et al.*, 2017; Table 1), respectively referred to as "Mao
79 PCR", "Meng PCR", "Brunner qPCR" and "Leung qPCR". All assays targeted the viral
80 major capsid protein (MCP) gene, the main viral coat protein, conserved among all members
81 of the *Iridoviridae* (Eaton et al. 2007). Oligonucleotide sequences (primers and probes) were
82 compared to an alignment of 21 ranaviruses with published whole genomes (Table S1) to
83 obtain orientations and annealing positions and to check for polymorphisms in binding
84 regions. Assay performance was compared using both cultured ranavirus isolates and field-
85 collected samples.

86 DNA was extracted alongside extraction controls (nuclease free water as a negative control
87 and cell-cultured ranavirus as a positive control) using an ammonium acetate protocol and
88 purified using ethanol washes (Nicholls et al. 2000). Samples were stored at -20°C until use.
89 Conventional PCRs were run in 8µl reactions and qPCRs in 20µl reactions. Two microliters
90 of template DNA were used in every reaction, regardless of assay used. A PCR positive
91 control consisting of DNA extracted from a cultured ranavirus isolated from a UK frog
92 (RUK13; GenBank accession KJ538546; Price, 2014) and a no-template negative control
93 consisting of nuclease-free water were run in duplicate on each PCR plate.

94 The Mao PCR reaction mixtures comprised forward and reverse primers MCP4 and MCP5
95 (Table 1; Mao *et al.*, 1997) at a final concentration of 0.5µM, 4µl of 2X DreamTaq Green
96 PCR Master Mix (Thermofisher Scientific, Massachusetts, USA), and nuclease-free water to
97 take the total volume excluding template to 6µL. The nested Meng PCR used the Mao PCR
98 as the first step but both primers were diluted to a final concentration of 0.05µM. The second
99 step of the nested PCR used primers MCP-IF and MCP-IR from Meng *et al.* (2013) at a
100 concentration of 0.4µM, but all other reagents and concentrations were the same as used for
101 the Mao PCR. The Brunner qPCR used the rtMCP-for and rtMCP-rev primers (Picco et al.
102 2007) at a final concentration of 0.9µM with 10µl TaqMan Universal 2x PCR Master Mix

103 (Thermofisher Scientific, Massachusetts, USA), rtMCP-probe at a final concentration of
104 0.25µM, and nuclease-free water to take the volume to 18µL (Brunner *et al.*, 2004 - cited in
105 Picco and Collins, 2007; Whitfield *et al.*, 2012, 2013). Reaction mixtures for the Leung
106 qPCR comprised forward and reverse primers (MCP_F and MCP_R) at a final concentration
107 of 0.5µM, 10µL TaqMan Universal 2x PCR Master Mix (Thermofisher Scientific,
108 Massachusetts, USA), MCP_probe at a final concentration of 0.25µM, and nuclease-free
109 water to take the volume to 18µL (Leung et al. 2017).

110 The Mao and Meng PCRs were run on a Techne PCRmax Alpha Cycler 1 (Fisher Scientific,
111 Loughborough, UK) and thermocycler settings comprised a 10-minute strand dissociation
112 step at 95°C, 23 cycles of 95°C for 30 seconds, 62°C (decreasing by 0.5°C per cycle) for 30s,
113 and 72°C for 30s, 25 cycles of 95°C, 50°C and 72°C each for 30s, and a final elongation step
114 of seven minutes at 72°C. The qPCRs were run on a StepOne Real-Time PCR system
115 (Applied Biosystems, California, USA) and began with a two-minute step at 50°C to prevent
116 carry-over contamination with Uracil-N glycosylase (UNG) followed by a 10-minute strand
117 dissociation step at 95°C. The Brunner qPCR was run for 50 cycles comprising steps of 95°C
118 for 20 seconds, 54°C for 20 seconds and 72°C for 30 seconds. The Leung PCR was run for
119 50 cycles comprising steps of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30
120 seconds. Conventional PCR products were visualized on 100ml 2% agarose gels containing
121 5µl SYBR Safe DNA Gel Stain (Invitrogen, California, USA) alongside a 100-base pair
122 ladder (Thermo Fisher Scientific, Massachusetts, USA). Positive results were defined in each
123 of two ways: 1) using a "majority-rules" approach where a consensus was taken by
124 considering the overall majority returned from replicates (Miller et al. 2015), 2) by taking
125 amplification in any replicate as evidence of a positive sample.

126

127 *2.1 Comparisson of assay sensitivity and specificity*

128 Assay analytical sensitivity was compared using laboratory isolates grown in culture in
129 epithelioma papulosum cyprini cells (Winton et al., (2010); ECACC 93120820). Isolates
130 were used from two of the lineages of the amphibian-associated ranaviruses (frog virus (FV)
131 3-like and common midwife toad ranavirus (CMTV)-like; (Price et al. 2017). A ten-fold
132 dilution series of DNA extractions of each isolate (1 to 1^{-10}), diluted in water, was used as
133 template DNA.

134 To investigate the relative diagnostic sensitivities and specificities of the assays, the Meng
135 PCR was considered a 'gold standard' on account of the high sensitivity of nested protocols
136 (Hafez et al. 2005, Miller & Sterling 2007) and the capacity to confirm virus amplification by
137 sequencing amplicons, as is recommended by the OIE (OIE 2017). DNA extractions of
138 samples from an archive comprising toe-clips and liver tissue from a diverse assemblage of
139 Costa Rican herpetofauna (Table S2) were screened using the Meng PCR and positive results
140 confirmed by sequencing at Macrogen Inc. (F. Wynne unpubl. data). Forty "known positive"
141 and forty "known negative" samples were selected at random from this larger set. Diagnostic
142 sensitivities of the Mao PCR, Brunner qPCR and Leung qPCR were calculated relative to the
143 Meng PCR (gold standard) as the proportion of the total known positive samples that
144 amplified. Diagnostic specificities were also calculated relative to the gold standard as the
145 proportion of known negatives that did not amplify.

146 *2.2 Intra-assay precision*

147 Intra-assay precision - defined as an assay's capacity to generate consistent results among
148 replicates within the same run - was investigated by using each assay to run six replicate
149 reactions per sample. Fifteen known positive and fifteen known negative samples were

selected at random from the sets used above. The intra-assay precision was calculated as the proportion of replicates that returned the expected result.

2.3 Phylogenetic analyses of amplicon sequences from Mao and Meng PCRs

Phylogenetic trees were generated and used to compare the MCP regions amplified by the Meng and Mao PCRs. A multiple-sequence alignment of the complete coding sequence of the MCP gene of 21 ranavirus isolates with published whole genomes (Table S1) was trimmed to the maximum possible length of both the Meng and Mao amplicon sequences by mapping the respective primer pairs of each assay to the alignment. Phylogenetic trees were then constructed with Mr Bayes v3.2.2 (Huelsenbeck & Ronquist 2001) using a generalized time-reversible model of nucleotide substitution with rate variation modelled as a gamma distribution with four rate categories. Two runs of four MCMC chains were run for 500,000 generations and the default program settings were used for other parameters.

3. RESULTS

3.1 Polymorphisms within binding regions of primers and probes

The Mao PCR primers were 100% sequence identical to all 21 ranavirus isolates but there were mismatches (single nucleotide polymorphisms) between the oligonucleotide sets of the other assays and some of the 21 isolates (Table 2). The Meng_IR primer had between one and four mismatches with eight of the isolates and appeared to be poorly suited for use with the whole group of *Ambystoma tigrinum* virus (ATV)-like ranaviruses (Price 2016, Price et al. 2017).

3.2 Analytical sensitivity of assays in detection of cultured ranavirus isolates

The Leung qPCR was two orders of magnitude more analytically sensitive than other assays regardless of the viral lineage of the cultured isolate (FV3 or CMTV; Table 3). The Meng

PCR and Brunner qPCR performed similarly, exhibiting slightly greater analytical sensitivity than the Mao PCR (Table 3).

3.3 Assay performance against field-collected samples

The assays did not perform equally against the field-collected samples (Fisher's exact test, $P = 2.1 \times 10^{-19}$). The Leung qPCR showed the highest diagnostic sensitivity, detecting all of the known positives and therefore demonstrating 100% comparative diagnostic sensitivity with the results from the Meng PCR confirmed by sequencing (the proposed gold standard; Figure 1a). The Brunner qPCR showed lower diagnostic sensitivity, only correctly identifying 11 of 40 (27.5%) positive samples if a majority-rules approach was applied. If amplification in any replicate was used to define a positive result, then an additional nine known positives were correctly identified taking the total sensitivity to 20 of 40 (50%; Figure 1a). The Mao PCR performed extremely poorly, correctly identifying only one known positive (2.5%) using a majority-rules approach with just two additional samples giving an ambiguous result, and the remaining 37 samples showing no amplification in either replicate (Figure 1a).

There was no significant difference between the assays in terms of diagnostic specificity against known negative samples (Fisher's exact test, $P = 0.106$). Using a majority-rules approach, all assays showed 100% diagnostic specificity, correctly identifying all of the known negatives. However, taking any amplification (either replicate) as a positive result reduced the Brunner qPCR's comparative diagnostic specificity to 92.5% (three of the known negative samples showed amplification in one replicate; Figure 1b). Since the Brunner qPCR had a significantly lower diagnostic sensitivity than the Meng PCR or Leung qPCR in screens of known positive samples it is very unlikely that this amplification was explained by a greater capacity to detect ranavirus than exhibited by those assays. Instead, it is most likely

196 that this amplification was non-specific and that the Brunner qPCR generated false positive
197 results for these three (out of 40) samples.

198 *3.4 Intra-assay precision*

199 The Leung qPCR performed most consistently of the assays tested in terms of the proportions
200 of replicate reactions showing amplification with known positive samples: all samples (n =
201 15) were identified as positive when applying a majority-rules approach with only a single
202 replicate in a total of 90 (15 samples, 6 replicates each) showing no amplification (Fig. 2a).
203 The Brunner qPCR performed relatively poorly: none of the 15 positives would have been
204 called positive using a majority-rules approach and just 38.9% of all replicates showed
205 amplification (Fig 2a). The Mao PCR performed very poorly with just a single replicate out
206 of 90 yielding a band on gels (Fig 2a). The Brunner qPCR had the lowest intra-assay
207 precision for negative samples, however both qPCRs showed high intra-assay precision: 93
208 of 96 replicates (96.9%) of the Brunner qPCR and 94 of 96 replicates (97.9%) of the Leung
209 qPCR showed no amplification (Fig. 2b). The Mao PCR showed 100% consistency among
210 negative samples; no replicate produced bands on gels (Fig. 2b). When a majority-rules
211 approach was applied to these results, no false positive results were generated by any assay.

212 *3.5 Phylogenetic signal of PCR amplicons*

213 The phylogenetic signal of the Meng PCR amplicon (320 base pairs [bp]) was compared to
214 that of the longer Mao PCR amplicon (531 bp). The two amplicons returned well-supported
215 trees with very similar topologies (Fig. 3). Neither amplicon contained sufficient signal to
216 return the CMTV-like ranaviruses as the monophyletic group that is obtained when using
217 much larger datasets (Price 2016). The amplicon sequences of both assays incorrectly placed
218 ToRV1 in the FV3-like clade but this isolate is a mosaic of two highly divergent ranavirus
219 types which requires whole genome data to resolve its true position (Price 2016). The shorter

220 Meng PCR amplicon exhibited some loss of resolution relative to the Mao PCR amplicon,
221 especially in distinguishing CMTV-like viruses, but otherwise the two trees were highly
222 concordant and both amplicons yielded accurate information that could be used to infer the
223 ranaviral group (ATV-like, CMTV-like, FV3-like).

224 **4. DISCUSSION**

225 Ranaviruses are important pathogens which cause an emerging disease, spread by humans in
226 some cases, that has had community-level impacts on amphibians (Picco & Collins 2008,
227 Schloegel et al. 2009, Price et al. 2014, 2016, 2017). Sensitive and reliable diagnostics are
228 required to yield accurate estimates of incidence and prevalence. We found considerable
229 variation in the performance of four molecular assays that are routinely used for ranavirus
230 diagnostics (both surveillance of wild populations and experimental laboratory studies).

231 Assays varied in their suitability for ranavirus detection based on the viral genotype: in
232 particular, one of the Meng PCR primers contained multiple mismatches to a whole group of
233 amphibian-associated ranaviruses, the ATV-like viruses, affecting the optimal temperature
234 for primer annealing and increasing the probability of false negative results. Assays also
235 varied in their diagnostic sensitivity and specificity as well as precision of results among
236 replicates. The Leung qPCR showed 100% comparative diagnostic sensitivity and specificity,
237 but the Mao PCR showed poor diagnostic sensitivity against our set of field-collected
238 samples and the Brunner qPCR also resulted in false negatives as well as potential false
239 positives. Although the Leung qPCR performed well in all experiments, neither qPCR assay
240 allowed sequencing of amplicons as confirmation of results (OIE 2017) or as a basis for
241 inferences about viral genotype (Black et al. 2017), meaning that alternative assays (or the
242 use of a second assay) may be required when working with samples from regions or species
243 where ranavirus has not been previously detected. In this respect, the Meng nested PCR has

high diagnostic sensitivity and yielded reasonable phylogenetic signal compared to the Mao PCR, enabling viral isolates to be roughly placed within the major groups of amphibian-associated ranaviruses (Black et al. 2017), although it appeared to be poorly suited for amplification of a whole group of ranaviruses due to mismatches in primer regions.

The use of an inappropriate assay that yields false negative results is a significant problem for estimates of the current ranavirus distribution. Low intensity infections of individuals as well as whole populations/communities where ranavirus occurs at low prevalence may be falsely reported as negative, biasing estimates of ranavirus distribution. This is important for three main reasons: 1) patchy distributions can be taken as evidence of long-distance dispersal and can favor the inclusion of agents outside of the straightforward host-pathogen interaction in conceptual models explaining pathogen distributions. 2) If, for example, assay sensitivity is only sufficient for detecting infections from visceral organs of individuals experiencing active disease, perceptions of the impact, host range and virulence of these viruses could be biased, which may influence the focus of both management approaches and research effort. 3) Assays with poor sensitivity could also bias understanding of the genetic diversity among ranaviruses (in the context of virulence and other key viral life history traits) if some genotypes have low transmission rates or increased latency for example. As with false negatives, false positives bias estimates of distribution and impact but might also have important consequences for conservation and commercial activities. Considering amplification of any replicate indicative of infection led to probable false positives during this study, whereas using a majority-rules| approach reduced this, indicating it to likely be a more accurate approach, which should be adopted as standard. Given its OIE status, there are pressures to consider ranavirus presence when undertaking translocations of herpetofauna for both commercial and conservation purposes. False positive results could interfere with

268 important economic activities such as the culture and supply of amphibians for food or
269 conservation actions.

270 In spite of the notifiable status of ranavirus disease and OIE guidelines for diagnostic tests,
271 there is currently no standardized approach to diagnostics (OIE 2017, Black et al. 2017).
272 Many molecular assays have been used for ranavirus detection, often without checks on the
273 appropriateness or power of the assays (Jaramillo et al. 2012, Black et al. 2017). Our study
274 highlights the importance of selecting and optimizing methods that are compatible with study
275 aims.

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404 **Tables**

405 **Table 1.** Primer and probe sequences of ranavirus diagnostic PCRs compared for sensitivity
406 and specificity.

Assay	Amplicon size (bp)	Oligo ID	Sequence (5'->3')	Strand	Position in coding sequence
Mao PCR	531	MCP 4	GACTTGGCCACTTATGAC	Sense	43-60
		MCP 5	GTCTCTGGAGAAGAAGAA	Anti-sense	556-573
Meng (nested) PCR	320	MCP-IF	TCGCTGGTGTTGCCTATCAT	Anti-sense	457-476
		MCP-IR	CTGCCAAGATGTCGGGTAAC	Sense	158-177
Brunner qPCR	70	rt-MCP-for	ACACCACCGCCCAAAAGTAC	Sense	1298-1317
		rtMCP-rev	CCGTTCATGATGCGGATAATG	Anti-sense	1347-1367
		rtMCP-probe	CCTCATCGTTCTGGCCATCAACCAC	Sense	1320-1344
Leung qPCR	97	MCP_F	GTCCTTTAACACGGCATACCT	Sense	381-401
		MCP_R	ATCGCTGGTGTTGCCTATC	Anti-sense	459-477
		MCP_probe	TTATAGTAGCCTR*TGCGCTTGGCC	Anti-sense	432-455

407 **R* is the IUPAC nucleotide ambiguity code for the bases Adenine or Guanine

408

409

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411

412 **Table 2**. Single nucleotide polymorphisms (SNPs) within regions of the major capsid protein
413 (MCP) gene complementary to primer and probe sequences. Full names and accession
414 numbers for virus isolates are provided in Table S1.

Assay	Oligo ID	Isolate	SNP count
Mao PCR	MCP 4	Not applicable	100% identical to all isolates
	MCP 5	Not applicable	100% identical to all isolates
Meng PCR	MCP-IF	SERV	1
	MCP-IR	SERV	2
		Rmax	2
		CodIV	2
		TFV	1
		EHNV	2
		ESV	2
		ToRV1	4
		ATV	2
Brunner qPCR	rt-MCP-for	SERV	1
		PPIV	1

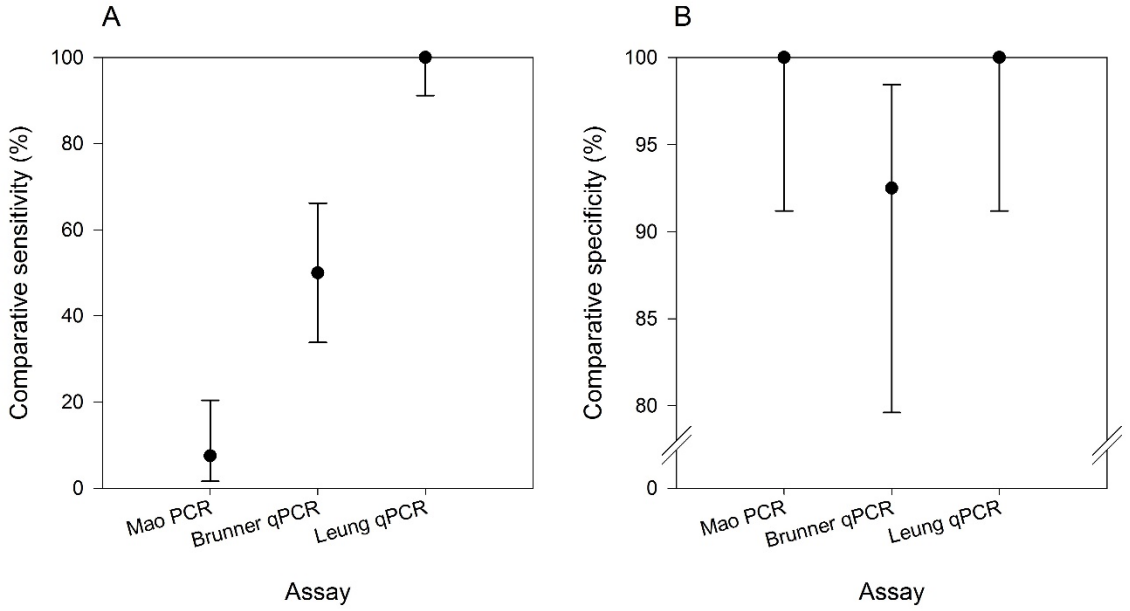
	rtMCP-rev	Not applicable	100% identical to all isolates
	rtMCP-probe	SERV	1
		GGRV	1
		PPIV	1
Leung qPCR	MCP_F	SERV	3
		ESV	1
	MCP_R	SERV	1
	MCP_probe	SERV	1
		GGRV	1
		ATV	1
		BIV	1

Table 3. Limit of detection of four PCR methods for detecting ranavirus isolates from two major groups of amphibian-associated ranaviruses (frog virus 3-like [FV3] or common midwife toad virus-like [CMTV]). DNA extracted from cultured isolates was diluted from 1 to $1e^{-10}$.

Isolate	Limit of Detection			
	Mao	Meng	Brunner	Leung
FV3	$1e^{-6}$*	$1e^{-6}$*	$1e^{-6}$	$1e^{-8}$
CMTV	$1e^{-6}$*	$1e^{-6}$	$1e^{-6}$	$1e^{-8}$

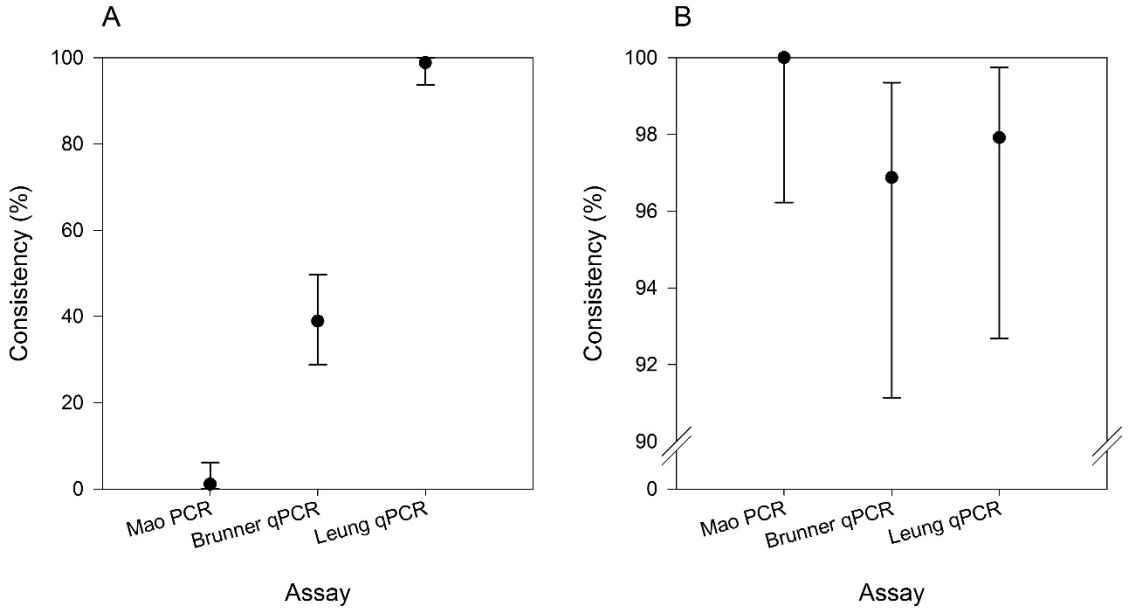
* amplification in one of two replicates at the dilution given

444 **Figures**



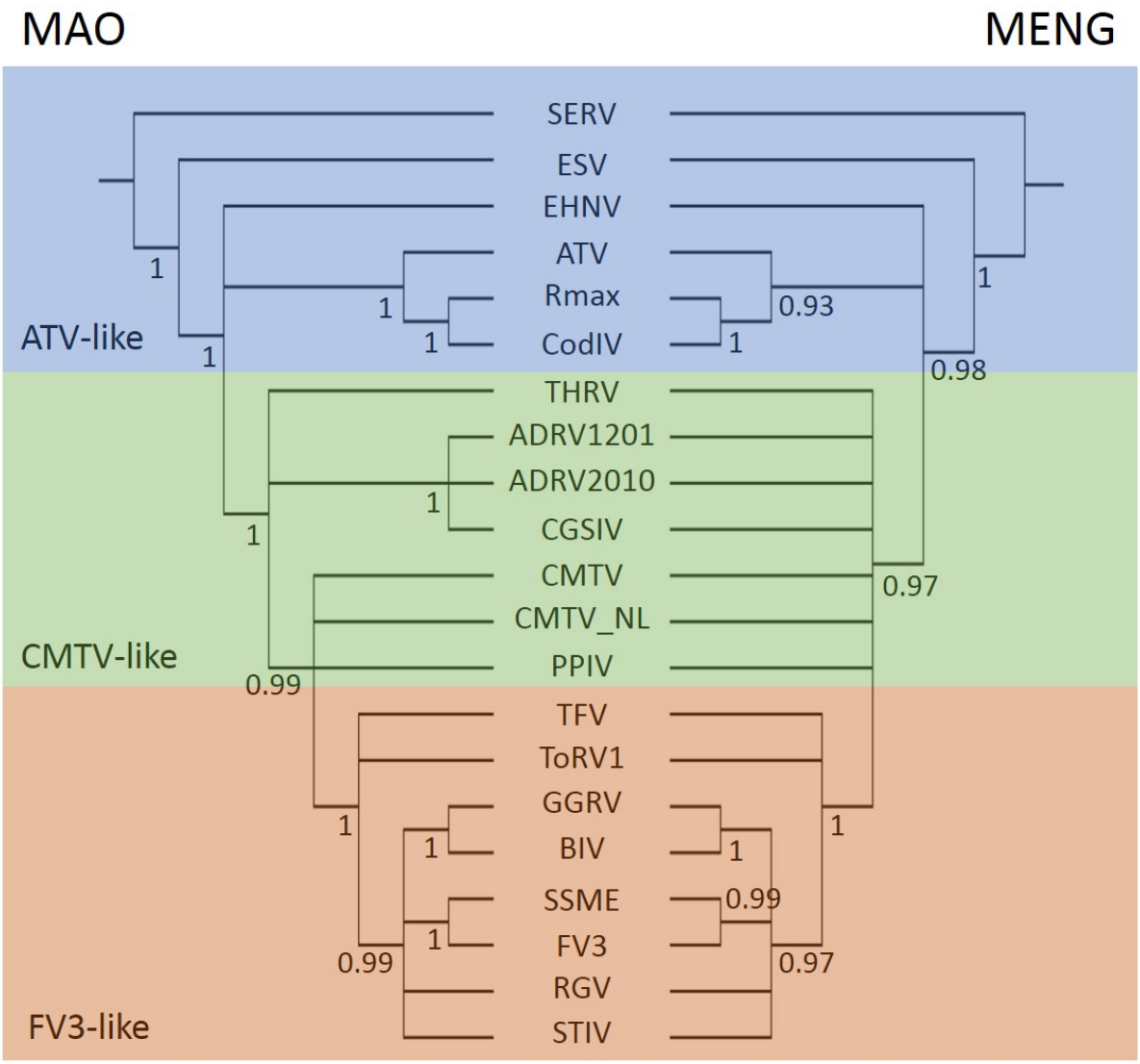
445

446 **Figure 1. Relative performance of four molecular assays in detecting ranavirus from**
447 **non-lethal samples of Costa Rican amphibians**. Assay performance is relative to a nested
448 PCR assay (Meng et al. 2013) used as a 'gold standard'. a) Relative sensitivity tested against
449 known positives, b) Relative specificity tested against known negatives. 95% confidence
450 intervals shown.



451

452 **Figure 2. Comparison of the capacity of ranavirus molecular assays to generate**
453 **consistent results among replicate reactions (intra-assay precision).** a) Proportion of
454 replicates that returned the expected result with known positive samples. b) Proportion of
455 replicates that returned the expected result with known negative samples. 95% confidence
456 intervals shown.



457

458 **Figure 3. Comparison of the phylogenetic signal contained in sequences from PCR**
459 **amplicons generated by two PCR methods (Mao PCR [531 bp] and Meng PCR [319**
460 **bp]).** Both assays target the viral major capsid protein gene and an alignment of the full locus
461 for amphibian-associated ranaviruses, with published complete genomes, was trimmed to the
462 lengths of the respective PCR amplicons. Trees were constructed using default settings in Mr
463 Bayes and drawn as cladograms (see main text). Support values at nodes are posterior
464 probabilities. Full names of viruses, GenBank accession numbers and citations are provided
465 in Table S1.