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Evidence for emergence of an amphibian iridoviral disease because of human-enhanced spread

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Abstract

Our understanding of origins and spread of emerging infectious diseases has increased dramatically because of recent applications of phylogenetic theory. Iridoviruses are emerging pathogens that cause global amphibian epizootics, including tiger salamander (*Ambystoma tigrinum*) die-offs throughout western North America. To explain phylogeographical relationships and potential causes for emergence of western North American salamander iridovirus strains, we sequenced major capsid protein and DNA methyltransferase genes, as well as two noncoding regions from 18 geographically widespread isolates. Phylogenetic analyses of sequence data from the capsid protein gene showed shallow genetic divergence (< 1%) among salamander iridovirus strains and monophyly relative to available fish, reptile, and other amphibian iridovirus strains from the genus *Ranavirus*, suggesting a single introduction and radiation. Analysis of capsid protein sequences also provided support for a closer relationship of tiger salamander virus strains to those isolated from sport fish (e.g. rainbow trout) than other amphibian isolates. Despite monophyly based on capsid protein sequences, there was low genetic divergence among all strains (< 1.1%) based on a supergene analysis of the capsid protein and the two noncoding regions. These analyses also showed polyphyly of strains from Arizona and Colorado, suggesting recent spread. Nested clade analyses indicated both range expansion and long-distance colonization in clades containing virus strains isolated from bait salamanders and the Indiana University axolotl (*Ambystoma mexicanum*) colony. Human enhancement of viral movement is a mechanism consistent with these results. These findings suggest North American salamander ranaviruses cause emerging disease, as evidenced by apparent recent spread over a broad geographical area.

Keywords: *Ambystoma tigrinum*, emerging disease, iridovirus, phylogeography

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Introduction

Emerging infectious diseases have captivated global attention because of recent events such as the spread of West Nile virus throughout North America and the rapid global spread of SARS (severe acute respiratory syndrome). To guide treatment strategies and eradication programs, it is critical to understand the origins and mechanisms of pathogen spread resulting in disease emergence. Emerging infectious diseases are broadly defined as those that are recently recognized, new in a population, or rapidly increasing in incidence, virulence or geographical range (Daszak *et al*. 1999).

Phylogeographical analyses can help identify the potential causes of disease emergence by, for example, revealing potential source hosts. A well-known recent application of this approach helped determine that SARS likely came from animals in the Chinese marketplace by switching hosts

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(Guan *et al*. 2003). Research can then focus on mechanisms by which the pathogen attacks the original hosts, as well as on evolved host defenses to identify potential treatment strategies. For example, the discovery of simian origins of HIV through phylogenetic analyses has been particularly helpful in guiding research programs and in developing potential treatment strategies (Hooper *et al*. 2000; Rambaut *et al*. 2001; Zimmer 2001).

Another key application of phylogenetic methods is testing whether epidemiologic patterns of disease spread are recent or occurred more distantly in the past. Distinguishing current vs. historical spread is particularly relevant for iridoviruses, which cause emerging diseases implicated in global decline in amphibian population (Collins & Storfer 2003; Collins *et al*. 2003). If iridoviruses emerged recently, perhaps by jumping hosts, management strategies can incorporate reduced or restricted movement of the source (original) host. Alternatively, if iridoviruses spread in the past, research efforts might focus on cofactors that suppress amphibian immunity as a cause of recent epizootics.

Iridoviruses are a globally distributed family infecting invertebrates, fish, amphibians and reptiles, but are incapable of infecting endotherms (Chinchar 2002). The family Iridoviridae is divided into four genera: two genera that infect invertebrates (*Iridovirus* and *Chloridovirus*) and two that infect vertebrate hosts (*Lymphocystis* and *Ranavirus*) (Williams *et al*. 2000). *Ranavirus* genomes are large, linear, double-stranded, icosahedral DNA molecules (*c*. 100–150 kb) circularly permutated, and terminally redundant (Goorha & Murtri 1982; Goorha 1995; Williams *et al*. 2000).

Iridoviruses in the genus *Ranavirus* have been associated with amphibians in Great Britain, China, Venezuela, and Australia (Collins *et al*. 2003). In North America, although chytrid fungi are most closely associated with amphibian declines, ranaviruses were isolated from the majority of amphibian mortality events between 1996 and 2001 (Green *et al*. 2002). Ranaviruses caused widespread tiger salamander (*Ambystoma tigrinum*) epizootics in Saskatchewan and Manitoba, Canada [Regina Ranavirus (RRV)] (Bollinger *et al*. 1999), southern Arizona [*Ambystoma tigrinum* virus (ATV)] (Jancovich *et al*. 1997), North Dakota, Colorado, and Utah, USA (Docherty *et al*. 2003). Die-offs among several species of frogs and salamander populations in Massachusetts, Tennessee, and North Carolina were also associated with ranaviruses (Green *et al*. 2002).

The geographical distribution of iridoviruses and particularly their effects on tiger salamander populations throughout western North America has led to the question: Is the spread of pathogen that caused disease emergence recent? Conversely, have the viruses been present since prehuman settlement of the American West, and thus an environmental change (e.g. build-up of pollutants that compromise amphibian immunity) had led to recently observed epizootic events throughout western North America?

We used phylogeographical analyses to answer these questions. We sequenced four regions of the viral genome, including: the gene encoding major capsid protein (MCP) — a structural gene recently used to differentiate Australian, European, and American ranaviruses (Marsh *et al*. 2002); DNA methyltransferase gene; and two intergenic spacer regions from iridovirus strains isolated from 16 salamander epizootic events throughout western North America and two human-associated strains (one from a rearing facility and one from a laboratory).

Our goals were to determine relationships among tiger salamander *Ranavirus* isolates and their relatedness to other ranaviruses; test whether single or multiple introductions of tiger salamander *Ranavirus* occurred; and describe the relatedness of tiger salamander iridovirus isolates to distinguish between current or historical spread. Monophyly relative to other ranaviruses and complete lineage sorting of salamander *Ranavirus* strains would indicate past spread. That is, because viruses generally evolve quickly, they should sort to monophyly relatively quickly (Holmes 2004). If, however, there is incomplete lineage sorting and shallow genetic divergence among strains that form a monophyletic clade, a single introduction and recent spread is suggested. Nonmonophyly of salamander ranaviruses relative to ranaviruses isolated from other species would suggest that some salamander strains are more closely related to viruses isolated from other host species rather than other salamander strains. In this case, multiple introductions of ranaviruses to salamanders or host-switches would be indicated. This result would be similar to that of HIV, which is nonmonophyletic. As a result, HIV-1 is thought to have resulted from a host switch of SIV from chimpanzees (Gao *et al*. 1999), and HIV-2 is thought to have jumped to humans from sooty mangabees (Lemey *et al*. 2003).

Methods

Viral isolate collection and isolation

Viral-associated salamander epizootics were sampled at six locations in Arizona, five in Colorado, three in Canada, and one each in Utah, North Dakota, and Indiana (Indiana University Axolotl Colony; Davidson *et al*. 2003) over a 6-year (1996–2002) period (Table 1; Fig. 4). Virus was also isolated from salamanders purchased at a bait shop in Phoenix, AZ, for a total of 18 isolates. All diseased salamanders collected from die-off sites were frozen at –70 °C until processed for virus isolation. Care was taken to ensure no cross-contamination during DNA isolation and polymerase chain reactions (PCR).

We homogenized salamander body wall in Eagle's minimum essential medium (MEM) (Cellgro, USA) supplemented with 2% fetal bovine serum (FBS) (HyClone,

Table 1 Sources, location information and GenBank accession numbers for salamander *Ranavirus* isolates examined in this study

Isolate name* (abbreviation)	Subspecies virus isolated from	Location	County	State	GPS coordinates	Year of die-off	Accession numbers
Ambystoma tigrinum virus (ATV)	*Ambystoma tigrinum stebbinsi*	San Rafael Valley	Santa Cruz	AZ	N 31°24′ W 110°27′	1995	AY150217
Axolotl *Ranavirus* (AXO)	*A. mexicanum*	Indiana University axolotl colony	Bloomington-Monroe	ID	Unknown origin	2001	AF517678 AY548337 AY548327
Cap mountain pond virus (CAP)	*A. t. nebulosum*	Cap Mountain pond	Fremont County	CO	N 38°38′ W 105°24′	2000	AY548301 AY548340 AY548316
Cunningham reservoir virus (CRV)	*A. t. nebulosum*	Cunningham reservoir	Gunnison County	CO	N 38°41′ W 107°2′	2000	AY548303 AY548344 AY548318
Dalmeny virus (DAL)	*A. t. diaboli*	Dalmeny, Saskatchewan	Rural Municipality of Corman Park	SK, Canada	N 52°20′ W 106°45′	2000	AY548304 AY548339 AY548328
Donut tank *Ranavirus* (DOT)	*A. t. nebulosum*	North Rim, Grand Canyon	Coconino	AZ	N 36°34′ W 112°12′	2000	AY548305 AY548331 AY548319
Guffy pond virus (GRV)	*A. t. nebulosum*	Guffy Pond	Fremont County	CO	N 38°37′ W 105°22′	2001	AY548306 AY548342 AY548320
Joe's mud hole (JMH)	*A. t. nebulosum*	North Rim, Grand Canyon	Coconino	AZ	N 36°34′ W 112°12′	2000	AY548313 AY548332 AY548321
Manitoba virus (MAN)	*A. t. diaboli*	White Water Marsh, Manitoba	Rural Municipality of Winchester	MB, Canada	N 49°14′ W 100°10′	1998	AY548314 AY548330 AY552612
Mud Lake virus (COV)	*A. t. mavortium/ nebulousm (hybrid)*	Mud Lake County	Boulder	CO	N 39°58′ W105°31′	2000	AY548302 AY548341 AY548317
North Dakota virus (ND)	*A. t. diaboli*	Waterfowl production area	Burleigh County	ND	N 46°69′ W 100°28′	1998	AY552611 AY548345 AY548315
Orchinik *Ranavirus* (ORV)	*A. t. mavortium?*	ASU animal care facility	Maricopa	AZ	Unknown– (purchased from bait shop)	2000	AY548307 AY548338 AY548322
Pat's pond virus (PPV)	*A. t. nebulosum*	Pat's pond	Gunnison County	CO	N 38°54′ W 107°12′	2000	AY548308 AY548343 AY548323
Prescott *Ranavirus* (PRV)	*A. t. nebulosum*	Poland Junct., Prescott Valley	Yavapai	AZ	N 36°31′ W 112°12′	1999	AY548309 AY548333 AY548324
Regina *Ranavirus* (RRV)	*A. t. diaboli*	Regina, Saskatchewan	Rural Municipality of Grand Coulee	SK, Canada	N 50°30′ W 104°49′	1997	AF080218 AF368229 AF368228
Snipe Lake *Ranavirus* (SLR)	*A. t. nebulosum*	North Rim, Grand Canyon	Coconino	AZ	N 36°31′ W 112°12′	2000	AY548310 AY548334 AY548325
Sulphur Springs virus (SSV)	*A. t. mavortium*	White water draw	Cochise County	AZ	N 32°03′ W 109°29′	1998	AY548311 AY548335 AY548326
Utah *Ranavirus* (UT)	*A. t. nebulosum*	Lake Desolation	Wasatch Mtns	UT	N 40°39′ W 111°36′	1998	AY548312 AY548336 AY548329

*All isolate names are ATV-abbreviation-isolate number-year, according to ICTV requirements; however, for simplicity, only the abbreviation names will be referenced in the text.

USA), centrifuged the material at 5000 × g for 10 min, and filtered the supernatant through 0.45 μm membranes. *Epithelioma pyprini cyprini* (EPC) cells were infected at 18 °C for 1 hour with 100 μL of tissue homogenates, and culture flasks (75 cm^2) were rocked every 10 min. After one hour, MEM with 10% FBS was added to the flasks, and cells were incubated at 18 °C–21 °C. When the cell monolayer was completely lysed, virus and infected cells were harvested and processed for PCR. A virus isolated from *Ambystoma mexicanum* from the Indiana University Axolotl Colony was also included (Davidson *et al*. 2003), as was frog virus 3 (FV3), which was obtained from the American Type Culture Collection (VR-567).

DNA isolation

We isolated viral DNA from infected cells using a modified Hirt (1967) procedure. Five 75 cm^2 flasks of *E. p. cyprini* cells were infected with each viral isolate at infection multiplicity of approximately 0.1 plaque forming unit/cell. After cytopathic effects reached approximately 80%, cells were centrifuged at 16 000 × g for 60 min, and the pellet containing cells and virus was resuspended in 2.5 mL of hypotonic lysis buffer (10 mM Tris-HCl, pH 8, 10 mM EDTA, 0.5% NP40, 0.25% sodium deoxycholate) and incubated on ice for 5 min. The mixture of lysed cells and virus was centrifuged at 3000 × g for 10 min at 4 °C to remove cellular debris. The supernatant was transferred to a new tube and sodium dodecyl sulphate (SDS) and Proteinase K were each added to achieve final concentrations of 0.1% and 1%, respectively, and incubated at 50 °C for 1 h. We then added an additional 1 mg/mL Proteinase K and incubated the mixture at 50 °C for 2 h or 16 h at 37 °C. DNA was extracted with phenol:chloroform:isoamylalcohol (26:24:1) and ethanol precipitated overnight at –20 °C with 0.3M sodium acetate. DNA was concentrated by centrifugation at 13 000 × g for 10 min, washed twice with ice cold 70% ethanol, and resuspended in nanopure water. We then treated resuspended DNA with 40 μg/mL of RNaseA (Promega, USA) at 37 °C for 30 min.

Polymerase chain reaction and sequencing

The 5′ end of the MCP was amplified by PCR using primers #4 (5′-GAC TTG GCC ACT TAT GAC-3′) and #5 (5′-GTC TCT GGA GAA GAA GAA-3′) developed by Mao *et al*. (1996) for FV3. A 187 bp region of the methyltransferase gene (Kaur *et al*. 1995) was amplified using primers Mtase reverse (5′-AGA GTA GGC GCA GTA GTC GAC GAC G-3′) and forward (5′-TGT GGG CCA GTC CCC CGT GCA G-3′). Comparison of the complete genomic sequence of ATV with approximately 40% of the genome of the RRV isolate revealed greater than 95% overall identity, but also some variable intergenic spacers (Jancovich *et al*. 2003). We examined two such variable regions: one that contained a different number of 16 bp repeats between the two genomes and a 100-bp region deleted in RRV as compared to ATV. The 16-bp repeat region was located between bp 6448 and 6707 of RRV clone *Pst*I-6.7 and bp 55 249 and 55 424 of the genomic sequence of ATV. The 100-bp deletion was found between bp 474–653 of RRV clone *Pst*I-1.3 and bp 64 659 and 64 836 of ATV genome (Jancovich *et al*. 2003). We designed primers 16F (5′-AGA AAT CTT GCG AGA CCG-3′) and 16R (5′-AAC TGT TGG TGA TTG CAG C-3′) to produce a PCR product of approximately 250 bp that encompassed the 16-bp repeat region and primers 100F (5′-CTT TAC TCT CCA TAT CGC C-3′) and 100R (5′- AAG ACT GGT GTC TCG TGG-3′) to amplify a 280-bp region that contained the 100-bp deletion.

PCR reactions were performed with 50–100 ng of template DNA, 250 nmol of each primer, 0.6 mM $MgCl_2$, 250 μM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25 °C), 0.1% Triton® X-100, and one unit of *Taq* polymerase (Promega, USA) at 94 °C (2 min) denaturing, 50 °C (2 min) annealing, and 72 °C (3 min) extension, for 25 cycles. PCR products were visualized by electrophoresis on 0.7% agarose gels, except for the 16-bp polymorphism products, which were visually compared using 3% Metaphor® agarose (BioWhittaker Molecular Applications). PCR products were gel purified and sequenced using automated equipment (ABI 377) (Sanger *et al*. 1977).

Phylogeographical analyses

Our viral isolates were compared with other ranaviruses present in the GenBank by multiple alignment of the capsid protein and methyltransferase sequences using CLUSTAL X (Thompson *et al*. 1994). We used PAUP*4b10 (Swofford 1998) for phylogenetic analyses and conducted neighbour-joining (Saitou & Nei 1981) maximum parsimony, and Bayesian analyses on major capsid protein sequences. Here, unrooted trees were used because all viral isolates were within the genus *Ranavirusi;* rooting the tree with members outside the genus is problematic because of low homology (often below 50%) and thus, alignment difficulties. MODELTEST 3.06 (Posada & Crandall 1998) was used to determine the most appropriate model of evolution for Bayesian analyses. This model was then used to generate trees in MRBAYES (Ronquist & Huelsenbeck 2003), where five chains were used, and 10 000 000 generations were run with uniform priors. After determining the generation at which the chains reached stationarity of log likelihood values (i.e. 'burn in'), trees were then run in PAUP*4.0 to generate a 50% majority rule consensus tree and posterior Bayesian probabilities for internal nodes. For neighbour-joining, we conducted 10 000 bootstrap replicates to determine support for internal nodes. We also conducted 10 000 bootstrap replicates with random addition and 10 addition

sequences per replicate for unweighted parsimony analyses. To compare our viral isolates among themselves, we first conducted partition homogeneity tests in PAUP* to determine if the capsid protein sequences and the two polymorphic repeat deletion region partitions were significantly different from random partitions (Farris *et al*. 1995). Next, we conducted phylogenetic analyses as above, except that FV3 (the type *Ranavirus*) was used as the outgroup because of its high homology with tiger salamander isolates. For parsimony analyses, because of a variable number of 16-bp repeats, gaps were coded as a single event (reflecting the size of the deletion) rather than as a fifth character.

To differentiate between recent introduction and spread of North American tiger salamander iridoviruses vs. more ancient spread and disease emergence because of environmental changes or recent changes in pathogenicity, we conducted a nested clade analysis (Templeton *et al*. 1995; Templeton 1998, 2004) of tiger salamander virus 'supergenes' formed from concatenated sequences used above. First, we created a haplotype network using TCS 1.13 (Posada *et al*. 2000). Individual haplotypes within this network were combined to form nested clades following rules in Templeton & Sing (1993). In short, haplotypes that had DNA sequences that differed by a single mutation were nested together in one-step clades, moving from the tips (exterior) to the interior of the haplotype network. In the case where no haplotypes occurred within one mutational step of a sampled viral haplotype, a '0' was used to designate an unsampled or nonexistent haplotype. Following nesting of all haplotypes into one-step clades, haplotypes in which DNA sequences differed by two mutational steps were nested in two-step clades, and haplotypes separated by three mutational steps were nested in three-step clades. Nesting continued until all haplotypes were included.

This haplotype network was then inputted, along with latitude-longitude data for each sampling locality (Table 1), into GEODIS 2.0 for the nested clade analysis (Posada *et al*. 2000). In a nested clade analysis, grouping of data based purely on DNA sequence is then compared with geographical spacing of haplotypes on the landscape to determine if there have been patterns of historical or current gene flow. Tests for geographical associations of haplotypes treat each geographical location as a categorical variable. Exact permutational contingency analyses (with 1000 permutations) of categorical variation in nested fashion were computed as in Templeton & Sing (1993). Geographical distances between sampling sites are calculated from latitude-longitude data input into GEODIS, and two distances are calculated. The first distance, D_C, measures how geographically widespread the individuals are that constitute each particular clade (Templeton *et al*. 1995). The second distance, D_N, is calculated based on the geographical distances of all individuals (with haplotypes that place them) in a particular clade from the geographical centre of another such clade (Templeton *et al*. 1995). When D_C, D_N, or both measures are significantly large or significantly small based on permutation tests, inferences can be made about whether the geographical pattern of haplotypes was representative of current or historical evolutionary processes. The inference table in Templeton (1998, 2004) was used to make determinations about such processes in nested clades.

Results

Sequencing analyses

When we aligned 514 bases of the MCP from the salamander *Ranavirus* isolates with available *Ranavirus* sequences from GenBank, we found little variation among salamander *Ranavirus* sequences. There are nine amino acid changes when comparing the sequence of ATV with that of FV3 (95.9% identity), and only one amino acid difference between ATV and RRV. The ATV, from San Rafael Valley, AZ, has identical MCP sequence to isolates CRV and PPV (from south-central Colorado). Isolates ATV, CRV, PPV, RRV (from Saskatchewan, Canada) and UT (from Utah) have an amino acid change at position 66 that is identical to FV3, but is not observed in the remaining viral isolates. Six viral isolates from Arizona (DOT, JMH, ORV, PRV, SLR, SSV), three from Colorado (CAP, COV, CRV), and one from Indiana University (AXO) have identical amino acid sequences.

Contrary to the MCP data, which showed some variability (< 1% divergence) among tiger salamander viral isolates, all salamander viruses examined have identical nucleotide sequence in the methyltransferase gene, and thus were uninformative for phylogeographical analyses. Alignment of the salamander *Ranavirus* methyltransferase amino acid sequence with those available in GenBank revealed 97.8% identity with European catfish virus, 96.7% with FV3 and 96.1% with *Rana tigrinum* viruses, and approximately 65% identity with doctorfish virus and largemouth bass viruses. However, because of the lack of nucleotide sequence data in the GenBank, along with the smaller number and non-overlapping taxa relative to the available MCP data, it is difficult to compare the two datasets.

The intergenic sequence data yielded more variability than the methyltransferase gene. There were four isotypes among salamander viral strains in the region containing 16-bp polymorphism. These isotypes were differentiated by insertion or deletion of a 16-bp repeat (5'-AATACTATTATCTTAA-3') and characterized by four distinct sizes: 262 bp (isotype 1), 246 bp (isotype 2), 230 bp (isotype 3), and 212 bp (isotype 4). The type ranavirus (FV3), Canadian isolates (RRV, MAN), and the North Dakota (ND) isolate were isotype 1. Isotype 2 was found in the Arizona isolates

(ATV, DOT, JMH, PRV, SLR, SSV), and the Utah (UT) isolate. Isolates collected from the bait shop in Phoenix, AZ (ORV), in Canada (DAL), in Colorado (CAP, COV, GRV), and in the Indiana University (AXO) were isotype 3. The two Colorado isolates (CRV, PPV) were isotype 4.

In the second intergenic region, alignment of the 280 bp sequences showed a 100-bp deletion among two Canadian isolates (RRV, MAN) and the one from North Dakota (ND), relative to the rest of the isolates. The third Canadian isolate (DAL), however, did not contain the 100-bp deletion. Differences in PCR product size was easily determined using 0.7% agarose gel electrophoresis. Several single nucleotide polymorphisms were also detected among isolates after sequence alignment. Three Arizona isolates from the Kaibab plateau (DOT, JMH, SLR) have a single substitution (transversion) at position 44 (C-A), relative to the rest of the isolates. The DAL isolate has a single substitution (transition) at position 54 (C-T) when compared with all other isolates. FV3, the type ranavirus, has one deletion and seven nucleotide substitutions relative to the tiger salamander iridovirus isolates. Note that *Ranavirus* isolates classified as isotype 1 for the 16 bp repeat region also had the 100-bp deletion, while isotypes 2–4 lacked the 100 bp deletion.

All new viral isolate sequences (for MCP, the 16 bp repeat and the 100 bp deletion) were deposited to GenBank (accession nos. AY548301–AY548345), as well sequences from Jancovich *et al*. (2003) and Docherty *et al*. (2003).

Phylogeographical analyses

Using PAUP*4b10, we constructed a gene genealogy of iridoviruses using MCP sequence data from salamander isolates and available homologous *Ranavirus* MCP sequences acquired from GeneBank (Fig. 1). Based on MODELTEST results, the most appropriate model of evolution corresponded with that of the HKY85 + Γ (Hasegawa *et al*. 1985; Yang 1994), which was used for Bayesian analyses, and which took approximately 7000 generations to reach stationarity in log-likelihood scores. The tree topology was consistent, regardless of search method (Fig. 1). The viruses associated with salamander mortalities formed a monophyletic clade, supported by bootstrap values of 94% and 86% from parsimony and neighbour-joining, respectively, and a posterior probability of 92% from Bayesian analyses (Fig. 1).

Viruses not isolated from salamanders (i.e. fish, other amphibians, and reptiles) are nonmonophyletic. MCP data suggest that salamander virus isolates may be more closely related to viruses isolated from sport fish, including epizootic haematopoietic necrosis virus [EHNV — from redfin perch, *Perca fluviatilis* (Langdon *et al*. 1986), and rainbow trout, *Onchorhynchus mykiss* (Langdon *et al*. 1988)] and viruses isolated from largemouth bass (Mao *et al*. 1999) and grouper (*Epinephelus tauvina*; Qin *et al*. 2003) than other

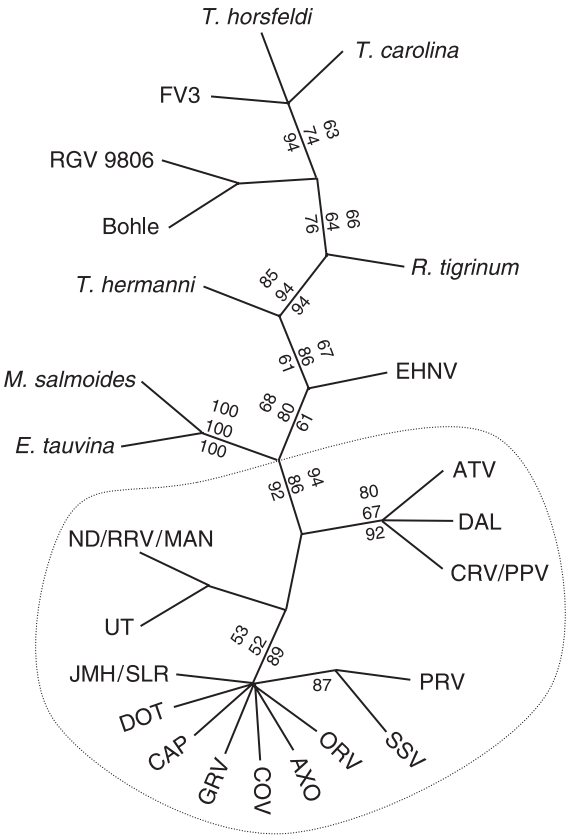

Fig. 1 Unrooted phylogenetic trees of 514 nucleotides of major capsid protein sequence from salamander iridovirus isolates and other *Ranavirus* isolates. Upper numbers at internal tree nodes are bootstrap support values for unweighted maximum parsimony analysis (50% majority rule consensus; 10 000 bootstrap replicates; length = 257; consistency index = 0.930), middle numbers are bootstrap support values for neighbour-joining analyses, and bottom numbers are Bayesian (50% majority rule consensus of 48978 trees) posterior support values. Dashed circle outlines salamander isolates; salamander isolate abbreviations follow designations in Table 1 and are as follows: ATV — *Ambystoma tigrinum* virus (Santa Cruz Co., AZ); AXO — *Axolotl* ranavirus (Indiana University); CAP — Cap Mountain Pond (Fremont Co., CO); COV — Mud Lake (Boulder Co., CO); CRV — Cunningham Reservoir (Gunnison Co., CO); DAL — Dalmeny (Saskatchewan, Canada); DOT — Donut Tank (Coconino Co., AZ); GRV — Guffy Pond (Fremont Co., CO); JMH — Joe's Mud Hole (Coconino Co., AZ); Manitoba virus (Manitoba, Canada); ND — North Dakota (Burleigh Co., ND); ORV — Orchinik (ASU, Maricopa Co., AZ); PPV — Pat's Pond (Gunnison Co., CO); PRV — Prescott (Yavapai Co., AZ); RRV — Regina (Saskatchewan, Canada); SLR — Snipe Lake (Coconino Co., AZ); SSV — Sulphur Springs (Cochise Co., AZ); UT — Utah (Wasatch Mts., UT). Non-salamander ranavirus strains include (with GenBank Accession numbers and references following in parentheses): Bohle (AY187406; March *et al*. 2002); EHNV (AF157667; Hyatt *et al*. 2000); Frog virus 3 (FV3) (U36913 *et al*. 1995; U15575 *et al*. 1996); grouper (*Epinephelus tauvina*; AF364593; Qin *et al*. 2003); RGV-9806 (AF192509.1; Zhang *et al*. 2001); largemouth bass (*Micropterus salmoides*; AF080250; Mao *et al*. 1999); *Rana tigrinum* (NC_003407; He *et al*. 2002); *Testudo carolina* (TCU82553; Mao *et al*. 1997); *Testudo hermanni* (AF114154; Marschang *et al*. 1999); and *Testudo horsfeldi* (THU82554; Mao *et al*. 1997).

amphibian viruses. Although sequence divergence is high between tiger salamander isolates and bass and grouper viruses (*c*. 25%), salamander isolates share several synapomorphies with these viruses relative to other amphibian isolates [Bohle (Hengstberger *et al*. 1993) e.g. FV3 (Granoff *et al*. 1966), *Rana tigrinum* virus (He *et al*. 2002), and RGV-9806/9807 (Zhang *et al*. 2001)].

When salamander isolates were compared among themselves, partition homogeneity tests did not yield significant differences between the datasets for MCP and both the 16-bp and the 100-bp intergenic regions. Consequently, we combined these data sets into a concatenated 'supergene' and analysed the entire 1069 bp sequence. Overall genetic divergence among salamander isolates was low (< 1.1%); however, there were some well-supported clades (Fig. 2). A Canadian isolate from Dalmeny (DAL — from *A. t. diaboli*) formed a clade with isolates from north-central Arizona (PRV and SSV), and two of four isolates from Colorado (PPV and CRV). Notably, one clade was formed with the bait shop isolate (ORV), and a virus from Colorado (COV) (Fig. 2). This clade was wellsupported, suggesting the virus found in bait may have come from Colorado. In addition, the isolate obtained from Indiana University (AXO), formed a clade with two other strains from Colorado (GRV/CAP). Viruses from Arizona were polyphyletic, with strains from the North Rim of the Grand Canyon (JMH/SLR, DOT) forming a well supported clade with the Colorado strains (GRV/CAP, COV). Colorado strains are also polyphyletic, forming an additional clade with Arizona strains as mentioned above. Viruses from Manitoba, Saskatchewan, and North Dakota (except the Dalmeny isolate) were of the same basal haplotype, and these viruses were all from the subspecies figer salamander *A. t. diaboli*. The isolate from Utah was not well resolved (only one support value — Bayesian — was above 50%; Fig. 2).

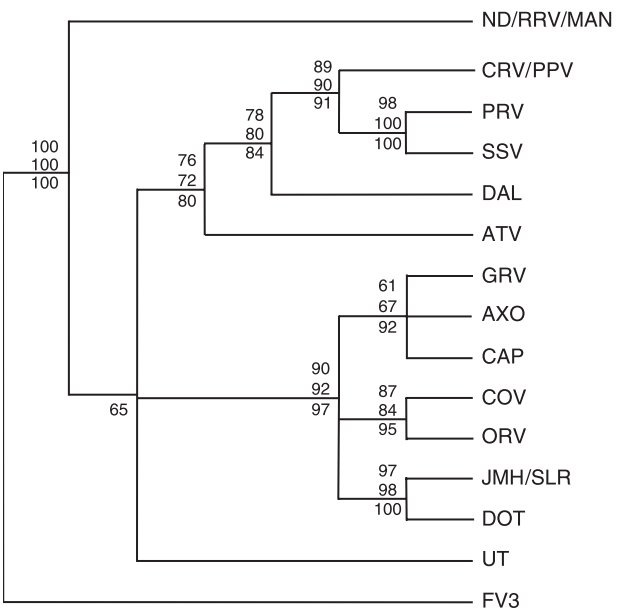

Fig. 2 'Supergene' phylogenetic analysis of concatenated sequences consisting of 1056 nucleotides from 18 tiger salamander iridovirus isolates ranging from Saskatchewan, Canada to southern Arizona, USA. Abbreviations follow designations in Fig. 1 and Table 1. FV3 (frog virus 3) was used as outgroup because it is the type *Ranavirus* isolate and has high homology with tiger salamander strains. In addition, monophyly of tiger salamander isolates was supported in the previous analysis. Upper numbers at internal tree nodes are bootstrap support values for unweighted maximum parsimony analysis (50% majority rule consensus; 10 000 bootstrap replicates; length = 100; consistency index = 0.700), middle numbers are bootstrap support values for neighbour-joining analysis (10 000 replicates), and bottom numbers (below lines) are Bayesian posterior support values (50% majority rule consensus of 6791 trees).

The nested clade analysis (Figs 3 and 4) showed a few major trends. First, there were several 'missing' haplotypes in the study (represented by 'zeros, Fig. 3). That is, several intermediate haplotypes were either not sampled or did not exist. Second, the nested clade analysis in Table 2 shows six clades from which biological inferences could be made. These six clades fulfilled the criteria of having significantly large or small distance values (D_C or D_N) based on permutation tests, as well as containing both interior (internal to the network) and tip (on the outsides of the network) clades in the haplotypes network (Table 2). For clades 1.2 and 1.9, results were inconclusive. Clade 1.4, which consists of haplotypes of virus isolates found in the proximity of the North Rim of the Grand Canyon, showed evidence of restricted gene flow. Clade 2.1 shows evidence of contiguous range expansion; it is notable that both the bait strain (ORV; clade 1.1) and the Indiana University strain (clade 1.2) are in this clade. At the three-step clade level, clade 3.1, which includes these two isolates, as well as isolates from Colorado (COV, CAP, GRV), Saskatchewan and Manitoba (RRV, MAN), North Dakota (ND), Arizona (SLR, JMH, DOT), and Utah (UT), shows evidence of long-distance colonization (Table 2). Clade 3.2, which includes isolates from central (PRV) and south-central (SSV, ATV) Arizona, Colorado (PPV, CRV), and Saskatchewan (DAL), shows results that are indeterminate between long-distance colonization, contiguous range expansion and past fragmentation. The two three-step clades are shown in a map (Fig. 4). Note that there is a disjunct distribution of clade 3.1, which is separated by clade 3.2. That is, haplotypes in clade 3.1 are genetically more similar to one another than those contained in clade 3.2, despite the fact that haplotypes in clade 3.1 are geographically separated by individuals in clade 3.2.

Discussion

Our data support the conclusion that the salamander isolates are one viral quasi-species, supported by low overall divergence in MCP sequence and two intergenic

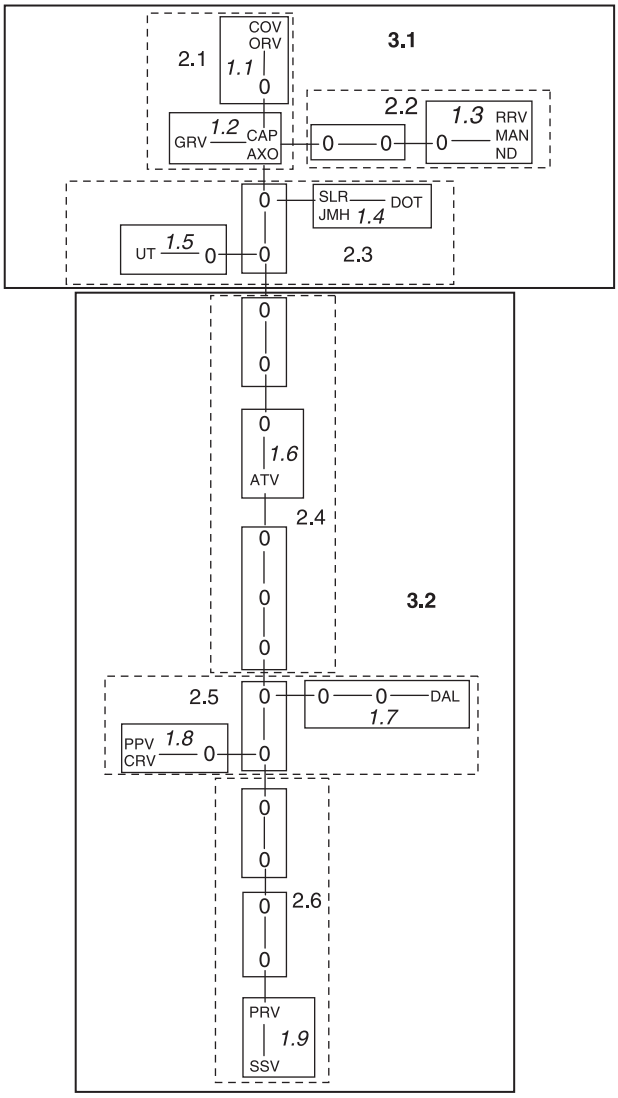

Fig. 3 Nested haplotype design that includes concatenated 'supergene' tiger salamander virus sequences from Fig. 2. Two or more viral isolate designations in the same place means that they share the same haplotype. Each line represents a single mutation. One-step clades include groups of haplotypes that are within one mutational step from one another, and thus their DNA sequence differs by a single mutation. Similarly, two-step clades include groups of haplotypes that are two mutational steps from one another, and three-step clades contain groups within three mutational steps. If a particular virus isolate in this study did not have another virus isolate within one mutational step, an open circle was assigned, which represents a missing or unsampled haploype. One-step clades are illustrated in thin line boxes; two-step clades are contained within dashed-line boxes, and three-step clades are outlined with bold line boxes.

spacers (< 1.1%; Fig. 1), as well as identical methyltransferase sequences. Although the methyltransferase gene was used in previous studies to classify unknown iridoviruses (Mao *et al*. 1999), it was not useful among the salamander ranavirus isolates.

Classification of North American salamander iridoviruses in the genus *Ranavirus* is upheld by a high degree of homology (> 90%) with other ranaviruses in both MCP and methyltransferase genes (Fig. 1) (Williams *et al*. 2000). Further, salamander iridoviruses form a well-supported monophyletic clade within the genus (Fig. 1). Taken alone, the monophyly of tiger salamander viruses suggests either a single introduction and radiation, or complete lineage sorting. However, shallow divergence of concatenated sequences spanning a structural gene and two noncoding regions among tiger salamander isolates from Arizona to Saskatchewan suggest a single introduction and recent spread. Our results also show a lack of clear geographical pattern and thus, suggest incomplete lineage sorting among phylogeographical groupings of tiger salamander virus strains (Fig. 2). Strains both from Arizona and Colorado are polyphyletic, whereas the strain from Canada forms a clade with viruses from Arizona and Colorado, rather than with other strains from the same region and salamander subspecies. Dating the spread using a molecular clock is problematic because evolutionary rates are not available for salamander iridoviruses or iridoviruses in general. Nonetheless, because we observed monophyly, incomplete lineage sorting, and shallow divergence over a large geographical area, a single introduction and recent spread of tiger salamander *Ranavirus* strains is supported.

Two lines of evidence suggest human involvement in the recent spread of salamander ranaviruses throughout the western US. First, the capsid protein data suggest that salamander isolates are more closely related to those from sport fish than to other amphibian strains; however, statistics that supports this conclusion is not very strong. Alignment of salamander methyltransferase sequences with other available ranavirus sequences in the GenBank shows higher homology (> 97%) compared with other amphibian (tiger frog virus and FV3) and fish (European catfish virus) viruses, and thus do not confirm or reject the above result. Nonetheless, because fish, such as rainbow trout and largemouth bass have been introduced throughout much of western North America (Knapp & Matthews 2000; Kats & Ferrer 2003), it is logical to suggest that ATV may have originated from a fish ranavirus and underwent a host switch to salamanders. Note that the largemouth bass virus was isolated from the southeastern US, whereas the EHNV virus was isolated from rainbow trout from Australia and thus, more North American fish iridovirus isolates and further DNA sequence analysis are needed to test this hypothesis. For example, analysis of the complete MCP gene sequence may allow further phylogeographical resolution. In another study, examination of the entire MCP sequence allowed differentiation of Australian, European, and American ranavirus isolates (Marsh *et al*. 2002).

Second, anthropogenically associated strains cultured from salamanders purchased from a bait shop in Phoenix,

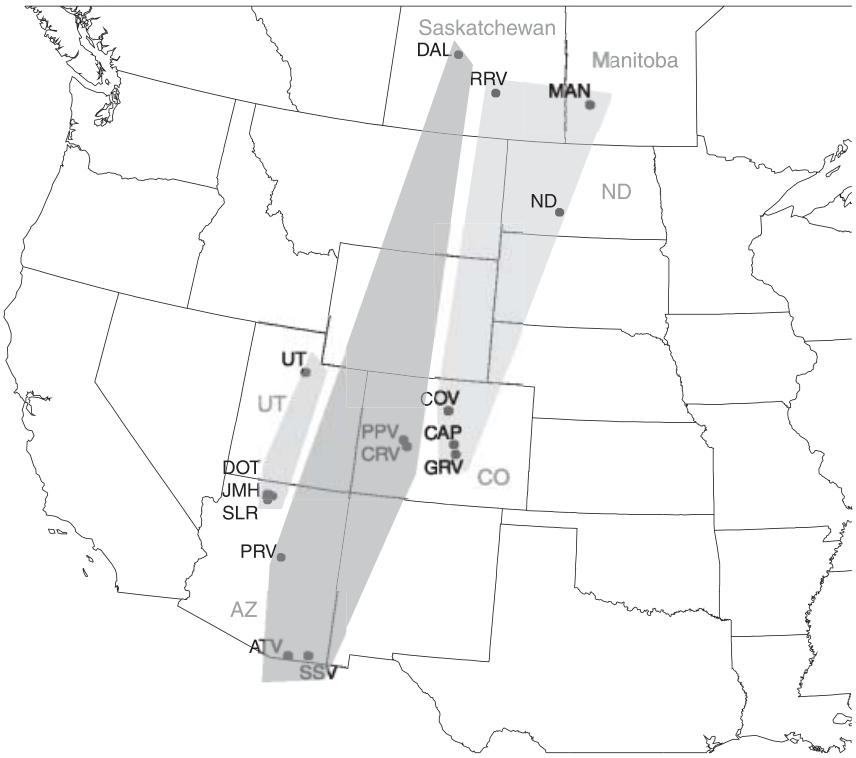

Fig. 4 Map of the two three-step nested clades (as identified in Fig. 3), and thus groups of haplotypes that are within three mutational steps of one another. Black dots indicate sampling localities with abbreviated virus isolate designations. Light text indicates state (US) or province (Canada). Light shading indicates nested clade 3.1 (disjunct; in two sections), and dark shading indicates nested clade 3.2.

Nested clade	Interior haplotypes/clades	D_C	D_N	Inference chain
1.2	CAP/AXO	0	2^S	
	Grv	0	2	
	I-T	0	0^S	1-2-11-12-No: CRE
1.4	SLR/JMH	2	2^L	
	DOT	0	1^S	
	I-T	2	1^L	1-2-11-17-4-No: RGF
1.9	PRV	0	276	
	SSV	0	278^S	
	I-T	0	-2^S	1-2-11-17-No: INC
2.1	1.1	0	100^L	
	1.2	2^S	50	
	I-T	2^S	50^S	1-2-11-12-No: CRE
3.1	2.1	66^S	377^S	
	2.2	221^S	888^L	
	2.3	172^S	703	
	I-T	16	34	1-2-11-12-13-21-Yes: LDC
3.2	2.4	0	780	
	2.5	917	872	
	2.6	13	152^S	
	I-T	674^S	697^L	1-2-11-12-13-14: CRE/LDC/PF

Table 2 Nested clade analysis results for ATV. Clade designations follow the nested design in Fig. 3; haplotype designations follow Table 1 results are only included for clades for which biological inferences could be made [i.e. those that contained both tip and interior nested clades as well as significant clade (D_C) and/or nested clade (D_N) distances]. I-T represents the average interior vs. tip clade distance. A 'S' or 'L' superscript designates a significantly small or significantly large distance measure, respectively. For each clade is an inference chain; the numbers in the inference chain represent the numbers of respective questions posed in Appendix 1 of Templeton (1998, 2004). Movement along the chain continued until a biological inference could be reached based on the final 'yes' or 'no' answer to the question posed. Biological inferences resulting from the 'yes' or 'no' answer were as follows: INC = inconclusive; CRE = contiguous range expansion; RGF = restricted gene flow; LDC = long-distance colonization, PF = past fragmentation

AZ, and from the Indiana University are found within clade 3.1, which shows evidence of long distance colonization (Fig. 4). Note that the strain isolated from moribund *A. mexicanum* from Indiana University (Davidson *et al*. 2003) has the same isotype as the Colorado isolates in 16 bp polymorphic region. The bait isolate (ORV) may also have originated from Colorado because the concatenated sequence was nearly identical to the Colorado strain (COV) from a state park that is easily accessible and frequently used as a recreational site. Alternatively, strains in Colorado may

have originated as a result of human introductions of bait salamanders.

Bait salamanders, commonly called 'waterdogs' (often the barred tiger salamander subspecies, *Ambystoma t. mavortium*) have been introduced throughout the western US (Collins *et al*. 1988). Waterdogs are introduced into non-native habitats by escape from fishing hooks or by human 'bait bucket' into ponds to be recollected and sold, comprising a multimillion dollar industry per annum (Collins *et al*. 1988). Although prohibited in some western states (e.g. Washington and California), bait salamanders are still legal in Arizona. The disjunct distribution of clade 3.1, which occurs in Colorado, northwestern Arizona and Utah, except central Arizona (Fig. 4), could reflect the east–west movement of bait, which commonly originate from in Colorado, Nebraska, and Oklahoma (Collins 1981).

A recent study supports the east–west movement of (bait) *A. t. mavortium* by demonstrating genetic introgression and hybridization with native *Ambystoma californiense* in California (Riley *et al*. 2003). Historical records also show that non-native *A. t. mavortium* have also been introduced into the Sulphur Springs Valley, AZ (source of ranavirus isolate SSV) (Collins 1981; Collins *et al*. 1988). Thus, bait salamander introduction by humans could be responsible for spreading virus around western North America. Although nested clade analyses have recently been the subject of controversy (see Knowles & Maddison 2002), recent reanalyses of multiple data sets have reinforced the value of the approach and helped refine the methods (Templeton 2004). In addition, we believe our approach here is reasonable because we have other ecological data (e.g. known movement of bait salamanders) that give background for interpreting the possible reasons for long-distance colonization.

It is possible, however, that human movement of bait does not explain recent virus movement. Our nested clade (Fig. 3) shows several missing haplotypes, and thus, the disjunct geographical signal may be a result of insufficient sampling or sequence data. Given that we are restricted to collection of iridovirus isolates during epizootics and only when fresh salamander tissue can be obtained, it is possible that we have missed intermediate haplotypes that will allow better resolution of our tree and, possibly alternative outcomes of our nested clade analysis. We are currently gathering more samples to investigate this possibility.

In summary, our data collectively suggest a single introduction and recent spread as the cause of emergence of salamander iridoviruses throughout the western US. The monophyly relative to other tiger salamander strains, low genetic divergence (particularly for a virus across a large geographical distance — Arizona to Manitoba), polyphyly of Arizona and Colorado isolates, and evidence of range expansion and long-distance colonization support recent spread.

There is also an evidence of human involvement based on the possible host-switch from sport fish and the cladistic analyses that show long-distance colonization and a disjunct distribution in clades that contain anthropogenically associated virus strains.

New hypotheses, including the possibility that salamander viruses originated as a host switch from fish, can be tested with additional sequencing and cross-infection experiments. Collectively, these experiments will shed light on the role human-enhancement on viral transport may play in global spread of amphibian pathogens and provide insights on the effects of pathogen transport in general.

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