

SHORT COMMUNICATION

Geographically variable selection in *Ambystoma tigrinum* virus (Iridoviridae) throughout the western USA

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Abstract

We investigated spatially variable selection in *Ambystoma tigrinum* virus (ATV) which causes frequent and geographically widespread epizootics of the tiger salamander, *Ambystoma tigrinum*. To test for evidence of selection, we sequenced several coding and noncoding regions from virus strains isolated from epizootics throughout western North America. Three of the sequenced regions contained homologues for genes putatively involved in host immune evasion and virulence: eIF-2 α , caspase activation and recruitment domain (CARD) and β -OH-steroid oxidoreductase. Selection analysis showed evidence of very strong purifying selection on eIF-2 α , purifying selection within certain viral clades on CARD and positive selection on β -OH-steroid oxidoreductase within certain clades. Analysis using MULTIDIVTIME and Tajima's relative rate tests indicate accelerated rates of evolution within clades associated with anthropogenic movement. These clades also demonstrate greater spatial variability in selection, suggesting a lack of local adaptation (i.e. locally adapted populations should exhibit little to no selection because of absent or reduced variation in fitness once a fitness optimum is reached). Increased transfer of non-native viral strains to naïve salamander populations, in conjunction with local maladaptation as a result of local selection pressures, may explain the spread and emergence of ATV epizootics in *A. tigrinum* in western North America.

Introduction

A central question in evolutionary biology is understanding spatial patterns in the variation of species' interactions. This geographic variability in species' interactions is in part due to underlying differences in local selection pressures (Thompson, 2005; Gomulkiewicz, *et al.* 2007). Emerging infectious diseases (EIDs) – such as avian influenza, HIV and West Nile virus which are of growing human concern – may originate through changes in local selection pressures.

Emerging infectious diseases have recently been implicated as an important hypothesis for the global decline of amphibian populations (Daszak *et al.*, 2003; Stuart *et al.*,

2004; Lips *et al.*, 2006; Rachowicz *et al.*, 2006). In particular, EIDs caused by the globally distributed chytrid fungus, *Batrachochytrium dendrobatidis*, and ranaviruses are considered important causes of amphibian population epizootics and declines (Marsh *et al.*, 2002; Daszak *et al.*, 2003; Stuart *et al.*, 2004; Fox *et al.*, 2006; Lips *et al.*, 2006). By studying the evolutionary dynamics of pathogens causing amphibian EIDs, we gain a general understanding of EIDs and the conditions necessary for the emergence of pathogens from their traditional host populations.

During the last 15 years, ranaviruses in particular have been associated with marked increases in globally distributed morbidity and mortality in fish, reptiles and amphibians (Chinchar, 2002). Based upon major capsid protein (MCP) sequence data, members of the genus *Ranavirus* in the family Iridoviridae are rapidly diverging (Marsh *et al.*, 2002). In North America, ranaviruses have been isolated from the majority of recent amphibian

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epizootics (Green *et al.*, 2002), including tiger salamander epizootics along the western cordillera. The ranavirus strains found in tiger salamanders are monophyletic relative to other ranaviruses and are now collectively called *Ambystoma tigrinum* virus (ATV) (Jancovich *et al.*, 2005). ATV was initially isolated and identified in 1996 from the endangered Sonora tiger salamander (*A. t. stebbinsi*) in the San Rafael Valley of Arizona, USA (Jancovich *et al.*, 1997), but closely related strains have since been identified from other tiger salamander epizootics throughout western North America (Bollinger *et al.*, 1999; Jancovich *et al.*, 2005).

To test for selection on ATV strains, we sequenced three open reading frames (ORF) homologous to genes involved in host immunosuppression. Such sequence data can be utilized to estimate selection via numerous approaches (e.g. Huelsenbeck *et al.*, 2006 or Subramanian & Kumar, 2006). Sequence-based selection analyses are particularly valuable for studying viral evolution because traditional phenotypically based estimations of selection are difficult. Furthermore, these analyses are particularly advantageous when combined with phylogeographic analyses because of the potential to identify spatially variable selection pressure. Information regarding spatial variability of selection gives insight into demographic dynamics (i.e. host or pathogen extinction risk or, conversely, the capacity of either to spread) and

local adaptation (Thompson, 2005). To this end, we sought to better resolve the ATV phylogeography by sequencing two presumably selectively neutral intergenic spacer sequences in addition to the three ORFs. Such phylogeographic analyses may also be used to lend insight into evolutionary rates and diversification within particular lineages of ATV.

By overlaying information about selection, geography and rates of evolution, we investigated how three gene homologues associated with host immune evasion are evolving and possibly affecting local adaptation in the host–pathogen interaction between *A. tigrinum* and ATV. The resulting evolutionary patterns give important insight into the genes central to the emergence of ATV and ranaviruses in amphibian populations.

Methods

We analysed the same 18 ATV viral strains studied in Jancovich *et al.* (2005) with the exception of Pat's Pond (38°54'N, 107°12'W) for which we could not obtain either functional viral isolates to culture, archived DNA or tissue samples. These 17 strains are broadly distributed throughout western North America (Fig. 1), but additional epizootics have been observed (Jancovich *et al.*, 2005). For this reason, we now include four additional strains of ATV from Bodie Tank (AZ, USA), Heidi's Tank (AZ, USA),

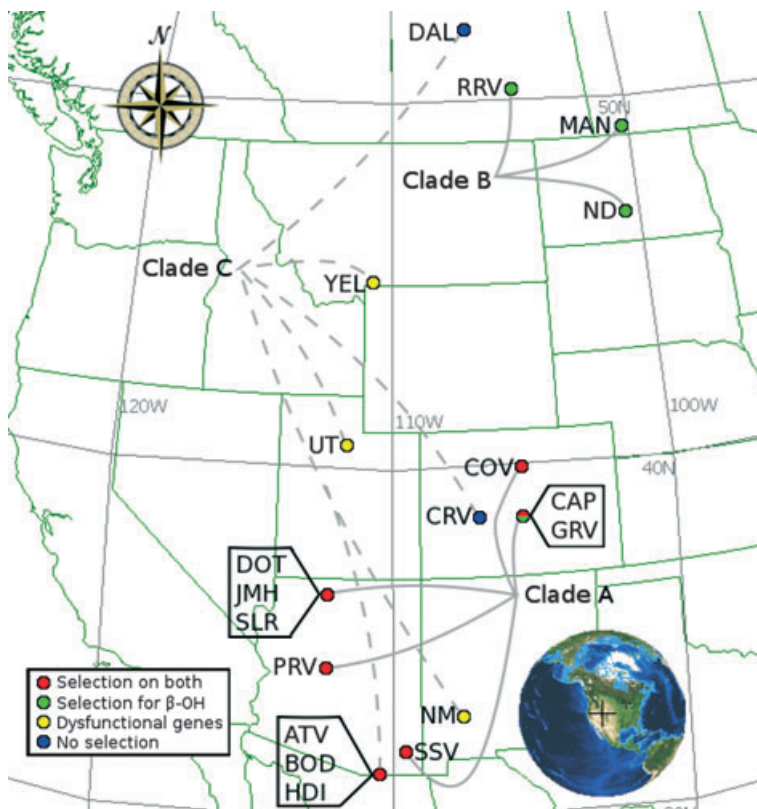


Fig. 1 A map of western North America showing the location of the epizootics from which our ATV isolates were derived. Dots are colour coded to indicate whether or not we found evidence of selection on the CARD gene and/or the β -hydroxy-steroid oxidoreductase gene. GPS coordinates may be found in the Supplementary material.

New Mexico (USA) and Yellowstone National Park (MT, USA). Latitude–longitude data for all strains analysed herein are provided in Table S1 (Supplementary material). When possible, we used either frozen, archived DNA samples or viral isolates that were used in previous studies. If neither of these were available, new viral isolates were cultured from frozen tissue samples of infected salamanders (see below) and the resulting DNA sequence data were compared with previous data to ensure the constancy of the strain. In all cases, where this quality control was necessary, identical sequences between the new and old strains indicated that there was no evidence of cross-contamination by other viral strains or viral evolution.

Viral culture, DNA isolation and sequencing

Individual moribund or recently dead salamanders were collected during *A. tigrinum* epizootics at each of the 21 studied localities (Table S1; Supplementary material) and stored at -80°C . We used the same viral isolation procedure as those of Jancovich *et al.* (2003, 2005). This method includes culturing virus on *Epithelioma papulosum cyprini* cells in Eagle's minimal essential medium. To isolate viral DNA, our harvested virus culture was aliquoted into 1.5-mL microcentrifuge tubes and centrifuged at $10\,800 \times g$ for 1 h. The resulting supernatant was discarded, and the pellet was resuspended using a hypotonic lysis buffer. The suspension was incubated on ice for 5 min and then centrifuged at $3000 \times g$ for 10 min at 4°C . We then placed the supernatant in a new microcentrifuge tube and added sodium dodecyl sulphate (SDS) to a concentration of 0.1% and proteinase K to a concentration of 1 mg mL^{-1} . The resulting mixture was incubated at 50°C for 3 h. Afterwards, we used a phenol–chloroform–isoamylalcohol (26 : 24 : 1) DNA extraction procedure (Sambrook *et al.*, 1989). Extracted DNA was resuspended and stored in buffered Tris–EDTA at a concentration of $100\text{ ng }\mu\text{L}^{-1}$. DNA stocks were later diluted to a concentration of $10\text{ ng }\mu\text{L}^{-1}$ using NANO-pure[®] (Thermo Scientific, Waltham, Massachusetts, USA) distilled H₂O for use in polymerase chain reactions (PCR). The PCR conditions and primer sequences can be found in Table S2 (Supplementary material). All primers were designed using the software package OLIGO[®] 6.1 (Molecular Biology Insights, Inc., Cascade, Colorado, USA).

Polymerase chain reaction products were sequenced using a BigDye[®] terminator cycle sequencing kit (v. 3.1; Applied Biosystems, Inc., Foster City, California, USA). We cleaned our cycle sequencing products using an isopropanol wash. An ABI 3730 high-throughput capillary sequencer (Applied Biosystems, Inc.) was used to visualize cycle sequencing products. The resulting electropherograms were inspected by eye and edited using SEQUENCHER[™] v. 4.7 (Gene Codes Corp., Ann Arbor, Michigan, USA) and aligned using CLUSTALX v. 1.8.2

(Thompson *et al.*, 1997). Afterwards, we inspected our sequence alignments by eye and corrected obvious alignment errors.

Selected and neutral sequences

In total, we analysed DNA sequences from eight different segments of the ATV genome. Three segments were previously analysed in Jancovich *et al.* (2005): the MCP, an intergenic spacer with a variable 16-bp insertion/deletion (16bp) and another intergenic spacer with a 100-bp insertion/deletion (100bp). Description of these primers and their PCR conditions can be found in Jancovich *et al.* (2005). The additional five regions were chosen based on the ATV genomic analysis of Jancovich *et al.* (2003); these regions – described in Table 2 of Jancovich *et al.* (2003) – were 40L/41R, 52R, 57R, 72L and 90R. The regions 72L and 90R have no predicted function and exhibit low homology with closely related ranaviruses. In combination with 16bp and 100bp, we expect these two segments to be more phylogenetically informative relative to ORFs because they are putatively neutral and thus more prone to mutation accumulation.

For sequences potentially under selection, we chose eIF-2 α , a caspase activation and recruitment domain (CARD) and 3- β -hydroxy- Δ 5-C27-steroid oxidoreductase homologues. The eIF-2 α protein subunit is involved in translational control and host interferon downregulation in eukaryotic cells. Phosphorylation of eIF-2 α leads to the shutdown of the translational machinery of the host which prevents viral replication and is therefore an efficacious method of antiviral defence. Viruses must therefore inhibit elevated levels of phosphorylated eIF-2 α within the cell to successfully replicate (Langland & Jacobs, 2004; Mohr, 2004; Mulvey *et al.*, 2006). CARD also functions in antiviral defences by initiating pathways leading to cell apoptosis. Interference with the CARD signalling pathway therefore prevents cell apoptosis and allows for replication of a virus within the host (Hiscott *et al.*, 2006; Johnson & Gale, 2006). Similarly, β -hydroxy-steroid oxidoreductases are thought to play an important role in evading host immune responses via inducing hormonal steroid synthesis in the host, thereby suppressing host immunity (Chinchar, 2002; Delhon *et al.*, 2006). Because of their putative importance in evading the host immune system, we hypothesize that these ORFs will be conserved via purifying selection or be changing through positive selection. We expect genes vital to ATV infection and replication should be conserved, by necessity, for all of the strains being studied. By contrast, genes important to local selection pressures and possibly local adaptation should be subject to geographically variable selection.

Bayesian and likelihood-based phylogenetic analysis

We began our phylogenetic analysis by using DT-MODEL (Minin *et al.*, 2003) to select the appropriate model of

nucleotide evolution for each of our eight sequenced regions and an additional 'supergene' sequence formed by concatenating the eight regions. Concatenation of multiple gene sequences has been shown to yield more accurate phylogenetic reconstructions in comparison with constructing a consensus tree from multiple gene phylogenies (Gadagkar *et al.*, 2005). To test whether sequence partitions provided congruent phylogenetic signals, we ran an incongruence length difference (ILD) test (Farris *et al.*, 1995) for all data partitions using the partition homogeneity test implemented in PAUP* v4.0b10 (Swofford, 2003). For the partition homogeneity test, we used 1000 replicates of a heuristic search with 100 random sequence additions and TBR branch swapping. We also assessed partition congruence of each sequence fragment in a pairwise manner by using the same tests except in these pairwise tests we used 100 replicates of the heuristic search.

After determining the appropriate nucleotide evolution model, we constructed phylogenetic trees using two different approaches: maximum-likelihood analysis and Bayesian analysis. In both analyses, we included data from tiger frog virus (TFV) and frog virus 3 (FV3); TFV was used as an outgroup in all cases due to the availability of its complete genome (GenBank accession no. AF389451). These two ranaviruses are closely related to ATV based on the analyses of Jancovich *et al.* (2003) which showed that: (1) ATV is clearly distinct from TFV and FV3; and (2) there is a high degree of ORF and sequence homology between these viruses.

Maximum-likelihood analyses were performed using PAUP* v.4.0b10. For each of the nine DNA fragments, we performed 200 bootstrap replicates using stepwise addition of random sequences for obtaining initial trees for branch swapping. For our Bayesian analysis, additional binary data about the presence (1) or absence (0) of sequence gaps were included; only the MCP region had no evidence of sequence insertions or deletions. The data containing sequence gap information were analysed using an F81 model of evolution and corrected appropriately for ascertainment bias (Felsenstein, 1992). We used MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) to perform our Bayesian phylogenetic analyses. We ran the default of two independent runs each with four Metropolis-coupled Monte Carlo Markov chains utilizing uniform prior distributions for all parameters. Chains were allowed to progress for 1×10^7 generations. To verify convergence of the independent runs, we examined the standard deviation of split frequencies and the potential scale reduction factor (PSRF). In all cases, convergence was attained in the allotted amount of generations (standard deviation of split frequencies ≤ 0.01 and PSRF ≈ 1). Chains were sampled every 1000 generations; the first 25% of all samples were discarded as burn-in. We allowed each sequenced subunit (e.g. 16bp, 100bp and 57R) to evolve independently when considering

multiple partitions at a time. Using the tree file created during our Bayesian analysis, we tested the hypothesis of monophyly of our resulting clades by searching for trees fitting a constrained topology where the groups being tested were paraphyletic. Thus, the number of trees fitting this constraint divided by the total number of trees estimates the support for the null hypothesis of paraphyly.

Tests of selection and rates of evolution

We conducted tests of selection on the eIF-2 α , CARD and β -hydroxy-steroid oxidoreductase homologues using MEGA v3.1 (Kumar *et al.*, 2004). For each gene, we tested whether there was evidence of purifying selection [the frequency of nonsynonymous (d_N) mutations is significantly less than the frequency of synonymous mutations (d_S), i.e. $d_N < d_S$] or positive selection ($d_N > d_S$). We tested these hypotheses at two levels: when considering all sequences simultaneously and when considering all pairwise combinations of sequences. Considering all sequences simultaneously tests for selection across the entire phylogeny and is a more conservative test (i.e. it is more difficult to find evidence of selection across all sampled strains). For estimating sequence distances, we utilized the 'Kumar' method based on codon position and degeneracy (see http://www.megasoftware.net/WebHelp/hc_kumar_comeron_method.htm for details). In short, this method is a slight modification of those of Comeron (1995) and Li (1993) (see Nei & Kumar, 2000, p. 64 for details) but further corrects for the arbitrary treatment of arginine and isoleucine codons in the Pamilo-Bianchi-Li method (Li, 1993; Pamilo & Bianchi, 1993). The difference between d_N and d_S was calculated as a Z-score, where $Z = (d_N - d_S) / (\text{var}(d_N) + \text{var}(d_S))^{0.5}$. The variance of our estimates was determined using 1×10^3 bootstraps. When considering all sequences simultaneously, gap data (insertions/deletions) were, by necessity, removed from all sequences. However, for pairwise comparisons, gap data were removed on a pair-by-pair basis.

To estimate relative divergence times of our isolates, we used MULTIDIVTIME v. 09.25.03 (Thorne *et al.*, 1998). MULTIDIVTIME uses a Bayesian probabilistic model to infer divergence times and changes in the rate of evolution over time. We also tested a hypothesis that rates of evolution are correlated between our genes (Thorne & Kishino, 2002). For our analysis using MULTIDIVTIME, we used the consensus topology from our MrBayes analysis and allowed our sequence partitions to evolve independently. We used the default normally distributed priors of MULTIDIVTIME, and allowed the Markov chain to proceed for 1×10^6 generations. To test for convergence of our divergence times at each node, we ran four independent runs and then calculated the repeatability (the intraclass correlation) of our node times. We also calculated relative rate tests on

each of our protein coding sequences to test consistency with evolution under a molecular clock (Tajima, 1993).

Results and discussion

Increasing the ATV genome sequence coverage provided a robust and completely resolved phylogeny of ATV within North America and allowed us to test for spatial variability in selection on potential host anti-immunity genes. Our eight sequenced regions totalled approximately 5800 bp after editing and inclusion of sequence gaps (insertions/deletions); these data represent > 5% of ATV genome. We found evidence of selection on all three putative virulence homologues, suggesting they are important to viral fitness and potentially involved in host anti-immune response. The eIF-2 α homologue was the only sequence where significant selection was detected across all strains; this selection was strongly purifying ($Z = 2.457$, $P = 0.008$). For the CARD and the β -hydroxy-steroid oxidoreductase homologues, there was no significant evidence of selection across all strains ($Z = 0.813$, $P = 0.209$ and $Z = 0.215$, $P = 0.415$ respectively). However, when comparing pairs of virus strains, there is support for purifying selection in the CARD sequence (Table S3; Supplementary material) and positive selection in the β -hydroxy-steroid oxidoreductase sequence (Table S4; Supplementary material).

All nodes of our ATV phylogeny are clearly resolved with good support values from both our Bayesian and maximum-likelihood reconstructions (Fig. 2a). Figure 2b shows the phylogram for the same isolates with branch lengths scaled to genetic distances. Our ATV isolates represent a monophyletic group ($P < 0.01$). Clades A and B (Fig. 2b) are monophyletic with respect to each other ($P = 0.01$) as is clade C with respect to clades A and B ($P = 0.01$). However, there was not significant support for the monophyly of the clade containing NM, UT and YEL with respect to the other members of clade C ($P = 0.37$). For convenience, we will refer to clades A, B and C throughout the rest of this article.

By inspecting pairwise selection estimates and mapping them onto the determined phylogeography, we see there are variable patterns of selection within particular clades for the CARD and β -hydroxy-steroid oxidoreductase homologues (Fig. 2b). Only the eIF-2 α homologue exhibited consistent patterns of selection across the entire phylogeny. We therefore believe eIF-2 α may play a critical role in ATV being able to successfully replicate within tiger salamander cells (Sanchez & Mohr, 2007). By contrast, spatially variable selection on CARD and β -hydroxy-steroid oxidoreductase could be due to differences in adaptive optima between populations. For example, β -hydroxy-steroid oxidoreductase is subject to selection in clade B, whereas CARD is not. This pattern may indicate that either: (1) only β -hydroxy-steroid oxidoreductase is important to local adaptation in these

populations; or (2) CARD is no longer evolving via natural selection. The second possibility could result from a lack of sequence variation or from having achieved a local fitness optimum.

Our analysis of evolutionary rates (Fig. 2a) gives some insight into the evolutionary history of the ATV virus. The repeatability of our node times for our four independent runs was very high (intraclass correlation $\rho^2 = 0.999$) which suggests convergence of our analysis. Unfortunately, enforcing a molecular clock to estimate divergence times is not possible because rates of molecular evolution are generally unknown in iridoviruses (Jancovich *et al.*, 2005) and thus we have no way to calibrate our chronogram. However, Thorne *et al.* (1998) have demonstrated that without calibration, the divergence times predicted using MULTIDIVTIME remain accurate for describing relative relationships among taxonomic units. Future research could utilize the timing of known outbreaks, transfer events or salamander phylogenetic information to attempt to calibrate our chronogram on a real-time scale.

From the chronogram (Fig. 2a), clades A and B diverged before the root node in clade C; however, divergence times within clade C are older than those within either A or B. Our relative rate test supports the hypothesis that evolutionary rates are different among clades A and B vs. C for the CARD ORF ($\chi_1^2 = 4.00$, $P = 0.046$); however, relative rate tests for the two other coding sequences were nonsignificant. Shallower divergence times within clades A and B in combination with a difference in relative evolutionary rates among clades suggest that the rate of evolution within clade C is slower than that of clade A or B.

Part of the observed difference in diversification rates could be due to observation bias (i.e. missing taxa from less heavily sampled regions) (Pybus *et al.*, 2002). However, when comparing San Rafael Valley viruses from clade C (ATV, BOD and HDI) with Kaibab plateau viruses in clade A (DOT, JMH and SLV), we see that the clade A group has shorter branch lengths (Fig. 2b). This suggests that our results are not due to observational bias because these two areas are of similar geographic scale and are arguably the most intensely studied and sampled regions for ATV epizootics.

The increased evolutionary rate within clade A could potentially be the result of increased contact rates with new salamander populations resulting from anthropogenic movement. Epizootics caused by the SSV strain are hypothesized to be the result of human-associated movement of salamanders used for fishing bait (Collins, 1981; Storfer *et al.*, 2007). Clade A also contains the two strains clearly associated with human movement (AXO from the Indiana University Axolotl Colony and ORV isolated directly from salamanders collected at a commercial bait shop). These facts support the hypothesis that clade A, at least in part, represents newly introduced, non-native or emerging virus strains.

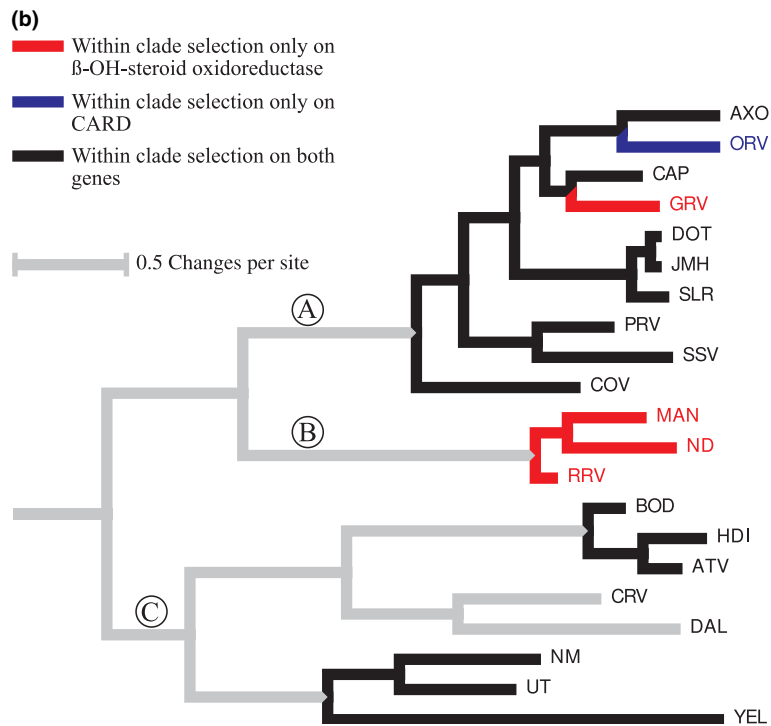
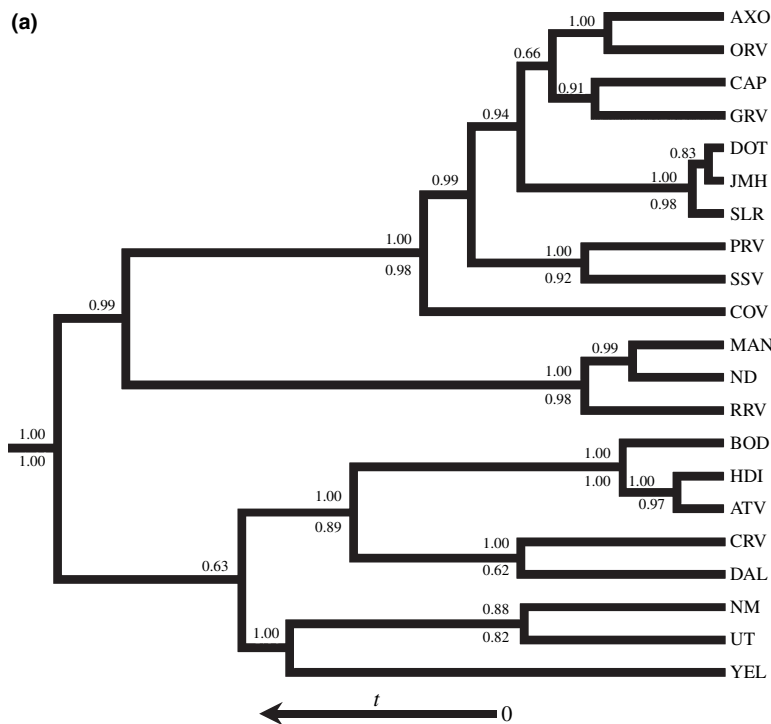


Fig. 2 The resulting phylogeography from Bayesian and maximum-likelihood analyses of the eight sequenced genome regions with the outgroups (TFV and FV3) pruned. (a) A chronogram with Bayesian support values for nodes shown above lines and maximum-likelihood support values shown below lines. Time on the tree is relative because of the lack of calibration points. (b) A phylogram of our ATV strains whose branches have been colour coded to reflect the presence or absence of selection on the CARD gene and the β -hydroxy-steroid oxidoreductase gene. Additionally, we found evidence of purifying selection on eIF-2 α across all strains (not depicted). Major clades have been indicated with letters A, B and C.

Interestingly, selection pressures are the most variable in clade A indicating a possible lack of local adaptation; this also fits with a hypothesis of recent movement of viruses into these populations. Variable selection pressure indicates local maladaptation because locally

adapted populations should exhibit reduced genetic variation for fitness (i.e. a population of individuals at their fitness optimum is locally adapted and has reduced variation in fitness); in turn, a lack of variation in fitness results in an absence of selection pressure (Fisher's

Fundamental Theorem). In contrast to clade A, in clade C, where the rate of evolution is slower, we see populations without selection on either CARD or β -hydroxy-steroid oxidoreductase (CRV and DAL) and populations where these homologues are most likely defunct due to frameshift mutations (NM, UT and YEL; Supplementary material). These populations of clade C could potentially represent endpoints of undisturbed local adaptation processes in ATV and tiger salamanders.

Our MULTIDIVTIME analysis further indicates that the rates of evolution for several of our sequence partitions are correlated. The presumably neutral noncoding sequences for 16bp, 100bp and 90R all seem to be evolving at correlated rates. More interestingly, the evolutionary rates of two of the three genes putatively involved in host immune evasion are correlated: CARD and β -hydroxy-steroid oxidoreductase (rank correlation $\rho = 0.817$, $P = 0.006$) suggesting these may act as a suite of traits involved in local adaptation of ATV to tiger salamanders.

An ILD test suggested that the sequence partitions were not congruent in their phylogenetic signal ($P < 0.001$). The pairwise ILD tests indicate that the source of the conflicting phylogenetic signal is in the 100bp and 52R partitions (Table S9; Supplementary material). Because we found significant selection on several regions and a significant ILD score, we re-estimated our phylogeny in two ways. First, if we considered only partitions giving congruent phylogenetic signal (i.e. excluding the 100bp and 52R regions), then the phylogenetic topology remained the same as in Fig. 2 except NM, UT and YEL, each formed independent ancestral lineages to clades A and B. Second, if strictly neutral data were utilized, the topology once again remained virtually unchanged with the exception of YEL grouping with clade B. As our topology changes little based on either of these re-analyses, and as utilizing all partitions gave the best branch support values, it is our opinion that Fig. 2 gives the truest picture of the phylogenetic relationship among ATV strains. Indeed, other studies have shown that it is often preferable to analyse the data as a whole due to increased resolution of a phylogeny (Cunningham, 1997a, b; Gadagkar *et al.*, 2005).

Conclusions

Increased gene flow resulting from human movement of viral strains may stimulate the ability of a pathogen to adapt to its host (Gandon, 2002; Morgan *et al.*, 2005; Gibbons *et al.*, 2006) and induce the observed spatial variability in selection on our virulence homologues. Gene flow may be favourable if the rate of gene flow is within a particular range and the underlying genetics of the system fits a particular model of interspecific interaction, such as a matching allele model (Gandon, 2002; Ridenhour & Nuismer, 2007). Future studies of relative

rates of viral and host gene flow can help determine whether movement of some ATV strains by humans favours pathogen local adaptation and, in turn, an increasing number of salamander epizootics. Other useful studies will focus on the relationships between underlying amino acid sequences of putative host immune evasion genes, their function and their role in viral virulence.

Regardless of future work, from a conservation standpoint, human transfer and release of infected bait salamanders into naïve populations needs to be stopped to afford tiger salamanders the opportunity to adapt to local ATV strains (which may in turn prevent epizootics). The process of coevolution and what shapes selection pressures between hosts and pathogens apply directly to our understanding of EIDs (particularly iridoviruses) and global amphibian decline.

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Supplementary Material

The following Supplementary material is available for this article:

Table S1 Locale information.

Table S2 Primer sequences and PCR conditions.

Table S3 Pairwise purifying selection tests for the CARD homologue.

Table S4 Pairwise positive selection tests for the β -OH-steroid oxidoreductase homologue.

Table S5 Pairwise purifying selection tests for the eIF-2 α homologue.

Table S6 Protein coding sequences for the CARD region.

Table S7 Protein coding sequences for the β -OH-steroid oxidoreductase region.

Table S8 Protein coding sequences for the eIF-2 α homologue.

Table S9 Pairwise ILD test significance values.

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