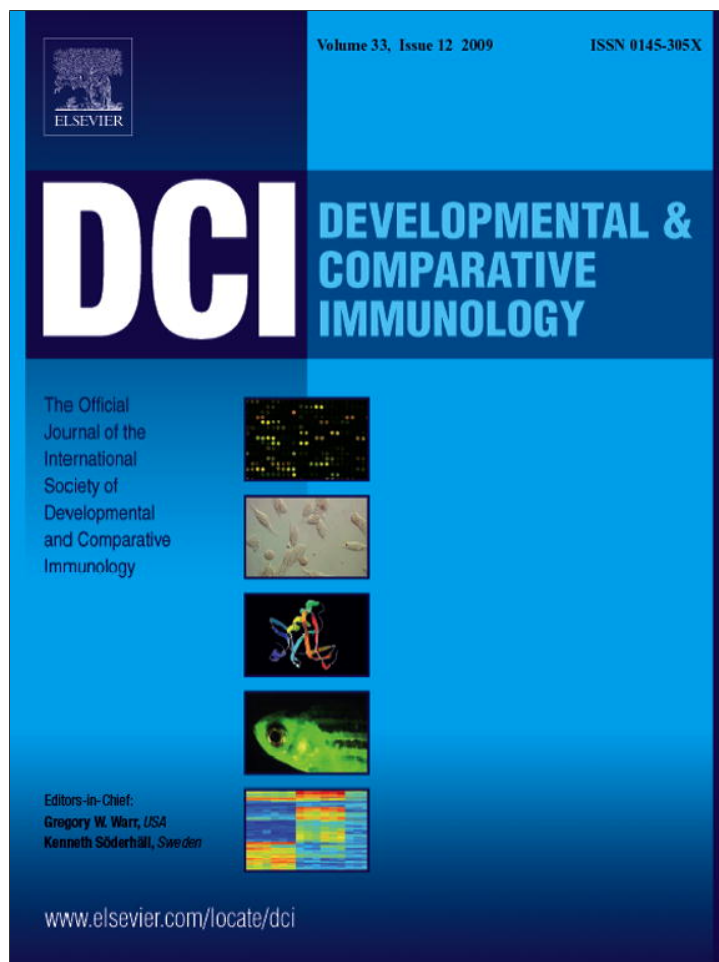


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## Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (*Rana pipiens*) populations suggest intraspecies differences in resistance to pathogens

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Ranatuerin-2

*Staphylococcus epidermidis*

Temporin

## ABSTRACT

The northern leopard frog (*Rana pipiens* or *Lithobates pipiens*) is historically found in most of the provinces of Canada and the northern and southwest states of the United States. In the last 50 years, populations have suffered significant losses, especially in the western regions of the species range. Using a peptidomics approach, we show that the pattern of expressed antimicrobial skin peptides of frogs from three geographically separated populations are distinct, and we report the presence of four peptides (brevinin-1Pg, brevinnin-1Pl, ranatuerin-2Pb, and ranatuerin-2Pc) that have not previously been found in skin secretions. The differences in expressed peptides reflect differences in the distribution of alleles for the newly described *Brevinin1.1* locus in the three populations. When enriched peptide mixtures were tested for their ability to inhibit growth of the pathogenic amphibian chytrid (*Batrachochytrium dendrobatidis*), peptides from Minnesota or Vermont frogs were more effective than peptides from Michigan frogs. Four of the purified peptides were tested for their ability to inhibit growth of two bacterial pathogens (*Aeromonas hydrophila* and *Staphylococcus epidermidis*) and *B. dendrobatidis*. Three of the four were effective inhibitors of *B. dendrobatidis* and *S. epidermidis*, but none inhibited *A. hydrophila*. We interpret these differences in expression and activity of antimicrobial peptides as evidence to suggest that each population may have been selected to express a suite of peptides that reflects current and past encounters with skin microbes.

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**Abbreviations:** 1,5 DAN, 1,5-diaminonaphthalene; 2,5 DHB, 2,5-dihydroxy-benzoic acid; a.u., arbitrary units; BCA, bicinchoninic acid; CHCA,  $\alpha$ -cyano-4-hydroxycinnamic acid; FDA, functional data analysis; HCL, hydrochloric acid; HPLC, high performance liquid chromatography; I.U., international units; MALDI MS, matrix-assisted laser desorption ionization mass spectrometry; MALDI MS/MS, tandem mass spectrometry to sequence selected peptides by analysis of fragment ions; MIC, minimal inhibitory concentration; MS, mass spectrometry; *m/z*, mass to charge ratio; OD<sub>490</sub>, optical density at 490 nm; TFA, trifluoroacetic acid.

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### 1. Introduction

Molecular variation is the raw material for evolution. Species need molecular variation in order to respond to environmental changes, including new infectious diseases and altered ecosystems. Traditionally, intraspecies variation is studied at either the macroscopic or the genetic level, and there is less emphasis on describing differences in expressed protein families. However, an understanding of functional differences among organisms at the protein level is essential for a complete picture of adaptive variation that connects genes to phenotypes.

Many amphibian species are experiencing steep population declines (reviewed in [1–5]), and emerging infectious diseases are thought to be a major cause (reviewed in [6–8]). It is important to assess and conserve genetic variation at immune system loci if amphibian species are to recover from these declines [9]. Leopard

**Table 1**Known antimicrobial skin peptides of *R. pipiens*.

| Peptides                   | Sequence                             | Theoretical monoisotopic MW | Observed and sequenced | References       |
|----------------------------|--------------------------------------|-----------------------------|------------------------|------------------|
| Brevinin-1Pa <sup>a</sup>  | FLPIIAGVAAKVFPKIFCAISKCC             | 2561.4                      | Yes                    | [35,36,39,40]    |
| Brevinin-1Pb <sup>a</sup>  | FLPIIAGIAAKVFPKIFCAISKCC             | 2575.4                      | Yes                    | [35,36,40]       |
| Brevinin-1Pc               | FLPIIASVAAKVFSKIFCAISKCC             | 2581.5                      | No                     | [35,36]          |
| Brevinin-1Pd               | FLPIIASVAAANVFSKIFCAISKCC            | 2567.5                      | Yes                    | [36]             |
| Brevinin-1Pe               | FLPIIASVAAKVFPKIFCAISKCC             | 2591.5                      | Yes                    | [36,40]          |
| Brevinin-1Pf               | FLPIIAGIAAKFLPKIFCAISKCC             | 2589.5                      | No                     | [40]             |
| Brevinin-1Pg <sup>a</sup>  | FFPIVAGVAGQVLKIFCTISKCC              | 2594.5                      | Yes                    | [39,40]          |
| Brevinin-1Ph <sup>b</sup>  | GIPLLPGLAANLCRPYCTITKNC              | 2543.3                      | No                     | [40]             |
| Brevinin-1Pi <sup>b</sup>  | GIPLLPGLAANLCRPINC                   | 1834.0                      | No                     | [40]             |
| Brevinin-1Pj               | FFPNVASVPGQVLRKIFCAISKCC             | 2651.4                      | No                     | [39]             |
| Brevinin-1Pk               | FLPIIAGVAAKVFPKIFCTISKCC             | 2593.5                      | No                     | [39]             |
| Brevinin-1Pl <sup>c</sup>  | FLPIIAGMAAKFLPKIFCAISKCC             | 2607.4                      | Yes                    | This manuscript  |
| Brevinin-1PLa <sup>a</sup> | FFPNVASVPGQVLKIFCAISKCC              | 2621.4                      | No                     | [38,39]          |
| Ranatuerin-2P              | GLMDTVKNAVAKNLGHMLDKLKCKITGC         | 2998.5                      | Yes                    | [36,37]          |
| Ranatuerin-2Pa             | GFLSTVVKLATNVAGTVIDTIKCKVTGGCRK      | 3176.8                      | No                     | [37]             |
| Ranatuerin-2Pb             | SFLTTVKLVNLAALAGTVIDTIKCKVTGGCRT     | 3520.0                      | Yes                    | [40]             |
| Ranatuerin-2Pc             | GLMDTVKNAVAKNLAAHMLDKLKCKITGC        | 3012.5                      | Yes                    | This manuscript. |
| Esculentin-2P              | GFSSIFRGVAKFASKGLKDLARLGVNLVACKISKQC | 3866.1                      | No                     | [36]             |
| Temporin-1P                | FLPIVIGKLLSGLL-CONH <sub>2</sub>     | 1367.9                      | Yes                    | [36]             |

<sup>a</sup> Encoded by the *Brevinin1.1* locus in *R. pipiens* and/or *R. palustris*; assayed antimicrobial activities of synthesized pure peptide.

<sup>b</sup> Divergent homolog predicted from DNA sequence; expression and antimicrobial activity is unknown.

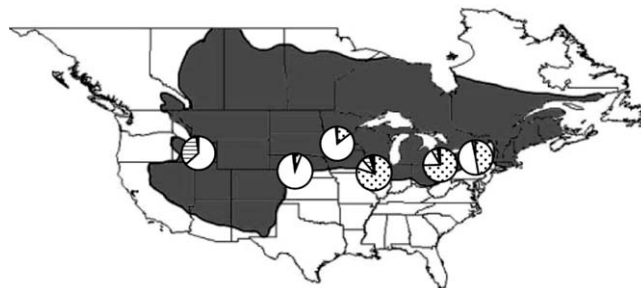
<sup>c</sup> Leucine, isoleucine, and lysine residues attributed based on sequence homologies with other brevinin-1 peptides.

frogs have long been model amphibians for studying genetic variation at the molecular level [10]. A recent taxonomic revision proposed by Frost et al. [11] places leopard frogs and related species (previously genus *Rana*) in the genus *Lithobates*. However, Hillis [12] argued persuasively that the newer classification scheme that replaces widely used species names is unnecessary. Because this issue is not resolved, we have chosen to retain the genus designation *Rana* throughout this paper which allows our work to be more directly connected to previous literature on this species. The northern leopard frog, *Rana pipiens*, has experienced population losses in the last several decades, especially in the western portions of its range. Some of the losses are associated with emerging infectious disease pathogens ([13–17], reviewed in [6,18]). Although the species is still abundant in many locations, its populations have experienced significant losses in comparison with historical numbers (reviewed in [18]). The status of the pickerel frog, *R. palustris* (or *Lithobates palustris*), with respect to emerging infectious disease is less clear, although it does not appear to be in serious decline [19].

Antimicrobial peptides (AMPs) are cationic, amphipathic molecules that kill pathogens by disrupting the cellular membrane or disrupting nucleic acids or protein synthesis within the cell (reviewed in [20–26]). Amphibian AMPs are produced in specialized granular glands (also called poison glands) in the skin. They are functionally diverse and thought to be an important component of innate defenses against pathogens that would enter by way of the skin ([27–31], reviewed in [32–34]). AMPs from many amphibian species have been isolated, sequenced, and tested for antimicrobial activity (reviewed in [25,32,34]), but very few studies have investigated AMP differences among individuals of the same species. Furthermore, the genetic basis for AMP diversity is not well-understood. Both *R. pipiens* and *R. palustris* are known to express complex mixtures of AMPs in their skin [35–38] (Table 1). In these species, a genetic locus encoding peptides of the brevinin-1 family, *Brevinin1.1*, shows a highly unusual pattern of genetic variation [39]. In *R. pipiens*, several divergent allelic lineages are segregating at *Brevinin1.1*, each encoding a different peptide. The most common peptides encoded by these alleles are brevinin-1Pa, brevinin-1Pb, brevinin-1Pg, and brevinin-1PLa (Table 1). These divergent alleles may have arisen through simple point mutations, through gene conversion from a paralogous locus, or through introgression from a different species. Regardless of the origin of the genetic diversity at

this locus, multiple tests of selective neutrality performed on the nucleotide sequences encoding these peptides indicate that the diversity is non-neutral and maintained by balancing natural selection [39]. Geographic variation in the frequencies of these alleles is shown in Fig. 1. Other loci in *R. pipiens* also encode brevinin-1Pa and brevinin-1Pb, as well as other peptides [40]. In contrast to this genetic diversity, the allele encoding brevinin-1PLa appears to be fixed at the *Brevinin1.1* locus in *R. palustris*, possibly owing to a selective sweep [39]. As with *R. pipiens*, *R. palustris* also expresses other AMPs encoded by other loci [38,41]. The peptides brevinin-1Pa and brevinin-1Pb have previously been shown to be active against bacterial and fungal microbes infecting humans [36]. Other *Rana* peptides are known to be active against amphibian pathogens [27–29,42–44]. Nothing is known about the antimicrobial activities of brevinin-1Pg, brevinin-1PLa, or many of the other peptides known to be produced by these frogs.

Given that the process of balancing selection appears to maintain divergent alleles for AMPs in *R. pipiens*, we hypothesized that peptides which differ among individuals would show substantial differences in antimicrobial activity. Presumably there are fitness trade-offs associated with the peptides encoded by the *Brevinin1.1* locus, resulting in their continued preservation by natural selection. One possibility is that individual peptides are specialized to inhibit a different class of pathogen (e.g. fungi, gram-positive bacteria, or gram-negative bacteria). A second possibility



**Fig. 1.** Map of allelic variation at the *Brevinin1.1* locus in *R. pipiens*. Each circle represents allele frequencies from at least 30 frogs in at least two populations within that region. White = brevinin-1Pa, spotted = brevinin-1Pg, horizontal stripes = brevinin-1PLa, black = all other alleles (including brevinin-1Pb). The dark gray background shading represents the range of *R. pipiens*.

is that families of peptides target the same class of pathogen, but some individual peptides within a family are specialized to target a unique species or a unique genetic strain of a single species, perhaps a species which is coevolving with the *Brevinin1.1* locus. A third possibility is that some peptides are universally more active than others at killing a broad spectrum of microbes, but there is a cost of production in the absence of deadly pathogens. The more effective peptide(s) may also damage host cells or beneficial skin bacteria [45–50] that would inhibit growth of skin pathogens.

We tested the activities of *R. pipiens* peptides against three amphibian pathogens: the fungus *B. dendrobatidis*, the gram-negative bacterium *Aeromonas hydrophila*, and the gram-positive bacterium *Staphylococcus epidermidis*. The most serious emerging infectious disease affecting amphibians at the present time is chytridiomycosis caused by *B. dendrobatidis* [51–53], reviewed in [6–8,32–34]. This chytrid fungus is transmitted by a swimming zoospore that attaches to amphibian skin and enters living skin cells of the epidermis [51–54]. It does not become systemic, but replicates within the skin. Infectious zoospores emerge and re-enter the skin of the same individual or a new host [51–54]. Amphibian AMPs vary remarkably in their ability to kill *B. dendrobatidis* [32,34], and current evidence suggests that AMPs in the mucus are an important component of innate defenses against this pathogen [28–31,43,55]. Populations of *R. pipiens* suffered some of the earliest recorded epizootics of *B. dendrobatidis* [6], although most populations today appear to be relatively resistant [56]. *A. hydrophila* can cause dermatosepticemia, commonly called “red-leg” disease in frogs [57,58]. This species has been isolated from both *R. palustris* and *R. pipiens* in the wild [59,60]. Although it occurs on the skin and in the digestive tracts of healthy animals, it can induce fatal disease in some anuran species when the animals are stressed [61,62]. Most amphibian AMPs tested thus far are not effective against *A. hydrophila*, so it represents the most challenging test of the brevinin-1 peptides [27]. *S. epidermidis* has been isolated from the skin of *R. pipiens* [63] and *R. catesbeiana* [64]. Although not thought to be a major epizootic agent, it represents the gram-positive bacteria, which often respond differently to AMPs than gram-negative bacteria [22].

Here we report on the patterns of expression of AMPs of the brevinin-1 family and other AMP families within three geographically separate populations of *R. pipiens*. By mass spectrometry, we show that each population (Michigan, Minnesota, and Vermont) expresses a unique suite of AMPs. The differences in expressed brevinin-1 peptides reflect differences in the distribution of alleles for the *Brevinin-1.1* locus [39]. Furthermore, enriched peptide mixtures from Vermont and Minnesota frogs were significantly more effective in growth inhibition of *B. dendrobatidis* than those of Michigan frogs. When the most abundantly expressed peptides (brevinin-1Pa, brevinin-1Pb, brevinin-1Pg, and brevinin-1PLa) were tested individually for antimicrobial activity against *B. dendrobatidis*, *A. hydrophila*, and *S. epidermidis*, some additional differences were observed. Taken together, the differences in antimicrobial activity of peptides from different populations suggest possible differences in resistance to pathogens that may reflect past and ongoing encounters with disease organisms.

## 2. Materials and methods

### 2.1. Frogs

*Rana pipiens* from Minnesota (USA) were obtained from BioCorporation, Alexandria, MN, and were locally collected. *R. pipiens* collected in Vermont (USA) were obtained from Connecticut Valley Biological Supply Co., Southampton, MA. Michigan (USA) *R. pipiens* were collected by D.C.W. near Mentha, MI. Scientific collection permits were provided by the Michigan Department of

Natural Resources. Commercially supplied frogs were obtained in the fall of the year. Michigan frogs were collected in the summer. The frogs were housed in groups of 5–10 frogs in polypropylene (opaque) plastic tanks with Plexiglas<sup>®</sup> covers containing a small amount of water. The tanks were inclined to allow the frogs to seek a wet or dry substrate. The water was changed, cages cleaned, and the frogs were fed live crickets two or three times weekly. All animal manipulations were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

### 2.2. Collection of skin peptides

Secretions containing skin peptides were collected by norepinephrine induction to avoid the more painful electrostimulation procedure. Frogs from Minnesota and Vermont were sampled in the laboratory in the fall or winter. Michigan frogs were sampled in the field in summer and again in the laboratory in the fall. Briefly, *R. pipiens* captured in the field or held in the laboratory were weighed within 0.1 g and injected with 10 nmoles (0.01 ml) per gram body weight (gbw) of norepinephrine (bitartrate salt, Sigma, St. Louis, MO). Animals were placed into 50 ml of collecting buffer (50 mM sodium chloride, 25 mM sodium acetate, pH 7.0) [29] and remained largely submerged for 10–15 min while skin secretions accumulated. Animals were then removed, and the buffer containing peptides was acidified to a final volume of 1% HCl or trifluoroacetic acid (TFA) to inactivate endogenous peptidases [29,30]. The acidified collection buffer with peptides was passed over C-18 Sep-Pak cartridges (Waters Corporation, Milford, MA USA). The peptides bound to Sep-Paks were eluted with 70% acetonitrile, 29.9% water, 0.1% trifluoroacetic acid (TFA) (v/v/v) and concentrated to dryness by centrifugation under vacuum. The total concentration of skin peptides recovered after Sep-Pak separation was determined by Micro BCA<sup>™</sup> (bicinchoninic acid) assay (Pierce, Rockford, IL, USA) according to manufacturer's directions except that bradykinin (RPPGFSPFR) (Sigma Chemical, St. Louis, MO) was used to establish a standard curve [29,30]. Consequently, concentrations of crude peptide mixtures are expressed as  $\mu\text{g}$  equivalents/ml with reference to the bradykinin standard.

### 2.3. Purified peptides

The sequences of peptides brevinin-1Pa and brevinin-1Pb were previously determined by automated Edman degradation [35,36] and confirmed from DNA sequences [37,40]. The sequences for brevinin-1Pg, and brevinin-1PLa were determined from DNA sequences [39,40] (GenBank accession numbers for all four peptides DQ276967, DQ276968, EU407142, EU407152). Each peptide is 24 amino acid residues long and contains a disulfide bridge between residues 18 and 24 (Table 1). At least 10 mg of each of these four peptides was synthesized at >90% purity by GenScript Corporation (Piscataway, NJ).

### 2.4. Analysis of peptides expressed in the mucus

To confirm that peptides encoded by the *Brevinin1.1* locus are expressed in the skin secretions and to examine geographic variation in expression profiles, we analyzed skin peptide profiles of *R. pipiens* using matrix-assisted laser desorption/ionization mass spectrometry [31,43] (MALDI MS). Peptides were induced from five and seven specimens of *R. pipiens* originating in Minnesota and Vermont, respectively. Peptides from six additional specimens were collected in the field in Michigan [56], and five of these were sampled again after three months in the laboratory. The molecular masses and relative concentrations of skin peptides were assessed using MALDI MS. For this study, MS measurements were performed using a Bruker Daltonics Ultraflex III time-of-flight

mass spectrometer (Billerica, MA). The instrument was operated in reflector, delayed extraction and positive ion mode. Instrument calibration was obtained using a mixture of standard peptides composed of leucine enkephalin with a mass-to-charge ratio ( $m/z$ ) of 556.277, human angiotensin II ( $m/z$  1046.542), human [Glu1]-fibrinopeptide B ( $m/z$  1570.677) and bovine oxidized insulin chain B ( $m/z$  3494.651). The matrix solution consisted of 10 mg/ml  $\alpha$ -Cyano-4-hydroxycinnamic acid (CHCA, LaserBio Labs, Sophia-Antipolis, France) in 50% volume acetonitrile and 50% volume of 1% TFA. Mass spectra were acquired across the range of  $m/z$  500–5000. Peptide solutions were diluted to a total peptide concentration of about 1 mg equivalent/ml in high performance liquid chromatography (HPLC)-grade water, mixed 1/1 with the matrix solution on target, and allowed to dry. Each peptide mixture was measured in triplicate. Automated data acquisition was performed by averaging signals from 1000 laser shots.

### 2.5. Confirmation of peptide sequences by MALDI MS/MS

Confirmation of some skin peptide sequences was obtained by tandem mass spectrometry (MALDI MS/MS). Measurements were obtained using the Ultraflex III time-of-flight mass spectrometer as well as a Shimadzu Corporation QIT time-of-flight mass spectrometer. Frog skin peptide samples were analyzed using the following matrices:  $\alpha$ -cyano-4-hydroxy cinnamic acid (CHCA, LaserBio Labs, Sophia-Antipolis, France), 1,5-diaminonaphthalene (1,5 DAN, Sigma-Aldrich, St. Louis, MO) and 2,5-dihydroxybenzoic acid (2,5-DHB, Sigma-Aldrich, St. Louis, MO). CHCA and 1,5 DAN were prepared at a concentration of 10 mg/ml in a 50/50 mixture of acetonitrile/0.2% trifluoroacetic acid. 2,5 DHB was prepared at a concentration of 10 mg/ml in a 50/50 mixture of methanol/0.2% TFA. MALDI samples were directly prepared on-target by mixing 0.5  $\mu$ l of frog skin peptides with an equivalent volume of matrix solution. The spots were allowed to dry prior to mass analysis. The MALDI MS/MS spectra were manually acquired by averaging signals from 5000 (Ultraflex III) or 2000 (QIT) consecutive laser shots. Alignment of the sequences with the

experimental MS/MS data was performed using the Bruker Daltonics BioTools 3.0 software.

### 2.6. Growth inhibition assays

*B. dendrobatidis* isolate 197 (originally isolated from the blue poison dart frog, *Dendrobates azureus*) [52] was maintained in culture as described previously [27,28]. Zoospores were harvested and cultured with or without peptides as described previously [29,43]. Briefly,  $5 \times 10^5$  zoospores in a volume of 50  $\mu$ l broth were plated in replicates of five in a 96-well flat bottom microtiter plate (Costar 3596, Corning Inc., Corning NY, USA) with or without addition of 50  $\mu$ l serial dilutions of peptides in sterile HPLC-grade water. The plates were covered, wrapped in plastic wrap to limit moisture loss, and incubated at 23 °C. To determine maximal growth (positive control for growth), some wells received 50  $\mu$ l of HPLC-grade water without peptide. To determine the value for maximal inhibition (negative control for growth) some cultures were treated by temperature shock (60 °C for 10 min) to induce death. Growth at seven days (23 °C) was measured as increased optical density at 490 nm (OD<sub>490</sub>) with an MRX Microplate Reader (Dynex Technologies, Inc., Chantilly, Virginia).

Minimal inhibitory concentration (MIC) is defined as the lowest concentration at which no growth was detectable. We tested the activities of the four purified peptides for growth inhibition activity against *B. dendrobatidis*. We also tested natural skin peptide mixtures originating from *R. pipiens* in Minnesota, Michigan, and Vermont using the same methods.

To compare the relative effectiveness of skin peptide mixtures from different populations of *R. pipiens*, we determined the percent inhibition of growth at a concentration of 50  $\mu$ g equivalents/ml and multiplied by the total  $\mu$ g equivalents/g of frog weight produced by each individual multiplied by one thousand [31].

*A. hydrophila* (isolate 43408) from the American Type Culture Collection (ATCC) was grown in nutrient broth at 30 °C. *S. epidermidis* (ATCC isolate 12228) was grown in tryptic soy broth at 37 °C. For growth inhibition assays,  $5 \times 10^6$  bacterial cells in a

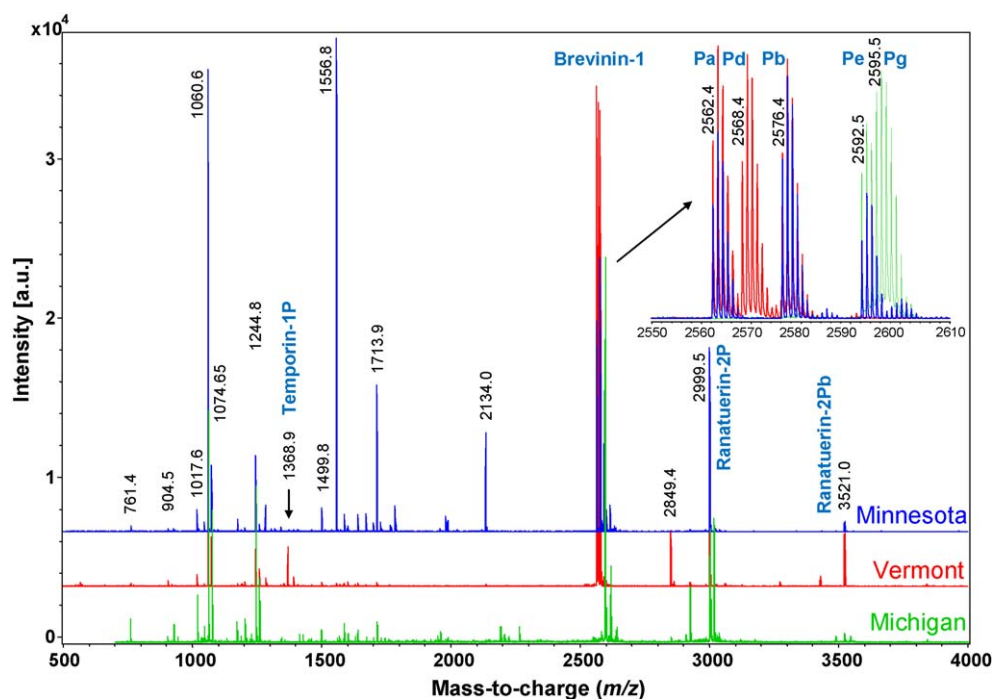


Fig. 2. Typical MALDI mass spectra of skin peptide mixtures from Vermont, Minnesota, and Michigan *R. pipiens*. The Minnesota pattern is shown in blue. The Vermont pattern is shown in red, and the Michigan pattern is shown in green.

volume of 50  $\mu$ l of broth were plated in replicates of five in a 96-well flat-bottom microtiter plate together with 50  $\mu$ l of either HPLC-grade water (positive control) or serial dilutions of purified peptide. As a negative control, we plated  $5 \times 10^6$  cells in a volume of 50  $\mu$ l of broth with 50  $\mu$ l of 20  $\mu$ g/ml streptomycin and 20 I.U. penicillin/ml in water. We covered the plates, wrapped them in plastic wrap to minimize loss of moisture, and incubated them at either 30 °C (*A. hydrophila*) or 37 °C (*S. epidermidis*) for 24 h. Growth was measured as increased optical density at 490 nm with a microtiter plate reader.

### 2.7. Statistical comparisons

Spectra pre-processing (FlexAnalysis 3.0, Bruker Daltonic) included baseline subtraction and de-noising. Using in-house written software (Wavespec), the mass spectra were also normalized (by total ion current) and recalibrated based on pre-selected peptides. Relative abundances of known antimicrobial peptides within *R. pipiens* populations were then obtained by comparing the signal intensities from MS profiles using methods of functional data analysis (FDA) [65]. Heuristically, this analysis approach uses a two-sample *t*-test at each *m/z* value to test for differences in the distribution means. The actual tests conducted use ranked normalized intensities, and thus correspond to a non-parametric Wilcoxon Rank Sum test. Because tests were conducted at multiple

*m/z* values, the predetermined pointwise *p*-values of 0.001 were judged significant. Previous simulation studies (unpublished) indicated that this threshold provides approximately 0.05 spectrum-wide significance. This method was used to compare the distribution of peptides from one geographic location to another.

In the growth inhibition assays, each data point represents the mean  $\pm$  standard error (SE) of five or more replicate wells. The means were compared by a one-tailed Student's *t*-test, and a *p*-value  $\leq$  0.05 was considered to be statistically significant.

The activity of natural mixtures of skin peptides (50  $\mu$ g equivalents/ml, multiplied by the total  $\mu$ g equivalents/g of frog weight  $\times 10^3$ ) was compared using a Kruskal–Wallis test.

## 3. Results

### 3.1. Skin peptide expression by geographical region

MALDI MS analysis of peptide mixtures from individuals in each geographic region revealed intraspecies variation in the brevinin-1 family of antimicrobial peptides and in other skin peptides (Fig. 2; Supplemental Fig. S1, Table 2). From the analysis of the MS profiles, the presence or absence and abundance (relative intensities) of several peptides were found to be significantly different among populations (Table 3). All seven Vermont frogs, and all five Minnesota frogs, had higher levels of brevinin-1Pa and -1Pb than

**Table 2**

Percent relative intensities of known antimicrobial skin peptides in skin secretions from *R. pipiens* originating in different geographical locations. The distributions of peptides, average values, and ranges are given for frogs from each location.

| Origin         | Peptides             |                     |                     |                     |                     |                     |                     |                      |                       |                       |
|----------------|----------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|----------------------|-----------------------|-----------------------|
|                | Temp-1P<br>Mass 1368 | Bv-1Pa<br>Mass 2561 | Bv-1Pd<br>Mass 2567 | Bv-1Pb<br>Mass 2575 | Bv-1Pe<br>Mass 2591 | Bv-1Pg<br>Mass 2594 | Bv-1Pl<br>Mass 2607 | Rana-2P<br>Mass 2998 | Rana-2Pc<br>Mass 3012 | Rana-2Pb<br>Mass 3520 |
| Minnesota lab  |                      |                     |                     |                     |                     |                     |                     |                      |                       |                       |
| MN-1           |                      | 22.4                |                     | 68.0                |                     |                     | 8.1                 |                      | 1.2                   |                       |
| MN-3           |                      | 32.1                |                     | 38.1                | 22.1                |                     | 8.5                 |                      | 1.0                   |                       |
| MN-4           |                      | 33.9                |                     | 39.9                | 24.8                |                     |                     |                      | 1.1                   |                       |
| MN-5           |                      | 34.2                |                     | 43.6                | 21.1                |                     |                     |                      | 1.1                   |                       |
| MN-6           |                      | 29.2                |                     | 56.2                |                     |                     | 10.5                | 2.9                  | 1.2                   |                       |
| Average        |                      | 30.4                |                     | 49.2                | 13.6                |                     | 5.4                 | 0.6                  | 1.1                   |                       |
| Range          |                      | 22.4–34.2           |                     | 38.1–68.0           | 0–24.8              |                     | 0–10.5              |                      | 1.0–1.2               |                       |
| Vermont lab    |                      |                     |                     |                     |                     |                     |                     |                      |                       |                       |
| VT-13          | 9.1                  | 33.2                | 11.9                | 20.5                |                     |                     |                     | 25.4                 |                       |                       |
| VT-14          | 4.4                  | 27.2                | 17.6                | 31.2                | 7.4                 |                     |                     | 8.1                  |                       | 4.1                   |
| VT-15          | 2.4                  | 31.4                | 35.2                | 15.5                | 13.2                |                     |                     | 0.4                  |                       | 1.8                   |
| VT-16          | 8.9                  | 28.6                | 10.3                | 22.7                |                     |                     |                     | 23.4                 |                       | 4.2                   |
| VT-17          | 3.4                  | 18.0                | 19.4                | 48.3                | 6.7                 |                     |                     | 4.0                  |                       | 1.1                   |
| VT-18          |                      | 24.0                | 6.7                 | 34.4                | 17.2                |                     |                     | 16.8                 |                       |                       |
| VT-19          | 3.7                  | 24.4                | 6.3                 | 36.5                |                     |                     |                     | 29.0                 |                       |                       |
| Average        | 4.6                  | 26.7                | 15.3                | 29.9                | 6.4                 |                     |                     | 15.3                 |                       | 1.6                   |
| Range          | 0–9.1                | 18–33.2             | 6.3–35.2            | 15.5–48.3           | 0–17.2              |                     |                     | 0.4–29.0             |                       | 0–4.2                 |
| Michigan lab   |                      |                     |                     |                     |                     |                     |                     |                      |                       |                       |
| MI-7           | 1.3                  |                     |                     |                     | 94.1                |                     |                     | 2.2                  | 2.4                   |                       |
| MI-9           |                      |                     |                     |                     | 73.1                | 16.6                |                     | 4.2                  | 5.6                   |                       |
| MI-10          | 5.7                  | 4.1                 |                     |                     | 62.1                | 17.0                |                     | 3.4                  | 5.5                   |                       |
| MI-11          |                      |                     |                     |                     | 30.2                | 45.0                |                     |                      | 24.8                  |                       |
| MI-12          |                      |                     |                     |                     | 30.1                | 50.1                |                     | 9.2                  | 10.7                  |                       |
| Average        | 1.4                  | 0.8                 |                     |                     | 57.9                | 25.7                |                     | 3.8                  | 9.8                   |                       |
| Range          | 0–5.7                | 0–4.1               |                     |                     | 30.1–94.1           | 0–50.1              |                     | 0–9.2                | 2.4–24.8              |                       |
| Michigan field |                      |                     |                     |                     |                     |                     |                     |                      |                       |                       |
| MI-1           |                      |                     |                     |                     | 36.1                | 46.1                |                     | 7.8                  | 10                    |                       |
| MI-2           | 2.0                  |                     |                     |                     | 34.3                | 45.1                |                     |                      | 18.5                  |                       |
| MI-3           | 4.0                  | 3.7                 |                     |                     | 34.5                | 44.8                |                     | 5.9                  | 7.2                   |                       |
| MI-4           | 1.9                  | 1.9                 |                     |                     | 54.9                | 32.5                |                     | 3.3                  | 5.0                   | 0.5                   |
| MI-5           | 3.9                  |                     |                     |                     | 30.6                | 46.6                |                     | 8.5                  | 10.4                  |                       |
| MI-6           | 1.9                  |                     |                     |                     | 32.4                | 49.0                |                     | 7.7                  | 9.0                   |                       |
| Average        | 2.3                  | 0.9                 |                     |                     | 37.1                | 44.0                |                     | 5.5                  | 10.0                  | 0.1                   |
| Range          | 0–4.0                | 0–3.7               |                     |                     | 30.6–54.9           | 32.5–49.0           |                     | 0–7.8                | 5.0–18.5              |                       |

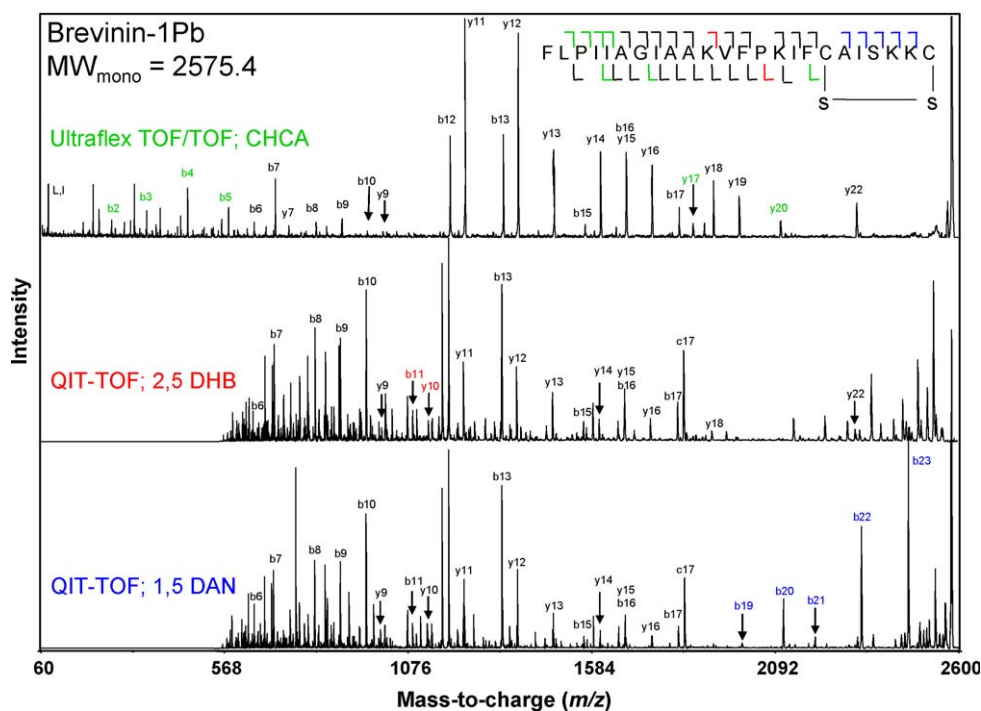
**Table 3**  
Pairwise comparisons of individual peptides from each geographic group of *R. pipiens*. Only peptides that differed significantly between groups are shown.

| Peptide        | Mass (Da) | Comparison                     | Nature of the difference                         |
|----------------|-----------|--------------------------------|--|
| Temporin-1P    | 1368      | Vermont lab vs. Minnesota lab  | Present in VT lab, absent in MN lab              |
| Brevinin-1Pa   | 2561      | Vermont lab vs. Michigan lab   | Greater intensity of signal in VT lab vs. MI lab |
| Brevinin-1Pa   | 2561      | Minnesota lab vs. Michigan lab | Greater intensity of signal in MN lab vs. MI lab |
| Brevinin-1Pb   | 2575      | Vermont lab vs. Michigan lab   | Present in VT lab, absent in MI lab              |
| Brevinin-1Pb   | 2575      | Minnesota lab vs. Michigan lab | Present in MN lab, absent in MI lab              |
| Brevinin-1Pd   | 2567      | Vermont lab vs. Minnesota lab  | Present in VT lab, absent in MN lab              |
| Brevinin-1Pd   | 2567      | Vermont lab vs. Michigan lab   | Present in VT lab, absent in MI lab              |
| Brevinin-1Pe   | 2591      | Vermont lab vs Michigan lab    | Greater intensity of signal in MI lab vs. VT lab |
| Brevinin-1Pe   | 2591      | Minnesota lab vs. Michigan lab | Greater intensity of signal in MI lab vs. MN lab |
| Brevinin-1Pg   | 2594      | Vermont lab vs. Michigan lab   | Present in VT lab, absent in MN lab              |
| Brevinin-1Pg   | 2594      | Minnesota lab vs. Michigan lab | Present in MI lab, absent in MN lab              |
| Brevinin-1Pl   | 2607      | Vermont lab vs. Minnesota lab  | Present in MN lab, absent in VT lab              |
| Brevinin-1Pl   | 2607      | Minnesota lab vs. Michigan lab | Present in MN lab, absent in MI lab              |
| Ranatuerin-2P  | 2998      | Vermont lab vs. Minnesota lab  | Greater intensity of signal in VT lab vs. MN lab |
| Ranatuerin-2P  | 2998      | Minnesota lab vs. Michigan lab | Greater intensity of signal in MI lab vs. MN lab |
| Ranatuerin-2Pc | 3012      | Vermont lab vs. Minnesota lab  | Present in MN lab, absent in VT lab              |
| Ranatuerin-2Pc | 3012      | Vermont lab vs. Michigan lab   | Present in MI lab, absent in VT lab              |

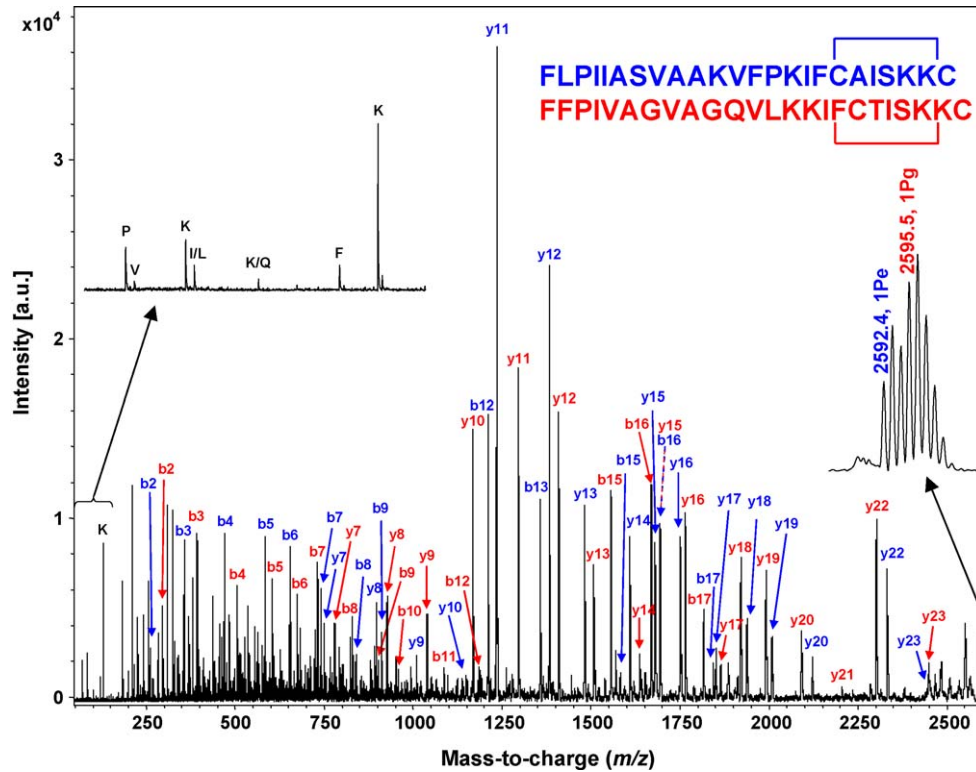
all Michigan frogs (Tables 2 and 3). Michigan frogs did not appear to express detectable levels of brevinin-1Pb. Conversely, all seven Vermont frogs, and all five Minnesota frogs, had undetectable levels of brevinin-1Pg with respect to all of the Michigan frogs (Tables 2 and 3). Thus, the presence of brevinin-1Pa and -1Pb was negatively correlated with the presence of brevinin-1Pg. Brevinin-1Pd was only detected in Vermont frogs (Table 2). Brevinin-1Pe was strongly expressed in the Michigan frogs but was detected with much lower abundances in the Minnesota and Vermont frogs. The newly discovered brevinin-1Pl (see below) was only observed in three of the five Minnesota frogs (Table 2). Temporin-1P was detected in Vermont and Michigan frogs but not detected in the Minnesota frogs (Table 2). Brevinin-1Pla, brevinin-1Pc, brevinin-1Pf, brevinin-1Ph, brevinin-1Pj, and brevinin-1Pk (Table 1) were not detected in any of these eighteen frogs. The six Michigan frogs

were sampled in the field and five of these again in the laboratory. Although the levels of temporin and brevinin-1Pg were observed to decrease, laboratory conditions did not significantly affect the pattern of known expressed antimicrobial peptides after three months.

The ranatuerin-2P peptides also showed different expression patterns across geographical locations. Ranatuerin-2P is encoded by a very common allele at the *Ranatuerin2* locus [40]. We hypothesize that the newly discovered ranatuerin-2Pc (see below) is encoded by a rare allele at this same locus. Both putative *Ranatuerin2* peptides (ranatuerin-2P and ranatuerin-2Pc) were clearly detected in the Michigan frogs whereas ranatuerin-2P was the only member of this locus detected in the Vermont frogs. Low levels of both of these peptides were detected in the Minnesota frogs (Table 1; Table 2). Ranatuerin-



**Fig. 3.** MALDI MS/MS spectra acquired for brevinin-1Pb ( $m/z$  2575.4) using different matrices and MS instrumentation. Fragment ions detected using the Ultraflex TOF/TOF instrument and CHCA as a matrix are shown in green. Fragment ions detected using the QIT-TOF and 2,5 DHB as a matrix are shown in red; and those detected using the QIT-TOF with 1,5 DAN as a matrix are shown in blue. The combined set of observed fragment ions allows for nearly complete verification of the sequence.



**Fig. 4.** MALDI MS/MS spectrum acquired with the Ultraflex TOF/TOF from the group of signals ranging from  $m/z$  2592 to 2600 using CHCA as a matrix. While similar in molecular weight, brevinin-1Pe and -1Pg could be distinguished by MALDI MS/MS. Both are present in high abundance in skin secretions of *R. pipiens* originating from Michigan. Fragment ions assigned to brevinin-1Pg are shown in red; and those assigned to brevinin-1Pe are shown in blue. Complete fragmentation schemes can be found in Scheme 1.

2Pb, encoded by a different locus [40], was only clearly detected in the Vermont frogs (Table 2).

Statistical analyses showed significant differences between populations in the expression of temporin-1P, brevinin-1Pa, brevinin-1Pb, brevinin-1Pd, brevinin-1Pe, brevinin-1Pg, brevinin-1Pl, ranatuerin-2P, and ranatuerin-2Pc (Table 3).

### 3.2. Confirmation of peptide identities

Using two different types of MALDI tandem mass spectrometers and three different matrices, we confirmed the identities of brevinins-1Pa, -1Pb, -1Pd, -1Pe, -1Pg, -1Pl and several other non-brevinin-1 peptides (Table 1, Figs. 3 and 4, Schemes 1 and 2). In several instances, the combination of overlapping and unique fragment ions observed from one or the other MS system has allowed us to significantly enhance the sequence coverage. In particular, we have used 1,5 DAN as a matrix, known to partially reduce disulfide bonds during the desorption/ionization process, to verify the portion of sequences contained within intrinsic disulfide bonds [66]. An example of sequence verification for brevinin-1Pb is presented in Fig. 3. Similar results were obtained for brevinin-1Pa, Pd and Pe, which could easily be isolated towards fragmentation in particular with the QIT-TOF system. Brevinin-1Pg was only clearly

| Brevinins                                 | Sequences and Observed fragmentation patterns |
|---|---|
| Brevinin-1Pa<br>MW <sub>mono</sub> 2561.4 | FLPIIAGVAAKVFPKIFCAISKKC<br>SS_____           |
| Brevinin-1Pb<br>MW <sub>mono</sub> 2575.4 | FLPIIAGVAAKVFPKIFCAISKKC<br>SS_____           |
| Brevinin-1Pd<br>MW <sub>mono</sub> 2567.4 | FLPIIASVAAKVFPKIFCAISKKC<br>SS_____           |
| Brevinin-1Pe<br>MW <sub>mono</sub> 2591.5 | FLPIIASVAAKVFPKIFCAISKKC<br>SS_____           |
| Brevinin-1Pg<br>MW <sub>mono</sub> 2594.5 | FFPIVAGVAGQVLKKIFCTISKKC<br>SS_____           |
| Brevinin-1Pl<br>MW <sub>mono</sub> 2607.4 | FLPIIAGVMAAKFLPKIFCAISKKC<br>SS_____          |

**Scheme 1.** Total fragmentation scheme obtained for all of the observed brevinin-1 peptides from MALDI MS/MS measurements performed as described in Fig. 3.

| Ranatuerins                                 | Sequences and Observed fragmentation patterns |
|---|---|
| Ranatuerin-2P<br>MW <sub>mono</sub> 2998.5  | GLMDTVKKNVAKNLAGHMLDKLKCKITGC<br>S_____S      |
| Ranatuerin-2Pc<br>MW <sub>mono</sub> 3012.5 | GLMDTVKKNVAKNLAHMLDKLKCKITGC<br>S_____S       |
| Ranatuerin-2Pb<br>MW <sub>mono</sub> 3520.0 | SFLTTVKKLVNLAALAGTVIDTIKCKVTGGCRT<br>S_____S  |

**Scheme 2.** Total fragmentation scheme obtained for all of the observed ranatuerin-2 peptides from MALDI MS/MS measurements performed as described in Fig. 3.

observed in the frog specimens coming from Michigan (Fig. 2, Tables 2 and 3) and had not previously been detected in skin secretions. In this case, its monoisotopic peptide could not be separated from the overlapping isotopic distribution of brevinin-1Pe. Both peptides were therefore isolated and fragmented simultaneously. The resulting tandem MS spectra clearly displayed fragment ions coming from both peptides (Fig. 4).

### 3.3. New antimicrobial peptides

In addition to confirming the sequences of brevinin-1Pa, brevinin-1Pb, brevinin-1Pd, brevinin-1Pe, and brevinin-1Pg, we sequenced a new brevinin-1 peptide. The signal at  $m/z$  2608 only detected in the Minnesota frogs (Tables 2 and 3) was identified as a new brevinin-1 sequence and was named Brevinin-1Pl (Supplemental Fig. S2). Notably, we also verified the presence of the ranatuierin-2Pb peptide (Supplemental Fig. S3), which had previously only been predicted from its nucleotide sequence [40]; and sequenced a new ranatuierin peptide detected at  $m/z$  3013.5 in the frogs coming from Michigan. A low intensity signal at  $m/z$  3013.5 was also observed in the peptides samples from Minnesota but not Vermont. This new peptide, named ranatuierin-2Pc, only differs in sequence with ranatuierin-2P by a single amino acid in position 15 (G → A) (Table 1, Supplemental Figs. 4 and 5, Scheme 2).

### 3.4. Assays of natural mixtures of peptides for growth inhibition of *B. dendrobatidis*

Natural mixtures of peptides from individuals in each population of *R. pipiens* were tested for growth inhibition activity against *B. dendrobatidis*. Populations differed significantly in the percent inhibition of *B. dendrobatidis* at 50  $\mu\text{g}$  equivalents/ml of peptide (Kruskal–Wallis test,  $\chi^2_3 = 9.895$ ,  $p = 0.019$ ; Fig. 5). Specifically, natural peptide mixtures from Minnesota and Vermont frogs were more effective against *B. dendrobatidis* than natural peptides from

### Effectiveness of mixtures of peptides against *Bd*

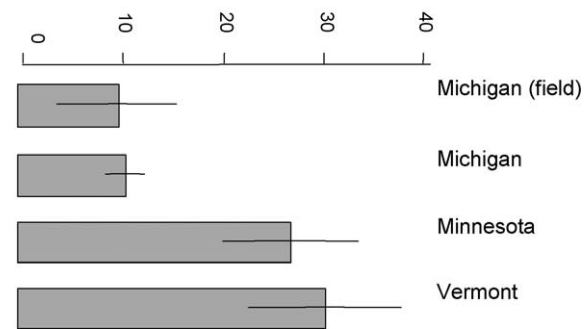


Fig. 5. Effectiveness of natural mixtures of skin peptides from different geographic populations of *R. pipiens* tested in growth inhibition assays against *B. dendrobatidis*. Antifungal activity is the total amount of peptides ( $\mu\text{g}$  equivalents) recovered per g of frog weight multiplied by the percent of growth inhibition at 50  $\mu\text{g}$  equivalents/ml of peptides  $\times 10^3$ .

Michigan frogs. Populations did not differ significantly in the total amount of peptide produced ( $\mu\text{g}$  equivalents) per gram body weight ( $p > 0.1$ ).

### 3.5. Assays of purified peptides for growth inhibition of *B. dendrobatidis*, *S. epidermidis*, and *A. hydrophila*

To investigate whether individual brevinin-1 peptides would show distinct patterns of antimicrobial activity, we chose three peptides that differed in their relative abundance in our samples (brevinin-1Pa, brevinin-1Pb, brevinin-1Pg) and one that is shared by *R. pipiens* and *R. palustris* (brevinin-1PLa) but was undetectable in the skin secretions of the populations we sampled [39]. The pattern of growth inhibition of *B. dendrobatidis* was very similar for brevinin-1Pa, brevinin-1Pb, and brevinin-1Pg; inhibiting significantly at concentrations above 3  $\mu\text{M}$  with minimal inhibitory concentrations (MICs) of 6.2–12.5  $\mu\text{M}$ . In contrast, the MIC for

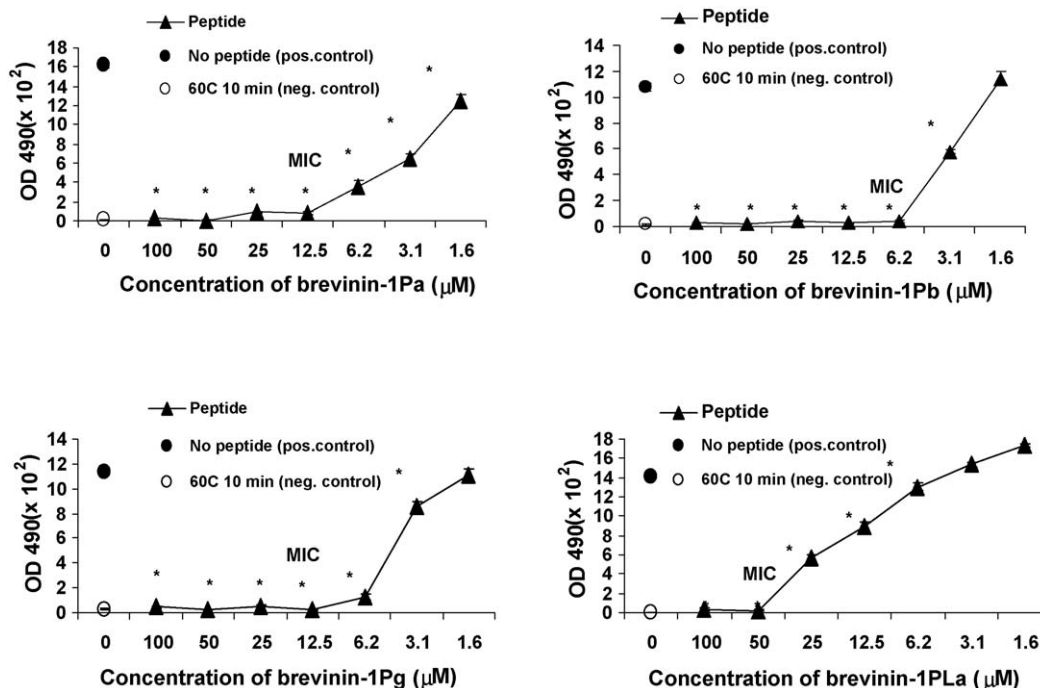


Fig. 6. Growth inhibition of *B. dendrobatidis* zoospores (isolate 197) after seven days of culture with various concentrations of purified brevinin-1Pa, brevinin-1Pb, brevinin-1Pg, and brevinin-1PLa. Each data point represents the mean  $\pm$  SE of five or six replicate wells. If no error bar is shown, the SE was less than the diameter of the symbol. \*Significantly less growth than positive controls (no added peptides) by a one-tailed Student's *t*-test;  $p \leq 0.05$ . MIC is the lowest concentration at which no growth was detected.

**Table 4**

Minimal inhibitory concentrations (MIC) of the four purified brevinin-1 peptides against three amphibian pathogens, in micromolar.

|               | <i>B. dendrobatidis</i> | <i>A. hydrophila</i> | <i>S. epidermidis</i> |
|---------------|-------------------------|----------------------|-----------------------|
| Brevinin-1Pa  | 12.5                    | >100                 | 12.5                  |
| Brevinin-1Pb  | 6.2                     | >100                 | 12.5                  |
| Brevinin-1Pg  | 12.5                    | >100                 | 12.5                  |
| Brevinin-1PLa | 50                      | >100                 | >100                  |

brevinin-1PLa was about 50  $\mu$ M (Fig. 6 and Table 4). MICs of the four purified brevinin-1 peptides against the two bacterial species are shown in Table 4. Brevinin-1Pa, -1Pb, and -1Pg all had nearly identical profiles, killing *S. epidermidis* with a minimal inhibitory concentration of 12.5  $\mu$ M, and showing no antimicrobial activity against *A. hydrophila*. Brevinin-1PLa showed no antimicrobial activity against either of the two bacterial species.

### 3.6. Discussion

We have demonstrated significant intraspecies variation in the constituents and activities of antimicrobial peptides expressed in the skin secretions of *R. pipiens*. Geographically separated populations secrete different suites of peptides with distinct antimicrobial potencies that could impact population responses to infectious diseases. In addition, this study is the first investigation in any taxon of differences in antimicrobial activities among peptides known to be allelic and maintained by balancing selection. We have shown that the *Brevinin1.1* gene is expressed in the skin, that skin expression profiles differ among populations, and that allelic peptides differ in their antimicrobial properties.

Some of the differences in AMP expression profiles correspond to variation at the *Brevinin1.1* locus. Although brevinin-1Pa and -1Pb were previously isolated from *R. pipiens* skin [36], there are multiple loci encoding these peptides [40]. Thus, it was not previously known whether the *Brevinin1.1* locus is expressed in the skin. However, no other locus is known to encode brevinin-1Pg. Thus, the identification of brevinin-1Pg in skin secretions by mass spectrometry and the negative correlation of brevinin-1Pg with brevinin-1Pa in the skin secretions of individual *R. pipiens*, shows that *Brevinin1.1* is expressed. Previous genetic work has shown that *Brevinin1.1* alleles vary geographically in *R. pipiens* (Fig. 1 and [39]). The frequency of the brevinin-1Pa allele is approximately 80% in Minnesota, approximately 10% near Michigan (sampled in Illinois, Ohio and the western shore of Lake Ontario in Ontario, Canada), and approximately 50% east of Lake Ontario [39]. Most of the remaining alleles encode brevinin-1Pg, which is more common than both brevinin-1Pb and brevinin-1PLa combined in all of these regions. This genetic geographic pattern roughly matches the geographic pattern of expressed AMPs observed using mass spectrometry. Namely, the frequency of brevinin-1Pa is high in Minnesota and Vermont and low in Michigan, and the frequency of brevinin-1Pg shows the opposite pattern. Since brevinin-1Pa and -1Pb are also encoded by other loci, it is possible that these loci could obscure the pattern of peptides encoded by *Brevinin1.1* alleles. Nevertheless, expression differences among individual frogs and among populations are apparent.

In addition to the *Brevinin1.1*-encoded peptides, several other peptides show differential expression among populations (Tables 2 and 3). Due to the small sample sizes in this study, the potential for random variation in mass spectrometry signal strength, and the lack of population genetic data for these peptides, we cannot conclude with certainty that any give peptide is more prevalent in a particular geographic region. Nevertheless, the large number of differences observed among individuals strongly suggests overall differences in peptide expression that vary broadly with geogra-

phy. Differences are also observed among individuals in the same population. It is unknown whether this intra- and inter-population diversity is due to natural selection, genetic drift, or phenotypic plasticity. If the differences are genetically based, it is possible that they may affect how populations respond to infectious diseases. If the differences are due to non-genetic causes, such as an individual's history of exposure to pathogens or abiotic factors, these results suggest that such environmental stressors may substantially influence amphibian skin peptide defenses, as suggested by other studies [44,67,68].

We observed significant differences in antimicrobial activities among peptides which vary within *R. pipiens*, both as purified peptides and as natural peptide mixtures (Figs. 5 and 6, Table 4). In purified form, brevinin-1Pa, -1Pb, and -1Pg were generally functionally equivalent against the three pathogens tested. Brevinin-1PLa was not strongly active against any of the pathogens tested. Furthermore, natural peptide mixtures from frogs of different geographic locations differentially killed *B. dendrobatidis*. This *in vitro* result was also reflected in the infection status of frogs. Michigan frogs collected in the field were not infected with *B. dendrobatidis*, whereas *R. pipiens* obtained from Minnesota and Vermont were found to be infected with *B. dendrobatidis* [56]. Infection status may reflect the prevalence of *B. dendrobatidis* in Minnesota and Vermont populations whereas some Michigan populations may be free of *B. dendrobatidis*. A survey of amphibians killed by vehicles (not disease) in the Northeastern USA showed that about 26% of Maine *Rana pipiens* were infected, and other amphibians from collection sites in Vermont were also infected [69]. Likewise, results from 2006 to 2007 surveys for *B. dendrobatidis* on recent metamorphs at breeding sites in the Upper Mississippi National Wildlife and Fish Refuge, the St. Croix National Scenic Riverway, and Voyageurs National Park in Minnesota showed that *B. dendrobatidis* was detected on some species at some sites in all areas, especially on more aquatic species (W. Sadinski, personal communication). Thus, the activity of natural skin peptide mixtures may be the result of selection pressure on populations due to past encounters with an infectious disease. It should be stressed that this link between peptide differences and disease is hypothetical, and we cannot rule out genetic drift and/or phenotypic plasticity as the cause of peptide differences among populations. Furthermore, because brevinin-1PLa was not observed in the skin expression profiles, the difference between brevinin-1PLa and the other brevinin-1 peptides could not be responsible for the difference in antifungal activity of natural peptide mixtures from different geographic regions. The antifungal differences among natural mixtures of peptides are probably due to differences in the concentrations of other AMPs, either of the brevinin-1 family or of other families (Table 1). Alternatively, it is possible that the peptides act synergistically, and their antimicrobial activities alone are not the same as their antimicrobial activities in peptide mixtures.

Our results do not support the hypothesis that each brevinin-1 peptide targets a different class of pathogen. We did not observe any trade-offs in antimicrobial activities; rather, brevinin-1Pa, -1Pb, and -1Pg were all very similar in activity, and brevinin-1PLa was universally less active. This result raises several questions. Why does balancing selection maintain these diverse variants in *R. pipiens*, when it would seem that a single type of peptide by itself would be sufficient? Why has brevinin-1PLa become fixed in *R. palustris* since it appears to be less effective against several different pathogens tested? The answer may be that these peptides have other properties which we did not observe. Rather than targeting completely different classes of microbes, presumably brevinin-1Pa and brevinin-1Pg differ in their ability to kill a specific pathogen that we did not test. Similarly, perhaps brevinin-1PLa is specialized for antimicrobial activity against a particular

pathogen, or perhaps it has low antimicrobial activity against all important pathogens, but it is otherwise advantageous in certain circumstances. Recently, several amphibians have been shown to have symbiotic bacteria associated with the skin [45–50], and some of them secrete metabolites that are inhibitory to the growth of fungal species including *B. dendrobatidis* [70,71]. Furthermore, many brevinin-1 peptides can lyse vertebrate blood cells, an ability which is often correlated with antimicrobial activity [72]. Therefore, the adaptive value of a peptide will depend on its propensity to harm disease pathogens, beneficial bacteria, and the host's own cells, as well as the prevalence of both pathogenic and beneficial microbes.

Interestingly, in *R. pipiens* both brevinin-1Pa and -1Pg appear to have recently increased in frequency, while brevinin-1PLa has decreased in frequency [39]. Chytridiomycosis, in particular, is a relatively new disease in North America, and therefore *B. dendrobatidis* is unlikely to be the specific pathogen that these peptides originally evolved to target. Given our results, it is tempting to speculate that *B. dendrobatidis* has selected for brevinin-1Pa and -1Pg in *R. pipiens* and reduced the frequency of brevinin-1PLa. Further research will be needed to generate support for this tentative hypothesis. If resistance to *B. dendrobatidis* is not provided by other immune defenses in *R. palustris*, this frog species would be susceptible to this fungal pathogen, since the brevinin-1PLa allele provides only very weak immunity. However, it should be noted that a rich array of AMPs (22 distinct peptides) from five AMP families have been detected in a pooled sample from three individuals of this species [38]. Thus, brevinin-1PLa may be less important than other skin peptides in protection from *B. dendrobatidis* in this species.

In conclusion, geographically correlated diversity in AMPs has functional consequences both within and between frog species. AMPs from distinct populations of *R. pipiens* differ in their activities against a serious fungal pathogen, *B. dendrobatidis*. The peptides of the *Brevinin1.1* locus are among these skin-expressed peptides, and they vary in both their antifungal and antibacterial activities, suggesting that populations of *R. pipiens* may differ in immunity from other conspecific populations and from *R. palustris*. Both inter- and intraspecific adaptive variation at AMP loci could be important for host-pathogen coevolution and the ability of amphibian species to resist emerging infectious diseases.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2009.07.004.

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