



**QUEEN'S
UNIVERSITY
BELFAST**

Identification of Miscellaneous Peptides from the Skin Secretion of the European Edible Frog, *Pelophylax kl. Esculentus*

Chen, X., Wang, H., Wang, L., Zhou, M., Chen, T., & Shaw, C. (2016). Identification of Miscellaneous Peptides from the Skin Secretion of the European Edible Frog, *Pelophylax kl. Esculentus*. *The Protein Journal*, 35(4), 291-299. <https://doi.org/10.1007/s10930-016-9672-z>

Published in:
The Protein Journal

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
[Link to publication record in Queen's University Belfast Research Portal](#)

Publisher rights
© Springer Science+Business Media New York 2016
The final publication is available at Springer via <http://link.springer.com/article/10.1007%2Fs10930-016-9672-z>

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Open Access
This research has been made openly available by Queen's academics and its Open Research team. We would love to hear how access to this research benefits you. – Share your feedback with us: <http://go.qub.ac.uk/oa-feedback>

Identification of miscellaneous peptides from the skin secretion of the European edible

frog, Pelophylax kl. Esculentus

Xiaole Chen ^{1,*}, He Wang ^{2,*}, Lei Wang ³, Mei Zhou ³, Tianbao Chen ³, Chris Shaw ³

1. School of Pharmacy, Fujian Medical University, Fuzhou, Fujian, China
2. School of Integrative Medicine, Fujian University of Traditional Chinese Medicine, China
3. Medicine Natural peptide discovery group, School of Pharmacy, Queen's University, Belfast BT9 7BL, Northern Ireland, UK

* Corresponding author. Tel: +86(0)59122862016; Fax:+86(0)59122862016

E-mail address: leochen5139@fjmu.edu.cn (Xiaole Chen); hwang11@qub.ac.uk (He Wang)

Abstract

The chemical compounds synthesised and secreted from the dermal glands of amphibian have diverse bioactivities that play key roles in the hosts' innate immune system and in causing diverse pharmacological effects in predators that may ingest the defensive skin secretions. As new biotechnological methods have developed, increasing numbers of novel peptides with novel activities have been discovered from this source of natural compounds. In this study, a number of defensive skin secretion peptide sequences were obtained from the European edible frog, *P. kl. esculentus*, using a 'shotgun' cloning technique developed previously within our laboratory. Some of these sequences have been previously reported but had either obtained from other species or were isolated using different methods. Two new skin peptides are described here for the first time. Esculentin-2c and Brevinin-2Tbe belong to the Esculentin-2 and Brevinin-2 families, respectively, and both are very similar to their respective analogues but with a few amino acid differences. Further, [Asn-3, Lys-6, Phe-13] 3-14 bombesin isolated previously from the skin of the marsh frog, *Rana ridibunda*, was identified here in the skin of *P. kl. esculentus*. Studies such as this can provide a rapid elucidation of peptide and corresponding DNA sequences from unstudied species of frogs and can rapidly provide a basis for related scientific studies such as those involved in systematic or the evolution of a large diverse gene family and usage by biomedical researchers as a source of potential novel drug leads or pharmacological agents.

Key words: Amphibian; Secretion; Mass Spectrometry; Peptide; Cloning

List of Abbreviation

<i>P.kl. Esculentus</i>	<i>Pelophylax kl. Esculentus</i>
MS	Mass spectrometry
RACE	Rapid Amplification of cDNA Ends
LCQ	Liquid chromatography quadrupole
ESI	Electrospray ionization

1. Introduction

Amphibian skin glands produce complex mixtures of bioactive compounds that have been used in traditional and folk medicines around the world for centuries. In modern times, it has become apparent that amphibians, whose living environments are full of various kinds of microorganisms, have developed a unique survival strategy for protecting themselves against potential pathogens. These highly-efficient host-defence compounds that are secreted from the skins of amphibians are attracting ever-increasing scientific attention, as it seems that they may be good templates for the development of new antibiotics designed to combat the emergence of pathogens that are resistant to conventional antibiotics. As many studies have shown, amphibian skin secretions not only produce potent broad-spectrum antimicrobial peptides, but also a number of peptides that have very similar structures and biological activities to mammalian neuropeptides and hormones, such as the bombesins, bradykinins and tachykinins [1-2]. In early research, hundreds or even thousands of amphibians were sacrificed to obtain enough material for biological and chemical analyses, though as modern methods and technologies have developed, this killing has become unnecessary. A good example of this advanced practice can be found in the mild electrical stimulation method that releases the secretions of the granular skin glands without damaging the host. This technique continues to play a key role in isolating and identifying the active peptides contained in skin secretions and in preservation of endangered species [1-3].

Members of the family Ranidae are widely distributed in Europe, Asia and North America, with an estimated 250 different species producing a large number of diverse antimicrobial peptides, more than 400 of which have been isolated so far [4-5]. Compared with the antimicrobial peptides isolated from other species that often contain a C-terminal amide, the peptides from ranid frog skin secretions are normally of 10-47 amino acid residues with a 6-9-membered cyclic loop region with a single disulfide bridge, called the Rana box, at the C-terminus [2, 6]. Based on the similarities of sequences between individual peptides, these can be classified into 13 peptide families comprising: brevinin-1s, brevinin-2s, esculentin-1s, esculentin-2s, japonicin-2s, nigrocin-2s, palustrin-1s, palustrin-2s, ranacyclins, ranatuerin-1s, ranatuerin-2s, and temporins. Generally, ranid frog antimicrobial peptides are cationic and adopt amphipathic α -helical structures in order to readily bind to bacterial cell membranes through which they induce cell lysis [7].

Esculentin-related peptides are regarded as the earliest characterised family and are the largest skin antimicrobial peptides, consisting of 46 amino acid residues, first isolated from the European edible frog, *P. kl. esculentus* [8-9]. After these reports, more families of peptides were described from this species, including bradykinins, brevinins and temporins. This edible frog species, *P. kl. esculentus*, which is a hybridogenetic

hybrid between *Rana ridibunda* and *Rana lessonae*, is a complex and special species for study, which not only represents a rich source for novel peptide discovery but also represents an important model for studying amphibian evolution [10].

Here, we report the structures of several skin secretion peptides identified in *P. kl. esculentus* by use of “shotgun cloning” and LC/MS/MS fragmentation sequencing. Brevinin-1E and Brevinin-1Ra were previously reported from other closely related species of ranid, though this is the first time they have been found through ‘shotgun’ cloning. [Asn-3, Lys-6, Phe-13] 3-14-bombesin (NLGKQWAVGHFM) was identified by molecular mass fingerprinting of reverse phase HPLC fractions of skin secretion and its structure confirmed following LC/MS. Brevinin-2Tb and Esculentin-2b were obtained in previous studies of this species, however, some primary structural modifications in precursors were found here and this may arise through natural variation between individual specimens or discrete populations. This molecular natural selection provides a good basis for the diversity in chemical structure that may eventually lead to functional development and/or optimisation.

2. Materials and methods

2.1 Preparation of *P. kl. esculentus* skin secretion

Pelophylax kl. esculentus ($n=30$) obtained from a local herpetological supplier were all adults and secretion harvesting was performed in the field after which frogs were released. Gentle transdermal electrical stimulation (5V; 3ms pulses) for 30s was employed to collect skin secretions from the frog’s dorsal skin. The stimulated secretions washed by deionised water from the skin were snap-frozen in liquid nitrogen and lyophilised, which was following stored at -20°C for further analysis.

2.2 “Shotgun” cloning of *P. kl. esculentus* skin secretion-derived cDNA

Five milligrams of lyophilised skin secretion were dissolved in 1ml of cell lysis/mRNA protection buffer supplied by Dynal Biotec, UK. By the use of magnetic oligo-dT beads as described by the manufacturer (Dynal Biotec, UK), polyadenylated mRNA was isolated and subsequently subjected to 5'- and 3'-rapid amplification of cDNA ends (RACE) procedures to obtain full-length peptide precursor nucleic acid sequence data using a Switching Mechanism At 5' end of RNA Transcript (SMART) -RACE kit (Clontech, UK) essentially. Briefly, the 3'-RACE reactions employed four pairs of primers, OL-Signal(5'-CCCAAAGATGTTACCTTGAAGAAA-3')/NUP, RA-Signal(5'-ATGTTCCACCATGAAGAAATC-3')/RA-AS(5'-CTATCCCACATCAGGAGACTTCC-3'), OL-Signal/C12-OAS(5'-GACATCTGTTGTGCATTAGCTAA-3') and OS-1(5'-GTTCCACCATGAAGAAATCCCTGTTACT-3')/NUP. These primers were designed to highly-conserved domains of the 5'-untranslated regions of previously

characterized peptide precursor cDNAs from ranid frogs. Based on a pGEM-T vector system (Promega Corporation), the gel purified 3'-RACE reactions were cloned and then sequenced by an ABI 3730 automated sequencer.

2.3 Identification and structural analysis of novel peptides

Five milligrams of lyophilised skin secretion were dissolved in 0.5 ml of 0.05/99.5 (v/v) trifluoroacetic acid (TFA)/water and centrifuged for clarification of microparticulate. A linear gradient formed from trifluoroacetic acid (TFA)/water; 0.1:99.9 (v/v), to trifluoroacetic acid (TFA)/water/acetonitrile; 0.1:19.9:80.0 (v/v/v) were pumped through a 1cm×25cm Jupiter 00G4052 semi-preparative C-5 reverse phase column (Phenomenex, UK) attached to a Cecil Adept Binary HPLC system (Adept Technology, Inc. USA) in 240 min at a flow rate of 1 ml/min for elution of collected supernatant. Samples (100µl) were removed from each fraction in triplicate, lyophilised and stored at -20°C prior to bioactivity assays. The fractions that exhibited specified activity were subjected to Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry (MALDI-TOF) MS analysis using a Perseptive Biosystems Voyager DE instrument (Framingham, MA, USA) in positive ion mode and α -cyano-4-hydroxycinnamic acid as matrix. Internal mass calibration of the instrument with peptide standards established the accuracy of mass determinations as $\pm 0.01\%$.

2.4 Tandem mass spectrometry sequencing

20 µl of the diluted skin secretion fraction pumped directly onto an analytical HPLC column (Phenomenex C-18; 4.6 × 150 mm) connected to an LCQ Fleet ESI ion trap mass spectrometer (Thermo Fisher, San Jose, CA, USA) in the positive detection mode. The linear elution gradient was formed from 0.1/99.9 (v/v) trifluoroacetic acid (TFA)/water to trifluoroacetic acid (TFA)/water/acetonitrile; 0.1:19.9:80.0 (v/v/v) in 135 min at a flow rate 20 µl/min. Mass analysis was performed in a positive ion mode with acquired spectra in the range of m/z 500–2000 with N50% relative intensity during HPLC-MS. Parameters for electrospray ionization ion-trap mass spectrometry (ESI/MS) were: spray voltage +4.5 kV, drying gas temperature 320 °C, drying gas flow 200 µl/min, and maximum accumulation time – for the ion trap – 350 ms. The first mass analysis was performed in full scan mode, then peptide ions with N50% relative intensity were selected for fragmentation by collision induced dissociation (CID), to generate b and y ions that were detected in a second mass analysis. The instrument was controlled by Xcalibur software (Thermo, USA) and data analysis was performed using Proteome Discover 1.0 (Thermo, USA). Sequest™ algorithm was employed to compare the acquired fragment ion profiles with the theoretical fragment ions generated from a FASTA database.

3. Result

3.1 Molecular cloning of novel peptide precursor-encoding cDNA

Brevinin-1, Brevinin-2, Esculentin-1, Esculentin-2 and bombesin, these five different families of bioactive peptide precursors were repeatedly cloned from the cDNA library constructed from the skin secretion of *P. kl. esculentus* using the primers that were designed from previously characterised ranid frog peptide precursor cDNAs. The nucleotide of open-reading frames of the cloned precursor transcripts and its translated amino acid sequences are illustrated in Fig 1. The deduced single copies of mature peptide sequences located at the C-terminal regions were analysed using the Basic Local Alignment Search Tool (BLAST) program of the US National Centre for Biotechnology information (NCBI) on-line portal.

Brevinin-1Ra and Brevinin-1E were previously obtained from the skin secretion of the marsh frog, *Rana ridibundus*, by high-performance liquid chromatography/ tandem mass spectrometry (HPLC/MS/MS) analysis, though according to the disadvantages of MS/MS, Lys/Gln and Ile/Leu, were not resolved as they are too close or indeed identical in molecular masses. This is the first time these two precursor sequences have been confirmed using a molecular cloning method and their first identification in *P. kl. esculentus*. Brevinin-2Tbe, belonging to the Brevinin-2 subfamily, has a precursor that displays significantly structural similarity to Brevinin-2Tb (98%) and Brevinin-2Ei (94%). The mature peptide sequence of Esculentin-2c, which belongs to Esculentin-2 subfamily, displays 95% identify with Esculentin-2b, where there are only two amino acids differences among total 37 amino acids that are Lys13 and Met29 that take the place of Ala13 and Ile29 of Esculentin-2b. All mature peptide sequences obtained in this study are compared with the most similar peptides in the database in Fig 2. Esculentin-2c and Brevinin-2Tbe has become available in Genbank Nucleotide Sequence Database though the accession code KT437660 and KT437661.

3.2 Identification and structural analyses of [Asn-3, Lys-6, Phe-13] 3-14-bombesin in reverse phase HPLC fractions of *P. kl. esculentus* skin secretion

[Asn-3, Lys-6, Phe-13] 3-14-bombesin was identified in the reverse phase HPLC fractions based on its singly-charged and mono-isotopic molecular mass $[M + H]^+$ m/z of 1386.59 as determined by Matrix-Assisted Laser Desorption/ Ionization Time of Flight (MALDI-TOF) mass spectrometric analysis and confirmed by LCQ ESI MS full scan. The spectrum corresponding to the primary structure of [Asp-3, Lys-6, Phe-13] 3-14-bombesin (Fig 3) was produced by entrapment of the doubly-charged ion of this peptide by the ion trap of the LCQ Fleet mass spectrometer with further determined using MS/MS fragmentation.

4. Discussion

In order to combat the increasing emergency of multiple drug-resistances in pathogenic bacteria all over world, scientists have been searching both chemical and natural product compound libraries for new lead compounds. The unique evolution of the amphibian host defence strategy not only provides a huge variety of bioactive peptides, but also their living environments and solutions to problems can supply clues for the development of possible therapeutics of medical or veterinary significance [11]. Amphibians are described as cold-blooded vertebrates covered by a skin that is rich in secretory glands [12-14] and it is these glands that manufacture, store and release the plethora of bioactive compounds. Members of the Ranidae ('true frogs') are such a good example that their skin secretions are constructed by a diverse range of bioactive compounds besides antimicrobial peptides. Generally, there are 10-20 unique peptides produced in one species which could have differences in sizes, sequences and spectrum of actions, etc., among frogs from different families, genera and species. Moreover, even members of the same species inhabiting different zones are able to create special peptides due to natural selection, such as Esculentin-2c that we report here. This phenomenon could explain why no two species have been found so far to produce the same antimicrobial peptides [2]. Such various peptides could be regarded as lead compounds as their potencies could be enhanced by chemical modification to promote the development of new drugs.

The European edible frog, *P. kl. esculentus*, is a hybrid originally produced between female *R. ridibunda* and male *R. lessonae*, whereas the lineages of *P. kl. esculentus* are maintained by mating females of *P. kl. esculentus* with males of *R. lessonae* [15]. Studies on the skin peptide precursor sequences of *P. kl. esculentus* by molecular cloning technology has indicated one common cDNA-encoding precursor structure, which has a highly conserved N-terminal preproregion composed of a 22 residues long hydrophobic signal peptide, either intra- or inter-specifically, and an 16-25 residues acidic propeptide that is followed by a typical prohormone processing signal Lys-Arg. Finally, a single copy of the mature peptide is encoded at the carboxyl terminus of the precursor sequence.

Compared with anti-bacterial mechanisms of conventional antibiotics that select intracellular targets and cellular processes such as DNA replication, protein and cell wall synthesis, the AMPs are able to rapidly disrupt the bacterial membranes directly with low selectivity making resistance evolution more unlikely. This fundamental mechanism of action supports AMPs as good candidates for new antibiotic drug development. Moreover, frogs from different species or subspecies produce various diverse antimicrobial peptides. Even the same species of frogs that live in different habitats or environments are capable of producing distinct repertoires of antimicrobial peptides to satisfy their special survival needs. The antimicrobial peptides modified by one or

several amino acids within their sequences could display differing sizes, net charges and hydrophobicity. Therefore, these analogues could exhibit differences in the spectrum of action and bioactivities to defend against the particular microbes that these species encounter. Esculentin-2c and Brevinin-2Tbe both have high similarity to the previously isolated antimicrobial peptides Esculentin-2b and Brevinin-2Tb, where just a few amino acid differences occur. These tiny changes inside peptide sequences, induced by either natural or artificial means, could create new or even higher potency antimicrobial peptides. For example, Brevinin-1BYa recently obtained from North American Foothill yellow-legged frogs, *Rana boylei*, belonging to the Brevinin-1 family, has broad-spectrum antibacterial and antifungal properties [16]. New research has discovered one portion of the residues of the full-length antimicrobial peptide sequences could also be the templates of new antibiotic development as they have potent abilities against many pathogens. Take Esculentin (1-21) for example, which is the N-terminal 1-21 region of the esculentin-1a isolated from *P. kl. esculentus*, exhibits the high antimicrobial activities against the most common mastitis-causing microbes in cattle [17,18].

Bombesin and its related homologues, which take part in the synthesis of neuropeptides and hormones and have widespread effects on the gastrointestinal tract and central nervous system's secretory functions, are widely distributed in the frog skin secretions, though its analogues obtained from *P. kl. esculentus* have been rarely reported before. [Asn-3, Lys-6, Phe-13] 3-14-bombesin, originally identified from the Marsh frog, *Rana ridibunda*, has an active core of eight amino acids at the C-terminus that is responsible for binding to receptors [19-20], and here, this peptide has been characterised in the skin secretion of *P. kl. esculentus* using an LC/MS technique. It was exciting to find another neuropeptide family, bombesin, represented in *P. kl. esculentus* skin in addition to bradykinin, that provides scientists with a better understanding of the bio-actions of the skin secretion of this species and an additional choice for neuropeptide study selection.

The skin secretions of *P. kl. esculentus* are a rich source of antimicrobial peptides that have high potency against bacteria including many pathogenic strains [16,21]. As more studies are performed on the isolation of peptides from amphibians, new peptides will be discovered and more additional bioactivities will be found that could supply great clues to improve therapeutic agents and drug development for human healthcare. Due to the development of molecular techniques, especially those that can provide comparisons of the nucleotide sequences of orthologous genes, new phylogenetic analysis of relationships between species has been made possible as an addition to the classic approach of using such aspects as the fossil record and morphological characteristics. Moreover, this improved understanding of amphibian evolutionary history should be more accurate, easier to understand and hence be more commonly accepted [22, 23].

Acknowledge

This work was supported by National Nature Science Foundation, China (Grant No. 81402842), Natural Science Foundation of Fujian Province, China (Grant No. 2015J05162), Natural Science Foundation of Fujian Province, China (Grant No. 2016J01374), Fujian Provincial outstanding talents for Education and Scientific research (Grant No. JK2014016) and The doctor startup foundation of Fujian Medical University (Grant No.2012bs002).

Conflict of Interest statement

The authors declare that they have no conflict of interest.

Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

1. Pukala TL, Bowie JH, Maselli VM, Musgrave IF, Tyler MJ (2006) Host-defence peptides from the glandular secretions of amphibians: structure and activity. *Natural Product Reports* 23; 3: 368-393
2. Vanhoye D, Bruston F, Nicolas P, Amiche M (2003) Antimicrobial peptides from hylid and ranin frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but a hypermutable antimicrobial domain. *European Journal of Biochemistry* 270; 9: 2068-2081
3. Tyler MJ, Stone DJ, Bowie JH (1992) A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. *Journal of Pharmacological and Toxicological Methods* 28; 4: 199-200
4. Won HS, Kim SS, Jung SJ, Son WS, Lee B, Lee BJ (2004) Research Articles - Structure-Activity Relationships of Antimicrobial Peptides from the Skin of *P. kl. esculentus* Inhabiting in Korea. *Molecules and Cells* 17; 3: 469
5. Simmaco M, Mignogna G, Barra D (1998) Antimicrobial peptides from amphibian skin: what do they tell us?. *Biopolymers* 47; 6: 435-50
6. Basir YJ, Conlon J M (2003) Peptidomic analysis of the skin secretions of the pickerel frog *Rana palustris* identifies six novel families of structurally-related peptides. *Peptides* 24; 3: 379-383
7. Conlon JM, Kolodziejek J, Nowotny N (2004) Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. *Biochimica Et Biophysica Acta* 1696; 1:1-14
8. Simmaco M, Mignogna G, Barra D, Bossa F (1994) Antimicrobial peptides from skin secretions of *Rana esculenta*. Molecular cloning of cDNAs encoding esculentin and brevinins and isolation of new active peptides. *The Journal of Biological Chemistry* 269; 16:11956-61
9. Simmaco M, Mignogna G, Barra D, Bossa F (1993) Novel antimicrobial peptides from skin secretion of the European frog *Rana esculenta*. *FEBS Letters* 324; 2: 159-61
10. Spolsky, C., Uzzell, T (1986) Evolutionary history of the hybridogenetic hybrid frog *Rana esculenta* as deduced from mtDNA analyses. *Molecular Biology and Evolution* 3; 1: 44-56
11. Clarke BT (1997) The natural history of amphibian skin secretions, their normal functioning and potential medical applications. *Biological Reviews of the Cambridge Philosophical Society* 72; 3: 365-79
12. Noble GK (1931) *The biology of the Amphibia*. New York
13. Csordás A, Michl H (1969) Primary structure of two oligopeptides of the toxin of *Bombina variegata* L. *Toxicon* 7; 2: 103-8

14. Zasloff M (1987) Magainins, a Class of Antimicrobial Peptides from *Xenopus* Skin: Isolation, Characterization of Two Active Forms, and Partial cDNA Sequence of a Precursor. *Proceedings of the National Academy of Sciences of the United States of America* 84; 15: 5449-5453
15. Engeler BRHU (2001) Choosy females and indiscriminate males: mate choice in mixed populations of sexual and hybridogenetic water frogs (*Rana lessonae*, *P. kl. esculentus*)
16. Pal T, Abraham B, Sonnevend A, Jumaa P, Conlon JM (2006) Brevinin-1BYa: a naturally occurring peptide from frog skin with broad-spectrum antibacterial and antifungal properties. *International Journal of Antimicrobial Agents* 27; 6: 525-529
17. Islas-Rodriguez AE, Marcellini L, Barra D, Mangoni ML, Orioni B, Stella L (2009) Esculentin 1-21: A linear antimicrobial peptide from frog skin with inhibitory effect on bovine mastitis-causing bacteria. *Journal of Peptide Science* 15; 9: 607-614
18. Marcellini L, Borro M, Gentile G, Rinaldi AC, Stella L, Aimola P, Barra D, Mangoni ML (2009) Esculentin-1b(1-18) - a membrane-active antimicrobial peptide that synergizes with antibiotics and modifies the expression level of a limited number of proteins in *Escherichia coli*. *The FEBS Journal* 276; 19: 5647-5664
19. Samgina TY, Artemenko KA, Gorshkov VA, Poljakov NB, Lebedev AT (2008) Oxidation Versus Carboxamidomethylation of S-S Bond in Ranid Frog Peptides: Pro and Contra for de Novo MALDI-MS Sequencing. *Journal of the American Society for Mass Spectrometry* 19; 4: 479
20. Samgina TY, Artemenko KA, Gorshkov VA, Ogourtsov SV, Zubarev RA, Lebedev AT (2008) De novo sequencing of peptides secreted by the skin glands of the Caucasian Green Frog *Rana ridibunda*. *Rapid Communications in Mass Spectrometry* 22; 22: 3517-3525
21. Kyle AA, Dahl MV (2004) Topical Therapy for Fungal Infections. *American Journal of Clinical Dermatology* 5; 6: 443-451
22. Hillis DM, Davis SK (1986) Evolution of Ribosomal DNA: Fifty Million Years of Recorded History in the Frog Genus *Rana*. *Evolution* 40; 6: 1275-1288
23. Conlon JM, Kolodziejek J, Nowotny N (2009) Antimicrobial peptides from the skins of North American frogs. *Biochimica Et Biophysica Acta* 1788; 8: 1556-63
24. Ali MF, Knoop FC, Vaudry H, Conlon JM (2003) Characterization of novel antimicrobial peptides from the skins of frogs of the *P. kl. esculentus* complex. *Peptides* 24; 7: 955-961

25. Wang Y, Knoop F, Remy-Jouet I, Delarue C, Vaudry H, Conlon J (1998) Antimicrobial Peptides of the Brevinin-2 Family Isolated from Gastric Tissue of the Frog, *P. kl. esculentus*. *Biochemical and Biophysical Research Communications* 253; 3: 600
26. Mangoni ML, Papo N, Mignogna G, Andreu D, Shai Y, Barra D, Simmaco M (2003) Ranacyclins, a New Family of Short Cyclic Antimicrobial Peptides: Biological Function, Mode of Action, and Parameters Involved in Target Specificity. *Biochemistry* 42; 47: 14023
27. X. Chen, H. Wang, Y. Shen, L. Wang, M. Zhou, T. Chen, C. Shaw (2016) Kunitzins: Prototypes of a new class of protease inhibitor from the skin secretions of European and Asian frogs, *Biochemical and Biophysical Research Communications* doi: 10.1016/j.bbrc.2016.06.062
28. Lebedev, A. T., Damoc, E., Makarov, A. A., Samgina, T. Y (2014) Discrimination of leucine and isoleucine in peptides sequencing with Orbitrap Fusion mass spectrometer. *Analytical Chemistry* 86; 14: 7017-22
29. Samgina, T. Y., Gorshkov, V. A., Artemenko, K. A., Vorontsov, E. A., Klykov, O. V., Ogourtsov, S. V., Zubarev, R. A., Lebedev, A. T (2012) LC-MS/MS with 2D mass mapping of skin secretions' peptides as a reliable tool for interspecies identification inside *Rana esculenta* complex. *Peptides* 34; 2: 296-302
30. Samgina, T. Y., Gorshkov, V. A., Vorontsov, Y. A., Artemenko, K. A., Zubarev, R. A., Lebedev, A. T (2011) Mass spectrometric study of bradykinin-related peptides (BRPs) from the skin secretion of Russian ranid frogs. *Rapid Communications in Mass Spectrometry Rcm* 25; 7: 933-40
31. Chen, X., Wang, L., Wang, H., Chen, H., Zhou, M., Chen, T., Shaw, C (2011) A fish bradykinin (Arg 0, Trp 5, Leu 8-bradykinin) from the defensive skin secretion of the European edible frog, *Pelophylax kl. esculentus*: Structural characterization; molecular cloning of skin kininogen cDNA and pharmacological effects on mammalian smooth muscle. *Peptides* 32; 1: 26-30
32. T. Yu. Samgina, V. A. Gorshkov, Ye. A. Vorontsov, K. A. Artemenko, S. V. Ogourtsov, R. A. Zubarev, A. T. Lebedev (2011) Investigation of skin secretory peptidome of *Rana lessonae* frog by mass spectrometry. *Journal of Analytical Chemistry* 66; 13: 1298-1306
33. Chen, X., Wang, H., Yang, M., Wang, L., Zhou, M., Chen, T., Shaw, C (2016) Identification and bioactivity evaluation of two novel temporins from the skin secretion of the European edible frog, *Pelophylax kl. esculentus*. *Biochemical and Biophysical Research Communications* doi:10.1016/j.bbrc.2016.05.163

Data deposition footnote

The nucleotide sequence of the Brevinin-2Tbe and Esculentin-2c precursors from the skin secretion of the European edible frog, *Pelophylax kl. esculentus*, have been deposited in the EMBL Nucleotide Sequence Database under the accession code KT437661 and KT437660.

Legends to Figures

Fig 1. Nucleotide sequence and open reading frame amino acid translation of full length prepro cDNA from the skin secretion of the European edible frog *Rana esculenta* encoding brevinin-1E (a), brevinin-1Ra (b), brevinin-2Ec (c), Esculentin-2c (d), brevinin-Ei (e), brevinin-2Tbe (f) . The double underlined sequence is the putative signal peptide. The single underlined sequence is the sequence of the mature peptide and the stop codon is indicated by an asterisk.

Fig 2. (A) A comparison of the primary structures of the Brevinin-1 and -2 related peptides isolated from frogs of the *R.esculenta* complex with those of brevinin-1 and-2 from *R.brevipoda porsa*. (B) Comparison of the amino acid sequences of Esculentin-1 and -2 related peptides from skin secretions of *R.esculenta*. The disulphide bonds formed by the identical cysteine residues were underlined. Gaps (---) were introduced to maximise the identities. Amino acids common to all the peptides in each of the two subfamilies present in skin secretions of *R.esculenta* are in shadow.

Fig 3. (A) LCQ ESI Electrospray (LCQ) MS/MS spectrum of putative bombesin-related peptide. (B) Predicted b- and y-ion MS/MS fragment ion series (singly- and doubly- charged) of [Asn-3, Lys-6, Phe-13] 3-14-bombesin. Observed ions are indicated in black typeface.

Table 1. Skin secretion peptides isolated from *Rana esculenta*

Name	Sequence	M.W.	Ref.
Brevinin-1E	FLPLLAGLAANFLPKIFCKITRKC	2676	8
Brevinin-1Ea	FLPAIFRMAAKVVPTIICSITKKC	2649	8
Brevinin-1Eb	VIPFVASVAEMQHVYCAASRKC	2480	8
Brevinin-1Ecb	FLPLLAGLAANFFPKIFCKITRKC	2712	8
Brevinin-1Ra	VIPFVASVAEMMQHVYCAASRRC	2640	19, 20
Brevinin-2E	GIMDTLKNLAKTAGKGALQSLLNKASCKLSGQC	3361	8
Brevinin-2Ea	GILDTLKNLAISAAKGAAQGLVKNKASCKLSGQC	3242	8
Brevinin-2Eb	GILDTLKNLAKTAGKGALQGLVKNKASCKLSGQC	3316	8
Brevinin-2Ec	GILLDKLNFAKTAGKGVLSLLNTASCKLSGQC	3519	8
Brevinin-2Ed	GILDSLKNLAKNAGQILLNKASCKLSGQC	2999	8
Brevinin-2Ef	GIMDTLKNLAKTAGKGALQSLVKMASCKLSGQC	3365	25
Brevinin-2Eg	GIMDTLKNLAKTAGKGALQSLLNHASCKLSGQC	3371	25
Brevinin-2Eh	GIMDTLKNLAKTAGKGALQSLLNHASCKLSKQC	3442	25
Brevinin-2Ei	GILDTLKNLAKTAGKGILKSLVNTASCKLSGQC	3309	25
Brevinin-2Ej	GIFLDKLNFAKGVASLLNKASCKLSGQC	3181	24
Brevinin-2Tbe	GILDTLKNLAKTAGKGALQSLLNHASCKLSGQC	3354	-
CPRF-Ea	GLGSILGKILNVAGKVGKTIGKVADAVGNKE	3007	24
CPRF-Eb	GLGSFLKNAIKIAGKVGSTIGKVADAIGNKE	3055	24
CPRF-Ec	GLGSFFKNAIKIAGKVGSTIGKVADAIGNKE	3091	24
Esculentin-1	GIFSKFGRKKIKNLLISGLKNVGKEVGMDVVRTGIDIAG CKIKGEC	4884	8
Esculentin-1a	GIFSKLAGKKIKNLLISGLKNVGKEVGMDVVRTGIDIA GCKIKGEC	4799	8
Esculentin-1b	GIFSKLAGKKLKNLLISGLKNVGKEVGMDVVRTGIDIA GCKIKGEC	4802	8
Esculentin-1c	GIFSKLAGKKIKNLLISGLKNIGKEVGMDVVRTGIDIAG CKIKGEC	4813	8

Esculentin-2a	GILSLVKGVAKLAKGLAKEGGKFGLELIACKIAKQC	3711	8
Esculentin-2b	GIFSLVKGAAKLAGKGLAKEGGKFGLELIACKIAKQC	3717	8
Esculentin-2c	GIFSLVKGAAKLLGKGLAKEGGKFGLELMACKIAKQC	3778	-
Ranacyclin E	SAPRGCWTKSYPPKPKCK	1904	26
Temporin-1Ec	FLPVIAGLLSKLF	1417	9,24
Peptides A1	FLPAIAGILSQLF	1388	9,24
Peptides B9	FLPLIAGLLGKLF	1400	9,24
Temporin-1Ee	FLPVIAGVLSKLF	1402	33
Temporin-1Re	FLPGLLAGLL-NH ₂	1012	33
[Asp ³ , Lys ⁶ , Phe ¹³]3-14- bombesin	NLGKQWAVGHFM	1386	20, 29, 32
kunitzin-RE	AAKIILNPKFRCKAAFC	1893	20,27,28
Arg ⁰ , Trp ⁵ , Leu ⁸ -bradykinin	RRPPGWSPLR	1221	29, 30, 31, 32

Fig 1.

(a)

M F T M K K S M L L L F F L G T I
1 ATGTTACCA TGAAGAAATC CATGTTACTC CTTTCTTCC TTGGGACCAT
TACAAGTGGT ACTTCTTTAG GTACAATGAG GAAAAGAAGG AACCTGGTA
N L S L F E E E R D A D E E E R
51 CAACTTATCT CTTTTGAGG AAGAGAGAGA TGCCGATGAA GAAGAAAGAA
GTTGAATAGA GAAAAACTCC TTCTCTCTCT ACGGCTACTT CTTCTTTCTT
R D N P D E S E V E V E K R F L P
101 GAGACAATCC AGATGAAAGT GAAGTTGAAG TGGAAAAACG ATTTCTTCCA
CTCTGTTAGG TCTACTTTCA CTTCAACTTC ACCTTTTTCG TAAAGAAGGT
L L A G L A A N F L P K I F C K I
151 TTGTGGCAG GTCTGGCTGC TAATTTCTTG CCGAAAATAT TTTGTAATAA
AACACCGTC CAGACCGACG ATTAAGAAGC GGCTTTTATA AACATTTTA
T R K C *
201 AACCAGAAAA TGTTGAAACT TTGGAATTGG AAATCATCTG ATGTGGAAAA
TTGGTCTTTT ACAACTTTGA AACCTTAACC TTTAGTAGAC TACACCTTTT
251 TCATTTAGCT AAATACACAT CAGATGTCTT ATAAAAATA AAGATATTGC
AGTAAATCGA TTTATGTGTA GTCTACAGAA TATTTTTTAT TTCTATAACG
301 ATACAGAATA TAAAAAATA AAAAAAATA AAAAAA
TATGTCTTAT ATTTTTTTTT TTTTTTTTTT TTTTTTA

(b)

M F T M K K S M L L L F F I G T I
1 ATGTTACCA TGAAGAAATC CATGTTACTC CTTTCTTTA TTGGGACCAT
TACAAGTGGT ACTTCTTTAG GTACAATGAG GAAAAGAAAT AACCTGGTA
N L S L C E E E R A A D E E E R
51 CAACTTATCT CTCTGTGAGG AAGAGAGAGC TGCTGATGAG GAAGAAAGAA
GTTGAATAGA GAGACACTCC TTCTCTCTCG ACGACTACTC CTTCTTTCTT
R D D Q A E T E V E V E K R V I P
101 GAGATGATCA AGCAGAAACA GAGGTTGAGG TGGAAAAACG AGTTATACCA
CTCTACTAGT TCGTCTTTGT CTCCAATCC ACCTTTTTCG TCAATATGGT
F V A S V A A E M M Q H V Y C A A
151 TTTGTGCAA GTGTGGCTGC CGAAATGATG CAGCACGTGT ATTGTGCAGC
AACACCGTT CACACCGACG GCTTTACTAC GTCGTGCACA TAACACGTCG
S R R C *
201 TTCCAGAAGA TGTTAAATTA AATTGGAAAT CATCTGCTGT GGAAAATCAT
AAGGTCTTCT ACAATTTAAT TTAACCTTTA GTAGACGACA CCTTTTAGTA
251 TTAGCTAAAT GCTAAATGTC TTATAAAAAA ATAAAGTTGT TGCATACACT
AATCGATTTA CGATTACAG AATATTTTTT TATTTCAACA ACGTATGTGA
301 GTTACAAAA AAAAAAATA AAAAAAATA AAAAAA
CAATGTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTT

(c)

M F T M K K S L L L L F F L G T I
1 ATGTTACCA TGAAGAAATC CCGTTACTC CTTTCTTC TTGGACCAT
TACAAGTGGT ACTTCTTTAG GGACAATGAG GAAAAGAAAG AACCTGGTA
S L S L C E E E R N A D E D D G
51 CTCCTTATCT CTCTGTGAGG AAGAGAGAAA TGCTGATGAG GATGATGGGG
GAGGAATAGA GAGACACTCC TTCTCTCTTT ACGACTACTC CTACTIONCC
E M T E E V K R G I L L D K L K N
101 AAATGACAGA GGAAGTAAA AGAGGTATCC TCCTGGATAA GCTGAAGAAT
TTTACTGTCT CTTTCATTTT TCTCCATAGG AGGACCTATT CGACTTCTTA
F A K T A G K G V L Q S L L N T A
151 TTTGCCAAGA CAGCAGGCAA AGGTGTGCTC CAGAGTCTGC TGAATACGGC
AAACGGTTCT GTCGTCCGTT TCCACACGAG GTCTCAGACG ACTTATGCCG
S C K L S G Q C *
201 ATCTGTAAA CTTTCTGGAC AATGTTAAA CATGAATTGG AAGTCATTG
TAGAACATTT GAAAGACCTG TTACAATTTT GTACTTAACC TTCAGTAAAC
251 ATGCAGAATA TCATTAGCT AAATGCTAAA TGTCTGATAA AAAATAAAA
TACGTCTTAT AGTAAATCGA TTTACGATTT ACAGACTATT TTTTATTTT
301 GATCACACAA AAAAAAAAAA AAAAAAAAAA AAAAAA
CTAGTGTGTT TTTTTTTTTT TTTTTTTTTT TTTTTTTT

(d)

M K K S L L L L F F I G T I
1 GTTACCATG AAGAAATCCC TGTTACTCCT TTTCTTTATT GGGACCATCT
CAAGTGGTAC TTCTTTAGGG ACAATGAGGA AAAGAAATAA CCCTGGTAGA
S L S L C Q E E R G A D G E E E G
51 CCTATCTCT CTGTCAGGAA GAGAGAGGCG CCGATGGAGA AGAGGAAGGG
GGAATAGAGA GACAGTCTT CTCTCTCCGC GGCTACCTCT TCTCCTTCCC
E E M K R G I F S L V K G A A K L
101 GAAGAAATGA AAAGAGGTAT TTTCTCGCTA GTCAAAGGTG CAGCCAAGCT
CTTCTTTACT TTTCTCCATA AAAGAGCGAT CAGTTTCCAC GTCGGTTCGA
L G K G L A K E G G K F G L E L
151 ACTGGGCAAA GGTTGGCCA AGGAAGGGGG CAAGTTTGGG CTGGAGCTTA
TGACCCGTTT CCAAACCGGT TCCTTCCCCC GTTCAAACCC GACCTCGAAT
M A C K I A K Q C *
201 TGGCTTGTA AATTGCAAAA CAATGTAAA TCTCAATTG GAGTCATCT
ACCGAACATT TTAACGTTTT GTTACAATTT AGAAGTTAAC CTCCAGTAGA
251 GATGTGGAAT ATCATTAGC AAAATGCTAA TTGTCTAATA AAAAAATAG
CTACACCTTA TAGTAAATCG TTTTACGATT AACAGATTAT TTTTTTTATC
301 CAATGTCACA AAAAAAAAAA AAAAAAAAAA AAAA
GTTACAGTGT TTTTTTTTTT TTTTTTTTTT TTTT

(e)

 M F T L K K S L L L F F F L G T I
1 ATGTTACCT TGAAGAAATC CCTGTTACTC TTTTCTTTC TTGGGACCAT
TACAAGTGGA ACTTCTTTAG GGACAATGAG AAAAAGAAAG AACCTGGTA
 S L S L C Q E E R N A D E D D G
51 CTCCTTATCT CTCTGTCAGG AAGAGAGAAA TGCTGATGAG GACGATGGGG
GAGGAATAGA GAGACAGTCC TTCTCTCTTT ACGACTACTC CTGCTACCCC
 E M T E E E K R G I L D T L K N L
101 AAATGACAGA GGAAGAAAAA AGAGGTATCC TGGATACGCT GAAGAATTTA
TTTACTGTCT CTTCTTTTTT TCTCCATAGG ACCTATGCGA CTTCTTAAAT
 A K T A G K G I L K S L V N T A S
151 GCCAAGACAG CAGGCAAAGG TATACTGAAG AGTCTGGTGA ATACGGCATC
CGGTTCTGTC GTCCGTTTCC ATATGACTTC TCAGACCACT TATGCCGTAG
 C K L S G Q C *
201 TTGTAAACTT TCTGGACAAT GCTAAAACAT GAATTGGAAG TCATTGATG
AACATTTGAA AGACCTGTTA CGATTTTGTA CTTAACCTTC AGTAAACTAC
251 CAGCATATCA TTTAGCTAAA TACTAAATGT CTGATAAAAA ATAAAAATAT
GTCTTATAGT AAATCGATT ATGATTACA GACTATTTTT TATTTTATA
301 CACATGAAAA AAAAAAAAAA AAAAAAAAAA AAAA
GTGTACTTTT TTTTTTTTTT TTTTTTTTTT TTTT

(f)

 M F T L K K S L L L F F F L G T I
1 ATGTTACCT TGAAGAAATC CCTGTTACTC TTTTCTTTC TTGGGACCAT
TACAAGTGGA ACTTCTTTAG GGACAATGAG AAAAAGAAAG AACCTGGTA
 S L S L C Q E E R N A D E D D G
51 CTCCTTATCT CTCTGTCAGG AAGAGAGAAA TGCTGATGAG GACGATGGGG
GAGGAATAGA GAGACAGTCC TTCTCTCTTT ACGACTACTC CTGCTACCCC
 E M T E E E K R G I L D T L K N L
101 AAATGACAGA GGAAGAAAAA AGAGGTATCC TGGATACGCT GAAGAATTTA
TTTACTGTCT CTTCTTTTTT TCTCCATAGG ACCTATGCGA CTTCTTAAAT
 A K T A G K G A L Q S L L N H A S
151 GCCAAGACAG CAGGCAAAGG TCGCTCCAG AGTCTGCTGA ATCATGCATC
CGGTTCTGTC GTCCGTTTCC ACGCGAGGTC TCAGACGACT TAGTACGTAG
 C K L S G Q C *
201 TTGTAAACTT TCTGGACAAT GTAAAACAT GAATTGGAAG TCATTGATG
AACATTTGAA AGACCTGTTA CAATTTTGTA CTTAACCTTC AGTAAACTAC
251 CAGAATATCA TTTAGCTAAA TACTAAATGT CTGATAAAAA ATAAATAGAT
GTCTTATAGT AAATCGATT ATGATTACA GACTATTTTT TATTATCTA
301 CAC
GTG

Fig 2.

(a)

	1	24
Brevinin-1	(1)	FLPVLAGIAAKVVPALFCKITKKC
Brevinin-1E	(1)	FLPILLAGLAANFLPKIFCKITRKC
Brevinin-1Ea	(1)	FLPATFRMAAKVVPPTIICSITKKC
Brevinin-1Eb	(1)	VIPFVASVAAEMQ-HVYCAASRKC
Brevinin-1Ecb	(1)	FLPILLAGLAANFFPKIFCKITRKC
Brevinin-1Ra	(1)	VIPFVASVAAEMMQHVYCAASRRC

Formatted: Font: (Default) Courier New, 10 pt, Bold

Formatted: Font: (Default) Courier New, 10 pt, Bold, Font color: Red

	1	34
Brevinin-2	(1)	-GLDSLKGF AATAGKGVLSLLSTASCKLAKTC
Brevinin-2E	(1)	-GIMDTLKNLAKTAGKQALQSLLNKASCKLSGQC
Brevinin-2Ea	(1)	-GILDTLKNLAISAARKGAAQGLVKNKASCKLSGQC
Brevinin-2Eb	(1)	-GILDTLKNLAKTAGKQALQGLVKNKASCKLSGQC
Brevinin-2Ec	(1)	GILDKLKNFAKTAGKGVLSLLNTASCKLSGQC
Brevinin-2Ed	(1)	-GILDSLKNLAKNAG---QILLNKASCKLSGQC
Brevinin-2Ef	(1)	-GIMDTLKNLAKTAGKQALQSLVKNKASCKLSGQC
Brevinin-2Eg	(1)	-GIMDTLKNLAKTAGKQALQSLLNHASCKLSGQC
Brevinin-2Eh	(1)	-GIMDTLKNLAKTAGKQALQSLLNHASCKLSKQC
Brevinin-2Ei	(1)	-GILDTLKNLAKTAGKQALQSLVKNKASCKLSGQC
Brevinin-2Ej	(1)	GIFDKLKNFAK---GVAQSLLNKASCKLSGQC
Brevinin-2Tbe	(1)	-GILDTLKNLAKTAGKQALQSLLNHASCKLSGQC

Formatted: Font: (Default) Courier New, 10 pt, Bold

Formatted: Font: (Default) Courier New, 10 pt, Bold, Font color: Red

(b)

	1	48
Esculentin-1	(1)	GIFSKFGRKKIKNLLISGLKNVGKEVGMD--VVRTGIDIAGCKIRGEC
Esculentin-1a	(1)	GIFSKLAGKKIKNLLISGLKNVGKEVGMD--VVRTGIDIAGCKIRGEC
Esculentin-1b	(1)	GIFSKLAGKKIKNLLISGLKNVGKEVGMETDVVRTGIDIAGCKIRGEC
Esculentin-1c	(1)	GIFSKLAGKKIKNLLISGLKNIGKEVGMD--VVRTGIDIAGCKIRGEC

Formatted: Font: (Default) Courier New, 10 pt, Bold

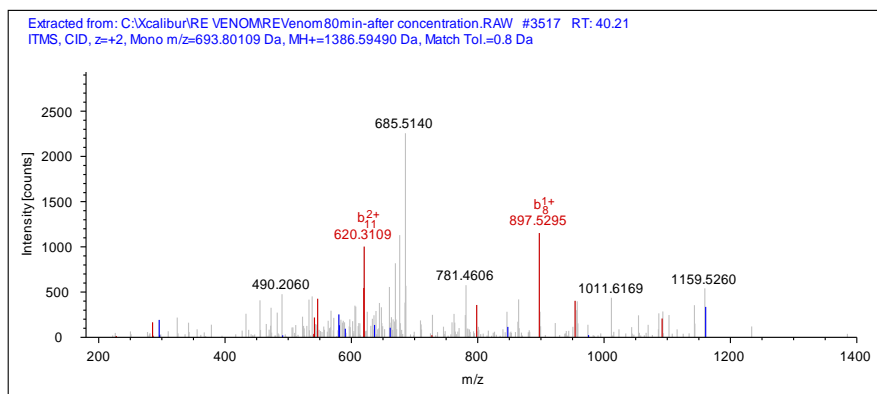
Formatted: Font: (Default) Courier New, 10 pt, Bold

	1	37
Esculentin-2a	(1)	GILSLVKGVAKLAGKGLAKEGGKFGLELIACKIAKQC
Esculentin-2b	(1)	GIFSLVKGAAKLAGKGLAKEGGKFGLELIACKIAKQC
Esculentin-2c	(1)	GIFSLVKGAAKLLGKGLAKEGGKFGLELMACKIAKQC

Formatted: Font: (Default) Courier New, 10 pt, Bold, Font color: Black

Formatted: Font: (Default) Courier New, 10 pt, Bold

Fig 3.



Formatted: Font: Calibri, 11 pt

#1	b(1+)	b(2+)	Seq.	y(1+)	y(2+)	#2
1	115.05021	58.02874	N	-	-	12
2	228.13428	114.57078	L	1273.65109	637.32918	11
3	285.15575	143.08151	G	1160.56702	580.78715	10
4	413.25072	207.12900	K	1103.54555	552.27641	9
5	541.30930	271.15829	Q	975.45058	488.22893	8
6	727.38862	364.19795	W	847.39200	424.19964	7
7	798.42574	399.71651	A	661.31268	331.15998	6
8	897.49416	449.25072	V	590.27556	295.64142	5
9	954.51563	477.76145	G	491.20714	246.10721	4
10	1091.57454	546.29091	H	434.18567	217.59647	3
11	1238.64296	619.82512	F	297.12676	149.06702	2
12	-	-	M	150.05834	75.53281	1