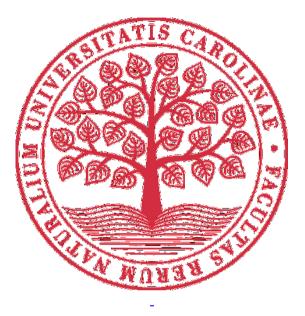
Charles University, Faculty of Science Department of Zoology

Doctoral study programme: Zoology

Summary of the Doctoral thesis



Mechanisms of the karyotype evolution in scorpions (Arachnida: Scorpiones)

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Abstract

Scorpions represent an ancient group of arachnids that have colonized a diverse range of terrestrial environments since Silurian times. Despite their long evolutionary history and ecological diversity, these animals are characterized by morphological stasis. However, the uniform morphology of extant scorpions may hide the true diversity of contemporary lineages. The study of cytogenetic traits thus has the potential to deepen our knowledge of the diversity of contemporary scorpion taxa and bring new insight into the processes underlying karyotypic changes in this group of arachnids. The subject of this thesis is to examine the diversity and dynamics of karyotypes of selected scorpion groups with holokinetic and monocentric chromosomes. Comparative cytogenetic analysis, including more than 110 species from five families, was based on standard cytogenetic techniques and mapping of the gene for 18S ribosomal RNA and telomeric (TTAGG)n repeats by fluorescence in situ hybridization (FISH). To elucidate the fundamental structural mechanisms underlying karyotype differentiation in the studied groups, the emphasis was placed on the interconnection of cytogenetic and sequence data of the analyzed species. The knowledge of the genetic structure and phylogenetic relationships of studied taxa played a crucial role in the interpretation of observed chromosomal polymorphism or polytypism. Moreover, this knowledge enabled us to propose the main trends involved in the reorganization of scorpion karyotypes. The present results demonstrate that scorpions exhibit exceptional chromosomal variability at different structural levels. The detected differences within individual scorpion lineages indicate a distinct degree of involvement of macro- and microstructural changes in karyotype differentiation.

Keywords: karyotype differentiation, holokinetic chromosomes, multivalent association, chromosome rearrangements, telomeric (TTAGG)n repeat

Abstrakt

Štíři představují starobylou skupinu pavoukovců, která od dob siluru kolonizovala rozmanitou škálu terestrických prostředí. Navzdory dlouhé evoluční historii a ekologické rozmanitosti je pro tyto živočichy příznačná morfologická stáze. Uniformní morfologie štírů nicméně může maskovat skutečnou diverzitu současných linií. Studium cytogenetických znaků tak pomůže prohloubit naše znalosti o rozmanitosti recentních taxonů štírů a současně přinést nový vhled do procesů stojících v pozadí karyotypových změn u této skupiny pavoukovců. Předmětem předkládané dizertační práce je studium rozmanitosti a dynamiky karyotypů vybraných zástupců štírů s holokinetickými a monocentrickými chromozomy. Komparativní cytogenetické analýzy více než 110 druhů z pěti čeledí zahrnovaly nejen standardních cytogenetické techniky, ale i mapování genu pro 18S ribozomální RNA a telomerických (TTAGG)n repetic metodou fluorescenční in situ hybridizace (FISH). Pro účely objasnění fundamentálních strukturních mechanismů stojících v pozadí diferenciace karvotypu u studovaných skupin byl kladen důraz na propojení cytogenetických a sekvenčních dat analyzovaných druhů. Znalost genetické struktury a příbuzenských vztahů studovaných taxonů sehrála nezastupitelnou roli jednak při interpretaci chromozomální polymorfismu, resp. polytypie, jednak pro navržení hlavních trendů uplatňujících se v reorganizaci štířích karyotypů. Předkládané výsledky demonstrují, že štíři se vyznačují nebývalou chromozomální variabilitou na různých strukturních úrovních. Detekované rozdíly naznačují různou míru zapojení makro- i mikrostrukturních změn při diferenciaci karyotypů v rámci jednotlivých evolučních linií štírů.

Klíčová slova: karyotypová diferenciace, holokinetické chromozomy, multivalentní asociace, chromozomální přestavby, telomerické (TTAGG)n repetice

1. Introduction

Order Scorpiones represents an iconic and evolutionarily successful group of arachnids that appeared in the Silurian period (Dunlop 2010). Despite the long evolutionary history and high adaptability to different terrestrial environments, scorpions are exceptionally uniform in their external morphology. However, such morphological stasis may result in discrepancies between systematics and taxonomy and hinder the discovery of the true diversity of scorpion species. Thus, the study of the evolution and diversity of this group of arachnids is currently focused on the employment of modern molecular methods with an emphasis on an integrative approach combining different data sources (e.g. Bryson et al. 2013; Ojanguren-Affilastro et al. 2017; Talal et al. 2015). Cytogenetic research of scorpions has also undergone significant progress over the last decade, which has led to a deeper understanding of the genome structure of the studied taxa (e.g. Mattos et al. 2018; Ubinski et al. 2018). The complex evolutionary history of the species could be imprinted not only on the genetic variability of current lineages, but it can also leave traces in their karyotype structure. The study of cytogenetic traits can thus deepen our knowledge of scorpion diversity and bring new insight into the processes involved in karyotypic changes. However, the integrative approach, combining the study of genetic and karyotypic diversity of contemporary lineages in the context of their geographical distribution, is still lacking in scorpions.

Current knowledge of scorpion cytogenetics is based on narrowly-focused studies of a different nature. Thanks to these works we have fundamental knowledge about spermatogenesis (e.g. Sokolow 1913), formation of synaptonemal complexes (e.g. Shanahan & Hayman 1990), achiasmatic meiosis in males (e.g. Almeida et al. 2019), and karyotype diversity of species (e.g. Shanahan 1989a,b; Schneider et al., 2009a,b). Scorpions are characterized by a wide range of chromosome numbers – from 2n = 5 in *Tityus bahiensis* (Buthidae) (Schneider et al. 2009a) to 2n = 186 in *Chaerilus stockmannorum* (Chaerilidae) (Kovařík et al. 2018). Moreover, the presence of chromosomal polymorphism, associated with the occurrence of structural heterozygotes, has been identified in a number of species (e.g. Mattos et al. 2013; Shanahan 1989a, b; Schneider et al. 2009a, b). Order Scorpiones includes evolutionary lineages whose chromosomes show a different kinetochore distribution. The occurrence of monocentric chromosomes characterized by a localized centromere was documented in a majority of cytogenetically studied families. In contrast, the presence of holokinetic chromosomes with a diffuse kinetochore is a characteristic cytogenetic trait of a single evolutionary scorpion lineage, family Buthidae (Shanahan 1989a). The unique

combination of cytogenetic features makes scorpions an attractive group for studying various aspects of karyotype diversity and differentiation. Moreover, the occurrence of evolutionary lineages with both monocentric and holokinetic chromosomes offers a great opportunity to compare the cytogenetic peculiarities of these two different systems.

The two aims of this thesis are: (i) to extend current knowledge of various cytogenetic aspects in scorpions and, (ii) to elucidate the main mechanisms of karyotype differentiation in selected scorpion groups with holokinetic and monocentric chromosomes. Karyotypic diversity was examined in more than 110 species at different structural levels using both standard and molecular cytogenetic approaches. The karyological data obtained were further interpreted in selected groups in the context of phylogenetic relationships of studied species, or used for taxonomic purposes.

2. Aims of the study

This thesis entitled "Mechanisms of the karyotype evolution in scorpions (Arachnida: Scorpiones)" focuses on the study of karyotype diversity and dynamics of genome arrangement in major scorpion evolutionary lineages which possess chromosomes with the distinct spatial distribution of the kinetochore [i.e. holokinetic and monocentric chromosomes]. Examination of cytogenetic aspects in scorpions with holokinetic chromosomes included a comparative analysis of more than 80 species belonging to the family Buthidae which is unique among other scorpion families by having chromosomes with diffuse kinetochore. To elucidate cytogenetic features in scorpions with monocentric chromosomes, representatives belonging to four families, Euscorpiidae, Hormuridae, Scorpionidae and Scorpiopiodae, were studied. In total, comparative data from more than 110 species was obtained for cytogenetic analyses based on standard and molecular cytogenetic methods. Selected scorpion groups were subsequently included in molecular phylogenetic analyses or morphological analyses. In this thesis, I aimed to resolve several specific tasks:

- 1. To describe karyotypes of studied species using conventional and molecular cytogenetic methods and to use the data obtained for comparative karyotype analysis emphasizing detection of chromosomal polymorphism or polytypism.
- 2. To integrate cytogenetic and genetic data in order to elucidate the main trends of karyotype differentiation in selected groups of scorpions.
- 3. To utilize cytogenetic data for taxonomic purposes.
- 4. To identify the main mechanisms involved in karyotype differentiation specific for groups with holokinetic and monocentric chromosomes

3. Material and methods

Examined specimens were obtained during field trips organized by lab members, in collaboration with other colleagues, or from the exotic pet trades. The material included more than 110 species from major biogeographic regions (except Australasia and the Nearctic realm). The karyotypes of studied individuals were examined using standard cytogenetic techniques (Giemsa staining, C-banding) and molecular cytogenetic methods (fluorescence *in situ* hybridization with 18S rDNA and telomeric repeat (TTAGG)*n* probes). Molecular techniques included DNA isolation and amplification, PCR, and probe synthesis. Sequence editing was performed in Geneious R11 and phylogenetic trees were reconstructed using

Bayesian inference (BI) and Maximum Likelihood (ML). The BI analyses were conducted in MrBayes v.3.2 and the ML analyses were conducted in raxmlGUI 1.3. The graphical visualizations and image analysis were performed using appropriate software (FIJI, Affinity Designer, Affinity Photo).

4. Results and discussion

Using a combination of several methodological approaches, I have studied aspects of karyotype differentiation at various structural levels in selected scorpion groups. Publications included in this thesis show the importance of the integrative approach in tackling questions of karyotypic dynamics and scorpion systematics.

Summary of included publications:

I. Šťáhlavský, F., Nguyen, P., Sadílek, D., Štundlová, J., Just, P., Haddad, C. R., Koç, H., Ranawana, K.B., Stockmann, M., Yağmur, E.A., Kovařík, F. Evolutionary dynamics of rDNA clusters on chromosomes of buthid scorpions (Chelicerata: Arachnida). *Manuscript*.

This study significantly broadens the knowledge of chromosomal distribution and the dynamics of major ribosomal DNA loci in buthid scorpions. Chromosomal mapping of 18S rDNA was carried out in 75 species from 19 genera, covering all the main evolutionary lineages within Buthidae. As such, it represents the most robust sampling set yet studied in scorpions using the rDNA-FISH method. The data were interpreted in the phylogenetic context proposed by Fet et al. (2003, 2005) and Esposito et al. (2018). Our results demonstrate that the number and position of rDNA across buthid species are more variable than previously suggested. In addition, a specific rDNA phenotype shows a positive phylogenetic signal. Identified changes in rDNA distribution probably resulted from structural mechanisms, such as inversions or fissions. On the other hand, the dispersion of rDNA sites in the genome, which was restricted to particular evolutionary lineages, is still of unknown origin.

II. Just, P., Šťáhlavský, F., Kovařík, F., Štundlová, J. Tracking the trends of karyotype differentiation in the phylogenetic context in *Gint* Kovařík et al., 2013, a scorpion group endemic to the Horn of Africa (Scorpiones: Buthidae). *Manuscript*.

This integrative study is the first of its kind in scorpions with holokinetic chromosomes. In order to elucidate fundamental mechanisms of karyotype differentiation in the scorpion genus *Gint*, we conducted an integrative approach combining cytogenetic data and sequence-based phylogeny for the species of interest. Our results show that *Gint* is exceptionally diverse in its karyotype macrostructure and also in the distribution of rDNA. Moreover, most of the analyzed individuals were heterozygous for reciprocal translocation and/or fusions/fissions. These findings point to the ongoing changes in genome organization in this scorpion group. The compilation of different data sources has allowed us: (i) to verify species and kinship identity of polymorphic individuals; (ii) to reveal that significant intra- and interspecific chromosomal differences strikingly contrast with the low level of genetic divergence of the species studied; (iii) to propose the main trends of karyotypic differentiation in the phylogenetic context.

III. Kovařík, F., Košulič, O., Šťáhlavský, F., Plíšková, J., Dongkhamfu, W., Wongprom,
P., 2015. Two new species of *Euscorpiops* Vachon, 1980 from Thailand and
Myanmar (Scorpiones: Euscorpiidae: Scorpiopinae). *Annales Zoologici* 65, 109–122.
(IF₂₀₁₅= 1.136)

Euscorpiops represents a diversified scorpion genus inhabiting Southeast Asia. From the cytogenetic point of view, this group is virtually unexplored. Karyotype characteristics were known at that time in a single species of this genus, *Euscorpiops neradi* [2n = 48] (Kovařík et al. 2013). For this reason, we analyzed the karyotype of newly described species *Euscorpiops orioni* using standard cytogenetic techniques (Giemsa staining and C-banding). We found that this species having 2n = 103 shows distinctive differences in karyotype macrostructure compared to *E. neradi*. Moreover, both analyzed specimens were heterozygous for fusion/fission resulting in the odd number of chromosomes in this species. Our results extend the knowledge of karyotype diversity in *Euscorpiops* and also demonstrate the high potential of using karyotype characteristics in the cytotaxonomy of this scorpion group.

IV. Plíšková, J., Kovařík, F., Košulič, O., Šťáhlavský, F., 2016. Description of a new species of *Heterometrus* Ehrenberg, 1828 (Scorpiones: Scorpionidae) from Thailand with remarks about the utilization of cytogenetic data in taxonomy of the genus. *Annales Zoologici* 66, 467–476. (IF₂₀₁₆= 0.699)

Genus *Heterometrus* represents one of the better explored scorpion groups from a cytogenetic point of view. Various studies have shown that *Heterometrus* species exhibit striking interspecific karyotype differences, suggesting cytogenetic features could act as a useful cytotaxonomic marker in this scorpion group. In this study, we described the holotype of *Heterometrus minotaurus* using traditional morphological criteria and karyotype analysis (based on Giemsa staining and C-banding). We found that this species possesses the lowest number of chromosomes within its genus, 2n = 54. Surprisingly, two heterozygous associations were present in its karyotype - a trivalent and multivalent chain composed of 13 chromosomal elements. Our findings provide the first evidence that multivalent associations can also achieve high complexity in scorpions with monocentric chromosomes. Moreover, we demonstrate that the previous cytogenetic records for a number of *Heterometrus* species, unfortunately, can not be used to address current taxonomic issues of the genus. *Heterometrus* has undergone large-scale changes in the taxonomic status and delimitation of the species over the years. As such, it is therefore uncertain to which taxa the published cytogenetic characteristics belong.

V. Šťáhlavský, F., Štundlová, J., Lowe, G., Stockmann, M., Kovařík, F., 2018. Application of cytogenetic markers in the taxonomy of flat rock scorpions (Scorpiones: Hormuridae), with the description of *Hadogenes weygoldti* sp. n. *Zoologischer Anzeiger* 273, 173–182. (IF₂₀₁₈= 1.601)

Hadogenes represents a typical element of the Afrotropical fauna reaching the highest species richness in South Africa. In this study, we carried out comparative cytogenetic analysis using both standard and molecular cytogenetic techniques in three *Hadogenes* species. Our results corroborate with findings by Newlands & Cantrell (1985) that members of this genus are exceptionally diversified in its karyotype macrostructure, which highly contrasts with shallow interspecific morphological differences. Moreover, we have deepened the knowledge of karyotype variability in *Hadogenes* by finding interspecific differences in the chromosomal location of rDNA loci. On the other hand, telomeric (TTAGG)*n* repeats were located exclusively at the ends of the chromosomes, suggesting that extensive changes in genome

organization in *Hadogenes* may not have included telomeric regions, or interstitial telomeric regions could be subsequently lost or reduced to a level that escapes detection by FISH. We conclude that karyotype characteristics, as a part of integrative taxonomic studies, may serve as helpful markers in delineating closely related, morphologically indistinguishable *Hadogenes* taxa.

VI. Štundlová, J., Šmíd, J., Nguyen, P., Šťáhlavský, F., 2019. Cryptic diversity and dynamic chromosome evolution in Alpine scorpions (Euscorpiidae: *Euscorpius*). *Molecular Phylogenetics and Evolution* 134, 152–163. (IF_{2018/2019}= 3.992)

This interdisciplinary study, the first of its kind in scorpions, combines cytogenetic methods with multilocus time-calibrated phylogenetic analyses in order to uncover hidden diversity and to elucidate the mechanisms of karyotype evolution in Alpine scorpions. An extensive sampling across the species range and a combination of different data sources helped us to reveal that Alpine scorpions are remarkably diversified at both chromosomal and genetic levels. Using an integrative approach, we have delineated ten deeply divergent lineages that exhibit the features of local endemics and indicate the presence of cryptic species. Based on molecular dating, we suggested that these lineages diversified during the Plio-Pleistocene. Our results indicate that macrostructural chromosomal changes most likely did not represent a pivotal driving force in diversification, but are, rather, a consequence of this process. Our findings served as a basis for the subsequent taxonomic revision of Alpine scorpions (Kovařík et al. 2019).

5. Conclusions

This PhD thesis contributes significantly to the expansion of current knowledge about karyotypic diversity at various structural levels in scorpions with holokinetic and monocentric chromosomes. In addition, we have combined, for the first time, cytogenetic and molecular phylogenetic data in scorpions, allowing us to follow the main trends involved in the reorganization of scorpion karyotypes.

An integrated methodological approach has not been routinely implemented in scorpions in the study of karyotypic differentiation mechanisms, although, as indicated by our results, it creates an effective tool for explaining the phenomena of interest. The results of particular projects included in this thesis demonstrate that knowledge of the genetic structure and phylogenetic relationships of studied taxa is a key element for the interpretation of chromosomal polymorphism or polytypism. Moreover, such information forms a necessary framework for proposing evolutionary trends in structural changes in the genome architecture of the species studied. In light of the morphological uniformity of scorpions, which may mask the true diversity of extant taxa, the importance of the combined analysis of cytogenetic and genetic traits is all the more important. In this regard, our outcomes of comparative analyses in Alpine scorpions also accentuate the importance of extensive sampling in the study of hidden diversity and karyotypic variability in taxa whose populations are sensitive to spatial isolation and accumulation of different types of chromosomal changes due to low vagility and stenotopy. Thus, integrative studies, which are the main basis of this thesis, may represent a new direction for the study of scorpion cytogenetics in the future.

The cytogenetic results obtained for representatives of evolutionary scorpion lineages with both holokinetic and monocentric chromosomes confirmed the assumption that these different systems exhibit specific karyotypic changes at different structural levels. Scorpions of the family Buthidae having holokinetic chromosomes are characterized by low diversity in chromosome numbers. Buthids are characterized by either relatively conservative or highly polymorphic karyotype macrostructure. According to the occurrence of structural heterozygotes, it is apparent that the reorganization of chromosomes in buthid scorpions occurs at a high frequency. Such changes arise primarily through two types of macrostructural rearrangements: prevalent reciprocal translocations and minor chromosomal fusions/fissions. At the same time, our results from 18S rDNA mapping indicate the possible involvement of inversions in the structural reorganization of the buthid karyotype. However, the occurrence of this type of cryptic rearrangement is difficult to detect, which may underestimate the importance of these phenomena in karyotype evolution. Surprisingly, frequent chromosomal changes in karyotypes of buthid species are not accompanied by the retention of telomeric (TTAGG)*n* repeats which would be apparent as interstitial telomeric sequences (ITS). Our results also indicate that involvement of structural rearrangements in alterations of rDNA distribution and dispersion is lower than expected.

Scorpions with monocentric chromosomes have not received much attention, although this group is very interesting from a cytogenetic point of view. These evolutionary scorpion lineages usually show highly differentiated karyotypes at the interspecific level. Macrostructural changes in the genome arrangement are likely to occur mainly as a result of centric (Robertsonian) fusions or fissions. This is indicated not only by the apparent differences in 2n, but also by a high incidence of structural heterozygotes for fusion/fission, or the presence of centromeric ITS. Reciprocal translocations, which were identified in a heterozygous state in one species, are probably less likely to be involved in the karyotype differentiation in scorpions with monocentric chromosomes. Moreover, the involvement of inversions in the karyotype rearrangement is also possible, as our data of distinct rDNA distribution among closely related species indicate.

This PhD thesis contributes significantly to the knowledge of karyotypic diversity at different structural levels among scorpions with holokinetic and monocentric chromosomes. However, we do not have additional information for particular observed phenomena, e.g. multiplication of rDNA in buthid scorpions, size heteromorphism of rDNA loci between homologous chromosomes or ITS distribution in scorpionids, to indicate the true background of the mechanisms causing these changes at the microstructural level. As such, the results of this project raise a number of questions that may be the subject of future projects aimed at elucidating the nature of cryptic chromosomal changes which may play an important role in the differentiation and evolution of scorpion karyotypes.

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Employment History

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Education

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2012 - 2014	■ M.Sc., Faculty of Science, Charles University in Prague, Czech Republic Thesis title: <i>Karyotype evolution of the subgenus Euscorpius (Alpiscorpius) in Alps.</i> (supervisor Dr. F. Stahlavsky). Graduated with Distinction
2009 – 2012	B.Sc., Bachelor grade in Biology, Faculty of Science, Charles University in Prague, Czech Republic Thesis title: Diversity and distribution of scorpions (Arachnida: Scorpiones). (supervisor Dr. F. Stahlavsky).

Research Publications

Journal Articles

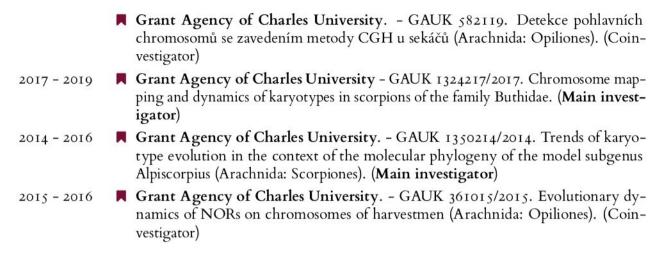
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Grants

since 2019	Grant Agency of Czech Republic GACR 19-22346Y. Cytogenomika
	druhových párů afrických anuálních halančíků: unikátní model p). (Coinvestigator)

Grants (continued)



Presentations

Oral presentations

- 2017 Utilization of molecular cytogenetic markers in the study of karyotype dynamics in the family Buthidae (Arachnida, Scorpiones). Plíšková J., Kovařík F., Nguyen P., Sadílek D., Šťáhlavský F. (30th European Congress of Arachnology, Nottingham, Great Britain)
- 2015 Cytogenetics is not dead! What kind of important information could bring us cytogenetic data in the century of progressive molecular studies?. Plíšková J., Kovařík F., Šťáhlavský F. (29th European Congress of Arachnology, Brno, Czech Repoublic).

Poser presentations

- 2018 Let's rearrange! Structural dynamics of holokinetic chromosomes in small buthid scorpions (Buthidae: Gint). Štundlová J., Just P., Kovařík F., Šťáhlavský F. (31st European Congress of Arachnology, Vác, Hungary).
- 2017 Cryptic diversity, endemism and biogeographical history in Alpine scorpions (Euscorpiidae: Euscorpius). Plíšková J., Šmíd J., Nguyen P., Šťáhlavský F. (30th European Congress of Arachnology, Nottingham, Great Britain). 1st place in the Poster Competition
- 2014 Unexpected karyotype diversity and chromosomal evolution of endemic alpine scorpions (Euscorpiidae: Euscorpius). Plíšková J., Šťáhlavský F. (20th International Chromosome Conference, Canterbury, Great Britain).
- 2012 Karyotype differentiation of Alpine scorpions of the subgenus Euscorpius (Alpiscorpius) (Scorpiones: Euscorpiidae). Plíšková J., Vallo P., Kovařík F., Šťáhlavský F. (27th European Congress of Arachnology Ljubljana, Slovenia). 1st place in the Poster Competition

Oral Presentations (in Czech)

2013 Možnost využití cytogenetických metod v taxonomii rodu Euscorpius (Scorpiones: Euscorpiidae). Plíšková J., Vallo P., Kovařík F., Novotný T., Šťáhlavský F (Congress of the Czech Zoological Society - Zoologické Dny 2013 - Brno, Czech Republic).

Presentations (continued)

Poser presentations (in Czech)

2015 Karyotypová variabilita odrážející existenci lokálního endemismu kryptických druhů štírů na území Alp. Plíšková J., Šťáhlavský F. (Congress of the Czech Zoological Society - Zoologické Dny 2015 – Brno, Czech Republic).

Popularization

2015 Diversity of Scorpions of the *Euscorpius* Genus – Why do They not Let Present-day Arachnologists Sleep? Plíšková J. (Živa). (in Czech)

Teaching activities

since 2014 Lecturer - Practice in Invertebrate Zoology (Faculty of Science, Charles University)

Major collaborations

2015 - ongoing	Dr. Jiří Šmíd (National Museum, Prague, Czech Republic)
2012 - ongoing	Dr. Petr Nguyen (University of South Bohemia, C. Budejovice, Czech Republic)

Membership

since 2010 Czech Arachnological Society

Skills

Languages	English – Good in spoken and written language (CEFR: B2 level) French – Passive knowledge
Conventional cytogenetic methods	chromosome preparations, C-banding, AgNO3 and Giemsa staining
Molecular cytogenetic methods	preparing probes for in situ hybridization, indirect FISH, TSA FISH, CGH
Molecular methods and phylogeny	PCR, purification, sequence editing and alignment (program Geneious), multilocus phylogenetic analyses in programmes raxmlGUI, MrBayes, basics in BEAST
Graphic softwares	Adobe Photoshop, Affinity Photo, INKscape

References

Available on Request