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Karyotype evolution of the family Araneidae
Karyotypová evoluce pavouků čeledi Araneidae

MSc. thesis

Supervisor: Assistant professor RNDr. Jiří Král, Dr.

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Prehlásenie:

Prehlasujem, že som záverečnú prácu spracoval samostatne a že som uviedol všetky použité informačné zdroje a literatúru. Táto práca ani jej podstatná časť nebola predložená k získaniu iného alebo rovnakého akademického titulu.

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Podpis

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Abstract

Orb-weavers (Araneidae) are a diversified spider family comprising more than 3,100 species in more than 170 genera. Together with 13 other families, they constitute to superfamily Araneoidea. The presented thesis focuses on karyotype evolution of Araneidae, including its comparison with a related family Tetragnathidae. The results obtained from 19 araneid and four tetragnathid species confirm previously postulated hypothesis that the ancestral karyotype of Araneoidea (including Araneidae) consists of 24 acrocentric chromosomes in males, including two acrocentric X chromosomes of system X_1X_20. However, there is a tendency of 2n decrease in some araneids due to centric fusions. In these cases, centric fusions affected most autosomes (and sometimes gonosomes as well); number of chromosome pairs decreased from 11 to six. Three independent reduction events were detected in this thesis. Furthermore, pattern of nucleolar organizer regions (NORs) was studied in this thesis using fluorescent *in situ* hybridization, since data on evolution of this marker are scarce in spiders. Striking variability in NORs number was discovered, ranging from one to 13 loci. Remarkably, multiple centric fusions were always accompanied by considerable increase of NORs number. In araneids and tetragnathids possessing ancestral karyotype, low frequency of chiasmata per chromosome was observed. On the other hand, this frequency was increased in species with reduced 2n.

Keywords: chromosome, evolution, karyotype, nucleolar organizer region, sex chromosomes, speciation, spiders

Abstrakt

Križiakovití (Araneidae) sú početnou čeľaďou pavúkov zahŕňajúcou viac ako 3100 druhov v 170 rodoch. Spolu s 13 ďalšími čeľaďami tvoria nadčeľaď Araneoidea. Predložená práca je zameraná na karyotypovú evolúciu čeľade Araneidae, vrátane jej porovnania s príbuznou čeľaďou čeľustnatkovití (Tetragnathidae). Výsledky získané z 19 druhov križiakov a štyroch druhov čeľustnatiek potvrdzujú publikovanú hypotézu, že pôvodný karyotyp nadčeľade Araneoidea (vrátane čeľade Araneidae) pozostáva z 24 akrocentrických chromozómov u samcov, vrátane dvoch akrocentrických chromozómov X systému X_1X_20. U rady križiakov však dochádza k výraznému zníženiu 2n prostredníctvom centrických fúzií. Centrické fúzie zasiahli väčšinu autozómov (a niekedy aj gonozómy); počet párov klesol z 11 na šesť. V predloženej práci boli zistené tri takéto redukčné udalosti. Súčasťou práce bol aj výskum nukleolárnych organizátorov (NOR) s použitím fluorescenčnej hybridizácie *in situ*, nakoľko dostupné údaje o evolúcii tohto znaku u pavúkov sú obmedzené. Počet NOR bol veľmi variabilný, s rozpätím jeden až 13 lokusov. Mnohonásobné centrické fúzie boli vždy sprevádzané významným zvýšením počtu NOR. Frekvencia chiazmat na chromozóm bola u križiakov a čeľustnatiek s ancestrálnym karyotypom nízka. Na druhej strane u druhov vykazujúcich centrické fúzie sa táto frekvencia zvýšila.

Kľúčové slová: evolúcia, chromozóm, karyotyp, nukleolárny organizátor, pavúky, pohlavné chromozómy, špeciácia

Contents

Abbreviations

BL	*Larinioides patagiatus* population "Borová Lada"
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
cccDNA	covalently closed circular DNA
Cy3	cyanine dye 3
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DSB	double-strand break
ETS	external transcribed spacer
FISH	fluorescent *in situ* hybridization
FITC	fluorescein isothiocyanate
HK	*Larinioides patagiatus* population "Horská Kvilda"
HK-A/HK-B	*Larinioides patagiatus* cytotype A/B of population "Horská Kvilda"
HRP	horse radish peroxidase
IGS	intergenic spacer
ITS	internal transcribed spacer
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
NOR	nucleolar organizer region
NTS	non-transcribed spacer
p-arm	short arm of a chromosome
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
q-arm	long arm of a chromosome
rDNA/rRNA	ribosomal DNA/RNA

RT	room temperature
SCP	pair of sex chromosomes
snRNA	small nuclear RNA
SO	*Larinioides patagiatus* population "Suchdol nad Odrou"
SSC	saline-sodium citrate
T_m	melting temperature
TNB	Tris-NaCl-blocking
TNT	Tris-NaCl-Tween
TSA	tyramide signal amplification

1 Introduction

Orb-weaver spiders (Araneidae) are one of the most numerous and diversified family among spiders comprising more than 3,100 species and more than 170 genera. They belong to the large evolutionary lineage referred to as Entelegynae, where they occupy rather derived position in terms of evolution. They occur worldwide, inhabiting almost all terrestrial biotopes except for polar regions. However, their diversity achieves its peak in tropical and subtropical regions. Araneids owe their title to the typical two-dimensional webs they spin, using them for all kinds of activities ranging from prey hunting to shelter construction. These webs work better in humid surroundings thanks to the sticky substances. Thus, the majority of orb-weavers lives near water bodies. After catching a prey, araneid spiders inject poison into it, often continuing with wrapping it by silk and digesting it afterwards.

Although family Araneidae is being studied extensively from the cytogenetic point of view, only less than 80 species are karyotyped at present. From this part, the utter majority of araneids exhibits the diploid number of 24 in males, with all chromosomes being exclusively acrocentric. Such type of karyotype probably constitutes ancestral state in araneid spiders. The other karyotyped species exhibit either lower or higher diploid number (which is not lower than 13 or higher than 49, though), or eventually different chromosome morphology. In almost three quarters of examined species, two male chromosomes represent sex chromosomes, forming so called X_1X_20 sex chromosome system, where "0" denotes the absence of Y chromosome. Instead, only two probably non-homological X chromosomes are present in males, while in females they are double, creating $X_1X_1X_2X_2$ constitution so. This difference in number of chromosomes between males and females can be also revealed using flow cytometry.

In terms of molecular cytogenetics, little is known about either araneids or spiders in general. Only a few laboratories worldwide are dedicated to the study of various spider molecular markers localised on chromosomes, choosing mainly repetitive genes as rRNA ones included in nucleolar organizer regions (NORs).

The presented thesis focuses on the karyotype evolution of Araneidae as well as its comparison with the related family Tetragnathidae serving as an outgroup. A particular emphasis is placed on the orb-weavers possessing derived karyotypes

composed of either lowered or increased number of chromosomes, but especially investigating the former. The main aim of the thesis is to elucidate mechanisms leading to a decreased or increased diploid number in araneids and based on this knowledge, it aims to reconstruct the karyotype evolution of this family. This knowledge is important for better understanding of mechanisms operating in karyotype evolution of Araneidae, which contributes to overall knowledge of spider cytogenetics as well as cytogenetics in general, since possible phenomena of karyotype differentiation include various mechanisms like pericentric inversions, Robertsonian translocations, tandem fusions, polyploidisation, and female meiotic drive.

2 Aims of the thesis

Since there is so little cytogenetic information available about the family Araneidae compared to its total amount of species, it is necessary to determine ancestral karyotype traits of this family. Furthermore, it is necessary to look into araneid species possessing derived karyotypes in order to reveal the mechanisms of increase or decrease of chromosome number. A part of the thesis includes also the comparison with the related family Tetragnathidae in order to get an idea about ancestral chromosome features of the superfamily Araneoidea as a whole. Hence the thesis aims are following:

- To assess diploid number and chromosome morphology of selected species using Giemsa staining
- To assess sex chromosome system of selected species using Giemsa staining
- To establish nucleolar organizer regions pattern of selected species using fluorescent *in situ* hybridization (FISH)
- To optimise detection of selected molecular markers (5S rDNA, U2 snRNA, histone H3 genes) and establish their pattern using FISH
- Based on the obtained results, propose the basic traits of karyotype evolution of the family Araneidae

3 Literature review

3.1 Biology and phylogeny of Araneoidea

Spiders (Araneae) are an extremely diversified animal order comprising 47,215 species in 113 families up to date (World Spider Catalog 2018). Traditionally, spiders are divided into three main evolutionary lineages: Mesothelae, Mygalomorphae and Araneomorphae, with both latter forming the suborder Opisthothelae. The utter majority of spider diversity belongs to Araneomorphae (Coddington and Levi 1991). This infraorder is further divided into basal clades (Hypochilidae, Filistatidae, and other basal araneomorphs belonging to clade Haplogynae) and more advanced group Entelegynae, which contains the most representatives (Coddington 2005; Garrison et al. 2016; Wheeler et al. 2016).

The presented research applies mainly to family Araneidae and in part to its related family Tetragnathidae, whereby both of these families (along with other 13) form a big entelegyne superfamily called Araneoidea (Coddington 2005; Garrison et al. 2016; Wheeler et al. 2016). The evolutionary position of this superfamily is currently studied extensively (Fernández et al. 2014; Bond et al. 2014). Araneidae represents the second most numerous family of Araneoidea (after Lyniphiidae), as it includes 3,125 species in 174 genera. With such number of species, it is the third most numerous family of spiders in general after Salticidae (6,046 species) and mentioned Lyniphiidae (4,552 species) (World Spider Catalog 2018). Araneid spiders are cosmopolitan, occurring basically worldwide except for polar regions. However, they are most abundant in tropical and subtropical areas. Their most characteristic trait is construction of two-dimensional spiral wheel-shaped webs perfectly accommodated for catching flying prey. Such webs contain sticky substances and their fibres are incredibly strong, yet remarkably elastic. That is the reason for long-term human effort to achieve the mass production of such fibres, emphasizing so the big importance of orb-weavers. Furthermore, orb-weavers (like all spiders) are natural predators that maintain balance in ecosystems (Buchar and Kůrka 2001).

There are many other spider families building similar webs, either related families within the Araneoidea superfamily (notably Tetragnathidae), or other families, such as Uloboridae. Regarding Tetragnathidae, they are sometimes referred to as long-jawed orb-weavers and much like araneid spiders, they are cosmopolitan, too,

inhabiting most parts of the world excepting polar regions, southern part of Arabian Peninsula and New Zealand. They have typical elongated body and bold chelicerae (Buchar and Kůrka 2001).

The Araneoidea phylogeny is still not satisfactorily resolved and there are some minor disagreements between certain studies. The most recent molecular phylogenetic study (Wheeler et al. 2016) is, however, rather consistent with other recent studies (Garrison et al. 2016), suggesting relatively close relationship between Araneidae and Tetragnathidae, therefore supporting the choice of Tetragnathidae as an outgroup for this research. Concerning the family Araneidae itself, its internal phylogeny has been recently studied in order to determine status of the subfamily Zygiellinae only, which presents the basal group of the family (Fig. 1) (Gregorič et al. 2015).

3.2 Cytogenetics of entelegyne spiders

3.2.1 Evolution of diploid number

At present, there are only a few spider species karyotyped (819, Araujo et al. 2017) compared to the entire spider diversity. The bulk of cytogenetic knowledge belongs precisely to the Entelegynae lineage (692 karyotyped species, based on the data of Araujo et al. 2017). Overall, spiders exhibit considerable range of diploid number (2n), ranging from 7 (*Ariadna lateralis*, Segestriidae and *Dasumia carpathica*, Dysderidae; Suzuki 1954; Král and Kořínková 2013) to 128 (*Cyclocosmia siamensis*, Ctenizidae, Král et al. 2013). The spider with the highest 2n belongs to Mygalomorphae and indeed, this infraorder is much more variable in 2n range in comparison to Entelegynae, with the lowest 2n being 14 in males of *Atypus affinis* (Atypidae, Řezáč et al. 2006) and *Ischnothele caudata* (Dipluridae, Král et al. 2013). On the contrary, 2n range in Entelegynae is narrower, ranging from 10 (*Uloborus danolius*, Uloboridae, Sharma and Parida 1987) to 49 (*Araneus ventricosus*, Araneidae, Youju et al. 1993). Furthermore, 2n diversity within particular entelegyne families is low in the context of spider cytogenetics.

It is likely that the ancestral karyotype of entelegynes is composed of 42 chromosomes in males (Král et al. 2006), which can be still frequently observed in certain clades. However, most entelegyne karyotypes contain 21 – 30 chromosomes, with 22, 24, and 28 occurring most commonly in males (Král and Kořínková 2013). This

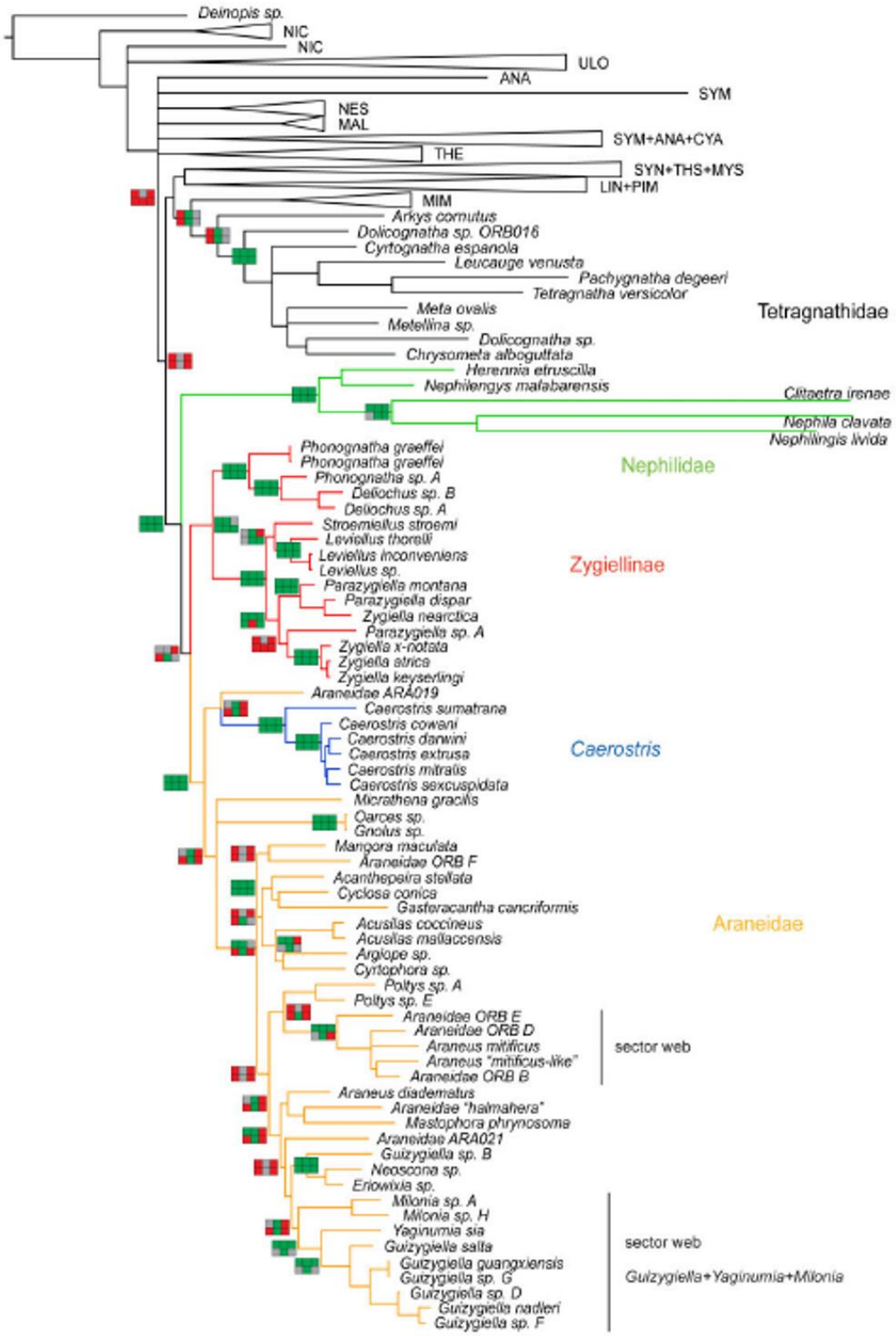

Fig. 1: Cladogram showing that Araneidae and Tetragnathidae are closely related families; internal phylogeny of Araneidae is not solved, but some relationships within this family are eventually becoming clearer, like the basal position of subfamilly Zygiellinae; other families are marked using the following codes: ANA, Anapidae; CYA, Cyatholipidae; DEI, Deinopidae; HOL, Holarchaeidae; LIN, Linyphiidae; MAL, Malkaridae; MIC, Micropholcommatidae; MIM, Mimetidae; MYS, Mysmenidae; NES, Nesticidae; NIC, Nicodamidae; PIM, Pimoidae; SYM, Symphytognathidae; SYN, Synotaxidae; THD, Theridiidae; THS, Theridiosomatidae; ULO, Uloboridae; adapted from Gregorič et al. (2015)

is true for both families studied in this thesis – the most common 2n in both Tetragnathidae and Araneidae is 24 in males (Araujo et al. 2017). This number was proposed to be the ancestral one in Araneidae (Araujo et al. 2011) and is very stable from the evolution point of view and similar to the situation in many other Araneoidea families as well (Araujo et al. 2015). However, it should be noted that only 22 tetragnathid and 73 araneid spiders have been karyotyped so far (Araujo et al. 2017). Additionally, 2n♂ = 24 is not observed in all tetragnathids studied so far – there are a few exceptional species possessing 22 or 25 chromosomes in males (Araujo et al. 2017). Regarding araneids, their diploid numbers range from 13 (*Neoscona* sp., Araneidae, Sharma and Parida 1987) to 49 (see above) – the situation entirely different from Tetragnathidae.

3.2.2 Evolution of chromosome morphology

Spiders are also characterized by a wide spectrum of chromosome morphologies. In the superfamily Dysderoidea, karyotypes are formed by holokinetic chromosomes (Král et al. 2006), i.e. chromosomes without a distinctive centromere. Such chromosomes possess a derived centromere, mitotic spindle microtubules attach to them over their entire length. In Dysderoidea, they probably arose from standard monocentric chromosomes as an adaptation to 2n reduction while maintaining considerable chromosome length. Otherwise long monocentric chromosomes could pose an issue in sister chromatids separation during both mitosis and meiosis (Diaz et al. 2010). The remainder of known spiders exhibits monocentric chromosomes with a clearly localised centromere. In Mygalomorphae and most basal araneomorphs, there is a predominance of biarmed chromosomes, i.e. metacentric or submetacentric (Král et al. 2011) and occasional predominance of monoarmed chromosomes in some clades (Král et al. 2013).

In contrast, the vast majority of entelegynes exhibits exclusively acrocentric chromosome morphology (Král et al. 2006). As mentioned in chapter 3.2.1, the ancestral entelegyne karyotype is probably composed of 42 acrocentric chromosomes, yet most entelegynes possess lower diploid numbers. Since such karyotypes are still composed of acrocentric chromosomes, the reduction of 2n could have happened as follows: either a series of tandem fusions took place (Suzuki 1954), or, alternatively, a series of centric fusions followed by subsequent pericentric inversions occurred (White 1973). Both scenarios lead to preservation of acrocentric morphology and at

the same time, 2n is reduced considerably. Nevertheless, the latter scenario is supported by the fact that centric fusions are the most common type of chromosome polymorphism in entelegyne spider populations (Král et al. 2006).

Remarkably, karyotypes of some entelegynes are mostly or exclusively biarmed, with 2n being usually a half (or approximately a half) of the original condition. By all means, such karyotypes are formed by centric fusions of all or most of the original acrocentric chromosomes. This phenomenon was at first observed in spider *Larinioides patagiatus* (Araneidae, Hackman 1948) and it was later described as the rule "all or nothing" (Rowell 1990). This phenomenon has been demonstrated in families Dyctinidae (Král et al. 2011), Oxyopidae (Stávale et al. 2011), Salticidae (Gorlova et al. 1997), and Sparassidae (Rowell 1985). Additionally, there are reports of entelegyne karyotypes consisting of biarmed chromosomes, but with the same or similar 2n as the related species with acrocentric karyotypes. In this case, such karyotypes are probably derived by pericentric inversions (Stávale et al. 2010).

The chromosome morphology of most araneid spiders examined so far is acrocentric, but there are certain species exhibiting predominance of biarmed chromosomes (Hackman 1948; Suzuki 1954; Araujo et al. 2017). In all tetragnathid spiders examined so far, there are acrocentric chromosomes exclusively (Araujo et al. 2017).

3.2.3 General trends of evolution of acrocentric chromosomes

Short arms of acrocentric chromosomes usually lack any coding genes. On the other hand, they are enriched in various types of repetitive DNA, which is apparent from both physical and cytogenetic mapping (Garagna et al. 1969; Jarmuz-Szymczak et al. 2014). Structural chromosome changes like translocations (including centric fusions) can be considered as consequences of recombination between ectopic loci during double-strand break (DSB) repair. Repetitive sequences are chosen as templates for ectopic recombination frequently. The occurrence of such sequences within p-arms of acrocentric chromosomes makes them prone to centric fusions (Schubert and Lysak 2011).

Centric fusions, or Robertsonian translocations, are fusions between two monoarmed chromosomes, forming a single biarmed chromosome. As mentioned in chapter 3.2.2, these fusions can pose a major driving force in karyotype evolution of certain clades. Although the importance of centric fusions in evolution is unquestionable, precise molecular mechanisms of this event are still unknown. Nonetheless,

mechanisms facilitating centric fusions have been proposed in mice and other mammals, including telomere shortening induced by deletion of telomerase RNA gene or breakage within minor satellite sequences. Additionally, other events like telomere inactivation can further stabilize products of centric fusions (Garagna et al. 1969; Slijepcevic 1998). Ectopic recombination between repetitive sequences leading to a centric fusion can take place as soon as telomeres fail to protect chromosome ends (Schubert and Lysak 2011), as is probably the case in all known human centric fusions (Jarmuz-Szymczak et al. 2014).

Animals possessing exclusively acrocentric chromosomes, like entelegyne spiders (discussed above), feral mice or geckos (Garagna et al. 1969; Castiglia et al. 2009) can be subjects of rapid karyotype evolution that sometimes even leads to speciation. A model describing such phenomenon counts with speciation by monobrachial centric fusions (Baker and Bickham 1986). In this model, two separated populations undergo different centric fusions, producing biarmed chromosomes with monobrachial homology, i.e. only one arm is homologous between these populations. A heterozygote for a single centric fusion has often a minimal problem during meiosis, as produced trivalents often segregate normally (Fig. 2a). However, if individuals of derived populations differ in multiple fusions, their hybrids are sterile because of formation of tetravalents or more complex multivalents (Fig. 2b) and thus, speciation may occur.

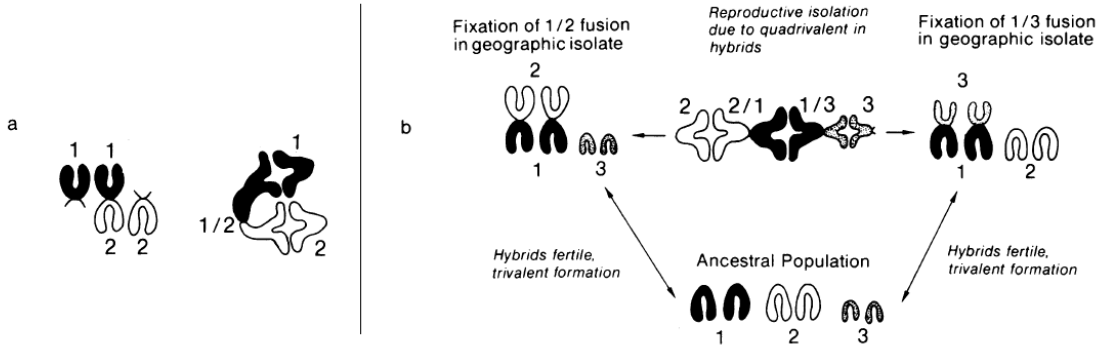

Fig. 2: (a) During meiosis of an individual heterozygous for a single centric fusion, fusion product and two original chromosomes form a trivalent, which causes some, but not dramatic, impairment of meiosis; (b) Hypothetical speciation event: chromosome pairs 1, 2, and 3 underwent centric fusions in different combinations in separated populations, which can normally cross with the ancestral population, but hybrids between them form tetravalents that impair meiosis; adapted from Baker and Bickham (1986)

Traditionally, it is said that formation of complex multivalents causes sterility in hybrids, which consequently leads to speciation. Indeed, such empirical data have been obtained in vesper bat *Rhogeessa* and mice populations (Capanna et al. 1985; Piálek et al. 2001) and centric fusions do play a role in speciation of certain animals. However, recombination suppression in rearranged chromosomes should be considered, too, because it decreases effectivity of these chromosomes in formation of reproductive barriers (Rieseberg 2001). Additionally, there is a peculiar example of spider *Delena cancerides* that overcomes the monobrachial centric fusion model thanks to the fact that its sex chromosomes are involved in fusions, too.

Compared to biarmed chromosomes, acrocentric chromosomes probably cannot undergo fissions in one step, producing two fission products that are able to segregate. Acentric fragments produced by fissions of acrocentric chromosomes need to gain new centromeres to be stable. Although some cases of epigenetic *de novo* formation of centromeres have been reported, this process is very rare (Schubert and Lysak 2011).

3.2.4 Evolution of spider sex chromosomes

Most karyotyped spiders possess a rare set of sex chromosomes ♂X_1X_2/♀$X_1X_1X_2X_2$, referred to as the X_1X_20 system (Benavente et al. 1982). Besides spiders, this system is rare in animals and has been discovered only in certain ostracods, nematods, and insects, but it is a derived mechanism in these cases (White 1973). On the contrary, it is probably the original sex chromosome system in spiders, as it is found in the most primitive living representatives of Mesothelae (Suzuki 1954). It is also vastly spread among entelegynes (Araujo et al. 2017). Since these X chromosomes are often unequal in size and do not exhibit any chiasmata during male meiosis, they are most likely non-homologous (Král and Kořínková 2013). There are two hypotheses of the X_1X_20 system origin. The first one presumes a fission of original metacentric X chromosome producing two acrocentric X chromosomes (Bole-Gowda 1950). The basis of the second hypothesis includes a nondisjunction of original X chromosome followed by a subsequent differentiation of the newly formed X chromosome (Postiglioni and Brum-Zorrilla 1981).

Despite its evolutionary stability in spiders (White 1973), the X_1X_20 system continues to develop into secondary systems. An example of such derived system is the X0 system often found in entelegynes, too, which could arise by several mechanisms.

The centric fusion of both X chromosomes of X_1X_20 system produces a single biarmed X chromosome of X0 system (Král et al. 2013). The tandem fusion leads to a single acrocentric X chromosome of X0 system (Bole-Gowda 1950). An alternative option features deletion of a single X chromosome in X_1X_20 system (Suzuki 1954). However, it is not likely that such deletion could be left without any effects on vitality. Therefore, successive segments translocations from one X to another is a more probable scenario.

Another derived sex chromosome systems found in entelegynes include $X_1X_2X_30$ or $X_1X_2X_3X_40$ systems, formed probably by nondisjunctions (Král et al. 2011; Král and Kořínková 2013). Systems with even more X chromosomes have been discovered (with maximum of 13), but only in mygalomorphs (Král et al. 2013). Neo-sex chromosomes are less frequent in spiders, but have been detected in almost all spider clades – Mygalomorphae, basal araneomorphs, and Entelegynae (Král et al. 2006; Král and Kořínková 2013; Král et al. 2013). Neo-sex chromosomes are formed by various rearrangements between actual sex chromosomes and autosomes, which are often reciprocal or Robertsonian translocations. A rearrangement between a single X chromosome and a single chromosome of an autosomal pair produces a neo-X chromosome, while the remaining autosome of the same pair turns into a neo-Y chromosome. Thus, neo-sex systems always contain a Y chromosome (Král and Kořínková 2013). Examples of mygalomorphs owning such systems are *Atypus affinis* (Atypidae) and *Ischnothele caudata* (Dipluridae), both with XY system (Řezáč et al. 2006; Král et al. 2013), or *Paratropis* sp. (Paratropididae) with $X_1X_2X_3X_4X_5X_6X_7Y$ system (Král et al. 2013). Among Entelegynae, we can find several species owning neo-sex systems, for instance $X_1X_2X_3X_4X_5Y$ system in *Tegenaria ferruginea* (Agelenidae, Král 2007) and X_1X_2Y or $X_1X_2X_3Y$ systems in certain jumping spiders of *Habronattus* (Salticidae, Maddison and Leduc-Robert 2013). However, perhaps the most outstanding example of neo-sex systems can be found in the family Sparassidae, namely *Delena cancerides*. Different centric fusions between autosomes and gonosomes within various populations of this spider led to permanent fusion heterozygosity in males, producing the chain of nine chromosomes (system $X_1X_2X_3X_4X_5Y_1Y_2Y_3Y_4$) in some hybrid populations, while others contain chain of less or more chromosomes (3 – 19). To make matters even more interesting, there are populations possessing two chains (Rowell 1985, 1987; Sharp and Rowell 2007).

Some basal araneomorphs possess X_1X_2Y system, namely members of families Drymusidae, Hypochilidae, Filistatidae, Sicariidae, and Pholcidae, but the origin of this system is unclear (Král et al. 2006).

In some spiders, karyotype analysis revealed the intriguing sex chromosome pair (SCP) morphologically indistinguishable from autosomes (Král et al. 2013). However, this pair behaves specifically during meiosis. Based on these observations and ultrastructural studies, Král et al. (2013) presumes that SCP pair presents original sex chromosomes of spiders. The SCP consists of X and Y chromosomes, which are structurally differentiated only slightly. The X chromosome of this pair could be responsible for multiple sex chromosome generation by non-disjunctions (Král 2007). Seeming absence of the SCP in many spiders (especially entelegynes) is due to inability to detect it by cytogenetic methods. Nonetheless, the SCP is probably differentiated on molecular level (Král and Kořínková 2013).

3.2.5 Meiosis: modifications and sex chromosomes behaviour

Specific modifications of meiosis are another feature typical for spiders. They occur mostly during prophase or metaphase I in males, but it is not always so. So called diffuse stage has been observed in males of mygalomorphs, basal araneomorphs, and some entelegynes (Král et al. 2006; Král and Kořínková 2013) and it is presumably a universal trait for all spider females (Král et al. 2011). It occurs during late prophase I, accompanied by extensive chromatin decondensation, probably reflecting enhanced transcriptional activity. Compared to decondensed autosomes, sex chromosomes are reversely heterochromatinised and inactive during this stage (Král et al. 2006).

During male diakinesis, a bipolarisation of nucleus has been discovered in Agelenidae (Král and Kořínková 2013) and Lycosidae (Chemisquy et al. 2008). In this case, chromosomes are divided into two unequal groups (one of them includes X chromosomes) arranged similarly to anaphase I, i.e. these groups are localised on the opposite nucleus poles. This phenomenon may be common for all entelegynes (Král and Kořínková 2013).

In some instances, male bivalents do not exhibit chiasmata until the end of prophase I. This delayed chiasmata expression is called cryptochiasmatic meiosis and has been discovered in some diplurid mygalomorphs (Král et al. 2011). It probably represents an intermediate stage between chiasmatic and achiasmatic meiosis (i.e.

the complete absence of chiasmata in heterogametic sex) (White 1973). Furthermore, there are examples of diplurid mygalomorphs exhibiting achiasmatic meiosis, for instance *Euagrus lynceus* (Král et al. 2013).

Yet another example of meiotic modification is so called inverted meiosis in males of *Dysdera crocata* (Dysderidae). The X chromosome chromatids of X0 system of this spider segregate to the opposite poles as early as in anaphase I rather than anaphase II. Such behaviour is naturally limited to organisms possessing holokinetic chromosomes only (Benavente and Wettstein 1980; Král and Kořínková 2013).

Overall, sex chromosomes in spider males tend to associate in a distinctive manner and behave differently compared to autosomes during meiosis. They are often specifically heterochromatinised, which results in either higher, or lower staining intensity, referred to as positive and negative heteropycnosis, respectively (Král et al. 2006, 2011). The majority of spiders exhibits the former (Araujo et al. 2012), which can be often helpful in the sex chromosome system determination. The pairing of sex chromosomes differs between various groups – in Mygalomorphae, basal Araneomorphae, and some Entelegynae, sex chromosomes typically pair at both ends, forming a loop. This pattern is likely ancestral in spiders (Král et al. 2006, 2011). In Entelegynae, sex chromosomes pair in parallel (Král 2007). Parallel association of spider sex chromosomes is achieved in a number of ways, one of them involves a structure called junction lamina, derived probably from synaptonemal complex (Benavente and Wettstein 1977). Spider females possess double dose of sex chromosomes and these behave specifically. They pair already during interphase, which presumably prevents recombination between chromosomes belonging to different X chromosome pairs (Král 2007; Král et al. 2011).

3.3 Structure and evolution of molecular cytogenetic markers

Molecular cytogenetics is a branch of cytogenetics which combines approaches of cytogenetics and molecular biology. A common approach used in this branch is to map particular DNA sequences to chromosomes. Generally, it is convenient to detect repetitive sequences and indeed, genes arrayed in repeats are first choice genes rather than single copy ones. Such genes often belong to so called multigene families.

3.3.1 Nucleolar organizer regions

Nucleolar organizer regions (NORs) represent domains of chromosomes which form a conspicuous structure of nucleolus, the fundamental factory for ribosome biosynthesis. Nucleolus is not a permanent part of a cell – it disappears in prophase and reappears in telophase. NORs reside inside secondary constrictions of chromosomes (achromatic gaps of stained chromosomes), surrounded by a specific form of heterochromatin. The DNA composition of many sequences of NORs is eminently conserved in eukaryotes and it includes tandem repeats of rRNA genes. These repeating units are separated from each other by the intergenic spacers (IGSs) and in most organisms, each unit is composed of genes coding 18S, 5.8S, and 28S rRNA in this order. There are also two internal transcribed spacers (ITS1 and 2, separating rRNA genes from each other) as part of each unit, as well as 5' and 3' external transcribed spacers (ETSs) circumscribing rRNA genes (Fig. 3). Together, these genes along with the transcribed spacers are targets for RNA polymerase I, which transcribes them into 45S rRNA. Further processing and removal of all spacers yields mature rRNA components, which then become parts of ribosomal subunits (Sumner 2003; McStay 2016).

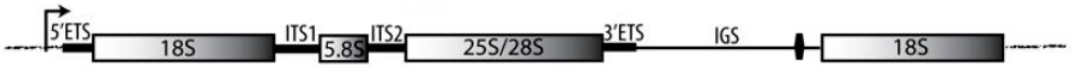

Fig. 3: Molecular composition of a single repeating NOR unit – 18S, 5.8S, and 25S/28S rRNA genes, together with the external (ETS) and internal (ITS) spacers, form a repeating unit separated from another one by an intergenic spacer (IGS); the transcription start for RNA polymerase I is indicated by an arrow; 28S rRNA gene is typical for many animals, 25S rRNA gene for yeasts; adapted from Richard and Manley 2009

In general, eukaryotes possess many copies of rRNA units comprised in NORs. Indeed, usually 80% of RNA in cells is present as rRNA. The exact copy number of NOR ribosomal genes varies greatly among different organisms, though. Organisms with smaller genomes can have as little as 100 copies of rRNA genes. On the contrary, some plants or vertebrates can have as much as thousands of copies of rRNA genes at their disposal. Overall, a karyotype can include a single NOR locus, but NORs are commonly spread among more chromosomes and typically occupy terminal

positions, as is true in human cells for instance. However, interstitial NOR positions exist as well (Sumner 2003; Nguyen et al. 2010).

It has been proved that NORs present an excellent marker for tracing karyotype evolution, since they are highly dynamic and often change their number and position within a genome. Nevertheless, no universal mechanism responsible for NORs spreading has been found so far and it is likely that many possible mechanisms are at play. The evidence for connection between NORs spreading and chromosome rearrangements is apparent for a long time. Eventually, it became clear that frequent NORs repatterning is the direct result of chromosome rearrangements, e.g. fission of a biarmed chromosome bearing a single pericentric NOR results in two chromosomes each bearing a terminal NOR. In other words, the multiplication of NORs occurred (Nguyen et al. 2010). The position change of NORs could be ascribed to centric fusions or pericentric inversions as well. Overall, such rearrangements are connected with ectopic recombination (i.e. recombination between non-homologous loci) that can lead to a DSB and so called crossover products (Nguyen et al. 2010). Besides chromosome rearrangements and ectopic recombination, transposition is yet another (and perhaps very common) mechanism of NORs spreading (Cabrero and Camacho 2008). Other mechanisms may involve spreading of NORs along with spreading of heterochromatin (Cabral-de-Mello, Oliveira, et al. 2011).

There are several methods for detection of NORs, of which the silver staining using $AgNO_3$ was performed as one of the first. This approach exploits natural affinity of particular proteins associated with NORs for silver, which is consequently deposited in NORs, effectively visualising them. However, it often fails to visualise the entirety of them, since only NORs transcriptionally active in a preceding interphase are detected (Sumner 1990). Although this detection may be still useful in some situations, it is possible to bypass it nowadays, using fluorescent *in situ* hybridization (FISH). This method is based on hybridization of a particular DNA probe with rDNA regions, often reliably revealing all NORs (Sumner 2003). However, this is not always true and at times, it is convenient to use both FISH and silver impregnation simultaneously (Cabrero and Camacho 2008).

In spiders, studies of NORs are scarce and mostly involve silver staining. The obtained results revealed the predominance of one or two NOR loci localised on autosomes in Mygalomorphae. The notable exceptions pose species *Ischnothele caudata*

(Dipluridae) and *Linothele megatheloides* (Dipluriade) with NORs on sex chromosomes, too (Král et al. 2013). Occurrence of NORs on sex chromosomes is also typical for basal araneomorphs (Král et al. 2006) and has been also detected in the entelegyne families Tetragnathidae and Nephilidae (Král et al. 2011). In other entelegynes, silver staining was conducted in family Sparassidae with representatives possessing two autosomal NOR pairs (Rodriguez-Gil et al. 2007), Lycosidae, whose representatives possess two autosomal NOR pairs (Dolejš et al. 2011), and Ctenidae, where one or two autosomal NOR pairs were detected (Araujo et al. 2014; Kumar et al. 2017). Only three studies have yielded information about NORs pattern in spiders using FISH so far: a single autosomal NOR pair in *Brachypelma albopilosum* (Theraphosidae, Král et al. 2013), one or three autosomal NOR loci in Ctenidae (Rincão et al. 2017), and up to ten autosomal NOR loci in *Wadicosa fidelis* (Lycosidae), which is the highest number of NORs reported in spiders so far (Forman et al. 2013).

3.3.2 5S rRNA genes

Ribosomal genes contained in NORs are not the only genes coding for rRNA. The large ribosomal subunit includes also 5S rRNA, which is coded by repetitive genes localised in other position of a genome compared to the rest of rRNA genes in many, but not all eukaryotes (Drouin and de Sá 1995). A common molecular composition of 5S rRNA genes in most eukaryotes involves hundreds to thousands of copies of these genes, with each gene separated by non-transcribed spacers (NTSs), as depicted in Fig. 4. In contrast to NOR genes, these genes do not form any characteristic chromosome structure and are transcribed by RNA polymerase III instead of I (Sumner 2003; Pinhal et al. 2008).

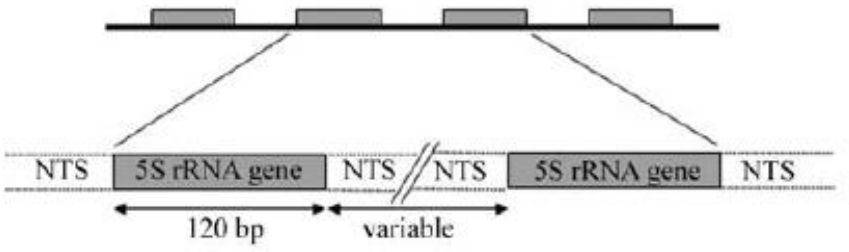

Fig. 4: Molecular composition of 5S rRNA gene clusters – many 5S rRNA genes (in higher eukaryotes usually each 120 bp long) are separated by non-transcribed spacers (NTSs) with variable size; adapted from Pinhal et al. 2008

However, it should be noted that in some eukaryotes, 5S rRNA genes are linked to other multigene families, like rRNA genes in NORs, histone genes or other repetitive DNA. At first, it seemed like it is the ancestral trait in lower eukaryotes, but later it was discovered in higher eukaryotes as well (Drouin and de Sá 1995). We can find such examples particularly in arthropods, including various crustaceans with 5S rRNA genes linked to NOR genes (Drouin et al. 1992; Drouin and de Sá 1995), spiders with 5S rRNA genes linked to NOR or histone genes (Drouin et al. 1992; Suzuki and Kubota 2011) or beetles with 5S rRNA genes linked to NOR or histone genes (Cabral-de-Mello, Oliveira, et al. 2011). However, there is no consensual opinion about the concerted evolution of these multigene families (i.e. paralogues in the same species are more similar than orthologues among species), nor there is a unified opinion about the primitive or derived state of this phenomenon in arthropods (Drouin et al. 1992; Drouin and de Sá 1995; Suzuki and Kubota 2011). Nonetheless, Drouin and de Sá (1995) hypothesize that two possible events can lead to such linkage formation: either insertion of cccDNA containing 5S rRNA genes, or, more likely, RNA-mediated transposition of 5S rRNA genes. Such events could also reversely lead to loss of these linkages and according to authors, this indeed probably happened repeatedly during evolution of Arthropoda.

There is only a single study showing the pattern of 5S rRNA genes in spiders using FISH, demonstrating that NTS region of 5S rRNA gene cluster includes intercalated histone H2B-like gene in *Oxyopes sertatus* (Oxyopidae, Suzuki and Kubota 2011).

3.3.3 Other molecular cytogenetic markers

In addition to rRNA genes, there are other multigene families suitable for cytogenetic mapping. Histones are proteins essential for formation of chromatin; they take part in formation of nucleosomes. Nucleosomal core includes histones H2A, H2B, H3, and H4, but further wrapping of DNA into higher structures also requires histone H1, which is not a part of the nucleosomal core (Sumner 2003). The reason that makes histone genes apt for cytogenetic mapping is their molecular composition. Similarly to rRNA genes, histone genes are also arrayed in tandem repeats in many eukaryotes. A cluster of histone genes includes either genes for all histones, or just some of them and their order is variable among organisms. In other cases, more than a single copy of a particular histone gene is present within a cluster. Furthermore, sole clusters dispersed throughout a genome have been detected. In most

eukaryotes, quartets or quintets of histone genes are most typically present within a cluster (i.e. without or with the histone H1 gene, respectively), as is the case for *Drosophila melanogaster* with quintet of histone genes within a cluster (Kedes 1979; Sellos et al. 1990).

At present, mapping of histone genes in invertebrates is limited mostly to insects (Cabral-de-Mello, Oliveira, et al. 2011; Bueno et al. 2013) and certain annelids (Sellos et al. 1990) and involves the histone H3 gene. From arachnids, the histone H3 pattern was investigated in *Tityus obscurus* (Almeida et al. 2017). Concerning spiders, no endeavour has been made to map histone gene clusters so far, except for the MSc. thesis of Hrubá (2017), who studied the pattern of histone H3 genes in *Macrothele* (Hexathelidae). These studies show that either a single large pair of histone H3 gene clusters is present on a single chromosome pair, or more chromosome pairs bear these clusters. This indicates that histone genes are dynamic markers able to change their pattern just like rRNA genes and similarly to them, ectopic recombination, transposable elements or cccDNA could be involved (Almeida et al. 2017).

Another example of class of multigene families is U snRNA. These RNAs are parts of either minor or major spliceosome responsible for introns excision from mRNA. The more studied major spliceosome dominant in metazoans includes U1, U2, U4, U5, and U6 snRNAs. In general, U snRNA genes are present in multiple copies within a genome and many genomes (mammalian in particular) contain a sizeable number of U snRNA pseudogenes as well. The exact copy number of U snRNA genes per genome varies among species, for example in case of U2 snRNA, there are probably six copies in *Drosophila melanogaster*, 17 copies in *Cenorhabditis elegans* or ten copies in *Rattus norvegicus*. However, tandems of U snRNA formed by several copies are often dispersed throughout a genome or linked to other multigene families (Marz et al. 2008).

Cytogenetic mapping of U snRNA genes has not been performed in spiders so far. The closest relatives where such analysis was performed are grasshopper *Abracris flavolineata* with a U2 snRNA cluster on B-chromosome (Bueno et al. 2013), cricket *Cycloptiloides americanus* with a subterminal U2 snRNA cluster on the largest autosomal pair (Palacios-Gimenez and Cabral-de-Mello 2015), and scorpion *Tityus obscurus* with a subterminal U2 snRNA cluster on a single autosomal pair (Almeida et al. 2017).

4 Material and Methods

4.1 Material

In the presented thesis, four species of family Tetragnathidae and 19 species belonging to family Araneidae were examined (Table 1). Target species were either obtained by individual hand collecting from particular locations of Czech Republic, Slovakia, Slovenia, and Australia, or kindly donated by fellows. Preferentially, subadult or adult specimens were collected (especially males) and processed immediately. If that was not possible, specimens were reared in plastic boxes with substrate and humidity adjusted to an exact species. Fruit flies, flies or crickets served as the food source according to size of spiders. Adult males could be distinguished due to the presence of palpal bulbs. Subadult males could be recognized thanks to the presence of swollen apical parts of pedipalps (in case of too young males, this swelling was not present and such spiders were kept until they reached subadult stages). All Australian species were collected by laboratory fellows as a part of expeditions in autumn of 2016 or 2017. Species identity of European spiders was confirmed by Dr. Petr Dolejš (Department of Zoology, National Museum – Natural History Museum, Prague), voucher specimens have been deposited in pure ethanol in National Museum, Prague. Material from Australia was deposited at Australian Museum, Sydney and determined by Australian colleagues Dr. Helen Smith (Australian Museum, Sydney) and Dr. Volker Framenau (University of Western Australia, Perth).

4.2 Composition of solutions

- Sörensen's buffer: 0.033M KH_2PO_4; 0.013M $Na_2HPO_4 \cdot 12H_2O$; pH = 6.8
- 2× SSC (saline-sodium citrate) buffer: 0.3M NaCl; 0.03M $C_6H_5O_7Na_3 \cdot 2H_2O$; pH = 7.0
- 50× Denhart reagent: 1% (w/v) Ficoll (type 400); 1% (w/v) polyvinylpyrrolidone; 1% (w/v) BSA
- 1× PBS (phosphate-buffered saline) buffer: 0.015M NaCl; 0.015M KCl; 0.015M KH_2PO_4; 0.005M $Na_2HPO_4 \cdot 12H_2O$; pH = 7.3
- Antifade DABCO: 0.21M 1,4-diazabicyclo[2.2.2]octane; 1M Tris-HCl (pH = 8.0); 0.9% (v/v) glycerol
- 1× TNT buffer: 0.1% (v/v) Tween 20; 1M Tris-HCl (pH = 8.0); 1.5M NaCl

- 1× TNB buffer: 0.5% (w/v) blocking reagent (PerkinElmer); 1M Tris-HCl (pH = 8.0); 1.5M NaCl
- Physiological solution: 0.15M NaCl; 5.6mM KCl; 2.4mM $NaHCO_3$; 3mM $CaCl_2$; pH = 7.0
- Fluormix: 0.25mM dATP; 0.25mM dCTP; 0.25mM dGTP; 0.17mM dTTP; 0.2mM dUTP-biotin/FITC (fluorescein isothiocyanate)

4.3 Chromosome preparations

Gonads of males were used for preparations predominantly, since they contain frequently dividing cells, yielding both meiotic and mitotic plates. Sometimes, it is possible to gain mitotic figures from other tissues with high frequency of dividing cells as well, such as ovaries, heart or intestines. However, these tissues were used exceptionally. Chromosome preparations were obtained using the modified plate spreading method (Traut 1976). After paralysing a spider in a freezer (usually 5 – 10 min, depending on size of an animal), abdomen was separated from cephalothorax by cutting a petiolus and embedded in a dissection dish filled with cold physiological solution (Lockwood 1961). Tissue was dissected under a stereomicroscope using a pair of tweezers and subsequently transferred to hypotonic solution (0.075M KCl) for 15 min, then fixed in freshly prepared fixative (mixture of ethanol – event. methanol – and acetic acid in ratio 3:1). Fixation was performed twice, for 10 min and 20 min. An appropriate piece of fixed tissue was cut and transferred onto a clean microscopic slide and immediately two to three drops of 60% acetic acid were dripped on it. Using a pair of tungsten needles sealed in glass rods, cell suspension was made inside a drop. Afterwards, the slide was placed on a histological plate set on 35°C or 40°C (depending on further applications). The drop was drawn on the slide using the tungsten needle until almost evaporated. The rest of the drop was removed and the slide was quickly inspected under a microscope using phase contrast in order to check the preparation quality and cell density. According to the preparation condition, the procedure resumed with either smaller, or bigger piece of the tissue until the tissue was completely depleted. Since most of the studied spiders were rather small, usually an entire gonad was used for a single slide. Preparations were then stained using 5% Giemsa solution in Sörensen's buffer for 26 min and observed. A fraction of slides was dehydrated using ascending

Species	Locality and coordinates	Methods applied
Araneidae		
Aculepeira ceropegia Walckenaer	Nový Jičín – Žilina, Ignácův vrch hill, CZ (49°35'N, 18°01'E)	Giemsa staining, FISH (18S)
Araneid gen. and sp. indet.	Canberra, Chinese church, AUS (35°15'N, 149°5'E)	Giemsa staining
Araneus circe Audouin	Detva, Square of SNP, SK (48°33'N, 19°25'E)	FISH (5S, **H3**, **U2**)
Araneus diadematus Clerck	Brozany, CZ (50°03'N, 15°47'E)	FISH (18S, H3, U2)
Araneus ginninderra Dondale	Canberra, Haydon Drive, AUS (35°15'N, 149°6'E)	Giemsa staining
Araneus nordmanni Thorell	Horská Kvilda, forest, CZ (49°03'N, 13°32'E)	Giemsa staining, FISH (18S)
Araneus psittacinus Keyserling	Canberra, swamp, AUS (35°15'N, 149°5'E)	Giemsa staining
Araneus quadratus Clerck	Suchdol nad Odrou, CZ (49°38'N, 17°57'E)	FISH (18S, 5S, H3/**H3**, U2)
Araneus saevus L. Koch	Horská Kvilda, forest, CZ (49°03'N, 13°32'E)	Giemsa staining, FISH (18S)
Araneus ventricosus L. Koch	Tokyo, JPN (35°60'N, 139°86'E)	FISH (5S, **H3**, **U2**)
Cyclosa fuliginata (L. Koch) Dondale	Canberra, swamp, AUS (35°15'N, 149°5'E)	Giemsa staining, FISH (18S)
Dolophones sp. Walckenaer	Canberra, swamp, AUS (35°15'N, 149°6'E)	Giemsa staining
Larinioides patagiatus (Clerck) Grasshoff	Suchdol nad Odrou, near Odra river (49°38'N, 17°57'E)/Borová Lada, peat-bog (49°00'N, 13°39'E)/Horská Kvilda, forest (49°03'N, 13°32'E)/Prague, Trója bridge (50°06'N, 14°25'E); all CZ	Giemsa staining, FISH (18S, 18S+28S)
Larinioides sclopetarius Clerck	Pardubice, Husovka/train station, CZ (50°02'N, 15°46'E/50°01'N, 15°45'E)	Giemsa staining, FISH (18S)
Leviellus stroemi (Thorell) Gregorič	Peřimov, cottage, CZ (51°40'N, 15°30'E)	Giemsa staining, FISH (18S)
Leviellus thorelli (Ausserer) Wunderlich	Lednice, castle park, CZ (48°48'N, 16°48'E)	Giemsa staining, FISH (18S)
Plebs bradleyi (Keyserling) Butler	Canberra, swamp, AUS (35°15'N, 149°5'E)	Giemsa staining, FISH (18S)
Zygiella atrica (L. Koch) Simon	Pardubice, Husovka, CZ (50°02'N, 15°46'E)/Prague, Charles Square, CZ (50°04'N, 14°25'E)	Giemsa staining, FISH (18S, 28S, 18S+28S, 5S)
Zygiella montana (Koch) Gregorič	Horská Kvilda, forest, CZ (49°03'N, 13°32'E)	Giemsa staining, FISH (18S, 28S, 5S)
Tetragnathidae		
Meta menardi (Latreille) Thorell	Mrzla jama pri Ložu, cadastre number 79, SLO (46°16'N, 14°43'E)	Giemsa staining
Tetragnatha demissa C. L. Koch	Canberra, lake, AUS (35°14'N, 149°5'E)	Giemsa staining
Tetragnatha nigrita Lendl	Pardubice, Husovka, CZ (50°02'N, 15°46'E)	Giemsa staining
Tetragnatha valida Keyserling	Canberra, lake, AUS (35°14'N, 149°5'E)	Giemsa staining

Table 1: List of studied species, including their localities and methods applied; where FISH was conducted, all studied markers are indicated: 18S, 28S, 5S – rRNA genes; H3 – histone H3 genes; U2 – snRNA genes for subunit U2; 18S+28S – colocalization of these markers; markers written in bold – TSA-FISH variant

ethanol series (1 min in 70%, 80%, and 96% ethanol) and stored in deep-freeze (-75°C) for later FISH experiments. Should the need arose, the fraction of slides stained with Giemsa solution was bleached (using the same methanol fixative as described above) and used for FISH experiments afterwards.

Preparations were observed using microscope Olympus BX50. Photographs of mitotic or meiotic chromosomes were captured under 100× magnification (immersion oil was applied), using CCD camera DP71 and software Olympus Cell^D (version 5.1). Software ImageJ (version 1.51u, National Institutes of Health) was used for chromosome counting and measuring. The sex chromosomes ratio was established by measuring sex chromosomes in suitable plates (late prophase I, metaphase I). For biarmed chromosomes, it was necessary to assess the centromeric index as the ratio of short (p) and long (q) arms of a chromosome and classify them according to Levan et al. (1964). Lastly, it was necessary to determine relative chromosome length, which is the length percentage of a single chromosome to total length of all chromosomes in a diploid set. Using the combination of sex chromosome ratio, centromeric index, and relative chromosome size, homologous chromosomes were paired and karyotypes were constructed in software Corel Photo-Paint (version X5).

Frequency of chiasmata was established as the ratio of total chiasmata number counted in all examined plates to total bivalent number counted in all examined plates. Either five, or ten late prophases I were examined if possible (if less than five plates were at disposal, the appropriate number was used). Position of chiasmata was assessed based on the distance of chiasmata from a chromosome end or from a centromere. Chiasmata were characterized as pericentric (if the distance from a centromere was up to 25%), distal (if the distance from a chromosome end was up to 25%) or intercalary (everything between pericentric and distal).

4.4 Fluorescence *in situ* hybridization

In order to visualize gene markers on chromosomes, method of FISH was conducted to detect patterns of 18S rDNA, 28S rDNA, 5S rDNA, histone H3, and U2 snRNA loci. For such detection, it is necessary to generate complementary DNA probes. Basically, there are two types of detection approaches – a probe can be labelled indirectly, i.e. using a hapten, which itself is not fluorescent and an extra step is required to bind a fluorophore to this hapten in order to obtain a fluorescent

signal. The second approach utilizes directly labelled probes, i.e. nucleotides containing a fluorophore are used (Speicher and Carter 2005). Generally, all probes were at first labelled indirectly by biotin, followed by signal amplification during FISH using streptavidin-Cy3 – antistreptavidin – streptavidin-Cy3 system. However, in case of histone H3 and U2 snRNA genes, the intensity and quality of signals obtained by this method were poor. Hence, TSA-FISH modification (described later in chapter 4.5) was used to improve signals. In this case, probes were labelled directly with FITC (fluorescein isothiocyanate). Regarding species *Larinioides patagiatus* and *Zygiella atrica*, colocalization of 18S and 28S rDNA was studied too – in this case, a combination of indirectly (18S rDNA) and directly (28S rDNA) labelled probes was utilized.

4.4.1 FISH with indirectly labelled probe

Concerning probe preparation, 20 – 70 ng of probe per slide was mixed with competitive DNA (sonicated DNA of salmon sperm, Sigma-Aldrich), 25 ng per slide. The probe hybridization cocktail was precipitated using alcohol to remove unlabelled nucleotides and small labelled fragments, which can cause nonspecific hybridization. Therefore, 3M sodium acetate (0.1× of DNA volume) and 96% cold ethanol (2.5× of DNA volume) were added. The mixture was transferred to deep-freeze (-75°C) for at least 1 hour or overnight. After that, the mixture was centrifuged at 20,000 g (20 min, 4°C). Subsequently, supernatant was removed from test tube and 70% cold ethanol was added (at least 4× of DNA volume). The mixture was centrifuged at 20,000 g (15 min, 4°C), followed by precise supernatant removing and complete evaporation of residual ethanol at 37°C. Dry pellet was dissolved in 100% deionized formamide (Sigma-Aldrich) (10 µl per slide). Afterwards, 20% dextran sulfate was added in equal volume. The mixture was denatured (5 min, 90°C), following by immediate transfer onto ice for 3 min.

The first step of slides manipulation was their dehydration in ascending ethanol series (1 min in 70%, 80%, and 96% ethanol). Afterwards, it was necessary to remove all RNA from slides, which was accomplished by incubation of slides with solution of 2% RNase A (10 mg/ml, Biotech) in 2× SSC buffer (100 µl of solution per slide) for 1 hour at 37°C, followed by washing of slides in 2× SSC (repeated two times, 5 min, 37°C). For such repeated washing, fresh solutions were used after every single washing step and this applies for the remainder of this text. Slides were

incubated in 50× Denhart reagent (30 min, 37°C). The next step involved denaturation of DNA on slides – 100 µl of 70% deionized formamide (Sigma-Aldrich) in 6.7× SSC was applied on each slide, cover glasses were placed onto slides and slides were placed on thermoblock with slide adapter (3 min, 68°C). After that, slides were immediately rid of cover glasses and transferred to cold 70% ethanol for 1 min, followed by 1 min in 80% and 96% ethanol. Finally, 20 µl of denatured hybridization cocktail was applied on each slide and cover glasses were put onto slides. Cover glass edges were sealed using Rubber cement (Marabu). Slides were placed inside a wet chamber (with filtration paper infused with 2× SSC) and incubated at 37°C overnight (16 – 20 hours).

The next day, slides were rid of Rubber cement and cover glasses and subsequently washed to remove unspecific hybridization. Stringent washing included 5 min in 50% formamide (Penta) in 2× SSC (46°C, repeated three times), 2 min in 2× SSC (46°C, repeated five times), 5 min in 0.1× SSC (62°C, repeated three times), and at least 5 min in 0.1% Tween 20 (Gerbu)/4× SSC at room temperature (RT).

The next step involved reaction blocking. Slides were placed inside wet chamber and 500 µl of 2.5% BSA (bovine serum albumin, Gerbu) in 4× SSC was applied on each slide. Cover glasses were put onto slides and slides were incubated at RT in dark for 20 min. After that, cover glasses were removed and 100 µl of 0.1% solution of streptavidin-Cy3 (Jackson Immuno Research Laboratories) in 2.5% BSA/4× SSC was applied on each slide. Cover glasses were put onto slides, slides were placed inside wet chamber and incubated at RT in dark for 30 min. Cover glasses were then removed and slides were washed three times in 0.1% Tween 20/4× SSC (3 min, 37°C) to remove the unbound conjugate. Another round of reaction blocking followed under the same conditions. Afterwards, cover glasses were removed, 50 µl of 4% biotinylated antistreptavidin (Vector) in 2.5% BSA/4× SSC was applied on each slide. Cover glasses were put onto slides and slides were incubated in wet chamber (37°C, 20 min). After another round of washing in 0.1% Tween 20/4× SSC and reaction blocking with BSA under the same conditions, the last signal amplification using 0.1% solution of streptavidin-Cy3 in 2.5% BSA/4× SSC at 37°C for 20 min was performed. Subsequently, slides were washed in 0.1% Tween 20/4× SSC for the last time. Preparations were stained with DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich) in subsequent washing steps as follows: 5 min in 1% Triton X (Sigma-

Aldrich)/1× PBS buffer, 8 min in DAPI (72.12nM DAPI in 1% Triton X/1× PBS), 2 – 5 min in 1% PhotoFlo (Kodak)/1× PBS, and 1 min in 1% PhotoFlo/MilliQ H_2O. After gentle drying of slides, 25 µl of DABCO (1,4-diazabicyclo[2.2.2]octane) antifade was applied on each slide along with a cover glass and excessive DABCO was squeezed out using filtration paper. Lastly, cover glass edges were sealed with nail polish and slides were allowed to dry completely.

Preparations were observed under fluorescent microscope Olympus IX81 equipped with appropriate filters and photographed under 100× magnification (with immersion oil), using CCD camera Hammatsu ORCA C4742-80-12AG. Pseudo-coloration and super-imposition were processed by Cell^R (Olympus) software; additional editing of colour balance has been performed in ImageJ where necessary.

4.4.2 Construction of probes

DNA isolation

The list of markers used in this thesis is presented in Table 2. For construction of DNA probes, genomic DNA of species *Zygiella atrica* or *Araneus diadematus* was used. Their DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen). At first, two legs (approx. 25 mg of tissue) of a spider stored in pure ethanol were removed and transferred to a sterile test tube, where they were cut into small pieces, followed by complete evaporation of residual ethanol at 37°C. Afterwards, 180 µl of ATL buffer and 20 µl of proteinase K (20 mg/ml) were added, followed by incubation at 56°C until complete lysis of tissue. Subsequently, 200 µl of AL buffer was added and incubation at 56°C continued for another 10 min. After that, 200 µl of 96% ethanol was applied into the mixture, which was then transferred to a DNeasy Mini spin column placed in a 2 ml collection tube. The assembly was centrifuged at 6000 g for 1 min and flow-through with collection tube were discarded. Afterwards, the column was placed in a new collection tube, 500 µl of AW1 buffer was added, the assembly was centrifuged at 6000 g for 1 min, and flow-through with collection tube were discarded. In the next step, the column was placed in a new collection tube, 500 µl of AW2 buffer was added, the assembly was centrifuged at 20,000 g for 3 min, and flow-through with collection tube were discarded. The column was then placed in a new sterile test tube, 200 µl of AE buffer was added followed by incubation for 1 min (RT), and assembly was centrifuged at 6000 g for 1 min. Quality of isolated

DNA was checked on 2% agarose gel. Concentration of DNA was measured using Qubit® 3.0 Fluorometer (Thermo Fisher Scientific).

Probe generation

The isolated genomic DNA was used for amplifying the target sequence using PCR with primers designed in previous publications or by Petr Nguyen, Institute of Entomology, České Budějovice (Table 2) and synthesized by Generi Biotech.

A single PCR reaction of volume 20 – 25 µl contained: 50% of PPP Master mix (Top-Bio), 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 100 ng of genomic DNA, and PCR H_2O (Top-Bio) to add up to 20 – 25 µl of reaction volume. The PCR amplification was performed using PCR cycler (LifeEco, Bioer) with heated lid. Thermal profile starts with initial denaturation (3 min, 95°C), followed by 35 cycles of denaturation at 95°C/30 s, primer annealing (temperature gradient at 51°C, 56°C, and 60°C/30 s) and extension (72°C/2 min), and ends with final chain extension (15 min, 72°C).

Size and quality of the PCR products were gauged on 2% agarose gel, followed by excision of candidate bands from the gel in most cases. The PCR products or gel slices were then purified using Wizard® SV Gel and PCR Clean-Up System as follows: in case of gel slices, appropriate amount of Membrane binding solution was added to a gel slice in a test tube (10 µl of solution per 10 mg of gel slice), followed by incubation at 50 – 65°C until gel slice was completely dissolved. In case of PCR product, an equal volume of Membrane binding solution was added to the PCR product. Dissolved gel slice or PCR product was then transferred to an SV Minicolumn placed in a collection tube, followed by 1 min incubation at RT. The assembly was centrifuged at 16,000 g for 1 min and flow-through was discarded. Afterwards, 700 µl of Membrane wash solution (mixed with ethanol prior to use in ratio 1:1) was added, the assembly was centrifuged at 16,000 g for 1 min, and flow-through was discarded. This step was repeated, but with 500 µl of Membrane wash solution and centrifugation for 5 min. After flow-through was discarded, the assembly was centrifuged at 16,000 g for 1 min to completely evaporate remaining ethanol. Minicolumn was then transferred to a new test tube, 50 µl of Nuclease-free water was added and after 1 min incubation at RT, the assembly was centrifuged at 16,000 g for 1 min.

The next step involved DNA concentration measurement using Qubit® 3.0 Fluorometer (Thermo Fisher Scientific). When low yield of product was obtained, DNA

was precipitated using ethanol (as described in chapter 4.4.1) and dissolved in lower volume of PCR water or, alternatively, PCR was repeated prior to this step.

The 18S rDNA probe was not prepared as a part of this thesis. Already constructed probe was at disposal instead, provided by Petr Nguyen (Institute of Entomology, České Budějovice). The production process for this probe was identical to the text described above, with these modifications: DNA was isolated from *Dysdera erythrina* (Dysderidae). This DNA was used as a template for PCR reaction with these forward and reverse primers, respectively: 5´-CGAGCGCTTTTATTAGACCA-3´ and 5´-GGTTCACCTACGGAAACCTT-3´ (Forman et al. 2013), synthesized by Generi Biotech. Gel extraction was conducted using QIAquick Gel Extraction Kit (Qiagen). The probe was indirectly labelled with biotin-14-dATP using nick translation method with Bionick Labelling System Kit (Invitrogen, Life Technologies).

Sequencing and alignment

The method of DNA sequencing, for the first time invented simultaneously by Sanger and Maxam with Gilbert in 1974, is used for determining DNA sequences.

All samples presented in this thesis were sequenced by MiSeq Illumina, using services of Laboratory of DNA sequencing in Biocev. A single sequencing reaction of 8 µl volume included 1 µl of 5 µM primer, 5 ng of DNA per 100 bp, and PCR H_2O (Top-Bio) to add up to 8 µl of reaction volume. Quality of sequences was assessed using software BioEdit (version 7.2.1) or Chromas (version 2.6.5, Technelysium) as well as additional editing of sequences if necessary. Basic Local Alignment Search Tool (BLAST, NCBI) was used to search for regions of homology between the obtained sequences and sequences available in databases. If a significant similarity with database sequences of related arthropods was found, it could be said that the desired marker was obtained and it was ready for labelling.

Probe labelling

In case the correct expected sequence was obtained, such DNA could be used for PCR labelling. The probes were labelled either indirectly using biotin-16-dUTP (Sigma-Aldrich), or directly using fluorescein-12-dUTP (Sigma-Aldrich). A single PCR reaction of volume 25 µl contained: 2.5 µl of LA buffer (Top-Bio), 1.25 µl of modified dNTPs Fluormix, 1 µl of forward primer, 1 µl of reverse primer, 0.05 µl of LA DNA polymerase (5 U/µl, Top-Bio), approx. 100 ng of purified target DNA sequence,

and PCR H_2O (Top-Bio) to add up to final volume. The amplification/labelling was performed with initial denaturation (5 min, 95°C), followed by 40 cycles of optimized thermal profile with prolongation of extension step for 4 min to ensure proper incorporation of labelled nucleotides, and final chain extension (10 min, 72°C).

Marker and DNA source	Set of tested primers	Primer sequences	Ideal T_m [°C]	Product size [bp]	Note	Reference
28S rDNA *Zygiella atrica*	28S-I	**28Sa: 5´-GACCCGTCTTGAAACACGGA-3´** **28Sb: 5´-TCGGAAGGAACCAGCTACTA-3´**	56	300	Successful primer set	Whiting et al. 1997
5S rDNA *Zygiella atrica*	5S-I	5Sa: 5´-ACGTTGCTTGACTTCGGTGA-3´ 5Sb: 5´-GTCGGGCGTAGTCAGTACTT-3´	51, 56, 60	150	Meaningless sequence obtained	Watanabe et al. 2009
	5S-II	5Sa: 5´-AACAACAAGTCCGGAGAACG-3´ 5Sb: 5´-GACCACTGAGAGGGTCGAT-3´	51	100	Meaningless sequence obtained	Suzuki and Kubota 2011
	5S-III	**5Sa: 5´-GCCAACGTCCATACCAYGYTGA-3´** **5Sb: 5´-AAGCCAACGNCACGYGGT-3´**	56	150	Successful primer set	Institute of Entomology, České Budějovice
H3 gene *Araneus diadematus*	H3-I	**H3a: 5´-TANGCACGYTCNCCNCGGAT-3´** **H3b: 5´- ATGGCNCGTACNAARCARAC-3´**	56	300	Successful primer set	Institute of Entomology, České Budějovice
U2 snRNA gene *Araneus diadematus*	U2-I	U2a: 5´-TCTCGGCCTWWTGGCTAA-3´ U2b: 5´-GMGGTASTGCAATACCGG-3´	cannot be determined	cannot be determined	No products present on gel	Colgan et al. 1998
	U2-II	**U2a: 5´-ATCGCTTCTCGGCCTTATG-3´** **U2b: 5´-TCCCGGCGGTACTGCAATA-3´**	60	150	Successful primer set	Bueno et al. 2013

Table 2: The list of all constructed probes along with spider species serving as DNA source, sets of tested primers and their ideal annealing temperatures (or melting temperatures, Tm), resulting product sizes on gel, and references; in case of degenerated primers: N = A/G/T/C, Y = T/C, R = A/G, W = A/T, M = A/C, S = G/C; primer sets successfully amplifying desired sequences are shown in bold; notes provide reasons of primer sets failure

4.5 TSA-FISH

Tyramide signal amplification (TSA) FISH is a modification of standard FISH that yields more intensive fluorescent signal due to reaction between horseradish peroxidase (HRP) and tyramide. HRP, in environment of H_2O_2, catalyses activation of tyramide (conjugated with a fluorophore) and deposition of activated tyramide-fluorophore complex to its proximity to the surface of chromosomes. This reaction results to vast signal amplification while maintaining specificity of binding site, since activated tyramide-fluorophore complex is deposited to close proximity of immobilised enzyme (Schonhuber et al. 1997; Fominaya et al. 2016). This work presents the very first case of TSA-FISH usage on spider chromosomes.

Concerning probe preparation, the whole process was identical to the description in chapter 4.4.1, only probe was labelled directly using FITC instead of biotin and 30 ng of probe was used per slide.

As to slides, they were at first dehydrated in ascending ethanol series (1 min in 70%, 80%, and 96% ethanol) and then underwent so called aging – incubation at 60°C for 1 hour to ensure stability of chromosomes. After aging, remains of cytoplasm were removed by incubation in 10mM HCl (Penta) (10 min, 37°C) and then washed three times in 1× PBS at 24°C, followed by incubation in 1% H_2O_2 (Sigma-Aldrich) (30 min, 24°C). After another round of washing in 1× PBS (under the same conditions), RNase A treatment, incubation in Denhart reagent, slide denaturation, and hybridization were performed as described above.

The next day, Rubber cement and cover glasses were removed and slides underwent stringent washing as described in chapter 4.4.1, excepting the last step, where 0.1% Tween 20/4× SSC was replaced by 1× TNT buffer. Reaction blocking followed – 200 µl of TNB buffer was applied on each slide. Cover glasses were put onto slides, slides were placed inside wet chamber and incubated at RT in dark for 30 min. Signal provided by FITC was amplified using TSA® Plus fluorescein detection kit (PerkinElmer) as follows: after cover glasses were removed, 200 µl of antifluorescein-HRP conjugate diluted in TNB (1:100) was applied on each slide. Cover glasses were put onto slides and slides were placed inside wet chamber and incubated at 37°C for 1 hour. After the incubation, cover glasses were removed and slides were rinsed with 1× TNT using a Pasteur pipette, followed by washing three times in 1× TNT (5 min, 24°C). After that, TSA Plus Amplification Reagent® (tyramide included,

PerkinElmer) in a solid state was reconstituted with 150 µl of DMSO (dimethyl sulfoxide) to prepare TSA Plus Stock Solution. This solution was diluted with 1× amplification diluent® (1:50, PerkinElmer) and 100 µl of such mixture was applied on each slide. Cover glasses were put onto slides and slides were placed inside wet chamber and incubated at RT in dark for 5 – 15 min. Afterwards, cover glasses were removed and slides were rinsed with 1× TNT using a Pasteur pipette, followed by washing three times in 1× TNT (5 min, 24°C). Finally, DAPI staining and the following steps of procedure were performed as described in chapter 4.4.1.

5 Results

5.1 Conventional cytogenetic analysis

5.1.1 Araneidae

Araneus

In the presented thesis, eight species of this genus were examined. Some of these species have already been studied, so data of previous authors were revised as well.

Araneus diadematus

Diploid number of this species was 24 in males, all chromosomes were exclusively acrocentric. Sex chromosome system was X_1X_20 (Fig. 24A, page 60 and Fig. 32B, page 69).

Araneus quadratus

Karyotype of males consisted of 24 chromosomes with exclusively acrocentric morphology. Sex chromosome system detected in this species was X_1X_20 (Fig. 24B, page 60 and Fig. 31B, page 67).

Araneus ginninderra

Diploid number in males was probably 24, sex chromosome system was most likely X_1X_20 (Fig. 5A, B). Due to lack of suitable plates, it was not possible to reliably determine chromosome morphology or other karyotype features.

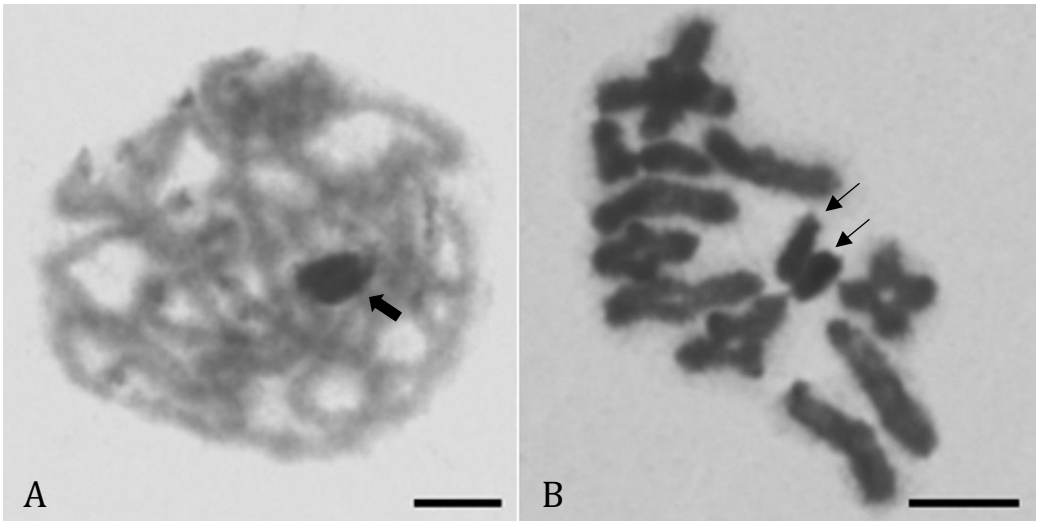

Fig. 5: *Araneus ginninderra* male. (A) Pachytene with sex chromosome body marked with a bold arrow, it is impossible to distinguish number of X chromosomes; (B) Metaphase I with 11 bivalents and probably two X chromosomes (arrows); scale – 5 µm

Araneus psittacinus

Unfortunately, preparations of this species were of poor quality and contained but a very few plates. Nevertheless, this species possessed probably 24 chromosomes in males and X_1X_20 sex chromosome system (Fig. 6A, B). Chiasma frequency was 1.27 with predominance of terminal chiasmata (measured from ten late prophases I).

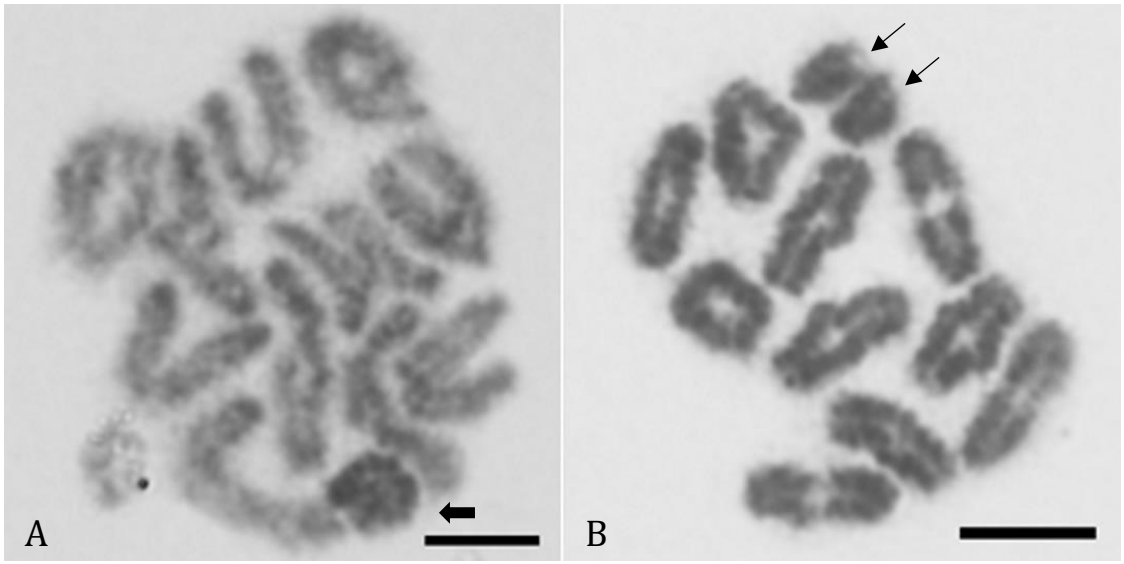

Fig. 6: *Araneus psittacinus* male. (A) Diakinesis showing 11 chromosomal bivalents and sex chromosome body marked with a bold arrow; (B) Incomplete metaphase I, sex chromosomes (arrows) are more separated than in the previous plate; scale – 5 μm

Araneus nordmanni

Diploid number in males was 23 with all chromosomes being acrocentric (Fig. 7B, C). The sex chromosome system was X0. The acrocentric X chromosome located at the periphery of nucleus and significantly longer than the rest of chromosomes exhibited low level of condensation and negative heteropycnosis throughout the meiotic division (Fig. 7A, B, C). Centromere of X chromosome was more condensed during diakinesis, which made this structure well visible during this period (Fig. 7A). During the second meiotic division in anaphase II, the X chromosome segregated to one of poles (Fig. 7B, C). Chiasma frequency was 1.11 with predominance of pericentric chiasmata (measured from five late prophases I).

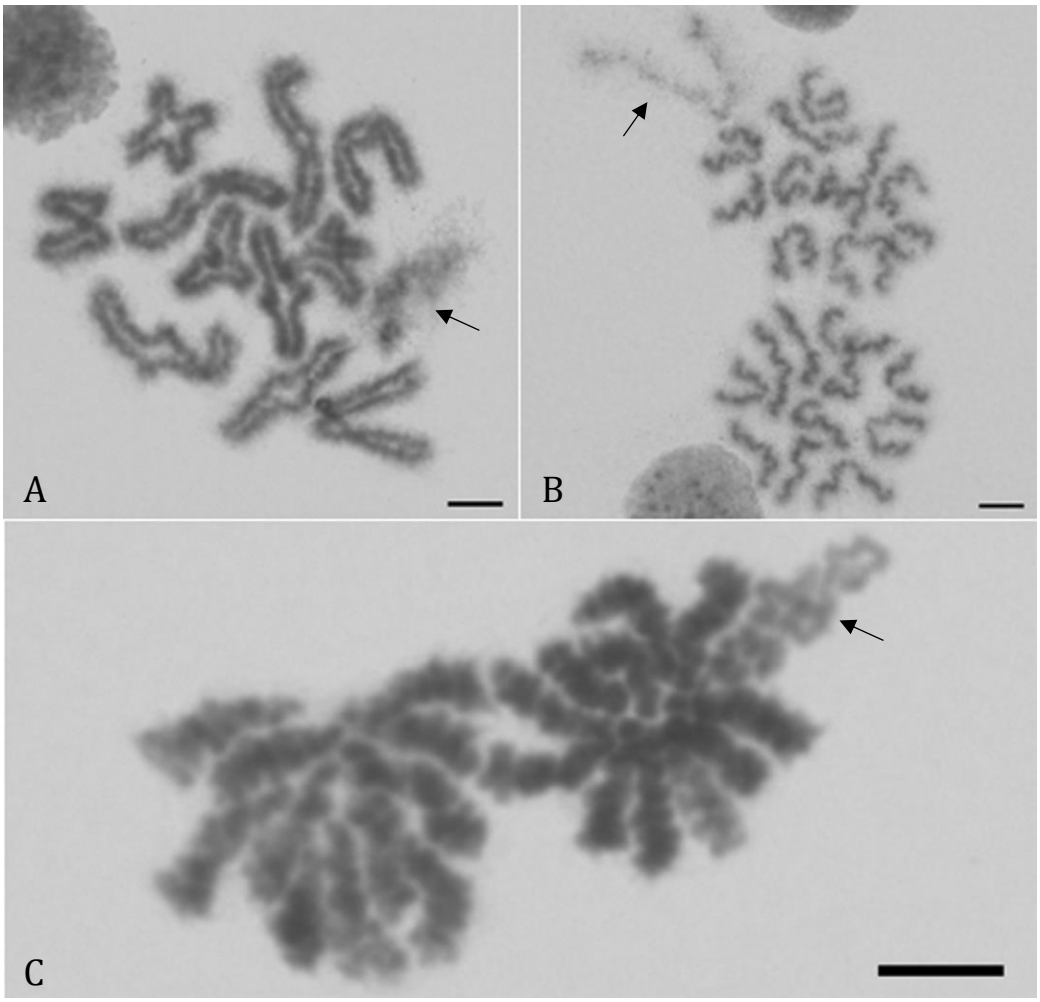

Fig. 7: *Araneus nordmanni* male, arrows mark the X chromosome. (A) Diakinesis; (B) Anaphase I; (C) Anaphase II; scale – 5 μm

Araneus saevus

Karyotype of this species resembled the situation of the previous species – *A. saevus* possessed 23 acrocentric chromosomes gradually decreasing in size (Fig. 8A) as well as X0 sex chromosome system. As in the case of *A. nordmanni*, the acrocentric X chromosome was weakly condensed and negatively heteropycnotic in both metaphase I and II. During metaphase II, it appeared to be longer than autosomes, located at one of poles (Fig. 8B, C). Chiasma frequency was 1.00 with predominance of pericentric chiasmata (measured from a single late prophase I).

Araneus circe

Males of this species exhibited 2n = 52, while all chromosomes possessed acrocentric morphology. The sex chromosome system detected in this species was $X_1X_2X_3X_40$ (Fig. 31C, page 67).

Araneus ventricosus

Karyotype of males was composed of 49 exclusively acrocentric chromosomes. The sex chromosome system was $X_1X_2X_30$ (Fig. 31D, page 67 and Fig. 32F, page 69).

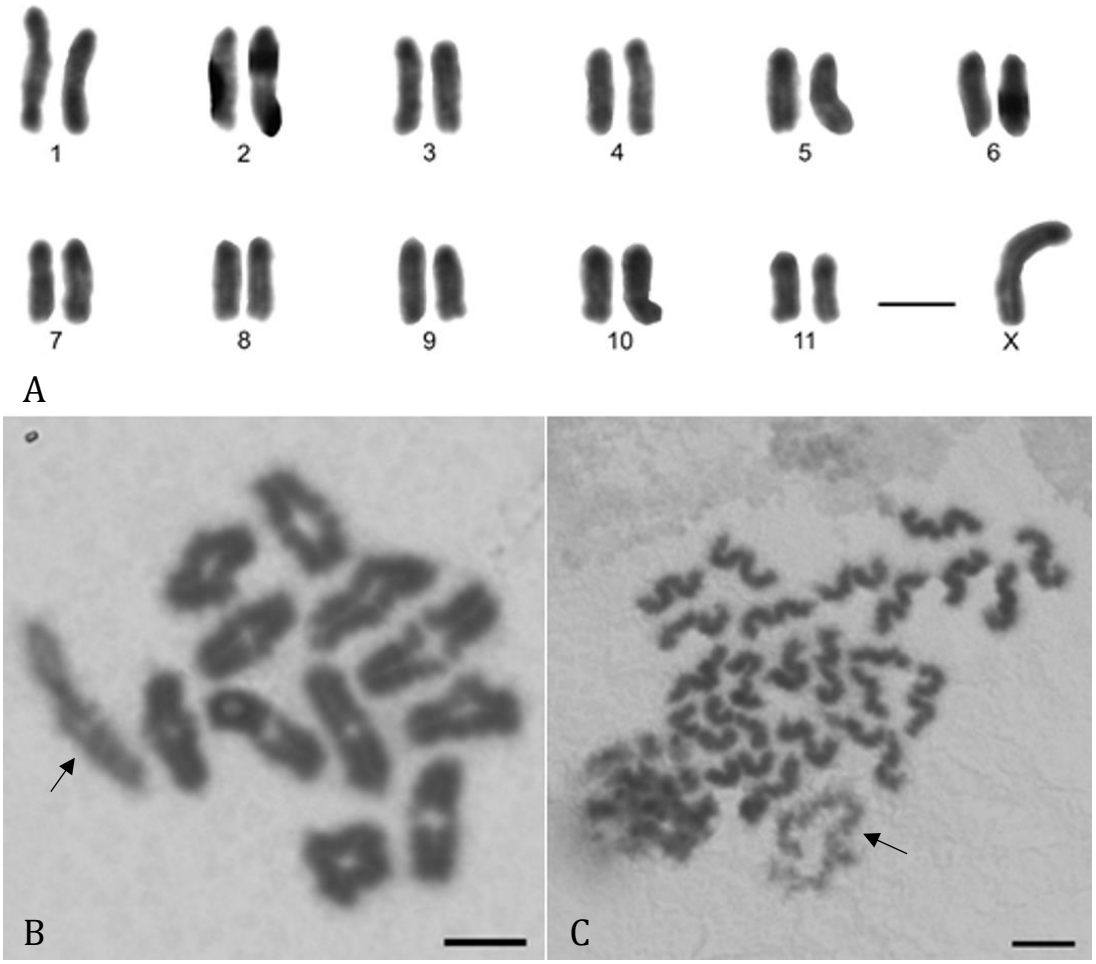

Fig. 8: *Araneus saevus* male, arrows point to the X chromosome. (A) Karyogram constructed from spermatogonial mitosis; (B) Metaphase I; (C) Two fused sister metaphases II; scale – 5 μm

Larinioides

Larinioides sclopetarius

Diploid number of this species was 24, all chromosomes were acrocentric and decreased gradually in size (Fig. 9A). The sex chromosome system was X_1X_20 and the sex chromosome ratio was 1:0.92 (s = 0.05, measured from five late prophases I and metaphases I). The X chromosomes formed a positively heteropycnotic sex chromosome body in pachytene (Fig. 9B). During metaphase I and anaphase I, they associated at centromeres and in anaphase I, they segregated together to one of

poles (Fig. 9C, D). Chiasma frequency was 1.00 with approx. equal occurrence of distal and pericentric chiasmata (measured from 10 late prophases I).

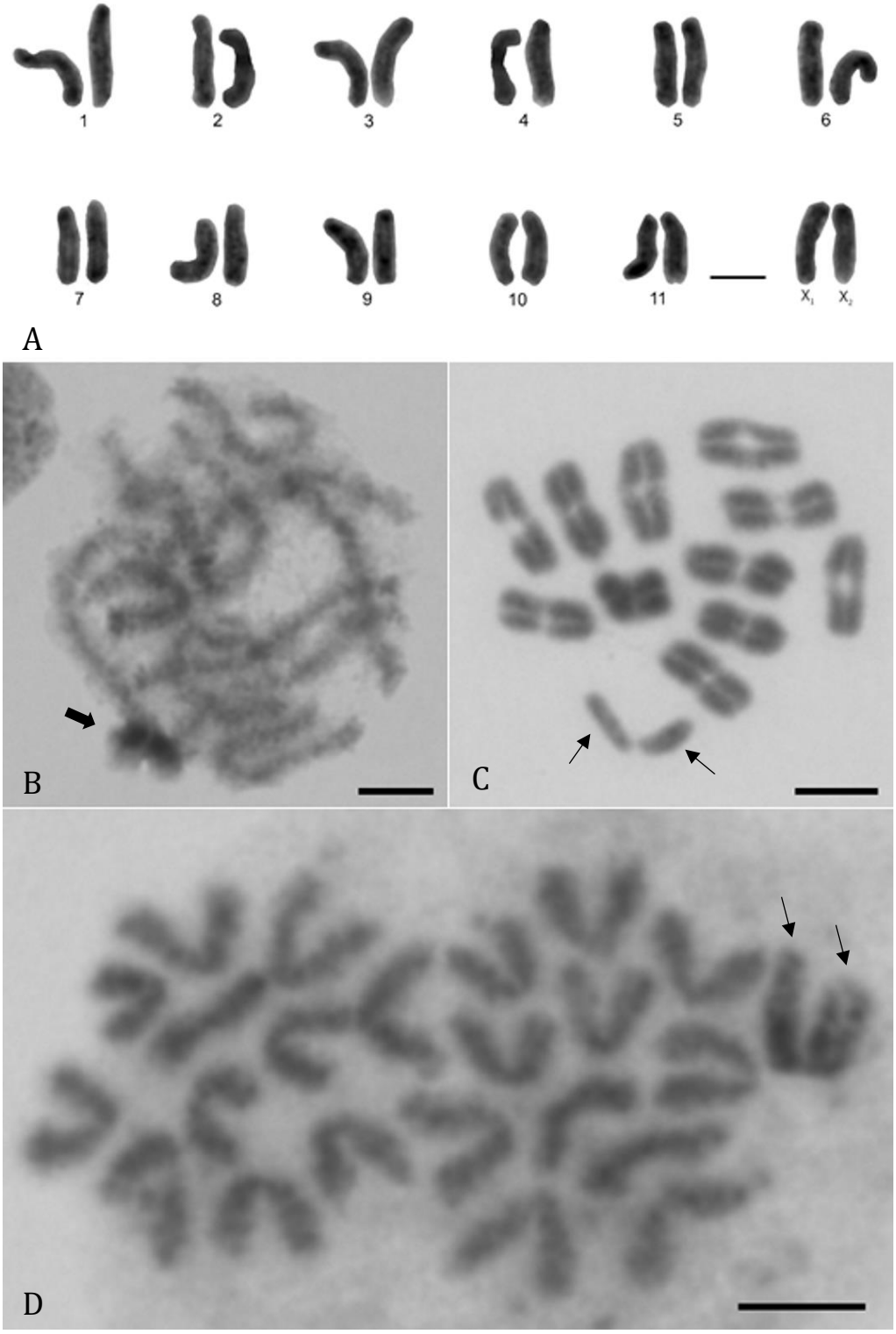

Fig. 9: *Larinioides sclopetarius* male, arrows point to X chromosomes. (A) Karyogram constructed from spermatogonial mitosis; (B) Pachytene with sex chromosome body (bold arrow); (C) Metaphase I; (D) Two fused sister anaphases I; scale – 5 µm

Larinioides patagiatus

Four various populations of this species, as well as both sexes, were studied in the presented thesis, revealing unexpected diversity of karyotypes. A single male of population from "Suchdol nad Odrou" (SO) had 16 chromosomes: three pairs of acrocentric chromosomes, four pairs of metacentric chromosomes, and two acrocentric gonosomes (Fig. 11A, B, C). The sex chromosome system detected was X_1X_20, with the sex chromosome ratio being 1:0.86 (s = 0.06, measured from ten late prophases I and metaphases I). As seen from Fig. 11A and B, all acrocentric and two pairs of metacentric chromosomes formed standard bivalents during diakinesis and metaphase I, but remaining four metacentric chromosomes were arranged in a tetravalent, which was sometimes circular (in 18 plates) and sometimes linear (in 11 plates). The sex chromosomes seemed to associate firmly at one end during diakinesis, exhibiting positive heteropycnosis in diakinesis, metaphase I, and metaphase II (Fig. 11A, B, C). Despite the presence of the tetravalent, segregation pattern seemed to be regular, with seven chromosomes segregating to one pole and seven chromosomes together with both gonosomes to another pole (Fig. 11C, the same observed in another eight plates).

Regarding a male of population from "Borová Lada, Šumava" (BL), diploid number was 14. Karyotype consisted of a single pair of acrocentric chromosomes, five pairs of metacentric chromosomes, and two acrocentric gonosomes of system X_1X_20 (Fig. 11D, E, F). The sex chromosome ratio was 1:0.90 (s = 0.06, measured from ten late prophases I and metaphases I). The acrocentric and three pairs of metacentric chromosomes formed standard bivalents, while remaining four metacentric chromosomes were again arranged in a tetravalent, which was either circular (in 23 plates) or linear (in three plates) (Fig. 11D, E). The sex chromosome pairing was the same as in the previous case and segregation pattern was probably regular (six autosomes + two gonosomes : six autosomes, Fig. 11F, the same observed in another three plates).

Population from "Horská Kvilda" (HK, 2 males) was even more complex, since it comprised two distinctive cytotypes, marked as cytotype A (HK-A) and cytotype B (HK-B). As to cytotype A, it was composed of 14 chromosomes: a single pair of acrocentric chromosomes, 11 biarmed chromosomes (impossible to determine centromeric index), and a single biarmed gonosome. Thus, the sex chromosome system

was X0 (Fig. 11G, H, I). In this case, both acrocentric and eight biarmed chromosomes formed normal bivalents, while remaining three biarmed chromosomes were arranged in a trivalent. It is very likely that this trivalent was composed of a single metacentric and two small submetacentric chromosomes (Fig. 11G). The segregation pattern was stable, with standard bivalents segregating regularly and the trivalent segregating in 2:1 ratio – the long metacentric chromosome always segregated with the X chromosome, two small submetacentric chromosomes were never present in such plates (Fig. 11H, I, observed in another single plate). Cytotype B consisted of 13 chromosomes: a single pair of acrocentric chromosomes, five pairs of metacentric chromosomes, and a single metacentric gonosome. The sex chromosome system was X0 (Fig. 10). All chromosomes behaved and segregated regularly (Fig. 11J). Chiasma frequency was 2.17 with predominance of distal chiasmata (measured from four late prophases I).

The last population from Prague involved three individuals, females only. Diploid number was 14 and karyotype was probably composed of 12 biarmed and two acrocentric chromosomes. However, only a few mitotic (and a few pachytene) plates were obtained and therefore, it was not possible to reliably assess chromosome morphology. Data on male chromosomes were lacking. Therefore, it was impossible to determine sex chromosome system (Fig. 11K, L).

Zygiellinae

Leviellus stroemi

Diploid number of this species was 24 in males, all chromosomes were acrocentric (Fig. 12B, C, D). The sex chromosome system was X_1X_20 (Fig. 12B, C) and the sex chromosome ratio was 1:0.91 (s = 0.07, measured from five late prophases I and metaphases I). These sex chromosomes were positively heteropycnotic during pachytene (Fig.12A). Nonetheless, this heteropycnosis disappeared in metaphase I. The X chromosomes segregated, as expected, together to one of poles (Fig. 12B, C). Chiasma frequency was 1.09 with predominance of pericentric chiasmata (measured from a single late prophase I).

Leviellus thorelli

Only females were collected, quality of preparations was low. Plates were formed by 26 chromosomes, as seen in mitotic metaphase. Chromosome morphology was probably exclusively acrocentric (Fig. 13).

Zygiella montana

Karyotype of this species consisted of 24 chromosomes in males, of which all were acrocentric. Their size decreased gradually (Fig. 14A, D, page 49). The sex chromosome system was X_1X_20 (Fig. 14B, C, E, page 49), the sex chromosome ratio was 1:0.79 (s = 0.09, measured from ten late prophases I and metaphases I). In pachytene, sex chromosomes formed a positively heteropycnotic body on the periphery of the plate and in metaphase I, they associated at the centromeres (Fig. 14B, C, page 49). As seen from Fig. 14E, segregation pattern of sex chromosomes was regular. Chiasma frequency was 1.01 with equal occurrence of pericentric and distal chiasmata (measured from ten prophases I).

Zygiella atrica

Diploid number in males was 14 and karyotype consisted of five metacentric pairs gradually decreasing in size, a single subtelocentric pair, and two acrocentric X chromosomes (Fig. 15A, page 50). Thus, the sex chromosome system was X_1X_20 with the sex chromosome ratio being 1:0.87 (s = 0.09, measured from five late prophases I and metaphases I). There was a firm association of sex chromosomes at centromeres in pachytene, diakinesis, and metaphase II (Fig. 15B – E, page 50). Two X chromosomes segregated together to one of poles (Fig. 15D, E, page 50). Chiasma frequency was 1.88 with predominance of distal chiasmata (measured from four late prophases I). In a half of examined plates, a bivalent with three chiasmata occurred.

Fig. 10: Karyogram of *Larinioides patagiatus*, male from population HK-B, constructed from two sister metaphases II; scale – 5 μm

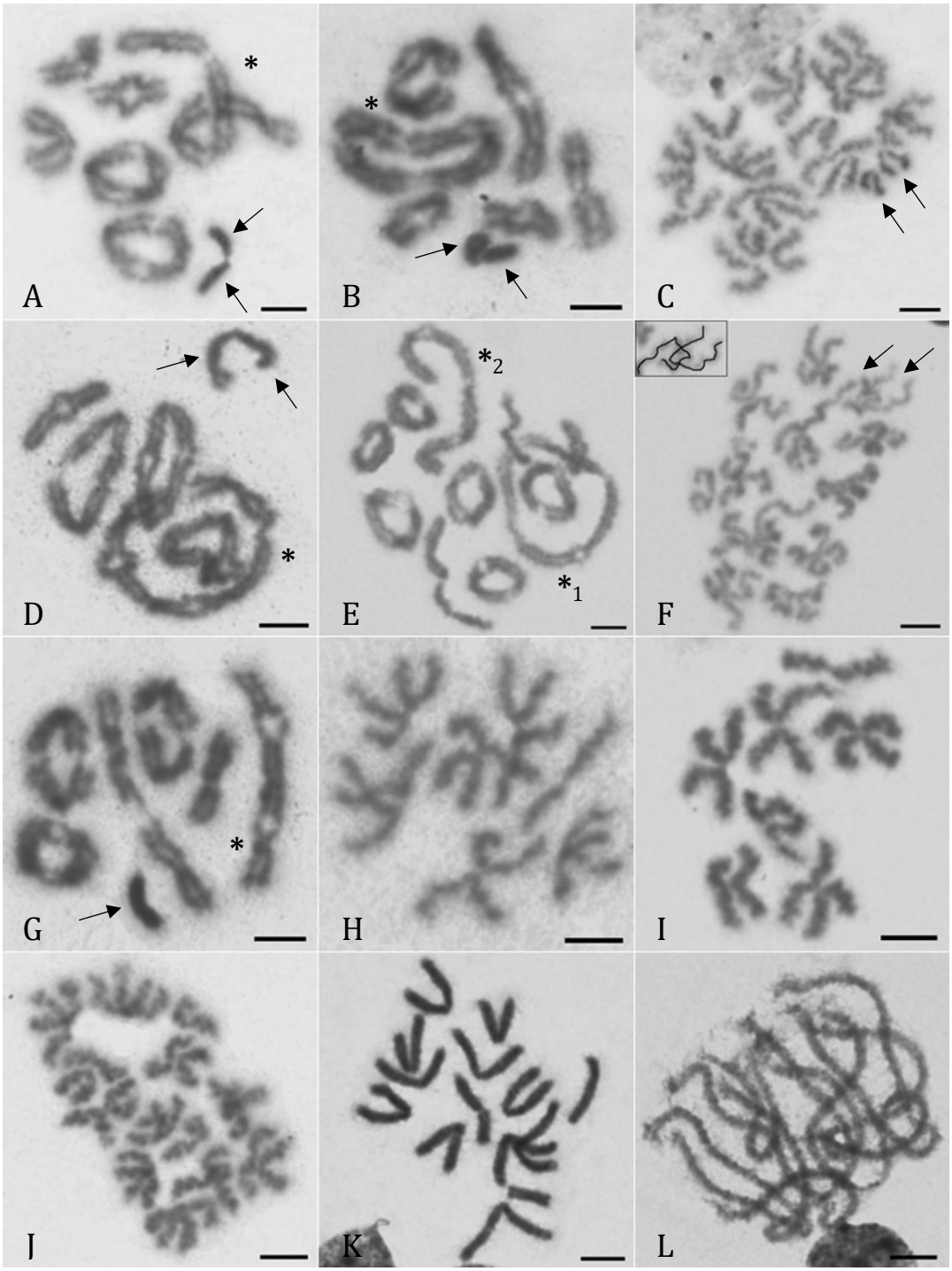

Fig. 11: *Larinioides patagiatus*, conventional analysis, unless otherwise stated, figures show male plates, arrows point to X chromosomes and asterisks mark multivalents. (A) Population SO, diakinesis containing a linear tetravalent; (B) Population SO, metaphase I with a circular tetravalent; (C) Population SO, two sister metaphases II; (D) Population BL, diakinesis with a circular tetravalent; (E) Population BL, plate formed by two fused diakineses – note circular (*1) and linear (*2) tetravalents; (F) Population BL, two fused sister metaphases II – note the scheme of sex chromosomes in the inset; (G) Population HK-A, metaphase I with a trivalent; (H, I) Population HK-A, metaphase II; (J) Population HK-B, two fused sister metaphases II; (K) Prague population, female mitotic metaphase; (L) Prague population, female pachytene; scale – 5 μm

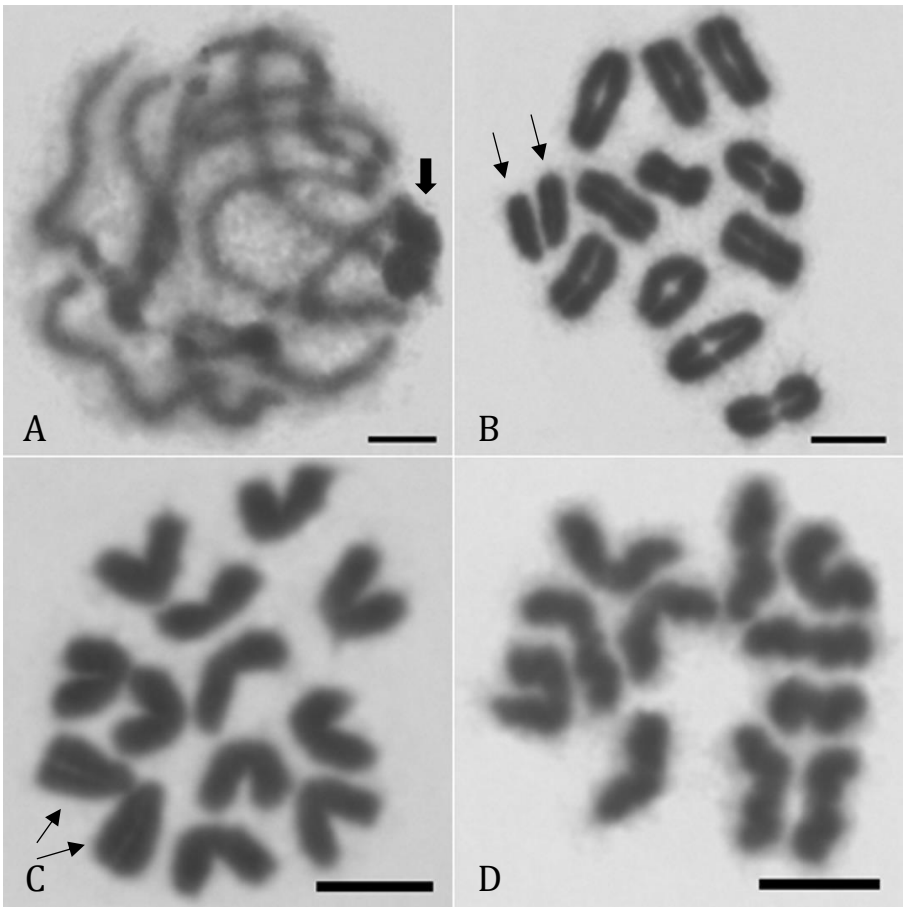

Fig. 12: *Leviellus stroemi* male, arrows mark two X chromosomes. (A) Pachytene, a bold arrow points to sex chromosome body; (B) Metaphase I; (C, D) Metaphase II plates, note acrocentric morphology of autosomes and sex chromosomes, (C) Metaphase II containing X chromosomes, (D) Metaphase II without X chromosomes; scale – 5 μm

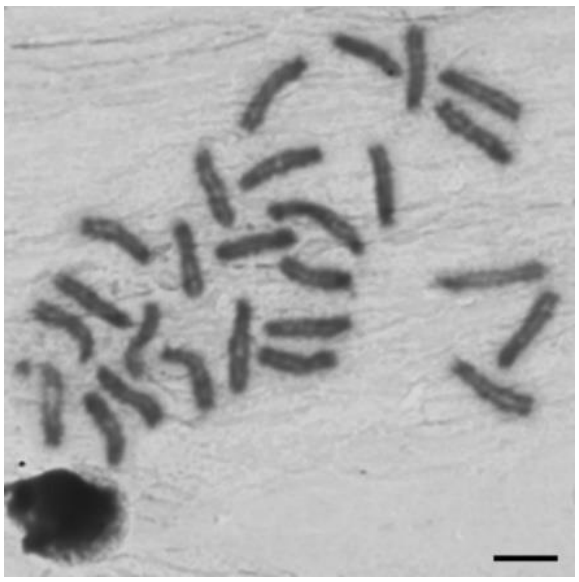

Fig. 13: *Leviellus thorelli*, female mitotic metaphase; scale – 5 μm

Other araneids

Aculepeira ceropegia

Similarly to many previous species, karyotype of males consisted of 24 exclusively acrocentric chromosomes gradually decreasing in size (Fig. 16A, page 51). The sex chromosome system was most probably X_1X_20. It was not possible to completely reliably distinguish sex chromosomes during pachytene and diakinesis. They formed very compact positively heteropycnotic mass during these stages (Fig. 16C, D, page 51). Furthermore, only metaphases and anaphases II with 11 chromosomes were obtained, i.e. without presumable two gonosomes (Fig. 16C, page 51). On the other hand, presumable X chromosomes spiralised more slowly than the rest of chromosomes during mitotic metaphases (Fig. 16B, page 51), which supports the existence of X_1X_20 system in this species. Chiasma frequency was 1.00 with predominance of distal chiasmata (measured from five late prophases I).

Plebs bradleyi

Karyotype of males consisted of 24 acrocentric chromosomes and sex chromosome system X_1X_20 (Fig. 17B, C, D, page 52). The sex chromosome ratio was 1:0.87 (s = 0.06, measured from ten late prophases I and metaphases I). The sex chromosomes were positively heteropycnotic from pachytene until metaphase II (Fig. 17A, B, C, page 52). In metaphase I, they associated at one end (Fig. 17B, page 52). In anaphase I and II, they segregated ordinarily (Fig. 17C, D, page 52). Chiasma frequency was 1.00 with equal occurrence of distal and pericentric chiasmata (measured from two late prophases I).

Dolophones sp.

Diploid number in males was 24, all chromosomes exhibited acrocentric morphology and decreased gradually in size (Fig. 18A, B, C, page 52). The sex chromosome system was X_1X_20, the sex chromosome ratio was 1:0.89 (s = 0.08, measured from ten late prophases I and metaphases I). The sex chromosomes associated at centromeres during metaphase I and segregated together to one of poles during anaphase I and II (Fig. 18B, C, page 52). Chiasma frequency was 1.00 with predominance of distal chiasmata, but intercalary chiasmata were rather frequent, too (measured from ten late prophases I).

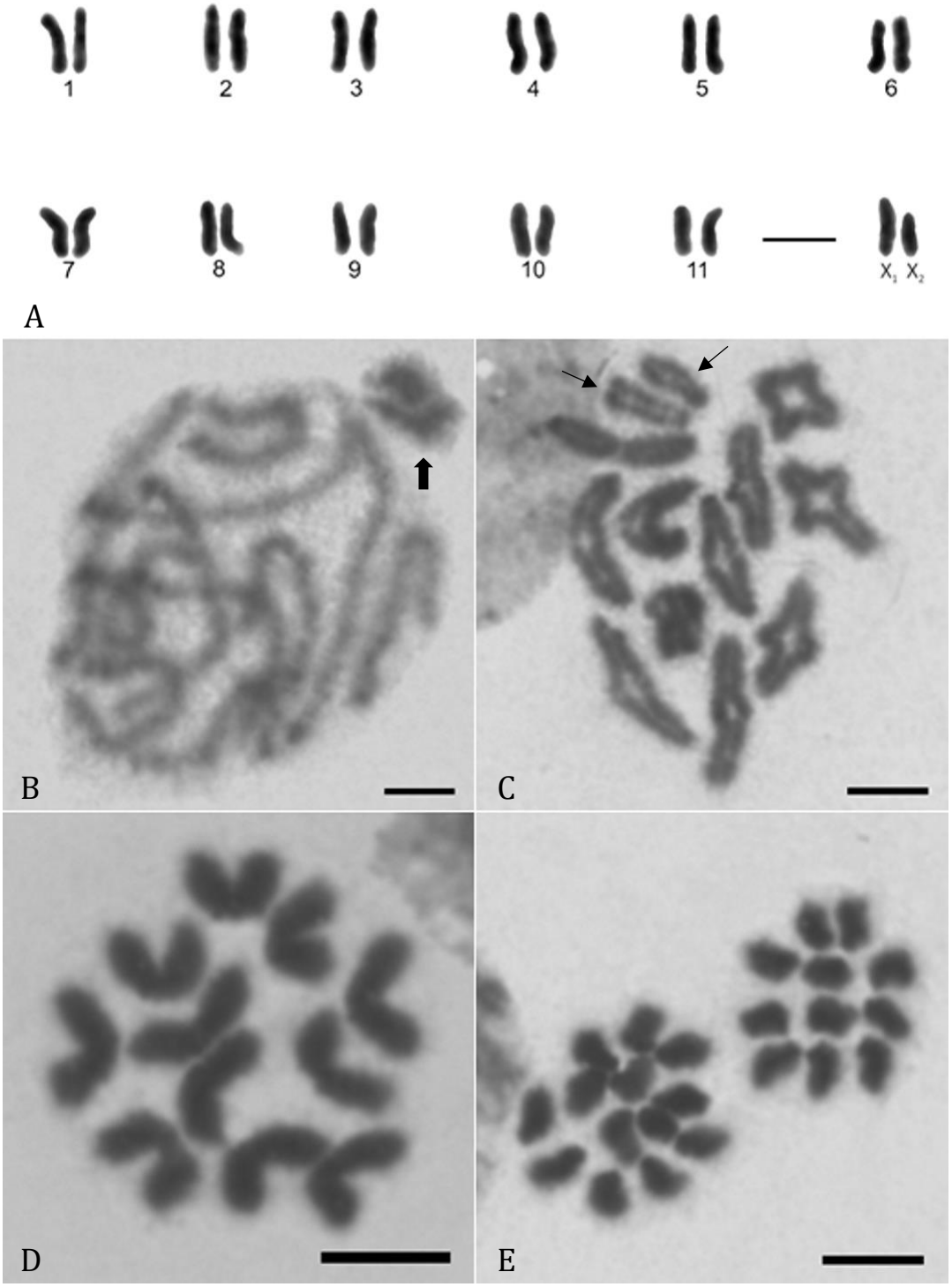

Fig. 14: *Zygiella montana* male, arrows mark X chromosomes. (A) Karyogram constructed from spermatogonial mitosis; (B) Pachytene with sex chromosome body marked with a bold arrow; (C) Diplotene; (D) Metaphase II without sex chromosomes, note acrocentric morphology of autosomes; (E) Anaphase II – it is not possible to recognize X chromosomes in the left half-plate containing them; scale – 5 μm

Cyclosa fuliginata

Karyotype of males consisted of 13 chromosomes, of which five chromosomal pairs were metacentric, a single chromosomal pair was acrocentric, and there was also a metacentric X chromosome (Fig. 19A, page 53). Hence the sex chromosome system was X0. The X chromosome was positively heteropycnotic from pachytene to anaphase II and exhibited standard segregation (Fig. 19B – E, page 53). Chiasma frequency was 1.94 with predominance of distal chiasmata (measured from five late prophases I). In four examined plates, a bivalent with three chiasmata occurred.

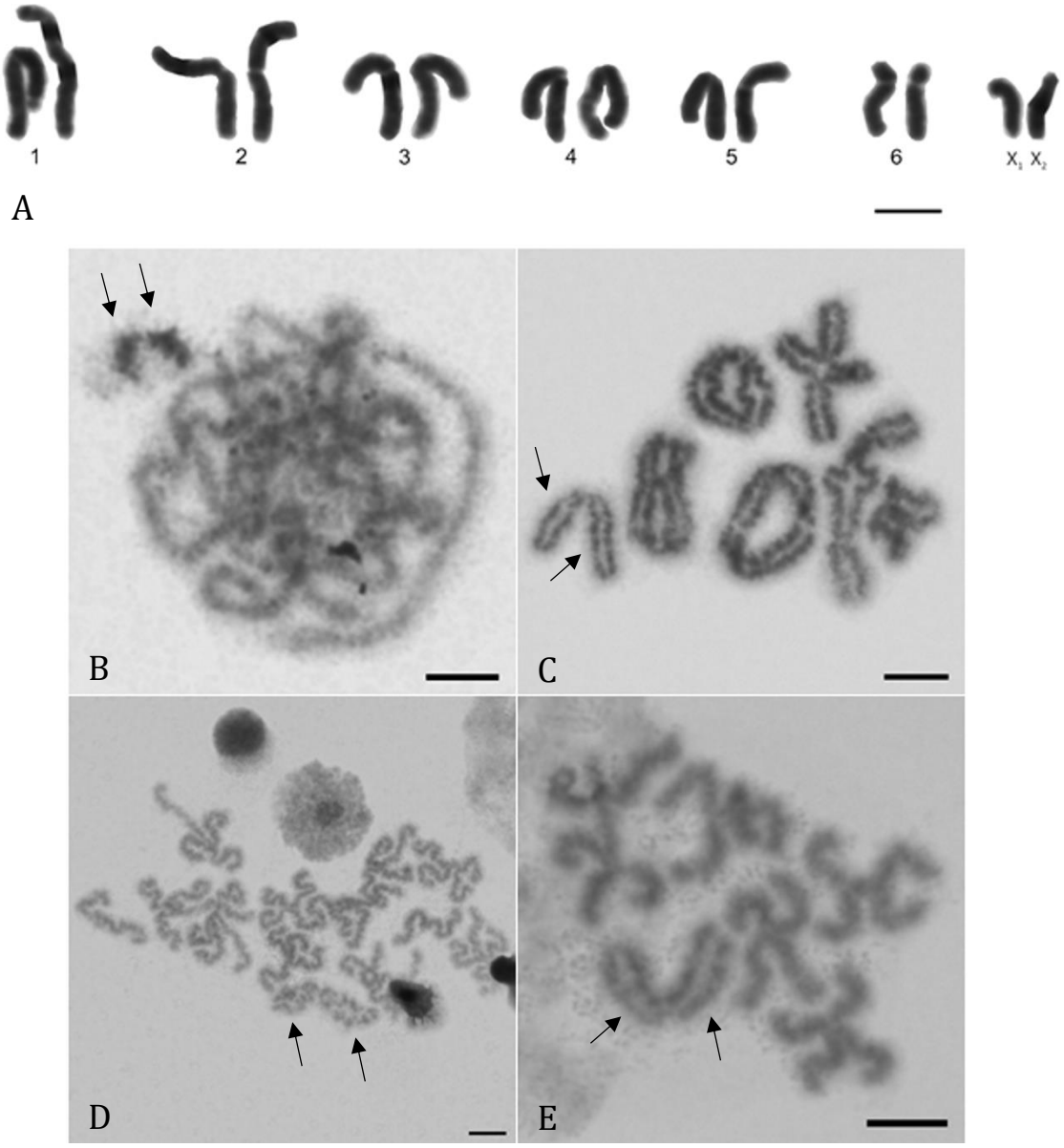

Fig. 15: *Zygiella atrica* male, arrows mark X chromosomes. (A) Karyogram constructed from spermatogonial mitosis; (B) Pachytene; (C) Diakinesis; (D) Two fused sister prometaphases II; (E) Metaphase II with X chromosomes; scale – 5 μm

Araneid gen. and sp. indet.

Karyotype of males of this yet undetermined Australian orb-weaver consisted of 28 chromosomes, of which 20 were metacentric, four were acrocentric, and there were also four acrocentric X chromosomes (Fig. 20A, E, F, page 54). The sex chromosome system was therefore $X_1X_2X_3X_40$, the sex chromosome ratio was 1:0.91:0.73:0.62 (s = 0.03, measured from five late prophases I and metaphases I). These X chromosomes were positively heteropycnotic during leptotene (each of them localised at a distinctive spot on the edge of a nucleus), pachytene (all four of them were separated, yet they were localised close to each other in form of the sex chromosome body), and metaphase II (they segregated together to one of poles) (Fig. 20B, C, E, page 54). During diakinesis, sex chromosomes associated at their ends (Fig. 20D, page 54).

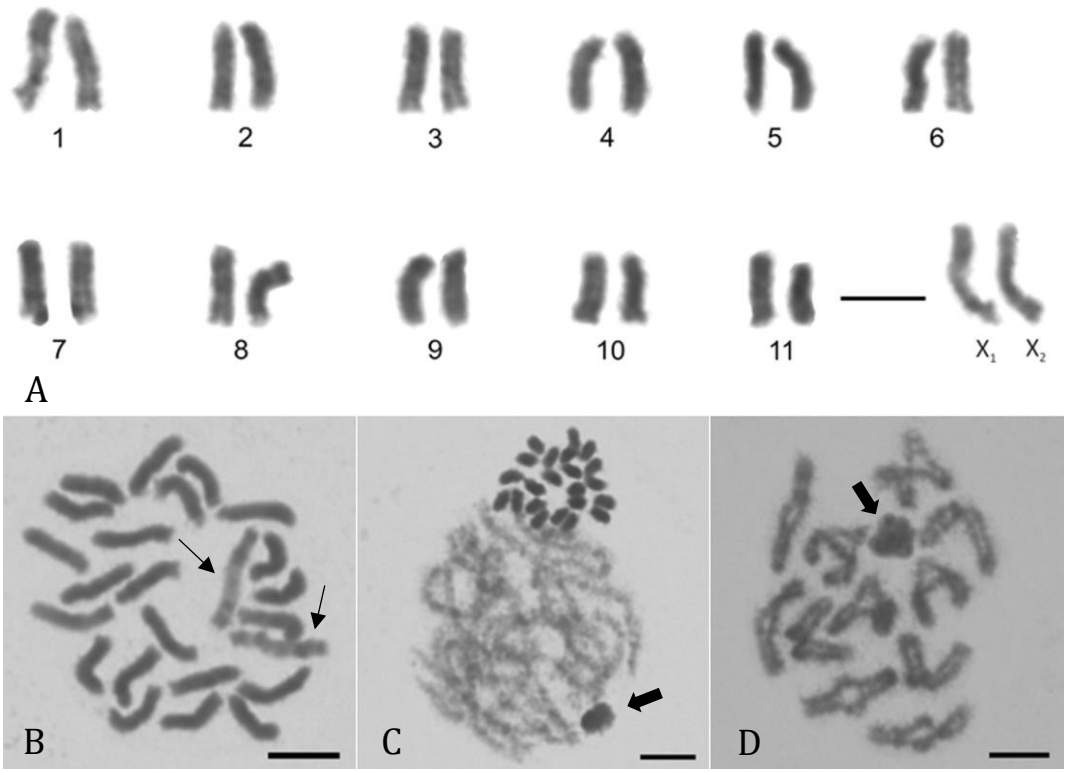

Fig. 16: *Aculepeira ceropegia* male, arrows point to sex chromosomes, bold arrows point to sex chromosome body. (A) Karyogram constructed from spermatogonial mitosis; (B) Mitotic metaphase; (C) Pachytene (bottom) and a half-plate of anaphase I (top); (D) Diakinesis; scale – 5 μm

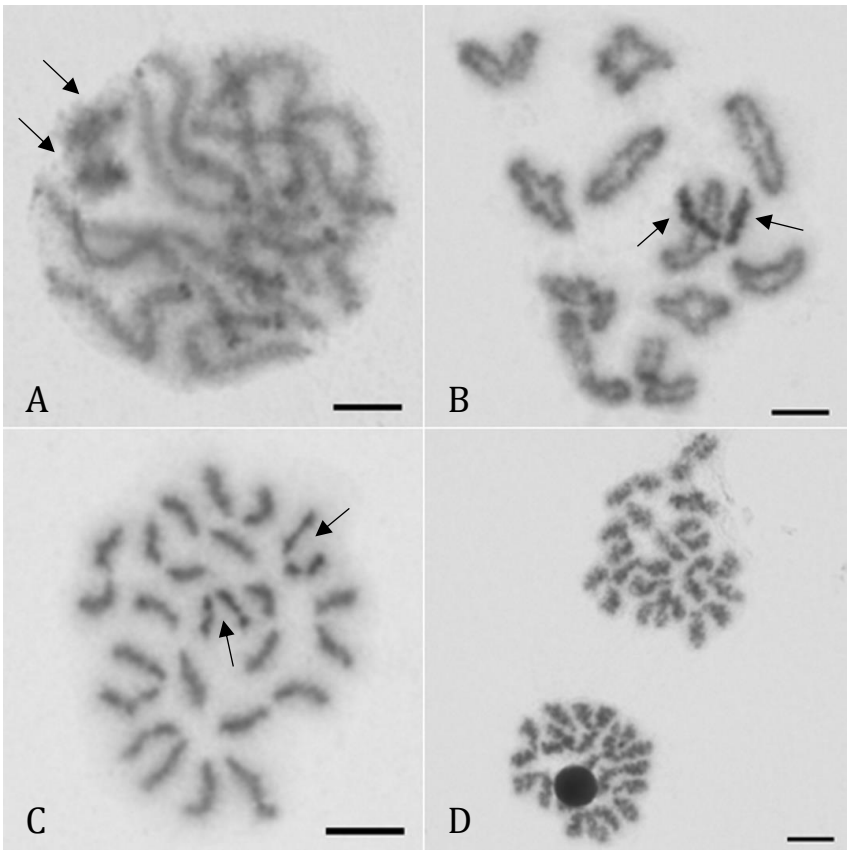

Fig. 17: *Plebs bradleyi* male, arrows indicate X chromosomes. (A) Pachytene; (B) Diakinesis; (C) Metaphase II with two X chromosomes; (D) Anaphase I – X chromosomes cannot be distinguished, but they are localised within the upper plate; scale – 5 μm

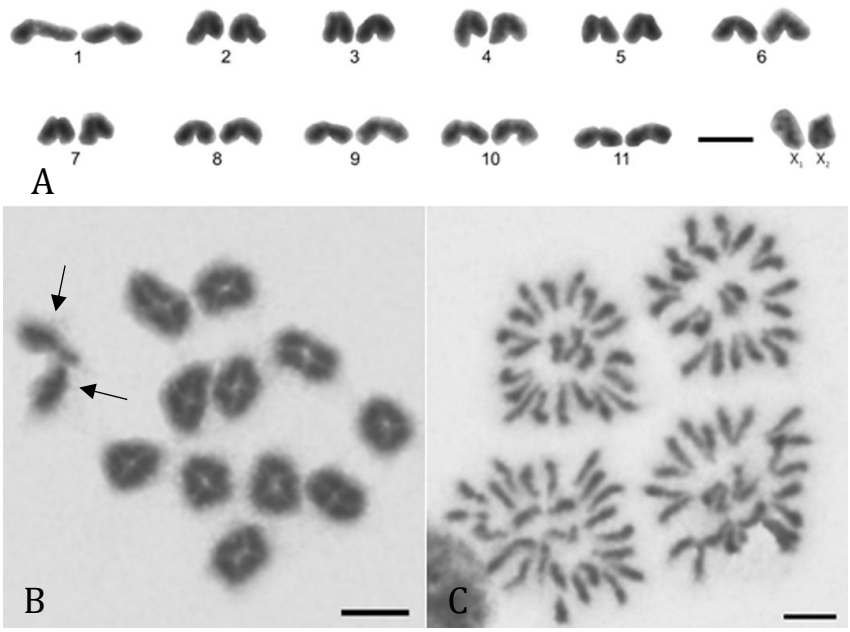

Fig. 18: *Dolophones* sp. male, arrows indicate X chromosomes. (A) Karyogram constructed from metaphase I; (B) Metaphase I; (C) Two anaphases I: top and bottom plates on the left are pair, top and bottom plates on the right are another pair, it is possible to count more reliably 11 chromosomes in the top right plate and 13 chromosomes in the bottom right plate; scale – 5 μm

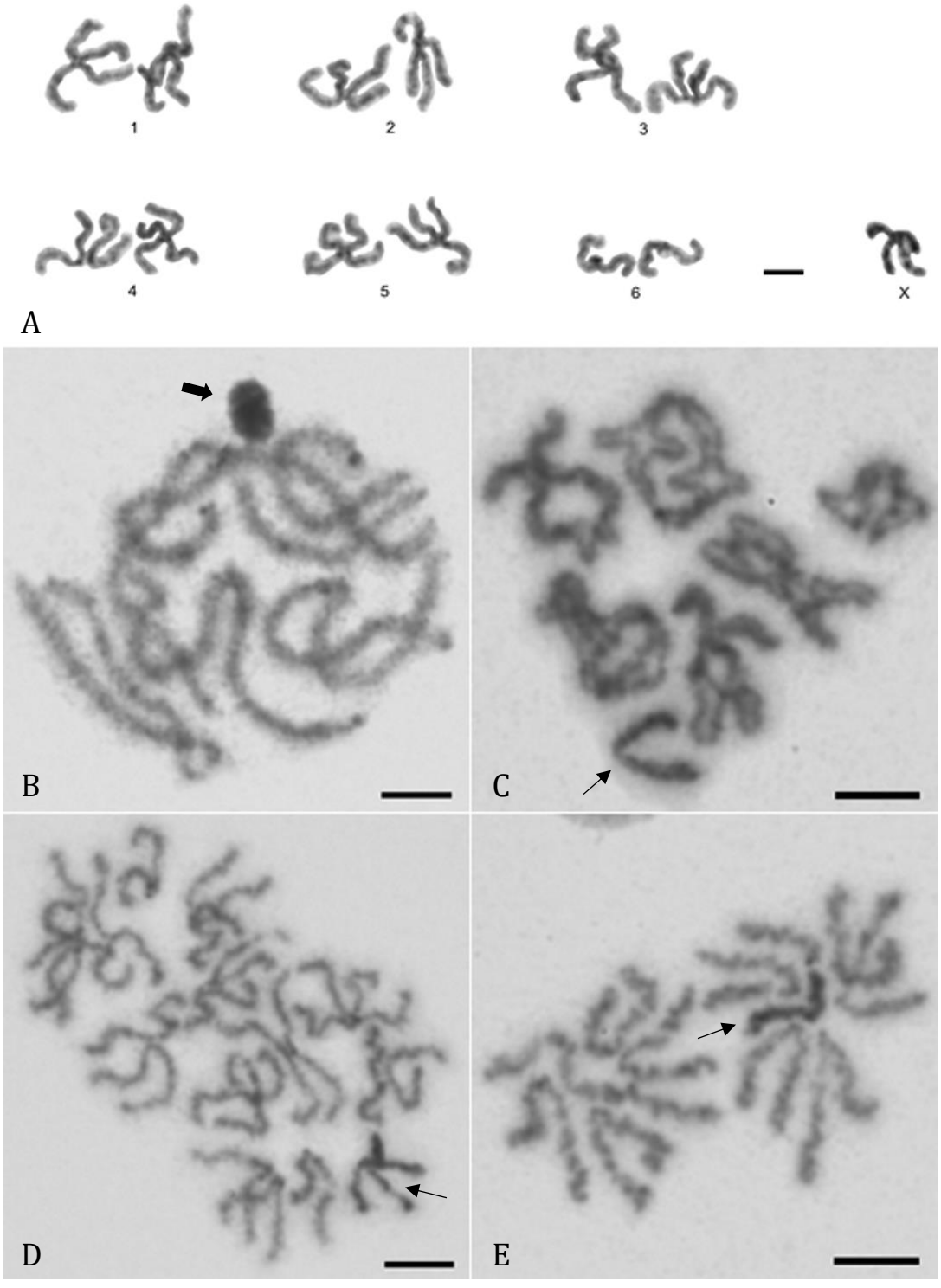

Fig. 19: *Cyclosa fuliginata* male, arrows indicate positively heteropycnotic X chromosome. (A) Karyogram constructed from two sister metaphases II; (B) Pachytene with sex chromosome body marked with a bold arrow; (C) Diplotene; (D) Two fused sister prometaphases II; (E) Anaphase II; scale – 5 μm

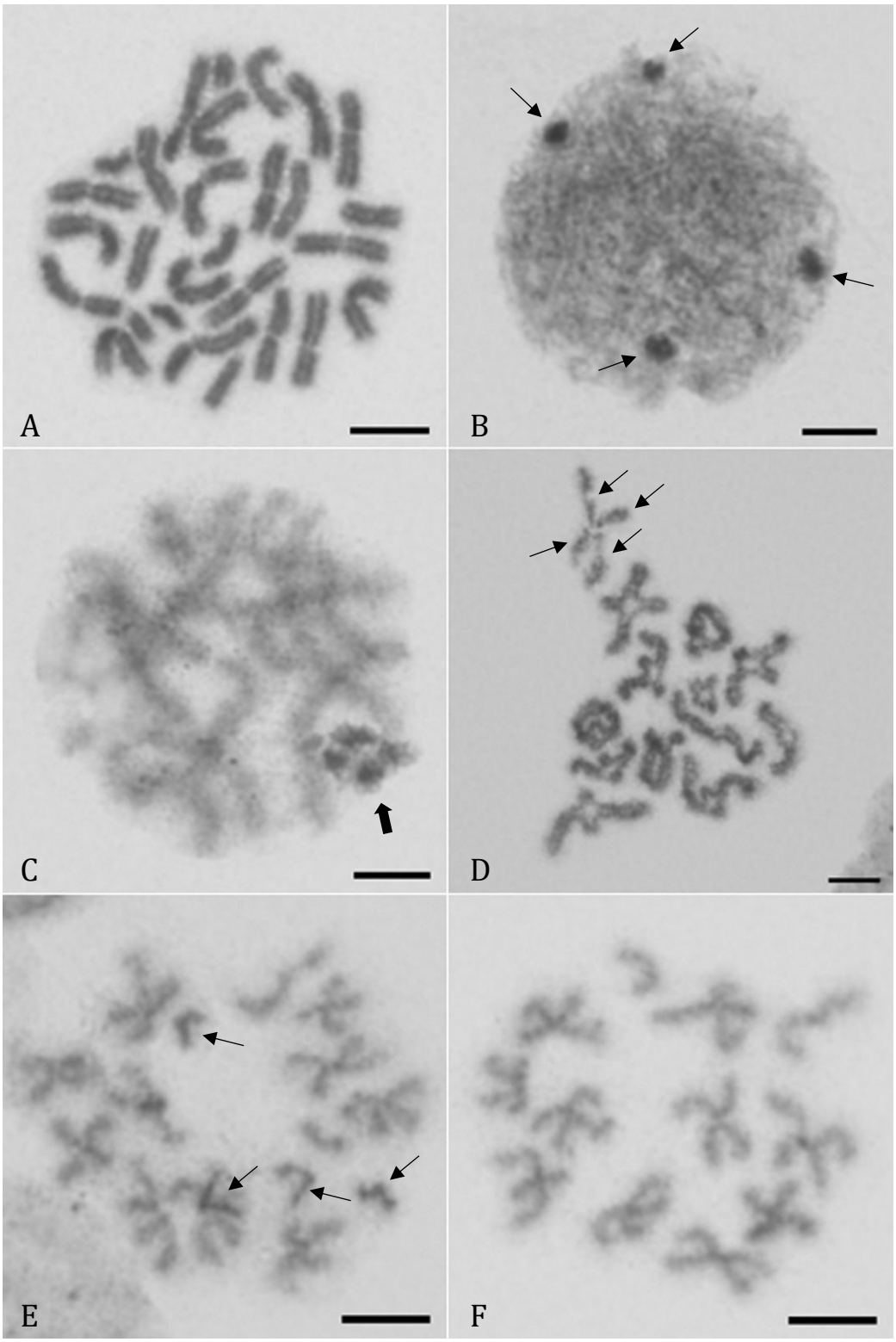

Fig. 20: Araneid gen. and sp. indet. male, arrows indicate X chromosomes. (A) Mitotic metaphase with 28 chromosomes; (B) Leptotene, note the distinctive positions of separated X chromosomes; (C) Pachytene with the sex chromosome body (bold arrow); (D) Diakinesis; (E) Metaphase II with sex chromosomes; (F) Metaphase II without sex chromosomes; scale – 5 μm

5.1.2 Tetragnathidae

In the presented thesis, a single species of *Meta* and three species of *Tetragnatha* were analysed.

Meta menardi

Males of this species possessed 24 acrocentric chromosomes which gradually decreased in size (Fig. 21). Sex chromosome system X_1X_20 was detected, as seen in metaphase I (Fig. 22). The sex chromosome ratio was 1:0.90 (s = 0.05, measured from five late prophases I and metaphases I). Chiasma frequency was 1.00 with predominance of pericentric chiasmata (measured from a single late prophase I).

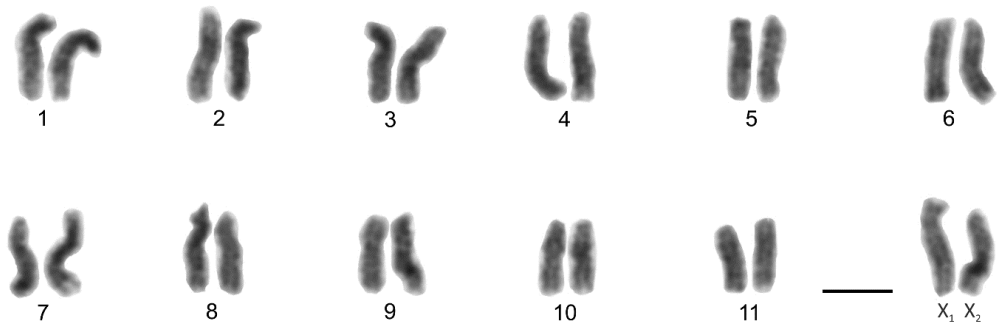

Fig. 21: Karyogram of *Meta menardi* male; constructed from spermatogonial mitosis; scale – 5 μm

Tetragnatha

Three species of this genus were analysed: *Tetragnatha nigrita* (Czech Republic), *T. demissa*, and *T. valida* (Australia). From these species, *T. nigrita* and *T. demissa* shared the following karyotype features: 2n♂ = 24, exclusively acrocentric chromosomes and the sex chromosome system X_1X_20 (Fig. 23B – E). The sex chromosome ratio was 1:0.95 in *T. nigrita* (s = 0.03, measured from three late prophases I and metaphases I) and 1:0.87 in *T. demissa* (s = 0.06, measured from ten late prophases I and metaphases I). It was not possible to assess this ratio in *T. valida* due to lack of suitable plates. In *T. nigrita* and *T. demissa*, the sex chromosomes exhibited positive heteropycnosis during pachytene, metaphase I, anaphase I, and metaphase II and regular segregation pattern was observed (i.e. both sex chromosomes segregating together to one of poles), as seen in Fig. 23A – E. In case of *T. valida*, only late prophases I were obtained and therefore, it was only possible to determine 2n♂ = 24. It is probable that the sex chromosome system was X_1X_20, with X chromosomes

being positively heteropycnotic during metaphase I (Fig. 23F). Chiasma frequency in *T. nigrita* was 1.00 with predominance of terminal chiasmata (measured from two late prophases I).

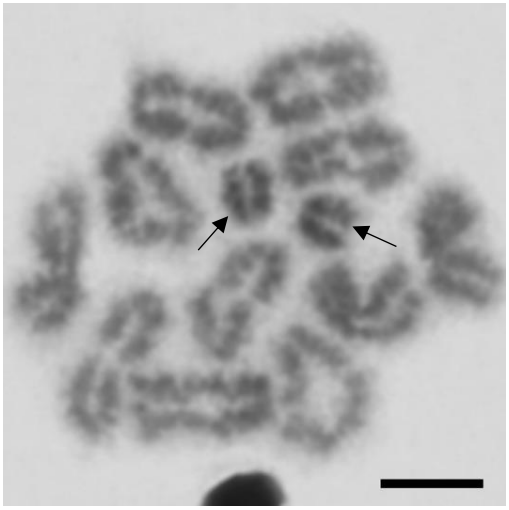

Fig. 22: *Meta menardi* male, metaphase I; arrows point to X chromosome univalents; scale – 5 µm

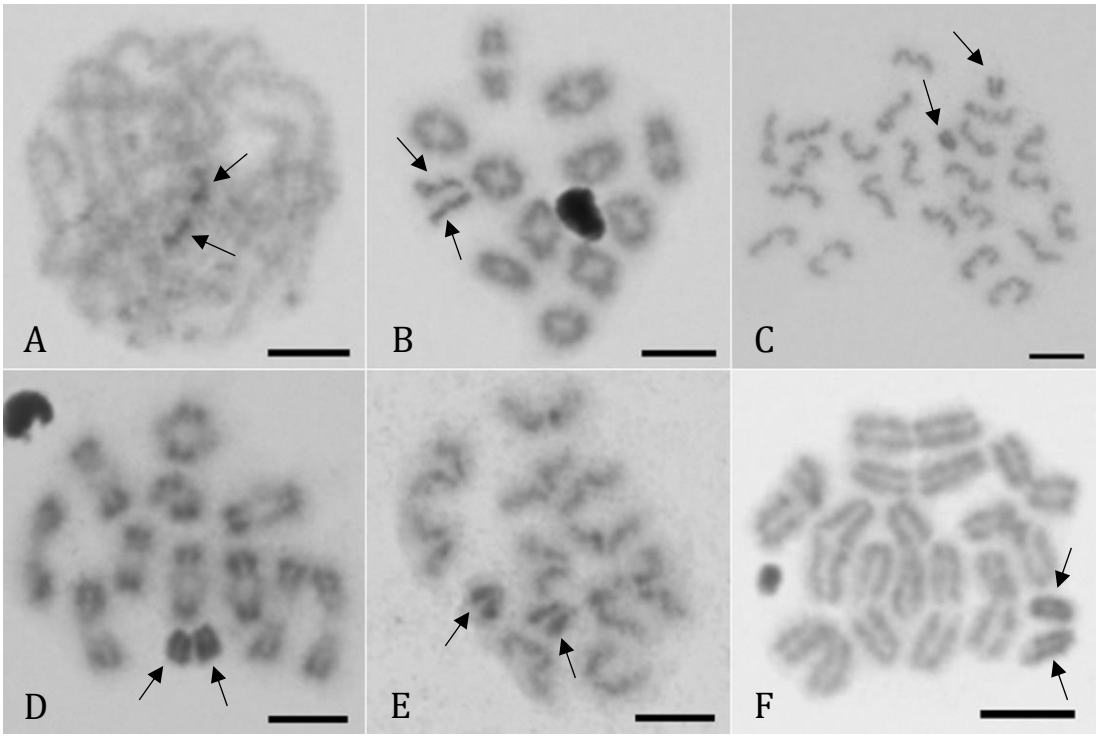

Fig. 23: Males of *Tetragnatha*, meiosis, arrows indicate X chromosome univalents, (A–C) *T. nigrita*, (D, E) *T. demissa*, (F) *T. valida*. (A) Pachytene; (B) Metaphase I; (C) Two fused sister metaphases II; (D) Metaphase I; (E) Metaphase II including X chromosomes; (F) Metaphase I; scale – 5 µm

5.2 Molecular cytogenetic analysis

5.2.1 Nucleolar organizer regions

Araneus

In all studied species of this genus, NORs were visualised using 18S rDNA FISH. This method revealed five terminal NOR loci in males of both *A. diadematus* and *A. quadratus* located on five chromosome pairs (Fig. 24A, B, page 60). However, in *A. diadematus*, one locus was heterozygous for the presence of 18S rDNA cluster. Moreover, one locus homozygous for the presence of 18S rDNA cluster exhibited signals on only one of the sister chromatids of each homologue (Fig. 24A, page 60).

In males of both *A. nordmanni* and *A. saevus*, four NOR-bearing bivalents were present, each with a single distal NOR locus (Fig. 24C – F, page 60).

Larinioides

In males of *L. sclopetarius*, six bivalents bore a terminal NOR locus. As seen in Fig. 25A and B (page 61), these loci were located at distal ends of bivalents.

Concerning *L. patagiatus*, NORs were visualised in males of populations SO and BL. In population SO, the pattern of NORs was found as follows: each of metacentric chromosomes belonging to the tetravalent bore a distal NOR locus at each arm; metacentric bivalent A bore two distal NOR loci; metacentric bivalent B bore a single distal NOR locus; acrocentric bivalent C bore a single distal NOR locus; both acrocentric bivalents D and E bore a single distal NOR locus as well as a single pericentric NOR locus; one of X chromosomes bore a single distal NOR locus (Fig. 26A, B, page 62). All autosomal loci were in a homozygous condition, excepting the distal locus on bivalent B and the pericentric locus on bivalent E, each of which were heterozygous for the presence of 18S rDNA cluster.

In population BL, NORs were detected as follows: each of metacentric chromosomes belonging to the tetravalent bore a distal NOR locus located on each arm; metacentric bivalent A bore a single distal NOR locus; both metacentric bivalents B and C bore two distal NOR loci; acrocentric bivalent D bore a single distal NOR locus and a single pericentric NOR locus; both X chromosomes bore a single distal NOR locus (Fig. 26C, D, page 62). All autosomal loci were in a homozygous condition, excepting the distal locus on bivalent A and the pericentric locus on bivalent D, each of which were heterozygous for the presence of 18S rDNA cluster. Note that

alphabetical markings of bivalents are formal, bivalents marked with the same letter in populations SO and BL do not correspond.

In population BL, NORs were also visualised using simultaneous colocalization of 18S and 28S rDNA probes. The distribution of all signals seemed to be consistent with the distribution of sole 18S rDNA signals. Colocalization occurred almost everywhere, excepting the acrocentric bivalent (the distal homozygous locus exhibited sole 18S rDNA signal and the pericentric heterozygous locus exhibited sole 28S rDNA signal) and a single metacentric bivalent (one locus exhibited sole 18S rDNA signal, one locus exhibited sole 28S rDNA signal) (Fig. 27, page 63).

Zygiellinae

Regarding males of *Leviellus stroemi*, two bivalents bore a distal NOR locus (Fig. 28A, B, page 64). In *L. thorelli* female, a single NOR locus was detected on a terminal part of a pachytene bivalent. Mitotic metaphases were consistent with this finding – two mitotic chromosomes bore a terminal locus (Fig. 28C, D, page 64).

Concerning *Zygiella*, NORs were visualised using both 18S and 28S rDNA probes. The obtained sequence of 28S rDNA probe was 99% consistent with the partial 28S rDNA sequence of *Z. atrica* (Araneidae), GenBank KY016935.1 (Wheeler et al. 2016):
GATCGCGTGCGAGTCATGGGTCTCGAATAAGCCCAGAGGCGCAATGAAAGCAAACGCCG
GGACTGATCCCGGCGGAGTTGGGATCCTCCCTCCTTCGCGGGGGAGGCGCACCAACGAC
CCGTCCTGTCCGATTTATCGGTTGGGCGGAGTCCGAGCATACACGTTGGGACCCGAAAG
ATGGTGAACTATGCCCGGGCAGGACGAGGCCAGAGGAAACTCTGGTGGAGGTCCGCAGC
GGTTCTGACGTGCAAATCGATCGTCAGATCCGGGTATAGGGGCGAAAGACTAATCGAA
CCATCTAGTAGCTGTTTCCTTCCGAAACCGTCTTTCGGGTCGGATGGTGTGTTCTCGGT
CTGCGGCGGACCGGTAGGGAGTTTAGTTCGGTTGGTGGGTGGGCCTCGTTCGGGAGGGA
GGGAGGCGCCCATCTCCGGGGGGATCAGGTCCGGGGTTGGCTTTCGTTGCGCCGCTGGG
CTTATTGGGGAGGATTGACTAGCGCACTTGTCCGCGCTCGTTGGTCCGTGTTTCCAGAC
GCCGCATCGGTCTGGGGTTGGCTTTCATTGCGCATCGGGGCTTAATCGAGACCCATTGA
CTCGCACACATGTTACACTCATTGGTCCGTGTCTTCCAGACGCCCCACGC.

In *Z. montana*, five bivalents bore a distal 18S rDNA locus (Fig. 28E, F, page 64). Consistently, five distal 28S rDNA loci were localised on five pachytene bivalents (Fig. 28G, page 64). In *Z. atrica*, all mitotic chromosomes bore 18S rDNA signals, including both X chromosomes. All metacentric chromosomes bore these signals at both ends except for three with 18S rDNA signal located at only one end. One

chromosome of the subtelocentric pair bore 18S rDNA signals at both ends, whereas other only at the end of its p-arm. Both X chromosomes bore a signal at one of their ends (Fig. 28H, page 64). Therefore, the total number of 18S rDNA loci was either 13 or 14, because there are more options of how these loci could be arranged according to how the mitotic metacentric chromosomes pair. In case of such metacentric chromosomes, it was not possible to determine which arm is which because of their almost equal lengths. Thus, it was not possible to see whether 18S rDNA signals were both localised on p-arms or q-arms of both homologues (and therefore forming a single homozygous locus) or one signal on p-arm of one homologue and other one on q-arm of other homologue (and therefore forming two heterozygous loci). Since no late prophases I or metaphases I were obtained on the slide, it was not possible to see how exactly these chromosomes paired. On the contrary, only five terminal 28S rDNA loci were detected on five pachytene bivalents (Fig. 28I, page 64).

In *Z. atrica*, NORs were also visualised using simultaneous colocalization of 18S and 28S rDNA probes. The distribution of all signals seemed to be consistent with the distribution of sole 18S rDNA signals. The quality of obtained signals was insufficient to completely compare the pattern of 18S and 28S rDNA signals. However, it seemed that contrary to the results above (i.e. 18S rDNA = 13 or 14 loci, 28S rDNA = five loci), colocalization occurred almost everywhere, perhaps excepting both ends of a single metacentric chromosome and both arms of a single subtelocentric chromosome. In the latter case, sole 28S rDNA signal was at the end of the p-arm and sole 18S rDNA signal was at the end of the q-arm (Fig. 29, page 65).

Other araneids

Regarding males of *Aculepeira ceropegia*, ten mitotic chromosomes bore a terminal NOR locus (Fig. 30A, page 66). In *Plebs bradleyi*, five bivalents bore a terminal NOR locus (Fig. 30B, page 66). In males of *Cyclosa fuliginata*, six NOR loci were detected as follows: a single distal locus on the acrocentric pair, two distal loci on a single metacentric pair, and three distal loci on three metacentric pairs (each pair bearing a single distal locus) (Fig. 30C, D, page 66).

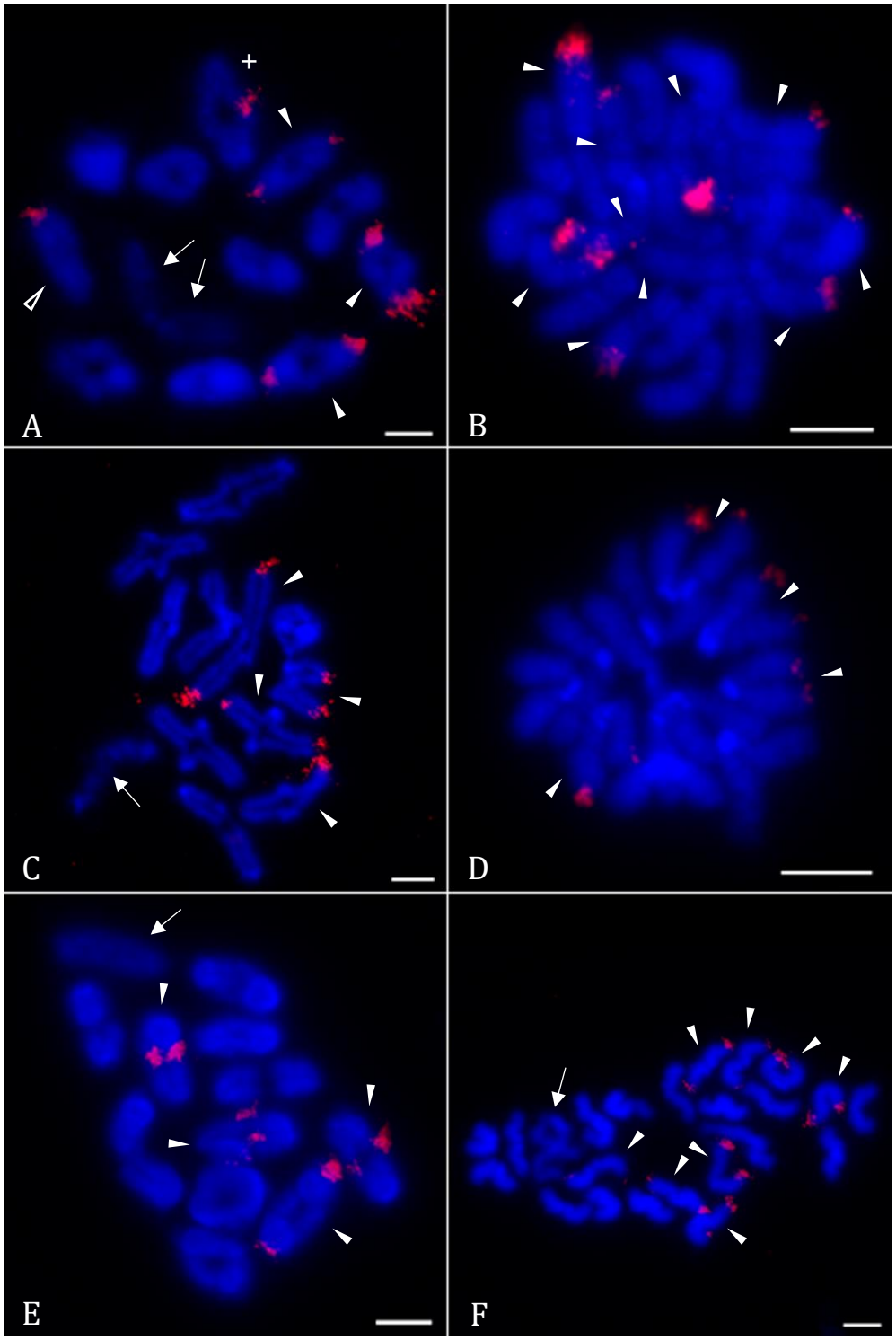

Fig. 24: Visualisation of NORs in males of *Araneus*. Unless otherwise stated, arrows indicate X chromosomes, full arrowheads mark chromosomes bearing NORs/bivalents homozygous for NORs, open arrowheads mark bivalents heterozygous for NORs. Centromeres are visible as DAPI positive structures in some plates (C, D). (A) *Araneus diadematus* metaphase I, signals are located on five bivalents – four of them are homozygous and one heterozygous for NORs, one homozygous bivalent bears signals on one of sister chromatids only (marked as +); (B) *A. quadratus* mitotic metaphase, note ten chromosomes with a terminal signal; (C) *A. nordmanni* metaphase I, note four NOR-bearing bivalents; (D) *A. nordmanni*, a half-plate of anaphase I without X chromosome – note signals located at distal ends of chromosomes; (E) *A. saevus* metaphase I, note four NOR-bearing bivalents, each of them include a single NOR locus; (F) *A. saevus*, two fused sister metaphase II, note signals at distal ends of eight chromosomes; scale – 5 μm

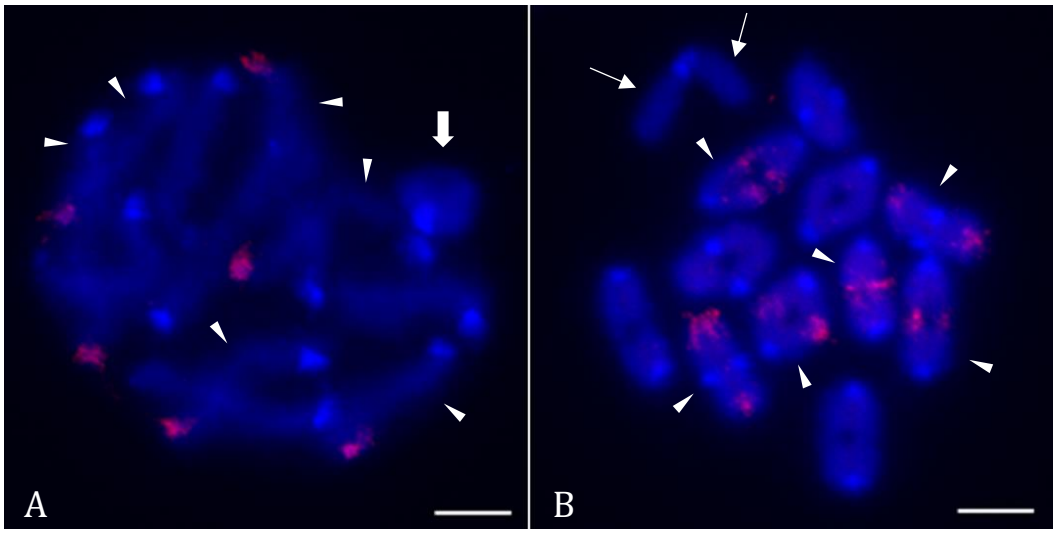

Fig. 25: Visualisation of NORs in males of *Larinioides sclopetarius*. Arrows mark sex chromosomes, full arrowheads mark bivalents homozygous for NORs. Centromeres are visible as DAPI positive structures. (A) Pachytene with sex chromosome body (bold arrow), note six bivalents each bearing a distal NOR locus; (B) Metaphase I, note six bivalents each bearing a distal NOR locus; scale – 5 µm

5.2.2 5S rDNA

Visualisation of 5S rRNA genes was conducted in *Araneus* and *Zygiella* only. The obtained sequence of 5S rDNA probe was 93% consistent with the 5S rDNA sequence of silkworm *Philosamia cythia* (Saturniidae), GenBank K02354.1 (Xian-Rong et al. 1982):

CCTTGCCTGTCGATCACCGTAGTTAGCATCATCGGGCGCGGTCAGTACTTGGGAGGGTG
ACCACCTGGGAACACCGCGTGCCGTTGGCTTGACTTCGGTGATCGGACGAGAACCGGTG
TTTTCAACATGGTATGGACGTTGGCCATAGTCTGATCGCC.

In case of *A. quadratus*, FISH unambiguously revealed a single subterminal locus. However, the fluorescence background was substantial, which prevented distinction between other candidate signals and the background. Thus, the presence of other loci of much weaker fluorescence could not be ruled out (Fig. 31A, B, page 67). The same situation occurred in all other examined species. Hence probably six or seven bivalents bore a terminal locus in *A. circe* (Fig. 31C, page 67). In *A. ventricosus*, probably eight mitotic chromosomes bore a terminal signal (Fig. 31D, page 67).

Regarding *Z. montana*, probably three bivalents bore a distal locus (Fig. 31E, page 67). In case of *Z. atrica*, the presence of three loci seemed to be most likely – a single metacentric bivalent bore a terminal signal at both ends and a single locus was located subterminally on a different metacentric bivalent (Fig. 31F, page 67).

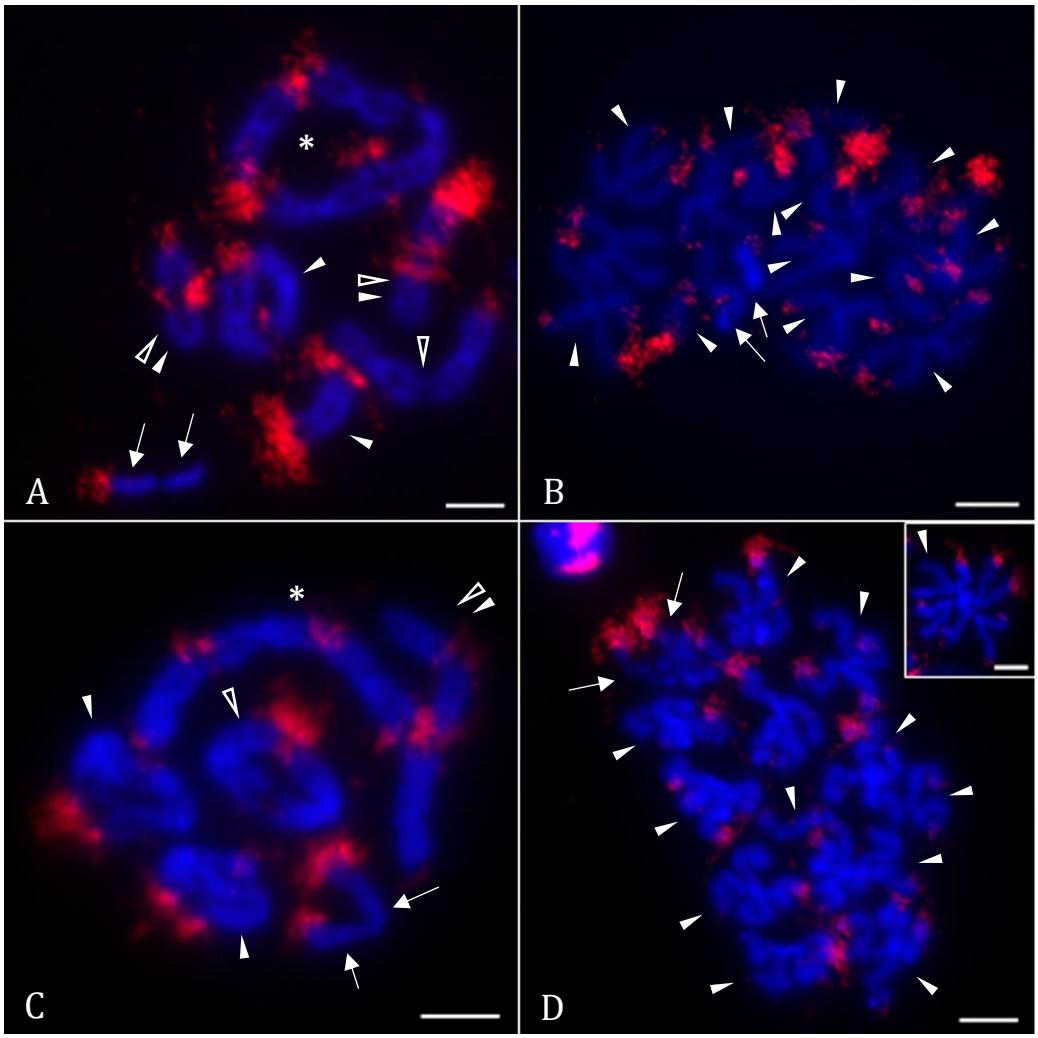

Fig. 26: Visualisation of NORs in males of *Larinioides patagiatus*. Unless otherwise stated, arrows indicate X chromosomes, full arrowheads mark chromosomes bearing NORs/bivalents homozygous for NORs, open arrowheads mark bivalents heterozygous for NORs, asterisks mark tetravalents. (A) Population SO, metaphase I, note the tetravalent with NOR loci at both ends of each chromosome, two bivalents homozygous for NORs, one bivalent heterozygous for NOR, two bivalents homozygous for one NOR locus but heterozygous for another (marked with both full and open arrowheads), and X chromosomes (one bearing a single NOR locus); (B) Population SO, two fused sister metaphases II, note 13 NOR-bearing chromosomes plus one NOR-bearing gonosome; (C) Population BL, metaphase I, note the tetravalent with NOR loci at both ends of each chromosome, two bivalents homozygous for NORs, a single bivalent heterozygous for NOR, a single bivalent homozygous for one NOR locus but heterozygous for other (marked with both full and open arrowheads), and X chromosomes each bearing a single NOR locus; (D) Population BL, two fused sister metaphases II, note 11 NOR-bearing chromosomes plus two NOR-bearing gonosomes – it may seem that there is no chromosome without NOR, but the existence of such chromosome is confirmed in the anaphase II inset (full arrowhead); scale – 5 μm

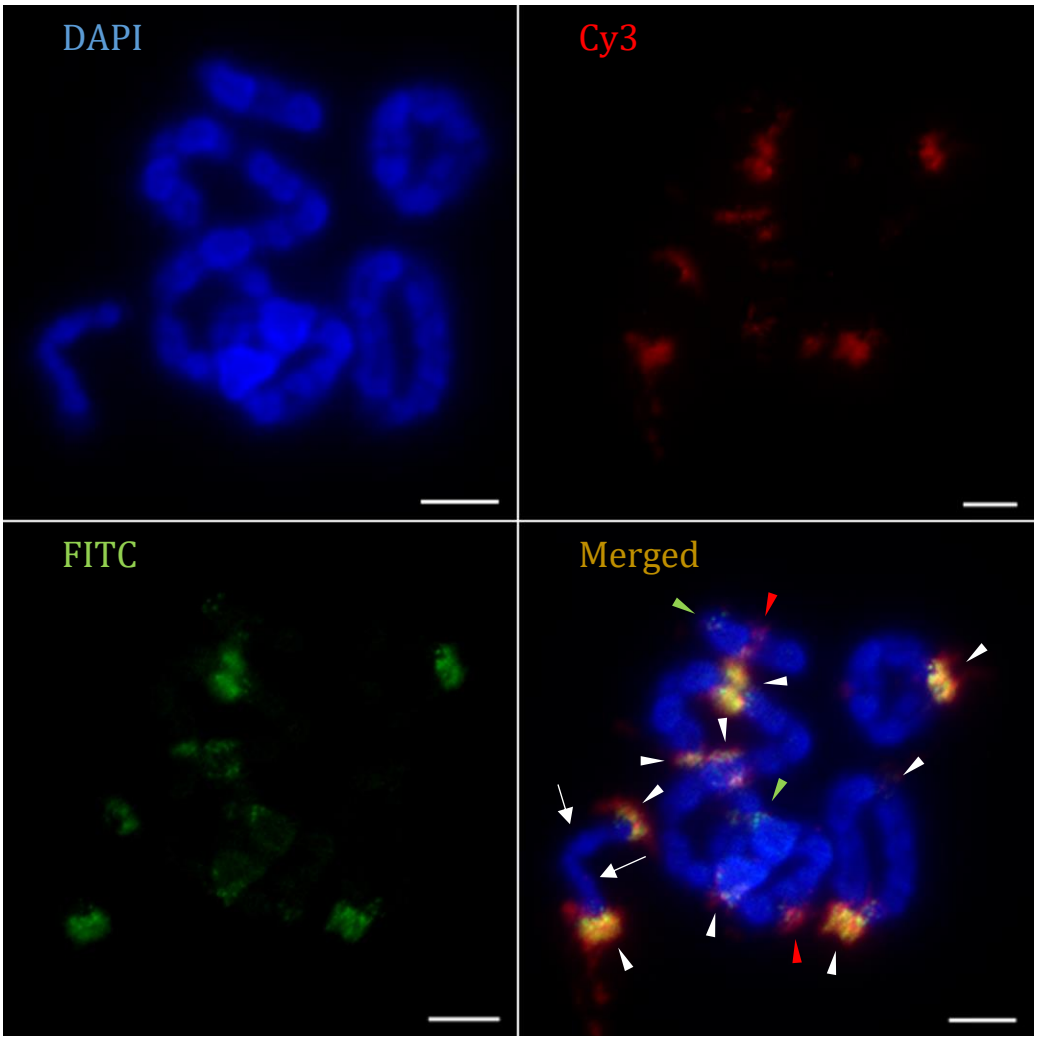

Fig. 27: *Larinioides patagiatus*, visualisation of NORs in males of population BL, metaphase I, colocalization of 18S and 28S rDNA clusters. 18S rDNA probe is detected using Cy3, 28S rDNA probe is detected using FITC. Note that most signals in merged figure are yellow (which means that both probes colocalise, white arrowheads), but there are also red and green signals (red and green arrowheads, respectively) indicating sole 18S and 28S rDNA clusters, respectively. Scale – 5 μm

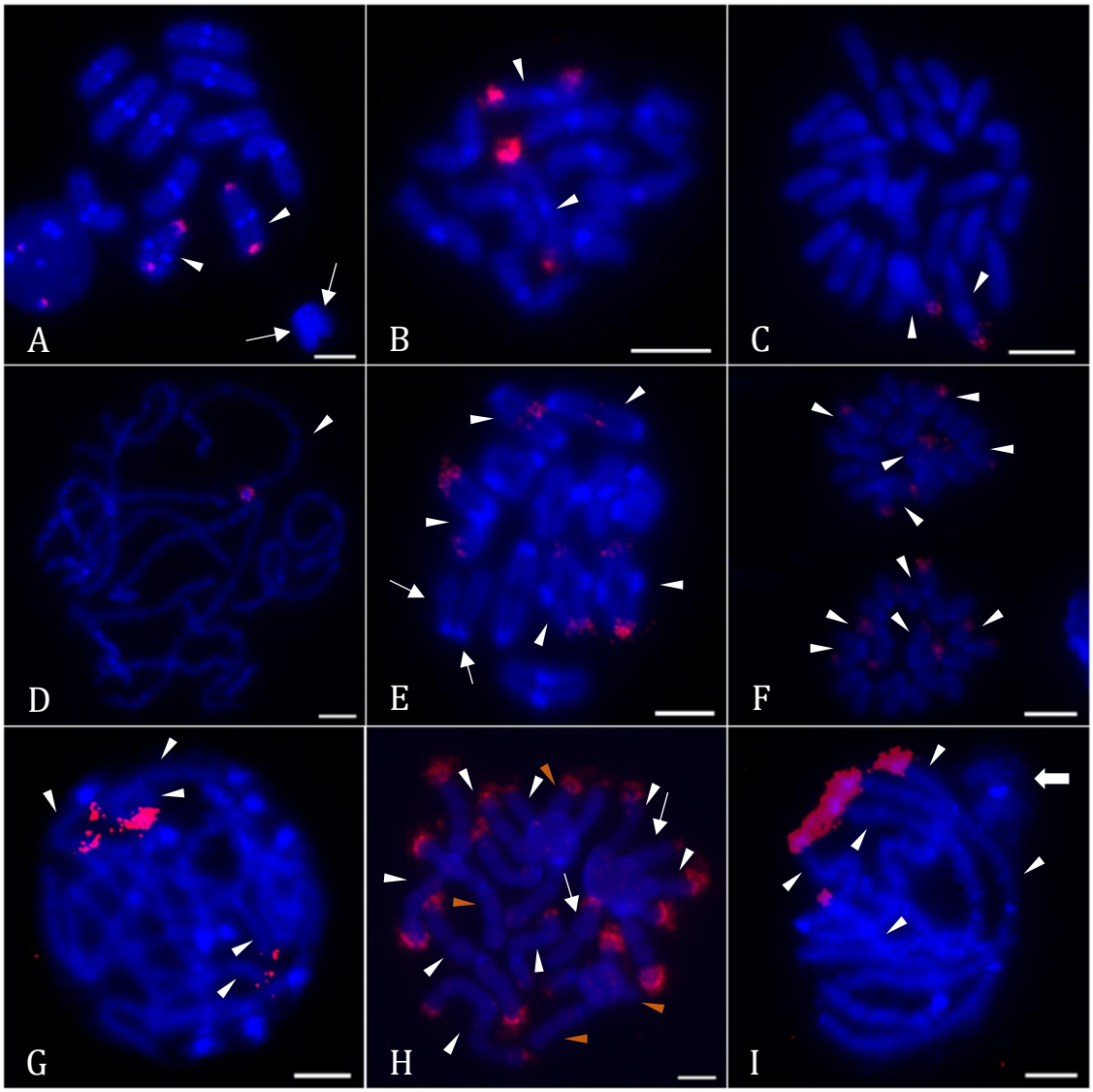

Fig. 28: Visualisation of NORs in Zygiellinae. Unless otherwise stated, arrows indicate sex chromosomes, full arrowheads mark chromosomes bearing NORs/bivalents homozygous for NORs, red signals represent clusters of 18S rDNA (A-F, H) or 28S rDNA (G, I), respectively. Centromeres are visible as DAPI positive structures in some plates (A, B, E, F, G). (A) *Leviellus stroemi*, male metaphase I, note two NOR-bearing bivalents containing a single distal NOR locus; (B) *L. stroemi*, male metaphase II without sex chromosomes, note signals at distal ends of two chromosomes; (C) *L. thorelli*, female mitotic metaphase, note two chromosomes with a terminal signal; (D) *L. thorelli*, female pachytene, note a terminal signal on a single bivalent; (E) *Zygiella montana*, male diakinesis, five bivalents contain a distal NOR; (F) *Z. montana*, male anaphase I, note signals at distal ends of ten chromosomes; (G) *Z. montana*, male pachytene, note five bivalents bearing signals; (H) *Z. atrica*, male mitotic metaphase, note that all chromosomes (including X chromosomes) bear signals –four autosomes and both gonosomes bear signals at one end only (marked with orange arrowheads for autosomes and white arrows for X chromosomes), the rest of chromosomes exhibits signals at both ends (white arrowheads); (I) *Z. atrica*, male pachytene, note five bivalents containing a terminal NOR locus and sex chromosome body marked with a bold arrow; scale – 5 µm

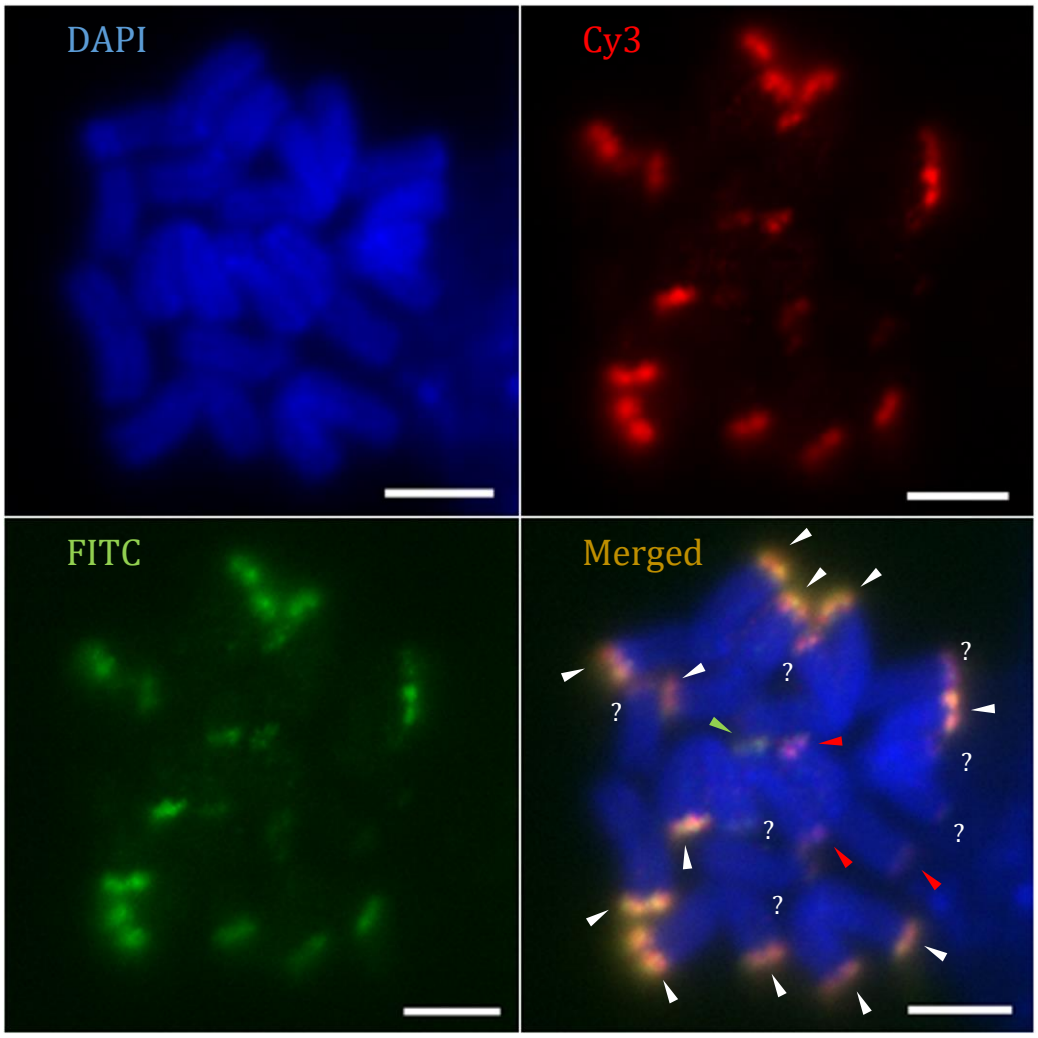

Fig. 29: Visualisation of NORs in males of *Zygiella atrica*, mitotic metaphase, colocalization of 18S and 28S rDNA clusters. 18S rDNA probe is detected using Cy3, 28S rDNA probe is detected using FITC. Note that most signals in merged figure are yellow (which means that both probes colocalise, white arrowheads), but there are also red and green signals (red and green arrowheads, respectively) – the low quality of signals, however, prevents completely reliable determination of such sole red (18S rDNA) or green (28S rDNA) signals, so question marks indicate unclear signals; scale – 5 µm

5.2.3 Histone H3 genes

Visualisation of histone H3 genes was conducted in *Araneus* only. The obtained sequence of H3 probe used was 81% consistent with the partial H3 gene sequence of mantis *Euantissa pulchra* (Hymenopodidae), GenBank EF384097.1 (Svenson and Whiting, unpublished data):

CAAATAGGGGGTGTTGGCTGATGGTCCCCTCTTCGCATGGATGGCGCACAAGTTCGTGT
CTTCAAGAGAGCCGACAAGTAGGCTTCCGTCGCCTCTTGCAAAGCCATCACAGCCGAAC
TTTGAAACCGCAGATCTGTCTTGAAGTCTTGAGCAATCTCTCTCACCAGGCGCTGGAAG

GGAAGTTTGCGAATCAGCAACTCCGTCGACTTCTGGTATCGTCGGATTTCTCACAAAGC
CACCGTTCCGGGTCTGTAACGATGAGGTTTCTTCACACCACCGGTGGCTGGAGCACTTT
TGCGAGCCGCTTTAGTCGCCAGCTGCTTCCTGGGGGCCTTTCCGCCTGTGCTTTTGCGA
GCAGTTTGCTTCGACGCGCCATGA.

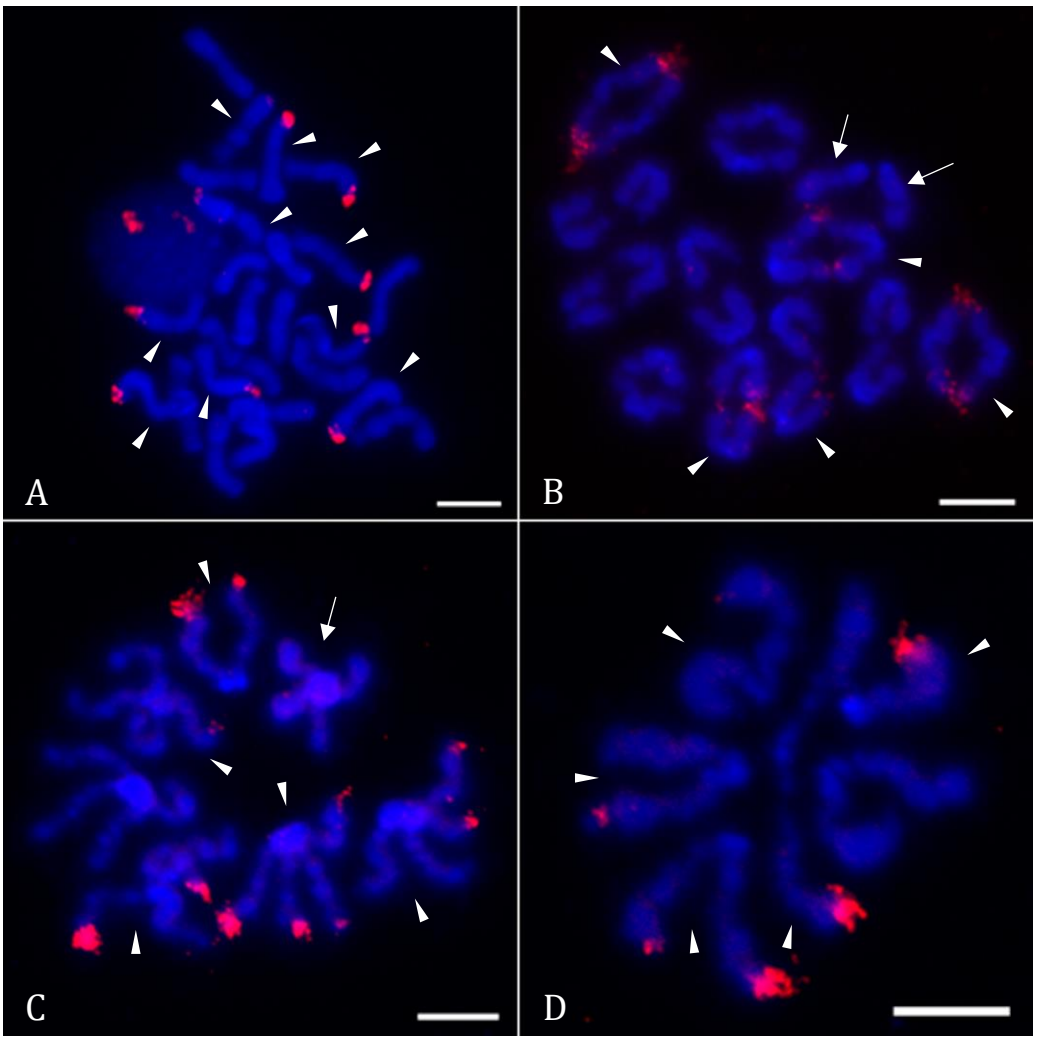

Fig. 30: Visualisation of NORs, unless otherwise stated, arrows indicate X chromosomes, full arrowheads mark chromosomes bearing NORs/bivalents homozygous for NORs. (A) *Aculepeira ceropegia* male, mitotic metaphase, note ten chromosomes with NOR signals; (B) *Plebs bradleyi* male, metaphase I, signals are located on five bivalents; (C) *Cyclosa fuliginata* male, metaphase II including X chromosome, note a single metacentric chromosome bearing two NOR loci, three metacentric chromosomes each bearing a single NOR locus (one of them exhibits very weak fluorescence, so it may seem like it is located on one chromatid only) and a single acrocentric chromosome bearing a single distal NOR locus; (D) *C. fuliginata* male, anaphase II without X chromosome, the NOR pattern is consistent with the previous picture, including the hardly detectable signal on a single metacentric chromosome; scale – 5 μm

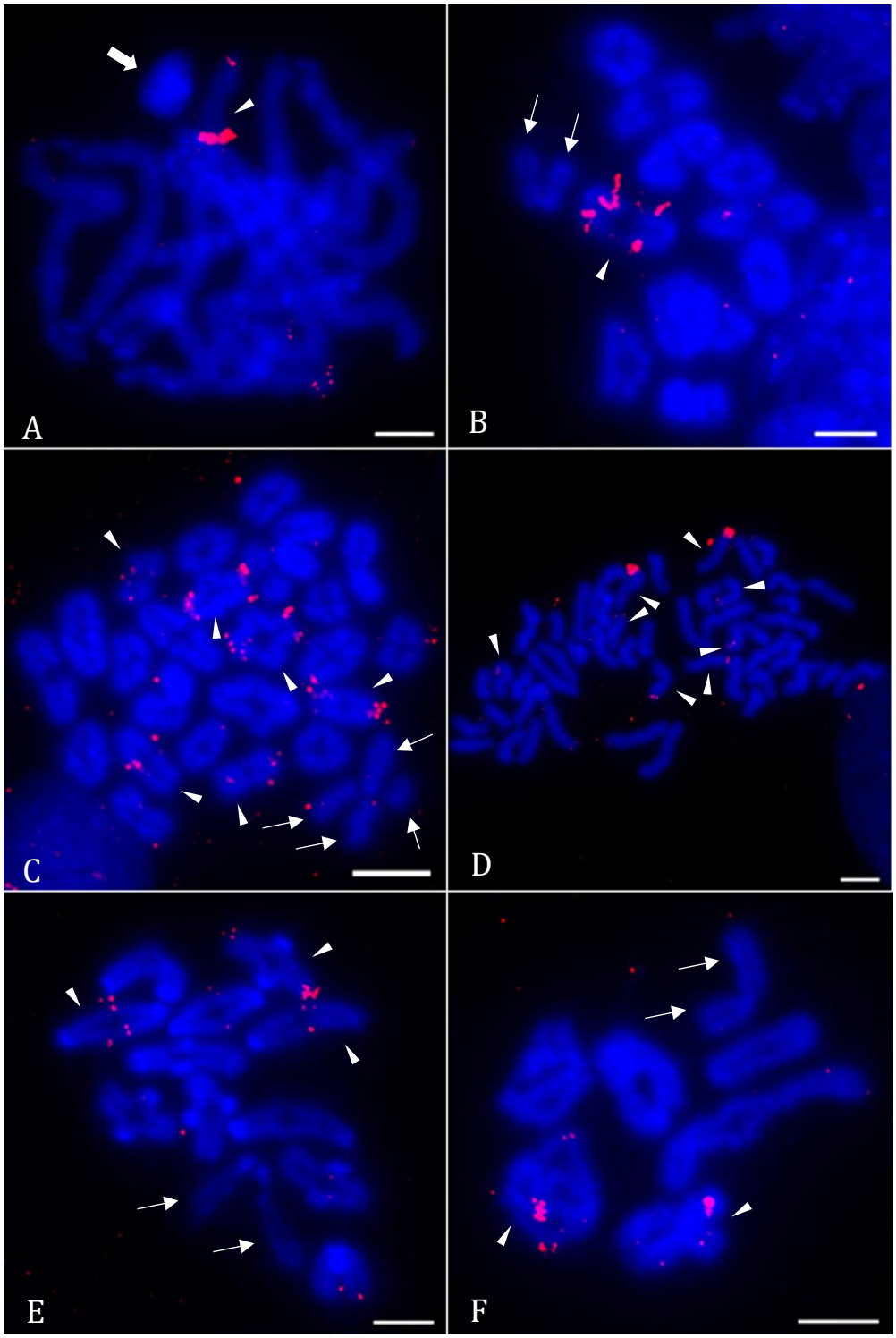

Fig. 31: Visualisation of 5S rDNA, unless otherwise stated, arrows indicate X chromosomes, full arrowheads mark chromosomes/bivalents bearing 5S rDNA and plates represent male individuals. (A) *Araneus quadratus*, pachytene with a single unambiguous subterminal signal (full arrowhead) and sex chromosome body (bold arrow); (B) *A. quadratus*, metaphase I, note a single bivalent with a strong signal; (C) *A. circe*, metaphase I, probably six bivalents bear a signal; (D) *A. ventricosus*, mitotic metaphase, note eight chromosomes bearing signals; (E) *Zygiella montana*, metaphase I, three bivalents bear a distal signal, centromeres are visible as DAPI positive structures; (F) *Z. atrica*, diakinesis, two signals are located at both ends of a single metacentric bivalent and a single signal is located subterminally on a different metacentric bivalent; scale – 5 μm

In case of *A. diadematus*, two bivalents bearing a terminal signal were detected using Cy3 (Fig. 32A). As can be seen from Fig. 32B, these signals were localised at distal ends of chromosomes. However, overall quality of signals was poor, background was substantial and the presence of another histone H3 loci could not be ruled out. Therefore, TSA-FISH was conducted in case of histone H3 genes as well, using FITC for visualising the signals. Unfortunately, this approach did not improve the results. Regarding *A. quadratus*, a single bivalent bearing a terminal locus was detected using the Cy3 (Fig. 32C). Again, due to the significant background and poor quality of signals, TSA-FISH was performed and this method revealed two pachytene bivalents bearing a terminal locus (Fig. 32D).

As to *A. circe* and *A. ventricosus*, TSA-FISH with FITC was used only. In *A. circe*, three unambiguous terminal loci were detected, each on a single bivalent (Fig. 32E). Moreover, additional (not so unambiguous) signals were present, but it was not possible to completely reliably distinguish them from the background. Therefore, the presence of more than three loci could not be ruled out. In *A. ventricosus*, four bivalents bearing a terminal locus were detected (Fig. 32F).

5.2.4 U2 snRNA genes

Visualisation of U2 snRNA genes was conducted in *Araneus* only. The obtained sequence of U2 snRNA probe used was 100% consistent with the gene for U2 snRNA of *Drosophila melanogaster* (Drosophilidae), GenBank X04256.1 (Alonso et al. 1983):

GGATGCAGGTCAGACCTACGCACTCATGACTTCCTCGGGAACTACGAGTGGCCTCCCGC
TCATCCATAGATGCCGAGAAGCGATAGCTGATAAGAACAGATACTACACTTTGATCTT
AGCCATAAGGCCGAGAAGCGATACGATAACGACC.

Unfortunately, informative results were obtained only in *A. diadematus*, using regular FISH and Cy3. In the rest of the species (*A. quadratus*, *A. circe,* and *A. ventricosus*), the results were not informative neither in case of FISH nor TSA-FISH. Regarding *A. diadematus*, two bivalents bearing a terminal locus were detected (Fig. 33A). The inset of Fig. 33A shows that the loci are located at the distal ends of chromosomes. Signals were not detected on sex chromosomes (Fig. 33B).

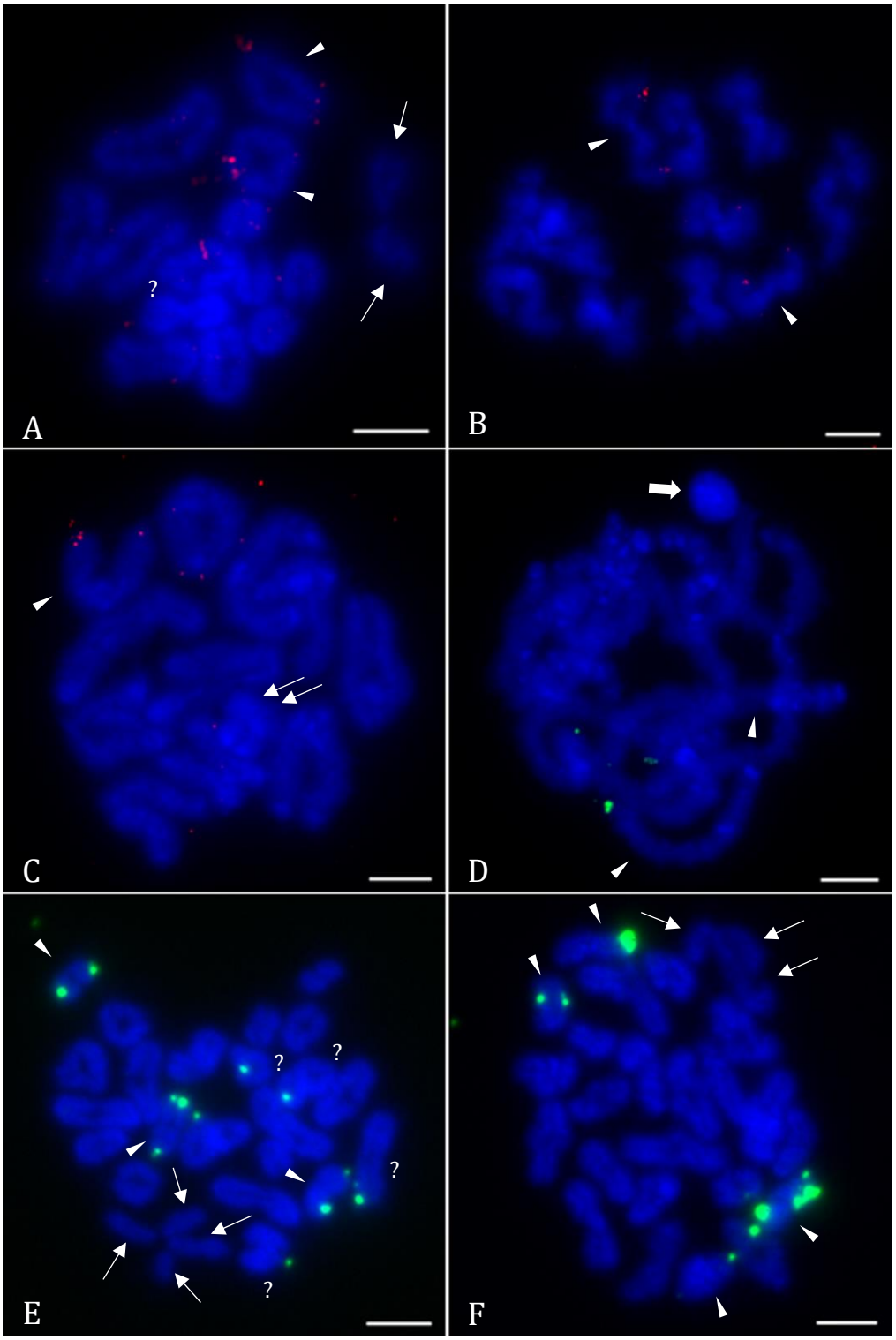

Fig. 32: Visualisation of H3 genes. Unless otherwise stated, arrows indicate X chromosomes, full arrowheads mark chromosomes/bivalents bearing H3 genes. Plates represent male individuals, (A-C) FISH, signals visualised using Cy3, (D-F) TSA-FISH, signals visualised using FITC. (A) *Araneus diadematus*, metaphase I, note two bivalents bearing a terminal locus and perhaps the third locus marked with a question mark; (B) *A. diadematus*, metaphase II without X chromosomes, note distal position of signals on two chromosomes; (C) *A. quadratus*, diakinesis, probably a single bivalent bears a terminal locus; (D) *A. quadratus*, pachytene, note two bivalents bearing a terminal locus and the sex chromosome body (bold arrow); (E) *A. circe*, metaphase I, note three unambiguous terminal loci on three bivalents and other unclear signals (marked with question marks); (F) *A. ventricosus*, metaphase I, note four bivalents bearing a terminal locus; scale – 5 μm

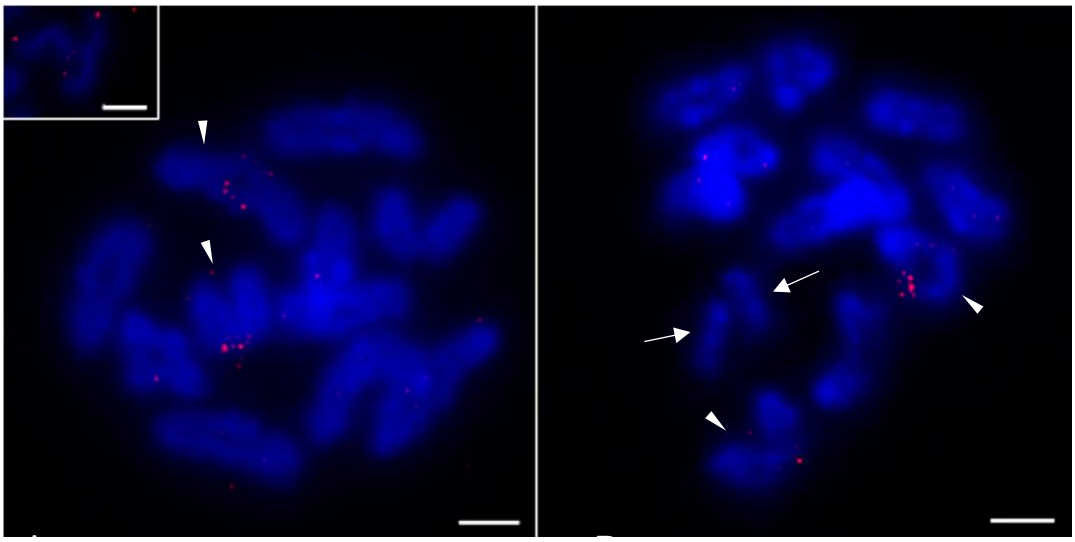

Fig. 33: Visualisation of U2 snRNA genes in a male of *Araneus diadematus*, arrows indicate X chromosomes, full arrowheads mark bivalents bearing U2 snRNA genes. (A) Incomplete metaphase I (without X chromosomes), note two unambiguous terminal loci on two bivalents – these loci are localised at distal ends of chromosomes, as shown in the metaphase II inset; (B) Metaphase I, including X chromosomes – note again two bivalents bearing signals, but there are no signals on the sex chromosomes; scale – 5 μm

6 Discussion

Although cytogenetics of family Araneidae has been studied rather extensively, the current knowledge is still insufficient, because only a slight portion of total araneid diversity has been investigated so far. Hence the presented thesis focuses on karyotype evolution of this diversified and numerous family. Furthermore, it is important to integrate araneid cytogenetics into the context of superfamily Araneoidea, to which araneids belong. For doing so, it is necessary to compare karyotype evolution of orb-weavers with another Araneoidea families, and for this purpose, related family Tetragnathidae was chosen. In the presented thesis, 19 araneid and four tetragnathid species were studied, of which 14 araneid and three tetragnathid species were analysed for the first time.

6.1 Evolution of diploid numbers and morphology of autosomes

Diversity of 2n and chromosome morphology in Entelegynae is relatively low (Král et al. 2013; Král and Kořínková 2013), which is also true for both Araneidae and Tetragnathidae. Diploid numbers in Araneidae males range from 13 (*Cyclosa fuliginata*, this study, *Neoscona* sp., Sharma and Parida 1987) to 52 (*Araneus circe*, this study), with the most common number being 24 in species studied here. Diploid number of *A. circe* is the highest one recorded in Entelegynae so far. Male diploid numbers in Tetragnathidae range from 22 (*Tetragnatha extensa* and *T. obtusa*, Hackman 1948) to 25 (*Leucauge celebesiana*, *L. decorata*, and *L. tesellata*, Datta and Chatterjee 1988); all species studied here share 2n♂ = 24. In both families, the utter majority of species studied here exhibits acrocentric morphology of chromosomes. These data are consistent with the overall range of 2n and chromosomal morphology typical for Entelegynae (see the database of Araujo et al. 2018), excepting mentioned *A. circe*, which exceeds the upper limit of 2n in Entelegynae known up to date (2n♂ = 49 in *A. ventricosus*, Youju et al. 1993). It has already been proposed that karyotype including 11 acrocentric pairs represents the ancestral state in Araneidae (Araujo et al. 2011). Indeed, the data obtained here support this argument, since 11 acrocentric pairs were detected in another nine species not studied before. This type of karyotype is also very frequent among other Araneoidea families (Araujo et al. 2015). If one assumes Araneoidea phylogeny according to Wheeler et al. (2016), it is indeed likely that 11 acrocentric pairs represent the ancestral state of this

superfamily – approx. 45% of karyotyped representatives exhibit this constitution, including basal clades (based on the data of Araujo et al. 2017). However, many more Araneoidea species, including representatives of all main clades, need to be examined (or revised, since some data in literature are incomplete or dubious) before unambiguous conclusions can be made.

6.1.1 Decrease of chromosome number

A considerable number of araneid spiders exhibits variant karyotypes composed of approx. half of chromosomes compared to the ancestral state. Most of such chromosomes exhibit biarmed morphology (mostly metacentric) and are uncommon for entelegynes in general. These unusual entelegyne karyotypes were at first discovered precisely in Araneidae, namely in *Larinioides patagiatus* (Hackman 1948) and then in *Neoscona* sp. (Sharma and Parida 1987) and *N. scylla* (Suzuki 1951). Other hints were brought as soon as such biarmed karyotype containing fewer chromosomes was discovered in Sparassidae, namely *Delena cancerides* (Rowell 1985). The mechanism responsible for formation of such karyotypes is centric fusion of original acrocentric chromosomes. Moreover, centric fusions exhibit exactly the same pattern in all other independent cases in which they occurred, including Dyctinidae (Král et al. 2011), Oxyopidae (Stávale et al. 2011), and Salticidae (Gorlova et al. 1997). That is, they are implemented according to the rule "all or nothing" (Rowell 1990), i.e. all acrocentric chromosomes fuse rather than just some of them, except for karyotypes with an uneven haploid number. In that case, one of acrocentric pairs has no other pair left to fuse with and it either maintains its acrocentric morphology (for instance mentioned *L. patagiatus*), or undergoes additional rearrangements leading to loss of acrocentric morphology, as will be discussed shortly. In other words, the total arm number in variant karyotypes is consistent with the total arm number in original karyotypes. In the presented thesis, three araneids with karyotypes struck by centric fusions were discovered, namely in *Cyclosa fuliginata*, *L. patagiatus*, and *Zygiella atrica*. In all these relatively unrelated species, centric fusions were carried out in exact accordance with the rule "all or nothing". i.e. all originally acrocentric autosomes fused, forming biarmed chromosomes, except for one pair, which remained acrocentric in *C. fuliginata* and a male of population HK-B of *L. patagiatus*. Regarding *Z. atrica*, it probably underwent a pericentric inversion, producing a subtelocentric pair. Hence results obtained here strongly support Rowell's

rule. Moreover, they point to the fact that centric fusions in orb-weavers are carried out in the same fashion in all independent cases and additionally, they seem to be more frequent in araneids than in other entelegyne families. This makes orb-weavers a suitable model for studying centric fusions.

It is not known why centric fusions in entelegynes happen according to the rule "all or nothing" and which factor is responsible for such effective fixation of variant biarmed karyotypes in populations. Furthermore, the reason for the wide-spread occurrence of the supposed ancestral karyotype of Araneoidea (11 acrocentric pairs) in these spiders is not known either. One of possible explanations I propose here is female meiotic drive. It has already been proposed that female meiotic drive plays a pivotal role in karyotype evolution of vertebrates exhibiting predominance of acrocentric chromosomes, like mice (Chmátal et al. 2014) or fishes (Molina et al. 2014) and it is responsible for maintaining both ancestral acrocentric karyotypes and variant biarmed karyotypes (Malik 2009). This is supported by many observations that in these organisms (as well as spiders studied here), cases with mixed karyotypes (i.e. containing both acrocentric and biarmed chromosomes in approx. equal ratio) are rare or absent. Recently, it has been shown that centromere strength affects the direction of female meiotic drive in mice. Chromosomes with strong centromeres preferentially segregate to an egg and it is naturally variable whether fused biarmed chromosomes or original acrocentric chromosomes possess strong centromeres. Basically, if a fused biarmed chromosome arises in a strain with strong centromeres of original acrocentrics, its fusion centromere is less effective than those of original acrocentrics and it preferentially segregates to the polar body (Fig. 34, top panel) (Chmátal et al. 2014). This could be theoretically applied on spider females, which could explain the conservativeness of acrocentric karyotypes not just in Araneidae, but in Entelegynae in general. Conversely, centric fusions originating on a weak centromere background of original acrocentrics may be strong relative to these acrocentrics and segregate preferentially to the egg (Fig. 34, bottom panel) (Chmátal et al. 2014). This, applied on spider females, could explain the rapid formation of completely biarmed karyotypes by centric fusions in some entelegynes.

Recently, the exact molecular mechanism of how strong centromere chromosomes can preferentially segregate to the egg has been discovered (Akera et al. 2017). It relies on spindle asymmetry – microtubules pulling chromosomes to the

polar body are differentiated from microtubules pulling chromosomes to the egg (they differ in levels of α-tubulin tyrosination). It is this molecular differentiation thanks to which strong centromere chromosomes can "distinguish" between given microtubules and attach preferentially to microtubules ensuring transport of chromosomes to the egg. Even if they at first attach to the polar body microtubules, this attachment is unstable and disrupts, giving chromosomes another attempt to attach to the egg microtubules (Fig. 35). I suppose that the same events might be happening in oocytes of spider females as well. However, further genetic and molecular experiments would be required to prove this hypothesis.

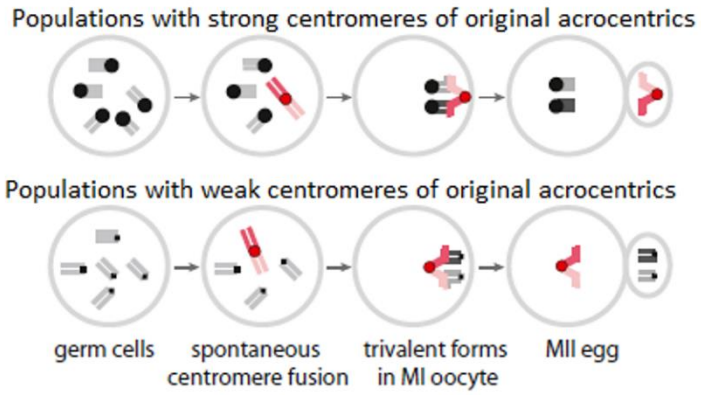

Fig. 34: Centromere strength affects direction of meiotic drive. In the top panel, fused metacentric chromosome possesses a weak centromere compared to strong centromeres of original acrocentrics, during the first meiotic division (MI), the fused chromosome forms a trivalent with the original acrocentrics and this trivalent segregates nonrandomly – since acrocentrics have strong centromeres, they are driven to the egg, while weak centromere metacentric chromosome is driven to the polar body. In the bottom panel, fused metacentric chromosome possesses a strong centromere compared to original weak centromere acrocentrics and thus, it is driven to the egg, while the acrocentrics are driven to the polar body; adapted from Chmátal et al. 2014

As to other male populations of *L. patagiatus*, their karyotypes are more complicated than population HK-B (which was homozygous for centric fusions of all autosomes except for one pair, as was discussed above). In males from populations SO and BL, a tetravalent composed of metacentric chromosomes was present. Such situation can naturally occur for example by crossing individuals homozygous for two different centric fusions in each one, leading to monobrachial homology of all fused metacentric chromosomes. The more fused monobrachially homologous chromosomes, the longer the chain (or ring) is formed during meiosis.

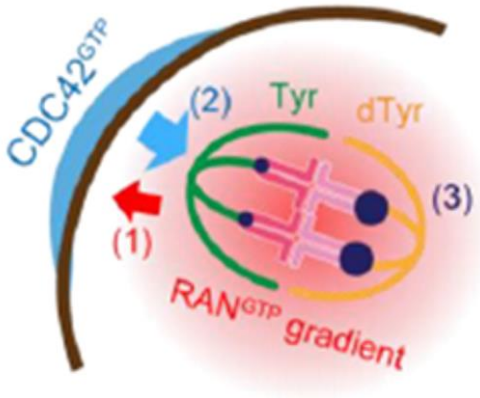

Fig. 35: Molecular mechanism of female meiotic drive. During oocyte division, spindle moves to the cortical site in perpendicular orientation, moving chromosomes along with it. Once chromosomes are close enough to the cortical site, chromatin-based RAN^{GTP} (Ras-related nuclear protein) gradient activates $CDC42^{GTP}$ (cell division control) cascade (1), which in turn leads to increased level of tyrosination (Tyr) on cortical microtubules (green in the picture) (2). In contrast, microtubules driving chromosomes to egg (yellow in the picture) are less tyrosinated (dTyr), which makes them marked for strong centromere chromosomes (chromosomes with big blue dots in the picture) – once strong centromere chromosomes are attached to the egg microtubules, they segregate exclusively to the egg, while weak centromere chromosomes (chromosomes with small blue dots in the picture) have no other option than to attach to the cortical microtubules and segregate to the polar body (3); adapted from Akera et al. 2017

Furthermore, *de novo* translocations can also arise (Rowell 1990; Wallace et al. 2002; Schmid et al. 2017). Besides this tetravalent, there are two other metacentric and three acrocentric pairs present in karyotype of a male from population SO. This situation occurred by centric fusions of four original acrocentric pairs (not involved in fusions leading to the tetravalent), but three remaining ones were left unfused. On the other hand, two of these three remaining acrocentric pairs of population SO fused in population BL, resulting to three metacentric pairs and a single acrocentric pair (besides tetravalent). In other words, population BL again confirms the rule "all or nothing", but two of all fusions occurred in the way that led to formation of a tetravalent in heterozygous progeny. These results are consistent with Hackman (1948), except for the tetravalent, which was not present in Finnish population of *L. patagiatus*. It is possible that population SO presents an intermediate step between the ancestral karyotype and a Finnish karyotype fully saturated with fused biarmed chromosomes, as there are three unfused pairs. Indeed, saturation of karyotypes with centric fusion biarmed chromosomes barely happens immediately on evolutionary scale.

Concerning population HK-A, centric fusions struck again all original acrocentric pairs excepting one. However, a male of HK-A is heterozygous for one of these fusions, which led to a trivalent formation. This occurs commonly in nature – trivalents usually cause some impairment to meiosis, but not so dramatic as in the case of tetravalent. Judging from morphology of diakinetic chromosomes in the trivalent, it seems that this trivalent is not composed of a metacentric chromosome and two original acrocentrics. Instead, two small biarmed (probably submetacentric) chromosomes circumscribe the central metacentric chromosome. If this is true, two small submetacentrics were probably formed by pericentric inversions.

6.1.2 Increase of chromosome number

The highest 2n known in Araneidae (and Entelegynae in general) up to date is 49 in *A. ventricosus* males (Youju et al. 1993). Indeed, the results obtained in *A. ventricosus* here are consistent with this. This species is a rather big orb-weaver with characteristic humps on abdomen, sharing this characteristics with another species *A. circe*. Although there is only morphological evidence supporting close relation of these spiders (namely similar morphology of palps), dramatically increased 2n = 52 was detected in this thesis also in males of *A. circe*. Another two species with humps and similar morphology of palps were studied here (*A. nordmanni* and *A. saevus*), but the original hypothesis (that these species have increased chromosome number as well) was not confirmed, since they possess original 11 acrocentric pairs. All chromosomes in *A. circe* and *A. ventricosus* maintain acrocentric morphology. Increase of 2n in *A. circe* and *A. ventricosus* further indicates their mutual relation, but analysis of molecular phylogeny of these species is required to make definitive conclusions.

Combination of increased 2n and acrocentric morphology of all chromosomes in *A. circe* and *A. ventricosus* indicates duplication of chromosome number. However, the number of autosomes in *A. circe* and *A. ventricosus* is not exactly double that of the ancestral state (expected 44 autosomes but observed 48 in *A. circe* and 46 in *A. ventricosus*). Such increase could occur by chromosome rearrangements. A simple mass fission of all autosomes is improbable, because acentric fragments formed after such process need to gain new centromeres *de novo*, which is a very rare event (Schubert and Lysak 2011). Initial pericentric inversions and subsequent centric fissions of newly formed biarmed chromosomes is a more likely scenario. Another option of chromosome number increase is polyploidisation. Although it is rare in

animals (due to existence of sex chromosomes, which pose an obstacle for polyploidisation), a considerable number of examples is known (Mable 2004). However, without other supportive data such as from flow cytometry, polyploidisation cannot be unambiguously confirmed. Moreover, polyploidisation alone cannot explain why the autosome number in both *A. circe* and *A. ventricosus* is higher than expected (i.e. expected 44 autosomes). Additional rearrangements might have followed polyploidisation, e.g. a pericentric inversion followed by a centric fission of a single metacentric pair in *A. ventricosus* (hence 46 observed autosomes) and two metacentric pairs in *A. circe* (hence 48 observed autosomes). Indeed, various rearrangements (such as centric fusions and fissions) occurred frequently after polyploidisation events in vertebrates, mainly fishes (Hufton and Panopoulou 2009).

Another case of increased 2n is yet undetermined Australian orb-weaver from Canberra, Australia (this study). In this case, however, only slightly increased 2n was discovered. Although male diploid number of this species was 28, its karyotype looks nothing like any other known araneid so far – it is composed of ten metacentric and two acrocentric pairs, and four acrocentric X chromosomes. In fact, there is no report of any other entelegyne with such mixture of metacentric and acrocentric chromosomes, but with predominance of the former. Perhaps the only similar karyotype can be found in *Strotarchus vittatus* (Eutichuridae), a spider possessing five metacentric and five acrocentric chromosome pairs along with two acrocentric X chromosomes (Mittal 1966). Unfortunately, the author does not propose any hypothesis on origin of this karyotype. In *Argyrodes elevatus* (Theridiidae), the number of chromosome pairs is the same as in its closely related species. In contrast to such species, however, its karyotype is formed by exclusively biarmed chromosomes. In this case, pericentric inversions of all original acrocentrics occurred (Stávale et al. 2010). Indeed, inversions of all autosomes could have happened in this Australian orb-weaver, too, followed by centric fissions of two autosomes, leading to formation of observed four acrocentrics. This theridiid and araneid indicate that pericentric inversions in spiders may also form karyotypes mostly or completely saturated by biarmed chromosomes just the way centric fusions do. However, there is also a possibility involving polyploidisation combined with subsequent centric fusions in the Australian orb-weaver.

6.2 Evolution of sex chromosomes

In the majority of species studied here, X_1X_20 sex chromosome system was detected (11 araneid and all four tetragnathid species). Indeed, this system is vastly spread among entelegyne spiders. Judging from its distribution, it may pose the ancestral state in Araneoidea, including Araneidae (Araujo et al. 2012, 2017). Both X chromosomes exhibit exclusively acrocentric morphology and during meiosis, no chiasmata form between them. Size of X chromosomes is not equal – one X chromosome is always 10 – 20% shorter than the other (12% in average, this study). These findings pinpoint to non-homology of X chromosomes of X_1X_20 system, as indicated by results of other authors (Araujo et al. 2012). However, definitive conclusions about the extent of homology of these X chromosomes cannot be made without sequencing (Ault et al. 2017). During male meiosis, X_1 and X_2 associate firmly at one end (proximal one in all cases where it was possible to distinguish ends) in almost all species possessing this system (this study). Such behaviour indicates possible involvement of constitutive heterochromatin in association of X chromosomes.

In four araneid species (and no tetragnathid ones), X0 system was detected. This system could be derived from X_1X_20 system by various mechanisms, including centric fusion (Král et al. 2011; Stávale et al. 2011; Araujo et al. 2012, and others) leading to metacentric morphology, or tandem fusion (or centric fusion followed by a subsequent pericentric inversion) (Araujo et al. 2012) leading to acrocentric morphology. In *A. nordmanni* and *A. saevus*, the single X chromosome of X0 system is acrocentric and considerably longer than autosomes. It is likely that such X chromosome was derived by a tandem fusion. On the contrary, metacentric X chromosome of X0 system in *C. fuliginata* and *L. patagiatus* (males from populations HK-A and HK-B) was presumably derived by a centric fusion. These results indicate that sex chromosomes, too, may be affected by meiotic drive – in species with the ancestral karyotype, acrocentric X chromosome of X0 system can be fixed just like X_1 and X_2 chromosomes, because original centromere of one of fused X chromosomes remains unaffected by a tandem fusion. Moreover, metacentric X chromosome of X0 system can be fixed in centric fusion karyotypes precisely due to alternation of its centromere strength after a centric fusion just like in case of autosomes, i.e. due to a centric fusion, strong centromere metacentric X chromosome is driven to the egg. Furthermore, this claim is supported by the fact that no araneid possessing the ancestral

karyotype and X0 system (with X chromosome being metacentric) at the same time is known (Araujo et al. 2017). On the other hand, there is X_1X_20 system present in centric fusion karyotypes of *L. patagiatus* (populations SO and BL) and *Z. atrica*. However, such opposite situation is not in conflict with this claim, because it is possible that sex chromosomes of these species are yet to be fused.

Another system detected here was $X_1X_2X_3X_40$, found in *A. circe* and Araneid gen. and sp. indet., all gonosomes were acrocentric. Like other systems with multiple X chromosomes in spiders, it is probably formed by non-disjunctions in X_1X_20 system, eventually $X_1X_2X_30$ system (Datta and Chatterjee 1988; Král et al. 2011). The second option is polyploidisation of X_1X_20 system. Indeed, this is probably the case in both *A. circe* and the undetermined Australian orb-weaver, but it is of course impossible to distinguish between non-disjunction and polyploidisation options from the data obtained. An alternative way in undetermined Australian araneid involves the same course of rearrangements as that of autosomes, i.e. a pericentric inversion of both X chromosomes of X_1X_20 system, followed by a centric fission of both newly formed metacentrics.

In *A. ventricosus*, system $X_1X_2X_30$ was present, with all gonosomes being acrocentric. A plausible mechanism of its origin in general includes non-disjunction of one X chromosome of X_1X_20 system and subsequent loss of homology (Araujo et al. 2012). In this case, however, one of three X chromosomes was twice as long as the other two. Therefore, it is probable that $X_1X_2X_3X_40$ system was formed as first (by non-disjunctions or polyploidisation in X_1X_20 system), followed by a tandem fusion of two X chromosomes.

The SCP was not discernible in a light microscope in any of spiders studied here, as is true for the entirety of Entelegynae (Král and Kořínková 2013). However, its existence in this group may be inferred from unusual behaviour of multivalents in males of *L. patagiatus*. Strikingly, the tetravalent of populations SO and BL seems to segregate in a balanced fashion, that is all sister metaphases II observed are composed of even number of autosomes. It is hard to imagine that a tetravalent would show regular segregation during meiosis – it is not only necessary to ensure balanced segregation of such tetravalent (in ratio 2:2), but chromosomes must segregate alternately as well, to ensure segregation of proper gene sets to gametes. Otherwise, mostly unbalanced gametes would be produced, leading to sterility of an

individual. In case of *Delena cancerides*, this problem was overcome by involvement of X chromosome in centric fusions in males as well as zig-zag orientation of the chain multivalent (Rowell 1990). In case of *L. patagiatus*, multiple X chromosomes are clearly not involved in centric fusions and the tetravalent is seemingly composed of autosomes. However, if proto-X and proto-Y chromosomes of the SCP were involved in centric fusions, they would ensure tetravalent segregation in alternate fashion, with two metacentric chromosomes including original proto-X and segregating to one pole, and two metacentric chromosomes including original proto-Y and segregating to another pole. If this assumption is correct, that would mean that *L. patagiatus* (populations SO and BL) possesses neo-sex system $X_1X_2X_3X_4Y_1Y_2$. What remains unclear is how exactly the alternate segregation of mostly circular tetravalent is achieved. One possibility is to take a shape of figure eight (∞) of the circular tetravalent by crossing chromosomes over themselves. Indeed, such shape was observed in more than 50% of plates with the tetravalent being circular. Another possibility is pairing of multiple X chromosomes with neo-X chromosomes in the tetravalent. Indeed, such interaction of original X chromosomes and neo-X chromosomes has been proved using transmission electron microscopy in *Tegenaria ferruginea* (Agelenidae, Král 2007). As to linear tetravalent, alternate segregation could be achieved again by interaction of original X and neo-X chromosomes or by taking a zig-zag shape of the linear tetravalent. However, no literally zig-zag structures were observed.

In a male from population HK-A of *L. patagiatus*, an analogous situation can be observed – the trivalent most probably segregates in stable ratio 2:1. This trivalent is composed of a central metacentric chromosome and probably two small submetacentric chromosomes. In all metaphases II observed, the central metacentric chromosome was always present in the same plate as the X chromosome and simultaneously, two small submetacentrics were never present in such plates. This may mean that similarly to populations SO and BL, the SCP is involved in this particular centric fusion leading to formation of the trivalent. If this is true, then the central metacentric chromosome of the trivalent acts as a neo-X chromosome, while two small submetacentrics act as neo-Y chromosomes. Thus, the sex chromosome system of population HK-A would be $X_1X_2Y_1Y_2$. Unfortunately, no plates containing two small submetacentrics were observed to further support this scenario.

Regarding female population from Prague, it is impossible to make definitive conclusions about sex chromosomes, because no suitable plates nor male data were obtained.

6.3 Evolution of nucleolar organizer regions

At present, only insufficient data about NORs is available in spiders. In Entelegynae, most results were obtained using silver staining, which was conducted in representatives of families Ctenidae (Araujo et al. 2014; Kumar et al. 2017), Lycosidae (Dolejš et al. 2011), Nephilidae (Araujo et al. 2015), Sparassidae (Rodriguez-Gil et al. 2007), and Tetragnathidae (Král et al. 2011; Araujo et al. 2015). Using FISH, NORs were studied in Ctenidae (Rincão et al. 2017) and Lycosidae (Forman et al. 2013) only. Based on available results, Dolejš et al. (2011) proposed the presence of two autosomal bivalents each bearing a single NOR to be the ancestral trait of Entelegynae. Additionally, results from Nephilidae and Tetragnathidae indicated that NORs present on X chromosomes of these two families are their synapomorphy (Král et al. 2011). Because NORs are highly dynamic in terms of their number, their ancestral pattern in Entelegynae is certainly hard to trace. However, Dolejš's proposal seems to be likely because the proposed pattern can be observed among various unrelated entelegyne families. On the other hand, it must be taken into account that most authors used silver staining which (compared to FISH) reveals only NORs transcriptionally active in a preceding interphase.

The range of NOR numbers in Araneidae revealed in the presented study is 1 – 13 NOR loci. Five araneid species possessing the ancestral karyotype and examined with FISH exhibited five autosomal NOR loci, while four species with the ancestral karyotype had a different number. Judging from its abundance and distribution among Araneidae, five autosomal NOR loci may present the ancestral pattern of this family.

In some animal groups (Artoni and Bertollo 2002; Rafael et al. 2006) including haplogyne spiders (Král et al. 2006), it is not uncommon to find NORs also on sex chromosomes. As to NORs localised on sex chromosomes in Entelegynae, however, such cases have been reported only in Nephilidae (Araujo et al. 2015) and Tetragnathidae (Král et al. 2011). Similarly, in the utter majority of Araneoidea examined here, NORs were localised on autosomes only, exhibiting exclusively terminal

(event. subterminal) position, mostly at distal ends of chromosomes. NORs on sex chromosomes were found only in two species (this thesis). In *Z. atrica* (X_1X_20), both X chromosomes bore a terminal NOR locus. In a male from population SO of *L. patagiatus* (X_1X_20), one X chromosome bore a distal NOR locus. On the contrary, both X chromosomes of X_1X_20 system bore a distal NOR locus in a male from population BL of *L. patagiatus*. Such X-linked NORs could arise by ectopic recombination between an autosome and a gonosome and later between both gonosomes themselves in case of NORs located on both X chromosomes.

The highest NORs number known in arachnids so far is ten NOR loci in *Wadicosa fidelis* (Lycosidae, Forman et al. 2013). All examined araneid species struck by centric fusions exhibit massive increase of NORs number to six NOR loci in *C. fuliginata*, 13 NOR loci in populations SO and BL of *L. patagiatus*, and probably 13 NOR loci in *Z. atrica* (this study), which surpasses the number detected by Forman et al. (2013). Since no prophase I or metaphase I plates were obtained in *Z. atrica* on slides after FISH, it was not possible to precisely determine pattern of 18S rDNA on particular chromosome pairs. One way to bypass such problem is to map position of NORs to an ideogram (Kirov et al. 2017). After doing so, the relative distribution of signals on mitotic chromosomes became more or less clear (Fig. 36). The data obtained here indicate connection between centric fusions and increase of NORs number.

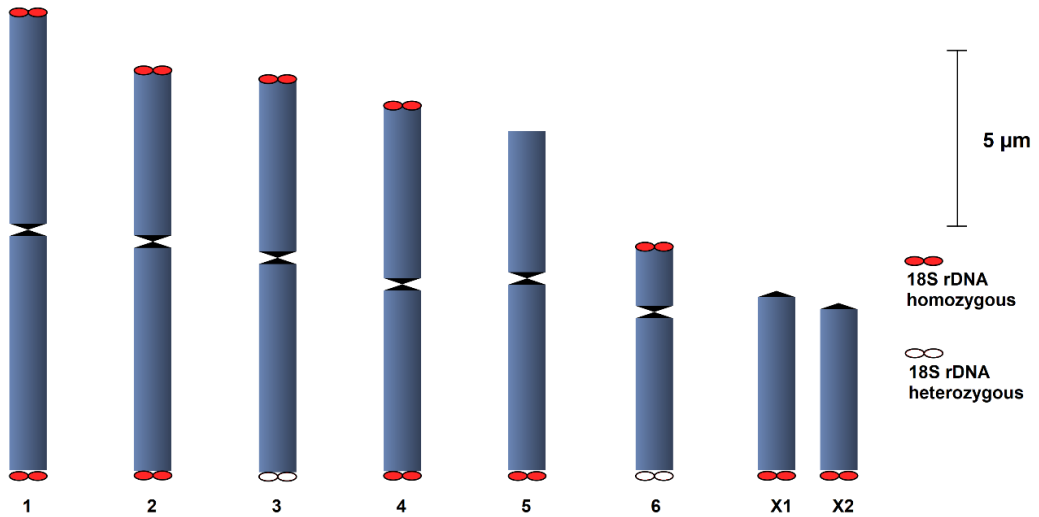

Fig. 36: Haploid ideogram of *Zygiella atrica* male (based on data from two mitotic metaphases); note chromosomes 3 and 6, whose 18S rDNA loci are shown in white at one end – it represents loci heterozygous for NORs, i.e. homological chromosome does not bear 18S rDNA locus at that end; also note that chromosome 5 is the only one with complete absence of 18S rDNA locus at one end; constructed in DRAWID software (v0.26)

A high number of NORs in araneid karyotypes exhibiting predominance of metacentric chromosomes raises a question if all NORs present are fully functional. Therefore, the distribution of 28S rDNA loci was studied in this thesis, too (based on assumption that a fully functional transcription unit should include all rRNA genes and that if both 18S and 28S rRNA genes are present, so is 5.8S rRNA gene between them). When 28S rDNA probe was applied separately in case of *Z. atrica*, only five signals were present on pachytene bivalents, indicating that only five NOR loci are fully functional and the rest of 18S rDNA signals are devoid of 28S rDNA, indicating non-functional NORs. There are perhaps two possible explanations: either presence of five NOR loci is indeed the ancestral state in Araneidae and after centric fusions, 18S rDNA loci spread massively by transposition; or gradual degradation of NORs occurs, which can lead to stabilization of a genome. Further colocalization results obtained here favour the latter – in *Z. atrica* and population BL of *L. patagiatus*, 18S and 28S rDNA probes colocalize almost everywhere excepting a few spots of sole 18S or 28S rDNA signals. However, there is also a possibility of populational polymorphism (since males of *Z. atrica* examined with 28S rDNA FISH separately come from a different population than males examined with colocalization FISH). Further FISH experiments are required, preferentially in combination with sequencing.

Regarding all species with the ancestral karyotype, all NOR loci were in a homozygous condition, excepting *A. diadematus*. In this species, a single NOR locus was heterozygous for the presence of 18S rDNA cluster. Moreover, a single chromosome pair homozygous for the presence of 18S rDNA cluster exhibited signal on only one of the sister chromatids of each homologue (observed repeatedly in all meiotic plates). Such situation can arise by a sister chromatid exchange or an unequal crossing over.

6.4 Evolution of 5S rRNA genes

In the presented thesis, optimisation of molecular markers other than 28S rRNA genes was conducted, including 5S rRNA genes. Concerning spiders, only a single study mapping 5S rRNA genes using FISH exists, demonstrating that NTS region of 5S rRNA gene cluster includes intercalated histone H2B-like gene in *Oxyopes sertatus* (Oxyopidae; Suzuki and Kubota 2011). In this study, detection of 5S rRNA genes has been performed to test hypothesis that karyotypes of araneids possessing high

diploid numbers were derived by polyploidisation. To test this hypothesis, 5S rDNA was detected in *A. quadratus* (a related araneid possessing the ancestral karyotype) and compared with 5S rDNA pattern in *A. circe* and *A. ventricosus*. It was expected that in case of polyploidisation, number of 5S rDNA loci should be two times higher compared to *A. quadratus*. A single unambiguous subterminal locus was detected in *A. quadratus*. However, due to substantial fluorescence background and low quality of signals, the presence of additional small loci exhibiting much weaker fluorescence cannot be ruled out. Indeed, further precise optimisation of this marker needs to be done in order to determine 5S rDNA pattern more reliably. The same applies to other examined species. Despite these problems, it was possible to detect eight mitotic chromosomes each bearing a single terminal 5S rDNA signal in *A. ventricosus* and six or seven bivalents bearing a terminal 5S rDNA locus in *A. circe*. Such results support the hypothesis stated above and point to a possibility that polyploidisation occurred indeed. However, it is necessary to support this hypothesis more, not just with precise optimisation of 5S rDNA probe, but with flow cytometry as well.

Concerning derived NOR pattern found in *Z. atrica* (this study), it was rational to detect pattern of 5S rRNA genes of this species as well and compare it with its relative *Z. montana* (because it possesses the ancestral karyotype). In both species, three 5S rDNA loci were present most likely. They differed only in their position (three bivalents bore a distal NOR locus in *Z. montana*, whereas a single bivalent bore a subterminal NOR locus and a different bivalent bore two distal NOR loci in *Z. atrica*). These results indicate that unlike NOR genes, 5S rRNA genes are probably not intertwined with centric fusions and maintain the ancestral number in the genus.

The results obtained here do not fit to what is known in other arthropods, mostly insects. In various examined insect groups, one or two 5S rDNA loci are common, often localised on sex chromosomes (Cabral-de-Mello, Martins, et al. 2011; Cabral-de-Mello, Oliveira, et al. 2011; Palacios-Gimenez and Cabral-de-Mello 2015). In species studied here, 5S rDNA loci were never localised on sex chromosomes and more than two 5S rDNA loci were always present. On the other hand, considering arthropods tend to possess one or two 5S rDNA loci, increased number of these loci in *A. circe* and *A. ventricosus* may further pinpoint to polyploidy hypothesis, too.

6.5 Evolution of histone H3 and U2 snRNA genes

Much like in case of 5S rDNA probe, histone H3 marker was optimised mainly to test the potential polyploidy in *Araneus*. In both *A. diadematus* and *A. quadratus*, FISH or TSA-FISH was used. However, further probe optimisation (as well as optimisation of TSA-FISH protocol used in spiders for the very first time) is necessary to improve quality as well as reliability of signals. The same applies to the remaining examined species – in *A. circe*, some H3 gene clusters were not reliably distinguishable from fluorescent background.

In other arthropods studied (insects and a scorpion), the pattern of histone H3 genes is rather conservative – usually, a single autosomal pair bears a H3 gene cluster (Cabrero and Camacho 2008; Cabral-de-Mello, Martins, et al. 2011; Cabral-de-Mello, Oliveira, et al. 2011; Almeida et al. 2017). Identical result was reported also in *Macrothele* in MSc. thesis of Hrubá (2017), which is the only work mapping histone H3 genes in spiders up to date. Whereas *A. diadematus* and *A. quadratus* exhibit probably two histone H3 gene clusters, number of these clusters is much higher in *A. circe* and *A. ventricosus*, since there are at least three (*A. circe*) and four (*A. ventricosus*) bivalents with a histone H3 cluster. This could be again ascribed to polyploidisation event.

The last marker optimised in this thesis was U2 snRNA. Again, the main purpose was to test the polyploidy hypothesis. Unfortunately, the probe hybridised only in case of *A. diadematus*, which was also expected since it was prepared using genomic DNA of this species. Further FISH experiments and optimisation are required. However, it is also possible that U2 snRNA genes within *Araneus* genus are rather diversified – consequently, a probe derived from DNA of one species does not hybridize to sequences in related species. In *A. diadematus*, two bivalents bore a distal signal. This is again not completely consistent with findings in other arthropods (insects and a scorpion), where only one autosomal pair bears a subterminal U2 snRNA locus. Eventually, B-chromosomes in a beetle *Aerenea flavolineata* (Cerambycidae) bear several U2 snRNA gene clusters, too (Bueno et al. 2013; Palacios-Gimenez and Cabral-de-Mello 2015; Almeida et al. 2017).

6.6 Analysis of chiasmata

Concerning studied species, overall range of chiasma frequency was 1.00 – 2.17. Generally, acrocentric chromosomes are typical for low chiasma frequency (John 1990). Indeed, results obtained in this study are consistent with this rule – in all species possessing the acrocentric karyotype, chiasma frequency was low, that is it ranged from 1.00 to 1.27. In araneid species with the ancestral karyotype, no universal pattern of chiasma position was observed – two species exhibited predominance of distal chiasmata and three species exhibited predominance of pericentric chiasmata. In *Larinioides sclopetarius*, *Plebs bradleyi*, and *Zygiella montana*, frequencies of distal and pericentric chiasmata were approx. equal, with just slight predominance of the latter. Excepting *Dolophones* sp., frequencies of intercalary chiasmata were negligible. In *Araneus psittacinus*, it was not possible to distinguish between pericentric and distal chiasmata because centromeres were not visible. If traditional phylogeny of Araneidae (Scharff and Coddington 1997) was taken into account and combined with molecular phylogeny according to Gregorič et al. (2015), all species studied here would be placed among subfamilies Araneinae and Zygiellinae (only positions of *Plebs* and *Larinioides* remain unclear). If such division is applied, then distribution and abundance of chiasmata among araneid clades suggest that perhaps pericentric pattern of chiasmata is ancestral in Araneidae.

Chiasma number per chromosome pair depends on chromosome size and particularly morphology, because morphology determines minimal number of chiasmata per chromosome. Indeed, at least one obligatory chiasma is required to ensure proper segregation of bivalents in anaphase I. Hence it is emphasized that chiasma frequency correlates with number of chromosome arms rather than whole chromosomes in mammals. Therefore, overall chiasma frequency should stay the same after formation of biarmed chromosomes due to centric fusions because total arm number remains unchanged. However, this is often not the case, as lowered chiasma number and recombination rate are reported in centric fusion mice or sheep (Dumas and Britton-Davidian 2002; Fröhlich et al. 2015). Indeed, compared to related species with the ancestral karyotype, chiasma frequency per chromosome arm is just slightly higher in case of *L. patagiatus* (population HK-B, 2.17), remains approx. the same in *Cyclosa fuliginata* (1.94), or is slightly lower in *Z. atrica* (1.88). Chiasma frequency in these species appears to be higher only if whole chromosomes

are taken into account. Raising chiasma frequency per chromosome can work as a mechanism compensating for decrease of chromosome number (John 1990). Anyhow, what changes is the chiasma position, which is almost exclusively distal in all three species with decreased 2n due to centric fusions. Occasionally, a bivalent with three chiasmata (two distal and one pericentric or intercalary) appears in *C. fuliginata* and *Z. atrica*. Increase of chiasma frequency per chromosome, as well as bivalents exhibiting more than two chiasmata, have been reported in *Oxyopes salticus* (Oxyopidae), which also underwent centric fusions (Stávale et al. 2011).

Interestingly, in both *L. sclopetarius* (related to *L. patagiatus*, but possessing the ancestral karyotype) and *Z. montana* (related to *Z. atrica*, but possessing the ancestral karyotype), distal and pericentric chiasmata occur almost equally, with slight predominance of the latter. Furthermore, *Leviellus stroemi* (another araneid with the ancestral karyotype belonging to Zygiellinae) exhibits complete predominance of pericentric chiasmata. Thus, it seems that at least within Zygiellinae (but probably *Larinioides*, too), there is a gradual shift from pericentric to distal position of chiasmata connected with centric fusions (this study). This situation is consistent with similar shifts observed in mice and pigs (Dumas and Britton-Davidian 2002; Mary et al. 2014), but more importantly, jumping spiders of *Habronattus* as well (Maddison and Leduc-Robert 2013). It supports the original model of White (1973) proposing that pericentric chiasmata present a constraint against centric fusions in general (including autosome – autosome fusions). Thus, the shift to distal chiasma positions may be necessary to promote centric fusions (White 1973). On the other hand, these observations are in conflict with results reported in *Delena cancerides* race homozygous for centric fusions, where no significant change of chiasma frequency or position is reported (Rowell 1991).

Shifts of chiasma positions in araneids possessing decreased 2n due to centric fusions may explain massive NORs spreading within their karyotypes. Distal position of chiasmata may support ectopic recombination between sequences located at chromosome ends. Ectopic recombination between distal regions of chromosomes would in turn lead to this massive spreading of NORs. Indeed, centromeric heterochromatin reduces recombination rate (Rowell 1991; Dumas and Britton-Davidian 2002). This is in agreement with the pattern observed in studied araneids. Concerning Zygiellinae, there are only two NOR loci in *L. stroemi* (exhibiting predominance

of pericentric chiasmata), whereas there are five in *Z. montana* (exhibiting equal ratio of pericentric and distal chiasmata) and 13 in *Z. atrica* (exhibiting almost exclusively distal chiasmata; this study). Such succession supports the hypothesis that spreading of NORs is indeed directly connected with chiasmata shift. Concerning *Larinioides*, the situation is very similar – there are six NOR loci in *L. sclopetarius* (exhibiting equal ratio of pericentric and distal chiasmata), but 13 in *L. patagiatus* (population BL, exhibiting almost exclusively distal chiasmata; this study). Unfortunately, no species analogous to *L. stroemi* is available in case of *Larinioides*.

7 Conclusions

Even though cytogenetics of Araneidae has been studied rather extensively, overall knowledge of araneid karyotype evolution is still insufficient compared to the vast diversity of these spiders. In the framework of the presented thesis, 19 araneid and four tetragnathid species were examined. Comparison with the related family Tetragnathidae is necessary to get an idea about ancestral chromosome features of the superfamily Araneoidea as a whole, since both Araneidae and Tetragnathidae belong to this superfamily and are closely related.

The results of araneid males revealed 2n ranging from 13 to 52. The most common 2n in araneids was 24, all examined tetragnathids exhibited this number. Most examined araneids and all tetragnathids exhibited acrocentric morphology of chromosomes. These findings strongly support already proposed ancestral karyotype of Araneoidea consisting of 24 acrocentric chromosomes. The ancestral karyotype of Araneidae is probably the same. However, there is also a trend of 2n decrease connected with change of chromosome morphology in some araneids. In males of *Cyclosa fuliginata*, *Larinioides patagiatus*, and *Zygiella atrica*, diploid numbers were approx. half of the ancestral state and most chromosomes were biarmed. Such karyotypes were formed by centric fusions of almost all original acrocentric chromosomes and are probably fixed thanks to female meiotic drive. On the other hand, some araneids exhibited considerably increased 2n, while maintaining acrocentric morphology of all chromosomes. Obtained data suggest that such karyotypes were formed by polyploidisation followed by additional rearrangements. In males of yet undetermined Australian orb-weaver, 2n was 28 with predominance of metacentric chromosomes. This karyotype was probably formed by pericentric inversions followed by centric fissions of some autosomes.

Proposed ancestral sex chromosome system in Araneoidea is X_1X_20. Indeed, this system was found in all tetragnathids and majority of araneids. In some araneid species, X0 system was detected, with X chromosome being either acrocentric in some cases (formed by a tandem fusion), or metacentric in other cases (formed by a centric fusion). Other systems detected were $X_1X_2X_3X_40$ (formed by non-disjunction, polyploidisation or rearrangements) and $X_1X_2X_30$ (derived from the previous one by tandem fusion of two X chromosomes). According to recent findings, spider karyotypes also contain a specific sex chromosome pair formed by little differentiated sex

chromosomes X and Y (i.e. proto-X and proto-Y), which could pose the ancestral sex chromosomes of spiders. Neo-sex systems $X_1X_2X_3X_4Y_1Y_2$ and $X_1X_2Y_1Y_2$ found in two populations of *L. patagiatus* were probably formed by centric fusions between the chromosomes of this pair and an autosomal pair resulting to monobrachial homology of involved chromosomes and formation of a trivalent or tetravalent segregating in balanced fashion.

Pattern of NORs was studied using FISH. Obtained results indicate that the ancestral pattern in Araneidae is five terminal autosomal NOR loci. Strikingly, multiple centric fusions were always accompanied by considerable increase of NORs number. Obtained data indicate that NORs spread by ectopic recombination between distal regions of chromosomes. Formation of multiple NORs by this process was probably accompanied by degeneration of some NORs.

In the framework of the presented thesis, other molecular markers (5S rDNA, histone H3 genes, and U2 snRNA genes) were optimised and visualised, too, using FISH or TSA-FISH (for the very first time in spiders) in order to reveal mechanisms leading to increased 2n in *Araneus*. Obtained data suggest polyploidisation, but data from flow cytometry are also required for completely unambiguous conclusions.

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