CHROMOSOME EVOLUTION IN THE GENUS LITORIA (ANURA, HYLIDAE)

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บทคัดย่อ

ชื่อเรื่อง : การศึกษาวิวัฒนาการของโครโมโซมในปาดสกุลลิทอเรีย

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ศัพท์สำคัญ : คาริโอไทป์มาตรฐาน ลิทอเรีย รูปแบบการย้อมแถบสี โครโมโซมเครื่องหมาย วิวัฒนาการของโครโมโซม

การศึกษาไมโทติกโครโมโซมของปาดสกุลลิทอเรียจำนวน 19 ชนิด โดยมี 4 ชนิดที่เป็นการศึกษาและรายงานเป็นครั้งแรกคือ *L. barringtonensis*, *L. genimaculata*, *L. nyakalensis* และ *L. personata* โครโมโซมที่ใช้ในการศึกษาได้จากการเตรียมจากไขกระดูก ด้วยวิธี *in vivo* colchicine treatments และย้อมสีโครโมโซมด้วยเทคนิคการย้อมสี Giemsa, C-banding, Ag-NOR staining, DAPI/Distamycin A, DAPI/Mithramycin, Q-banding และเทคนิค Telomere FISH พบว่าปาดทั้ง 19 ชนิดมีจำนวนโครโมโซมแบบดิพลอยด์ คือ 2n = 26 และจำนวนโครโมโซมพื้นฐานเท่ากับ 52 ยกเว้นใน *L. infrafrenata* ที่มีจำนวนโครโมโซมแบบดิพลอยด์ คือ 2n = 24 และจำนวนโครโมโซมพื้นฐานเท่ากับ 48 โครโมโซมของปาดทั้ง 19 ชนิดพบว่ามีความเป็นเอกลักษณ์สูงทั้งในขนาดและรูปร่างของโครโมโซม โดยโครโมโซมคู่ที่ 1 และ 4 เป็นชนิดเมทาเซนทริก คู่ที่ 2 และ 6 เป็นชนิดสับเมทาเซนทริก และคู่ที่ 3 และ 5 เป็นชนิดสับเทโลเซนทริก และพบโครโมโซมเครื่องหมายที่มีความจำเพาะในแต่ละชนิดในรูปแบบ secondary constriction ตำแหน่ง nucleolar organizer และการกระจายตัวของ constitutive heterochromatin ไม่พบโครโมโซมเพศในปาดสกุลลิทอเรียที่ได้ศึกษา ใน *L. barringtonensis* พบตำแหน่ง secondary constriction ขนาดใหญ่และเป็นตำแหน่งเดียวกันกับตำแหน่ง nucleolar organizer จากเทคนิคการย้อมแถบสี C-banding พบว่าแต่ละชนิดแสดงแถบสีแบบซีที่คล้ายคลึงกันมาก และพบว่าใน *L. meiriana* พบ C-block บนแขนของโครโมโซมคู่ที่ 12 ซึ่งเกิดจากกระบวนการ heterochromatin addition ทั้ง 19 ชนิดพบตำแหน่ง nucleolar organizer เพียงตำแหน่งเดียว ส่วนใหญ่พบบริเวณแขนข้างยาวของโครโมโซมขนาดเล็ก มีเพียง 4 ชนิดที่พบบริเวณแขนข้างสั้นของโครโมโซมขนาดใหญ่ ส่วนการศึกษาด้วยการย้อมแถบสีฟลูออเรสเซนต์พบว่าเทคนิค DAPI/Distamycin พบการเรืองแสงของโครโมโซมทั้งแท่งในปาดทุกชนิดที่ศึกษา เทคนิค

การย้อมสี mithramycin พบการเรืองแสงสว่างบริเวณตำแหน่ง nucleolar organizer เทคนิคการย้อมสี quinacrine mustard ไม่พบการเรืองแสงบริเวณตำแหน่ง centromere ของโครโมโซมของทุกชนิดที่ได้ศึกษา ส่วนเทคนิค Telomere FISH โดยใช้โพรบที่มีลำดับเบสบนตำแหน่งทีโลเมียร์ $(GGGTTA)_7$ และ $(TAACCC)_7$ พบการเรืองแสงบริเวณ telomere ของโครโมโซมทุกแท่งในทุกชนิดที่ศึกษา แล้วยังพบว่ามีการเรืองแสงบริเวณ centromere ของโครโมโซมคู่ที่ 4 ใน *L. eucnemis*, *L. genimaculata* และ *L. verreauxii* และคู่ที่ 1 2 และ 3 ใน *L. fallax* จากการศึกษาคาริโอไทป์ในปาดสกุลลิทอเรีย แสดงให้เห็นถึงอัตราการเกิดวิวัฒนาการของโครโมโซมที่ค่อนข้างต่ำ เนื่องจากโครโมโซมส่วนใหญ่เป็นชนิดเมทาเซนทริก และสับเมทาเซนทริก และไม่พบชนิดทีโลเซนทริกเลย ข้อมูลทางสัณฐานวิทยาและเซลล์พันธุศาสตร์ ทำให้ทราบสายสัมพันธ์ทางพันธุกรรมตามสายวิวัฒนาการของปาดสกุลลิทอเรียในออสเตรเลีย

ABSTRACT

TITLE : CHROMOSOME EVOLUTION IN THE GENUS LITORIA (ANURA, HYLIDAE)

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DEGREE : DOCTOR OF PHILOSOPHY

MAJOR : BIOTECHNOLOGY

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The mitotic chromosomes of 19 species of *Litoria* and the 4 new species (*L. barringtonensis*, *L. genimaculata*, *L. nyakalensis* and *L. personata*) studied here were prepared from bone marrow after *in vivo* colchicines treatment and analyzed by conventional staining, C-banding, Ag-NOR staining, DAPI/Distamycin A, DAPI/Mithramycin, Q-banding and Telomere FISH. All species were 2n=26, fundamental number (FN) =52 chromosomes, except *Litoria infrafrenata,* which was 2n=24, FN=48. In terms of arm ratios and centromere positions, the chromosome morphology of *Litoria* species was very characteristic. Pairs 1 and 4 were metacentric, pairs 2 and 6 were submetacentric and pairs 3 and 5 were subtelocentric. Species-specific chromosome markers were determined and included secondary constriction, location of NORs and heterochromatin distribution. Sex chromosomes could not be identified in the *Litoria* species studied. The secondary constriction showed major despiralization in *L. barringtonensis,* which is regarded as the nucleolar organizer. The C-banding studied revealed substantial differentiation in the heterochromatic component of the complement including the possession of whole arm C-blocks, some of which had evolved by chromosome addition; pair 12 of *L. meiriana*, while others involved a process of euchromatin information. None of the species analyzed shared the same C-banding pattern, although certain closely related species had very similar and highly

derived karyotypes. All species examined had only one pair of the nucleolar organizer regions in their chromosomes; the four *Litoria* species exhibited on the short arm of the large chromosome pair and the other species shown on the long arm of the small chromosome pair. Fluorescence banding in the distamycin A/DAPI counter stained the chromosomes of all species and showed a uniform fluorescence, the mithramycin-stained chromosomes of all species exhibited the brightest mithramycin fluorescence on the NOR regions, which can be used to verify the position of nucleolar organizer regions in each species and the quinacrine mustard showed negative fluorescence of the centromeric regions of all species of *Litoria*. In situ hybridization with the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers revealed, as expected, distinct hybridization signals at the telomeres of all chromosomes of *Litoria* species. Moreover, it was found that four species of *Litoria* showed signals not only at the telomeres of chromosomes, but also at the centromere of chromosomes (pair 4 in *L. eucnemis*, *L. genimaculata* and *L. verreauxii* and pairs 1, 2 and 3 in *L. fallax*). The karyotype of most species in the genus *Litoria* shown quite stable, characterized by a similar macrostructure, this seems to be evidence for a low chromosome evolution rate. Chromosome morphology, banding pattern and position of the nucleolar organizer regions (NORs) provide relevant characters for the understanding of the phylogeny and systemics of these *Litoria* tree frogs in Australia.

CONTENTS

CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

CHAPTER I

INTRODUCTION

Amphibians are cold-blooded animals, meaning they do not have a constant body temperature but instead take on the temperature of their environment. They have moist, scaleless skin that absorbs water and oxygen, but that also makes them vulnerable to dehydration (loss of bodily fluids). Without moist conditions, their skin dries out and they die. Therefore, amphibians are most often found near ponds, marshlands, swamps, and other areas where freshwater is available. Some amphibians become inactive when conditions are unfavorable for survival [1]. Amphibians are useful as excellent bioindicators. The three orders of living amphibians are caecilians (tropical worm like amphibians specialized for a burrowing mode of life), salamanders (a long tail, short legs and lung less) and frogs and toads (jumping tailless amphibians) are found in a variety of freshwater aquatic and terrestrial environments throughout the world [2]. Hylid frogs are one of the most species rich families of amphibians. With 885 species and 57 genera currently recognized they contain about 13% of all 6629 amphibian species [3]. Most hylid frogs are arboreal and are known colloquially as tree frogs [4]. Hylid frogs occur on all major continents except for Antarctica, but most species and genera occur in the New World tropics [3]. They are also relatively diverse in Australia, but have only a limited number of species in North America, North Africa, Europe and Asia [3]. The tree frogs of the family Hylidae are subdivided into five subfamilies: Phyllomedusinae, Hemiphractinae, Pelodryadinae, Amphignathodontinae and Hylinae. The 181 species of tree frogs in the genus *Litoria* belong to the subfamily Pelodryadinae of the anuran family Hylidae and are restricted to Australia and Papua New Guinea [3]. *Litoria* are arboreal, ground-dwelling and scansorial. The digits of arboreal species have dilated terminal discs and interdigital webbing on the hands. A small number of studies on the chromosomes of the genus *Litoria* have been performed using banding techniques and they are all very similar in their morphology and are typically hylid in format [5, 6]. King (1990) [6] observed chromosome evolution in anuran karyotypes and suggests that pericentric inversion has not been the sole basis

for structural change in the amphibian complement, for chromosome number reduction requires the fusion or translocation of elements and both chromosomal fusion and fission have also played a significant role in the evolution of anuran karyotypes. So, Phylogenetic analyses within the

Australian Hylidae have never considered more than a proportion of the component species of the recognised genera *Litoria*, *Nyctimystes* and *Cyclorana*. Resolution of phylogenetic relationships within *Litoria* awaits further data analyses. Until now, nothing has been reported in the literature on further banding analyses in these amphibians, although advances in cytogenetical techniques achieved during the last two decades have made it possible to perform very precise analyses of the fine structure of chromosomes. Nowadays, chromosomal homologies and chromosome rearrangements among different species can be recognized using multiple banding patterns. The aims of the present study are to describe the structural basis of chromosome number, size and morphology, the variation in the morphology of secondary constrictions, chromosome evolution and sex chromosome differentiation in species of the genus *Litoria*, using chromosome banding techniques such as conventional, Ag-NOR staining, C-banding and Fluorescence banding and to construct a phylogenetic tree of the *Litoria*'s tree frogs based on cytogenetic data. Thus, I expect the cytogenetic knowledge and the phylogenetic tree of the genus *Litoria* to be useful for helping accurately identify some amphibians and their relationships.

CHAPTER II

OBJECTIVES

The objectives of this study were:

(1) To study the number, size and morphology of chromosomes, chromosome markers and standard karyotypes of the Australian tree frogs in the genus *Litoria*.

(2) To determine the chromosome markers in the karyotypes of the *Litoria*'s tree frogs by using chromosome banding techniques.

(3) To study chromosome evolution and sex chromosome differentiation in the genus *Litoria*.

(4) To construct a phylogenetic tree of the *Litoria*'s tree frogs based on cytogenetic data.

CHAPTER III

LITERATURE REVIEW

3.1 Biology of amphibians

Amphibians, animals with moist, hairless skin through which water can pass in and out. Nearly all amphibians live the first part of their lives in water and the second part of land it so call two-stage or biphasic life cycle a double life reflected in the name amphibian, which comes from the Greek words amphi, meaning "both" and bios meaning "life" [2]. Amphibians were the first mammals with backbones to adapt to life on land. They are the/ ancestors of reptiles, which in turn gave rise to birds and animals. Scientists recognize more than 4,000 species of amphibians, all of which are members of one of three main groups: frogs and toads, salamanders, and caecilians [2]. Amphibians inhabit all landmasses except Antartica and some oceanic islands. They live in many environments, including grasslands, rain forests and even deserts. Most species require freshwater habitats such as ponds, swamps, streams or other wet environments for breeding. Some frog species rely on pools of water that collect in tree hollows or in the cup-shaped bases of epiphytes. Some burrowing frogs secrete a mucous cocoon around their body to prevent water loss while they are buried. Adult amphibians typically have body structures that enable them to move about on land as well as in the water. Most adult amphibians retain their teeth. Amphibians are cold-blooded or poikilothermic animals. They are not able to generate their own body heat. Instead, their body temperature is determined by their surroundings. Amphibian skin also contains numerous glands that secrete a slimy mucous layer to protect the skin from drying out and help draw in oxygen through the skin. In the water, these protective secretions help amphibians retain a healthy balance of salt and water within their internal tissues. In many amphibian species, mucus-secreting glands in the skin are modified to produce toxins and other substance that will repel or kill predators. Amphibians rely on their sense to find food and evade predators. Frogs and toads also use their keen hearing in communicating with one another. They produce a wide variety of vocalization, which they use in mating and territorial disputes. Frogs have bulging eyes that

protrude from either side of their head, enabling them to watch for danger and search for prey in nearly every direction. Amphibians use an organ in the roof of the mouth called Jacobson's organ for a sense of smell and taste. Most amphibians undergo a dramatic change in anatomy, diet and lifestyle known as metamorphosis after hatching into a larva form [1].

3.2 Importance of amphibians and problems

Amphibians are the great importance to humans. Most frogs live on a diet of insects, and in many areas they help control population of mosquitoes and crop damaging insects. In turn, they may be a food source of humans the legs of one type of frog are considered a delicacy in many parts of Europe. Frogs are also important in teaching and scientific research. Adult frogs are often used to teach students about the anatomy and physiology of vertebrates, or animals that have a backbone. Frog eggs, meanwhile, help scientists learn about embryonic development. Ecologist is interested in frogs and other amphibians because these animals are considered the excellent bioindicator. This means that the health of amphibian populations is thought to reflect the health of the ecosystem as a whole [7].

Since around 1980, scientists have reported starling declines in the populations of amphibians in many parts of the world [8]. Their two-stage life cycle and permeable skin make amphibians particularly sensitive to environmental disruptions such as drought and pollutants. This sensitivity makes them excellent bioindicator life forms whose well-being provides clues to the health of an ecosystem. Declines in amphibian populations may be due in part to natural fluctuations, but they more likely suggest that human are changing the environment more rapidly than amphibians can adapt. One such change is the destruction and modification of amphibian habitats, such as the cutting of forest and the draining of wetlands [7]. However, some amphibian groups are even disappearing mysteriously in areas where their habitat is not being destroyed such as two species of Australian gastric brooding frogs, the golden toad of Costa Rica and the red-legged frog of the North American Pacific coast. More recently, scientists have documented an alarming high occurrence of frogs with malformations such as missing or extra legs, abnormal webbing, and missing eyes. The causes of these malformations are still uncertain but may include disease from viral, bacterial, or fungal pathogens global warming and increased level of the ultraviolet B component of sunlight hilting the Earth as a result of depletion of the protective

ozone layer. Ultraviolet B light is particularly suspect in the decline of those amphibians that lay their eggs in shallow water, because eggs are exposed to sunlight for long periods. At a more local level, chemical pollutants, such as acid rain, pesticides, herbicides, and fertilizers, may be harming amphibians. In some regions, the introduction of alien or exotic species, or non-native competitors and predators has contributed to amphibian population declines. It is likely that an interactive of some or all of these factors may be exacerbating conditions for amphibians [1].

3.3 Definition and general description of Hylidae

Tree frogs of the family Hylidae have eight procoelous, non-imbricate, presacral vertebrae, the first two of which are unfused. The atlantyl cotyles of presacral I articulating with the skull are widely separate. Ribs are absent and the sacral diapophyses are dilated. The sacrococygeal articulation is bicondylar. The pectoral girdle is arciferal with a cartilaginous omosternum and sternum. Palatines are present, parahyoid absent and the cricoid ring is complete. The maxillae and premaxillae are dentate. The astragalus and calcaneum are fused proximally and distally. There are two tarsalia, and osseous or cartilaginous intercalary elements are present between the penultimate and terminal phalanges (except in *Cyclorana*) [9]. Amplexus is axillary. Larvae normally have keratinized beaks and denticles, and a sinistral, lateral or ventro-lateral spiracle. Diploid chromosome complement is 26, except for *Litoria infrafrenata* in which it is 24.

Currently the Hylidae includes five subfamilies. The Hylinae occurs in the Americas, Europe, Asia and North Africa, the Phyllomedusinae, Hemiphractinae and Amphignathodontinae are confined to South America, and the Pelodryadinae is confined to Australia, New Guinea and adjacent islands [3].

In the Australian region, the endemic subfamily Pelodryadinae comprises the genera *Litoria*, *Nyctimystes* and *Cyclorana*. Pelodryadines are arboreal, ground-dwelling, scansorial (*Litoria* and *Nyctimystes*) or fossorial (*Cyclorana*). The digits of arboreal species have dilated terminal discs and interdigital webbing on the hands, but terrestrial species have undilated fingers and lack webbing [9].

3.4 Natural history of Hylidae

Australian hylids are opportunistic predators on a wide variety of arthropods, primarily insects. Available data suggest dietary habits are non-specific, and that seasonal change in abundance of these groups [10]. Frogs are constrained by their need to obtain moisture from the environment. Despite this limitation some frogs are able to live in seasonally arid areas and avoid desiccation by burrowing. *Cyclorana* species and *L. alboguttata* burrow and form cocoons to avoid desiccation. Canopy-dwelling species such as *L. gracilenta*, *L. chloris* and *L. xanthomera* avoid desiccation by postural changes that protect the vulnerable ventral surface whilst "waterproofing" of the dorsal skin prevents water loss. These species descend to the ground to breed during heavy rains [10].

Humphries (1979) [11] studied a breeding guild of 11 sympatric *Litoria* species, including *L. lesueurii*, *L. aurea*, *L. flavipunctata*, *L. verreauxii* and *L. peronii*. He found that species respond predominantly to weather conditions to initate breeding, rather than to the presence or absence of other frogs. There was intense intraspecific competition for calling sites and non-calling males were tolerated only if they maintained their silence. The ability of frogs to disperse and/or migrate is dependent upon available moisture and suitable habitat for shelter. There is little doubt that some areas are constantly recolonised by frogs transported by flood waters and that many of these colonisations fail to become established South Australia [12]. Anecdotal evidence of amazing homing ability in *L. caerulea* is common, but little is known of the dispersal abilities of Australian hylids [12].

3.5 Cytogenetics of *Litoria* species

A small number of *Litoria* species have been analysed chromosomally at varying levels of resolution, ranging from chromosome number to relatively sophisticated banding techniques (NOR-staining, C-banding and fluorescence staining) and the *in-situ* hybridization of 18s+28s cRNA probes. All species have 2n=26, FN =52 chromosomes, but *L. infrafrenata* which has 2n=24, FN=48 [13, 14]. The metacentric and submetacentric karyotypes show a gradual diminution in size, and generally show similarities in both centromeric position and arm ratios. C-banding studies have revealed substantial differentiation in the heterochromatic components of

the complement, including the possession of whole arm C-blocks, some of which have evolved by chromosome addition while others have involved a process of euchromatin transformation [5]. None of the species analyzed share the same C-banding pattern, although certain closely related species have very similar and highly derived karyotypes [6].

The most striking variation is seen in the morphology of the secondary constrictions. Generally, one major nucleolus organizer constriction is present per genome, which may vary in its position between species. However, groups of closely related and often morphologically very similar species share the same type and location of their nucleolus organizing constrictions, which can therefore provide effective taxonomic markers [15]. King (1987b) [16] found that eight of the *Litoria* species had a single NOR site. However in *L. raniformis*, two sets of presumptive NORs were detected both by silver staining and C-banding [5], a finding which was confirmed by *in-situ* hybridization and 18s+28s rRNA probe [17, 6].

The fluorescence banding has rarely been studied and only in *L. infrafrenata*. In the DAPI/Mithramycin-stained chromosomes of *L. infrafrenata*, the centromeric heterochromatin in all chromosomes and the secondary constriction exhibits the brightest fluorescence. In contrast to the quinacrine mustard which demonstrates quenced fluorescence of the centromeric and most of the interstitial heterochromatic regions [18].

3.6 Secondary constrictions as a marker in the genus *Litoria*

King (1980) [5] reported the identification of a secondary constriction in the genus *Litoria* into five major groups on the basis of shared characteristics of chromosome morphology. The following description of these shared characters is illustrative and is not meant to be comprehensive:

Group A species: These species all possess a type 4 constriction on chromosome pair 10 [5]. They are including *L. lesueuri*, *L. latopalmata*, *L. inermis*, *L. tornieri*, *L. freycineti*, *L. nasuta*, *L. nigrofrenata*, *L. watjulumensis* and *L. coplandi*. They are generally small, grey, terrestrial frogs having very long legs, small discs on fingers and toes and a very pointed snout. They appear streamlined and can jump great distances. All species have a pronounced black eye stripe which curves down behind the shoulder.

Group B species: All species possess a type 2 constriction on chromosome pair 13 [5] and are including *L. aurea*, *L. raniformis*, *L. cyclorhynchus*, *L. moorei* and *L. dahlii*. These are very large, green, terrestrial animals which are basically ranoid in appearance. They have long, well-developed legs, long pointed snouts, and very small discs on their fingers and toes. They generally lack a pronounced eye stripe.

Group C species: All species in this group possess a terminally located type 1 constriction on chromosome pair 11. These species included in this karyotype group encompass a number of species groups which were recognized on morphological grounds by Tyler and Davies (1978) [9]. All species are arboreal and have very large discs on their fingers and toes. *L. caerulea*, *L. splendida*, *L. chloris* and *L. gracilenta* are very large, green tree frogs, whereas *L. peronii* and *L. rothii* are brown tree frogs. These species are characteristically thickset animals with short, blunt, rounded snouts. They lack any eye stripe coloration.

Group D species: Members of this group include the species *L. glandulosa*, *L. phyllochroa* and *L. pearsoniana* which share a type 5 constriction on pair 9. All species in group D have quite large toe and finger discs, are relatively blunt-nosed and deep-bodied frogs and share a dark eye stripe extending to the mid-abdomen. *L. glandulosa* is much larger than the other species.

Group E species: These animals all share a secondary constriction on chromosome pair 1 with *L. ewingi* and *L. verreauxi* having a constriction at the same site. This karyotypic group contains a series of species groups and may well be an artificial assemblage, although it should be noted that the members of this group are largely agreed upon by Tyler and Davies (1978) [9] on morphological criteria. All are small and relatively long-bodied frogs with proportionally short legs, well-developed finger and toe pads and a short rounded snout.

3.7 Heterochromatin distribution in the genus *Litoria*

King (1980) [5] studied chromosomes in Australian Hylid frogs by using C-banding techniques and found that in the C-banding patterns between species the most striking feature is that no two of them share the same pattern. In addition to C-bands associated with secondary constrictions there are four arbitrary classes of C-heterochromatin. These are (1) procentric bands,

(2) interstitial bands, (3) terminal grey bands and (4) major C-blocks that occupy most, if not all, of a chromosome arm.

(1) Procentric bands

There is remarkable variation in the quantity and distribution of these C-bands both between chromosomes and between karyotypes in *Litoria*. Species such as *L. raniformis* and *L. lesueuri* have uniformly small procentric bands, whereas *L. phyllochroa*, *L. peasoni, L. cooloolensis* and *L. moorei* have relatively large bands extending from the centromere into both arms. Other species, *L. peroni*, *L. chloris* and *L. infrafrenata* have small procentric C-bands in some chromosomes and large bands in other. In *L. infrafrenata* pairs 3, 5, 6, 7, 8 and 9 have large procentric blocks with extend preferentially into one arm rather than the other.

(2) Interstitial bands

Relatively few interstitial C-bands were encountered and when present, they occurred only as fine bands. They were found in *L. raniformis* pair 3, *L. peroni* pair 5, *L. lesueuri* pairs 3, 7 and 10 and *L. infrafrenata* pairs 4 and 6.

(3) Terminal grey bands

Lighter grey C-bands were presented in most chromosomes and appear in the telomeric regions. The expression of these bands was often variable and to some degree dependent on length of exposure to barium hydroxide. A large double barred grey C-band was presented on the long arm of pair 3 in all species. A similar band was presented on the long arm of pair 5 most species.

(4) Major C-blocks

The most common form of C-block occupies either a large proportion or else the whole of a chromosome arm and does not appear to have modified the external chromosome dimensions. In *L. lesueuri* pair 10, *L. pearsoni* pair 9, *L. peroni* pair 5 and *L. infrafrenata* pair 4 these showed major C-blocks. Small blocks exhibited in *L. lesueurii* pair 7, *L. chloris* pair 7 and *L. phyllochroa* pair 12.

3.8 Nucleolus organizer evolution in amphibians

Nucleolus organizer regions (NORs) are important marker for the study of chromosome evolution. The number and position of NORs are usually characteristic of species or

populations, although interindividual variability of these regions has been observed within populations of various organisms. In Anura, NOR analysis by silver staining has shown that most species, in both primitive and derived families, possess only one observation led King et al. (1990) [14] to suggest the presence of only a single pair of NORs in diploid karyotypes as an ancestral condition in Anura, a hypothesis previously propose by Schmid (1978) [19] for bufonids and hylids. The silver staining technique of Goodpasture and Bloom (1975) [20] has been widely used in anuran Amphibians as a means of determining the site of nucleolus organizer activity [19, 5, 21]. Schmid (1982) [22] was able to analyse the chromosome of some 260 anuran specimens from 23 different genera. This work confirmed the very high incidence of fixed heteromorphism in the size of NORs between specimens, wherein 67% of the individuals examined were heteromorphic. In addition, Schmid found that the overwhelming majority of species of anurans possessed only one pair of NORs in their diploid karyotypes. Mahony and Robinson (1986) [21], in the most complete analysis of any one the 99 species of the Myobatrachidae. A majority of species had a single NOR site; however, four species four the genus *Heleioporus* had up to five pairs of homologues characterized by silver staining regions.

Much emphasis has been placed on silver staining due to its claimed specificity for ribosomal cistrons [22]. While it may well be true that, because of its reaction to non histone proteins rich in sulphydril and disulphide groups, silver staining is attracted to newly transcribed rRNA (Varley and Morgan, 1978), there is now clear evidence that silver staining also binds to other chromosomal sites in Ampibians. This was most convincingly demonstrated by Nardie et al. (1978) [23] who compared the silver staining sites in *Triturus vulgaris* to those regions which were shown to contain 18S + 28S ribosomal cistrons by using *in-situ* hybridization. There is no doubt that the silver staining technique has been of immense value in demonstrating the position of NORs in those species which have only a single pair of these organelles. Nevertheless, when more than one pair of NORs is indicated by this technique, confirmation by in-situ hybridization with a specific ribosomal DNA probe appears to be mandatory. Indeed, the observation by Mahony and Robinson (1986) [21] of up to five pairs of presumptive NORs in four *Heleioporus* species highlights this dilemma. The possibility that the multiple sites in *Heleioporus* might be active NORs receives indirect support from the presence of multiple NORs in *Litoria raniformis* (King, 1987) as well as from the studies on *Triturus* [24]. While deletion of one of the NORs in a

pair of homologues has been documented in *Xenopus laevis* [22], the vast majority of fixed heteromorphisms appear to involve the amplification of the ribosomal DNA in one of a pair of homologues. That deletion has also been involved, is supported by the presence of a single silver stained NOR in one of two homologues [22], or else by a single chromosome showing grain accumulation at the NOR after 18S + 28S *in-situ* hybridization. When amplification is present it may take two forms. First, the amplification of only some ribosomal sequences producting subtle size variation between homologurs; second, the amplicfication of the entire NOR. In the latter case, an examination of the homologures may reveal a duplication, or in some cases triplication in size of the NOR [5, 22]. The great number of specimens and species which have fixed heteromorphic NORs; the very few specimens which show homomorphism for amplified NORs; and the fact that amplification is restricted to threefold size changes, suggest that severe constraints may be imposed on the extent of amplification.

3.9 Sex chromosome in amphibians

Most amphibian species present morphologically undifferentiated (homomorphic) sex chromosomes [6, 25, 26]. This means that in the heterogametic sex, the XY or the ZW sex chromosomes exhibit an identical morphology when studied with the classical cytogenetic techniques (uniform staining of chromosomes). Therefore, the early pioneering studies on amphibian karyotypes failed in the demonstration of differentiated sex chromosomes or yielded contradictory results [27]. Moreover, as no sex-linked genes with their characteristic mode of inheritance were known in the amphibians, other approaches were made to reveal the type of sex-determining mechanisms in these vertebrates. Such experiments were extremely time-consuming and difficult, but offer a most appealing and convincing method of proof [28].

The first certified highly heteromorphic sex chromosomes in the Anura were discovered in the South African Bull frog *Pyxicephalus adspersus* [29]. Male animals have ZZ chromosomes, females the ZW constitution. The W chromosome is considerably smaller than the Z and its short arm is completely heterochromatic. The same chromosome pair no. 8, which in *P. adspersus* represents the highly heteromorphic ZW pair, is still in an initial stage of morphological differentiation in the closely related *P. delalandii*. Although the chromosomes no. 8

of *P. delalandii* are still of the sample length in the female individuals, they differ from each other by a pericentric inversion and by the amount of heterochromatin [29].

Well-differentiated XY sex chromosomes characterize the male of several species of the American salamanders belonging to the family Plethodontidae. The five species of the American salamander genus Necturus have the most highly differentiated XY sex chromosomes yet discovered in the Urodela [30, 31]. Very distinct heteromorphic XY sex chromosomes were found in the South American marsupial frog *Gastrotheca riobambae* [32, 33, 34]. The Y chromosome is the largest element in the karyotype and almost complete heterochromatic. This is one of the very few vertebrate species having a Y larger than the X [28].

The evolution of heteromorphic sex chromosomes from one originally homomorphic chromosome pair was probably not the result of a single structural change, but most likely involved several subsequent steps [35]. Because evolutionary processes cannot be reproduced experimentally, the individual changes taking place over the course of chromosome evolution can only be reconstructed by means of comparative studies. A number of the known sex chromosomes of amphibians support the assertion that one of the initial steps in the evolution of sex chromosomes was an accumulation of repetitive DNA in the W and Y chromosomes. Thus in the primitive Y chromosomes of *Triturus* and *Gastrotheca pseustes*, the only visible difference between the X and Y is very small heterochromatic band in the Y [28]. In the more advanced Y and W chromosomes of the Amphibia, inversions are already present, as shown by *Hydromantes*, *Aneides*, *Pyxicephalus delalandii* and *Eupsophus migueli*. Finally, most of the highly evolved Y and W chromosomes are reduced to small, almost completely heterochromatic elements, as in *Necturus*. Nothing can yet be said about the location of these sex-determining genes within the Y and W chromosomes. It is possible that they are located in the few, small euchromatic regions still preserved in these chromosomes [26, 28]. With regard to the 0W/00 system found in *Leiopelma hochstetteri*, Green (1988) [36] proposed that it originated from a primordial ZW/ZZ type through loss of the Z chromosome. In the genus *Litoria* showed nonsexual dimorphism in its sex chromosomes [9].

3.10 Chromosome evolution in amphibians

Chromosome evolution rates have been identified in several organisms, ranging from genetic reduced values, as is the case for amphibians to high values, as is mammals [37]. Amphibian genomes differ greatly in DNA content and chromosome size, morphology and number (Stephen et al., 2011). The Anura and Caudata are massively dichotomous in terms of chromosomal organization and chromosome morphology, yet in many respects they are convergent in chromosome form. Thus, the primitive Anurans of the Archaeobatrachia may have high chromosome numbers (Leiopelmatidae 2n=46) with many microchromosomes (although some species in the Discoglossidae, Pipidae and Pelobatoidae have a low diploid number with many metacentric elements), whereas advanced families from the Ranoidea, Microhyloidea or Bufonoidea have metacentric karyotypes with low diploid numbers (2n=30 to 2n=22). Of course such a generalization glosses over the particular families which show high levels of repatterning associated with speciation such as the Pipidae, Arthroleptinae, Astylosterninae, Hyperoliidae and Eleutherodactylidae [38].

An analogous situation is found in the Caudata. Here the Cryptobranchoidea possess karyotypes with high chromosome number and many microchromosomes (2n=60), whereas, the advanced Ambystomatoidea generally have symmetrical metacentric karyotypes with 2n=28 in which all species share the same basic karyomorph. Morescalchi (1975) [39] referred to this phenomenon as evolution towards a symmetrical karotype and it appears to have occurred in both the Anura and the Caudata, although with some noteable exceptions.

Many species of Apoda show high chromosome numbers and chromosome morphology in many respects similar to that of the primitive Anurans and Caudates, other species have a much lower number. Many of the preparations studied to date are of very poor quality and the status of the smaller chromosomes in the genomes is uncertain. Undoubtedly, the Apoda should be regarded as a high priority area for chromosomal research [6].

The reduction in chromosome number and the symmetrization of the karyotypes in both Anurans and Caudates is in some respects more apparent than real. While there is no doubt that karyotypes of the derived families have a low chromosome number and similar chromosome morphology, the internal complexity of the variation found within these genomes is quite striking and this takes three basic forms. First, C-banding variation between species showed that no two

species in the most closely related complexes which have been analysed have an identical chromosome banding pattern. Thus, comparison of species from the genus *Triturus*, *Litoria*, *Hydromantes*, *Bufo* and *Rana*, show striking interspecific variation in taxa with essentially the same, or very similar, gross karyotypes. Second, in those few species which have been analysed by molecular techniques, it is apparent that externally similar chromosome morphology and chromosome number may mask quite marked changes in genome organization. The land mark study by Mizuno and Macgregor (1974) [40] on plethodontid Salamanders, while in need of updating, shows a substantial amplification of genome size between species, particularly in the repetitive component of the genome, without any change in overall karyomorphology. Comparisons of chromosome morphology and genome size suggest that these phenomena may be a quite common event in Amphibians [41].

Third, a detailed comparison of karyotypes involving species which have the same chromosome number and morphology, and are thus reported to have the same karyomorph, reveal that the overall similarity is in many respects illusory. For example, compare the haploid karyotypes found in the Microhyloidea with the same chromosome number: Phrynomerinae, Cophylinae and Brevicipitinae. Here multiple pericentric inversions or centromeric shifts have played a major role in chromosomal repaterning and this has occurred both between individual species as well as between groups of species. Nevertheless, the complements within these subfamilies may look superficially similar [6].

It is quite clear that pericentric inversion has not been the sole basis for structural change in the amphibian complement, for chromosome number reduction requires the fusion or translocation of elements. Both chromosomal fusion and fission have also played a significant role in the evolution of anuran karyotypes, and both of these rearrangements are associated with presumptive speciation in many taxa [6].

In general, there has been a significant underestimation of chromosomal repatterning in Amphibians. Thus, theoretical studies on rates of chromosomal evolution which have used amphibian data based on external chromosome morphology and chromosome number alone are massive underestivations of the changes which have taken place. Subsequent studies which have examined the distribution of heterochromatin in Amphibians reveal a high level of genome

reorganization by the processes of pericentric inversion, addition of heterochromation and euchromatin transformation [19, 29, 5].

3.11 Phylogeny of *Litoria* species

The diagnosis and contents of *Litoria* were reviewed by Tyler and Davies (1978b) [9]. It is unclear whether any of the character states included in their extensive diagnosis is synapomorphic. However, considering subsequent comments by several authors [42, 43, 44, 45], the available evidence suggests that *Litoria* is paraphyletic with respect to the other genera of Pelodryadinae, *Nyctimystes*: This genus was rediagnosed by Tyler and Davies (1979) [44]. Among the list of characters provided by them, the synapomorphies of *Nyctimystes* seem to be the vertical pupil and the presence of palpebral venation. Tyler and Davies (1979) [44] suggested that *Nyctimystes* was most closely related to some species groups of *Litoria* from New Guinea, implying that *Nyctimystes* is nested within *Litoria*. Specifically, they referred to the *L. angiana*, *L. arfakiana*, *L. becki*, *L. dorsivena*, *L. eucnemis*, and *L. infrafrenata* groups as the most likely to be related to *Nyctimystes*, because they share with *Nyctimystes* similarities in cranial structure (the *L. infrafrenata* and *L. eucnemis* groups) or the presence of large unpigmented ova and lotic tadpoles bearing large, ventral, suctorial mouths (the other groups). Tyler (1972) [46] first proposed its relationship to Australian hylids on the basis of the presence of a differentiated apical element of the m. intermandibularis. Subsequently, Tyler (1978) transferred *Cyclorana* to Hylidae. Tyler (1979) [43], King et al. (1979) [42] and Tyler et al. (1981) [10] considered it to be related to the *Litoria aurea* group, a result that was coincident with the analyses of albumin immunological distances generated by microcomplement fixation [48]. Wiens et al. (2006, 2010) [49, 50] studied on 35 species of *Litoria* and divided into two clades. The first clade 21 species of *Litoria* (species groups follow Tyler and Davies (1978) [9] and Frost (2010) [51], including species of the *rubella* group, *peronii* group, *dorsalis* group, *beckii* group, *arfakiana* group, *thesaurensis* group, *bicolor* group, *booroolongensis* group, *latopalmata* group and *coplandi* group. The second clade was subdivided into two subcaldes. One subclade includes *L. infrafrenata* and the genus *Nyctimystes*. The other subclade includes 15 species of *Litoria* including species of the *citropa* group, *caerulea* group, *chloris* group, *eucnemis* group, *lesueurii* group, *nannotis* group and *aurea* group. The tree supports monophyly of these groups.

CHAPTER IV

MATERIALS AND METHODS

4.1 Sample collection

One hundred and seventy-eight specimens of *Litoria* species were collected from Northeastern Australia (Table 1). The mature animals were collected in 1988 by Prof. Dr. Michael Schmid. The chromosomes of tree frogs of *Litoria* were prepared at the Evolutionary Biology Unit of the South Australian Museum in Adelaide. The fixed material was transferred to 1.8 ml plastic tubes, stored at -20 ^{o}C and transported to the laboratory in Würzburg (Germany) packed in dry ice for chromosome banding. Although the time interval between chromosome fixation in Australia and banding analyses in Würzburg was as long as twenty years, the quality of the chromosome preparations as well as the banding patterns was not affected [41].

4.2 Mitotic chromosome preparation

This procedure was performed by Prof. Dr. Michael Schmid et al. at the Evolutionary Biology Unit of the South Australian Museum, Australia. The mitotic chromosomes were prepared directly from bone marrow cells after *in vivo* colchicines treatment. The preparation of cell suspension, hypotonic treatment and fixation of the cells followed the methods described by Schmid (1978a) [52] with slight modification described as follows.

4.2.1 The *Litoria* specimens were injected intraperitoneally with colchicine solution (3 mg/ml; Gibco) and left for 16 hours before being paralyzed with diethyl ether. The amount of colchicine solution injected varied from 0.2-1.0 ml, depending on the size of the animals.

4.2.2 The limb bones such as femur, tibiofibula and humerus were freed from the musculature with a scalpel and the cartilageneous epiphyses cut off.

4.2.3 The bone marrow was flushed out into a centrifuge tube with 8-10 ml of hypotonic KCl solution (0.075 M) using a fine hypodermic needle. In the bone cavities of many species, fat

deposits had accumulated; these clumps had to be removed after the flushing out procedure from the upper layers of the hypotonic solution.

4.2.4 The bone marrow was then vigorously resuspended with a Pasteur pipette, and afterwards incubated in the hypotonic KCl solution for 20 minutes at room temperature.

4.2.5 After hypotonic treatment, the cell suspension was centrifuged at 1,800 rpm for 8 minutes and fixed with 8 ml of fresh-cold Carnoy's solution (1:3 glacial acetic acid : absolute methanol). The first 1-2 ml of fixative was added as drop-wise under constant shaking by vortex mixer.

4.2.6 The fixed materials were washed twice with freshly prepared fixative. The cells were then resuspended in 1 ml fixative, and 3 drops of this suspension were dropped on slides previously rinsed with distilled water. The slides were dried on a hot plate at 90 $^{\circ}$C overnight. One animal was used to prepare 5-6 slides.

4.3 Chromosome staining

Each of the prepared chromosome slides was examined before being stained using 20x and 40x objective lenses of a phase contrast light microscope. Well spread and good quality metaphase cells were then stained first with Giemsa dye for investigation of the chromosome number and chromosome marker. Afterwards, the slides were treated by the various chromosome banding methods within 1-2 weeks.

4.4 Giemsa staining

Giemsa staining technique was used to uniformly stain chromosomes and leave the centromeres constricted, thus enabling the measurement of chromosome length, centromeric position and arm ratio.

The slides were stained for 6 minutes in 5% Giemsa solution. Then, the slides were rinsed thoroughly with running tap water to remove excess stain. Afterwards, the slides were allowed to air dry at room temperature.

4.5 C-banding

To specifically stain the centromeric regions and other regions containing constitutive heterochromatin, the C-banding technique was performed according to the method of Sumner (1972) [53].

The air dried slides were placed in 0.2 N HCl at room temperature for 30 minutes, and then rinsed with distilled water before being incubated for 5-10 minutes at 30°C in saturated Ba $(OH)_2$ solution. Subsequently, rinsed slides were placed gently in a Coplin jar filled with fresh 0.2 N HCl at room temperature, then rinsed with distilled water before being placed in 2XSSC at 60°C for 1.3 hours and finally rinsed with distilled water at room temperature. Eventually, the slides were stained with 10% Giemsa solution for 5-15 minutes, and then the slides were rinsed with running tap water to remove excess stain and allowed to air dry at room temperature.

4.6 NOR-staining

Chromosomes were treated with silver nitrate solution which binds to the Nucleolar Organizing Regions (NOR), i.e., the secondary constrictions (stalks) of acrocentric chromosomes. The technique employed was that described by Goodpasture and Bloom (1975) [20].

The slides were flooded with 50% $AgNO_3$ solution (about 2 drops for each slide) and gelatin solution (about 2 drops for each slide), and then covered with a cover glass. Afterwards, the slides were incubated for 2 hours at 60°C, then rinsed very rapidly with distilled water and air-dried. Finally, the slides were stained with 2% Giemsa solution for 30 seconds, rinsed with tap water, air-dried and observed under the microscope.

4.7 DAPI/Distamycin A staining

The DAPI/Distamycin A staining technique is useful in identifying pericentromeric breakpoints in chromosomal rearrangements and in identifying chromosomes that are too small for standard banding techniques. The DAPI/distamycin A fluorescent staining technique was performed according to the method of Schweizer (1976) [54].

The slides were flooded with distamycin solution (2-3 drops for each slide), covered with a cover glass, incubated at room temperature for 15 minutes, and then rinsed briefly with pH 7.0 McIlvaine's buffer. The slides were then flooded with DAPI working solution, covered with a cover glass, incubated at room temperature for 15 minutes, and then rinsed briefly with pH 7.0 McIlvaine's buffer, air-dried and observed with a fluorescence microscope.

4.8 Distamycin A/Mithramycin banding

The Distamycin A/Mithramycin banding specifically reveals the GC-rich constitutive heterochromatin. Mithramycin labels the nucleolus organizer region very brightly in the karyotypes of amphibians. The Distamycin A/Mithramycin fluorescent staining technique was performed according to the method of Schmid et al. (1988) [55].

The slides were flooded with distamycin solution (2-3 drops for each slide), covered with a cover glass, incubated at room temperature for 15 minutes, and then rinsed briefly with pH 7.0 McIlvaine's buffer. The slides were then flooded with Mithramycin working solution, covered with a cover glass, incubated at room temperature for 30 minutes, and then rinsed briefly with pH 7.0 McIlvaine's buffer, air-dried and observed with a fluorescence microscope.

4.9 Q-banding

Chromosomes were treated with quinacrine mustard solution, a fluorescent stain, to identify specific chromosomes and structural rearrangements. The Q-banding technique was performed according to the method of Schmid (1983) [25].

The slides were rinsed briefly with 100% ethanol, 70% ethanol and 30% ethanol, respectively, and then rinsed briefly with Quinacrine-Mustard solution. Then, the slides were rinsed briefly with pH 7.0 McIlvaine's buffer, air-dried and observed with a fluorescence microscope.

4.10 *In situ* hybridization experiments

Fluorescence *in situ* hybridization (FISH) should be able to provide information on the telomere length of individual chromosomes. Directly labeled oligonucleotide probes are attractive

probes for such analysis because of their small size (good penetration properties), single strand nature (no denaturation of probe) and controlled synthesis. The fluorescence in situ hybridization experiments was performed according to the method of Schmid et al. (2003) [41].

The slides were placed in TBS1 buffer at room temperature for 2 minutes and then placed in 3.7% formaldehyde at room temperature for 2 minutes. Then the slides were placed in TBS2 and TBS3 buffer at room temperature for 5 minutes, respectively and then the slides were placed in Pre-treatment solution at room temperature for 10 minutes. The slides were placed in TBS4 and TBS5 buffer at room temperature for 5 minutes, respectively. The slides were placed in cold 70% ethanol, 85% ethanol and 95% ethanol for 2 minutes, respectively. Then, add 7 μl of Telomere PNA probe/FITC to the marked area on the slides and covered with a cover glass, placed on a hot plate at 80°C for 5 minutes and covered by a rubber cement and incubated for 2 hours at room temperature in the dark room. The slides were rinsed very rapidly with Rinse solution for 1 minute and incubated in Wash solution at 65°C for 5 minutes. The slides were rinsed briefly with cold 70% ethanol, 85% ethanol and 95% ethanol, respectively and air dried. The slides were flooded with counterstaining and covered with a cover glass.

4.11 Photomicrography and analysis

All microscopic analyses were conducted on Zeiss photomicroscopes III and Zeiss fluorescence microcopes equipped with incident HBO 50W mercury lamp illumination. Specific quinacrine mustard, Hoechst 33258 and mithramycin fluorescence was selectively obtained by exciting with UV light in the 450-490 nm wavelength range (filter combination BP450-490/FT510/LP520). DAPI fluorescence was analyzed under excitation with 360-400 nm UV light (filter combination G365/FT395/LP420). All black and white photographs were taken with Agfaortho 25 ASA film, and some color photographs of the restriction endonuclease-banded metaphase were made with Kodak Ektachrome 160 ASA film. For each banding technique applied at least 10 metaphases were prepared from each animal which exhibited the greatest banding clarity, size uniformity and straightness for each species were observed for analysis of the chromosome number and the demonstration of secondary constriction of chromosome marker.

4.12 Idiogram construction and karyotyping

Photomicrographs of at least 10 well-spread metaphase chromosome sets, which had the best banding clarity, size uniformity and straightness, were selected for each species of amphibians for ideogram construction and karyotyping. In order to construct ideogram and karyotype, the lengths of the short and long arms (characterized by relative length of chromosome) and centromeric ratio were measured using a standard ruler. Relative length of chromosome is a percentage of the total length of the entire chromosome complement and is given by,

$$\text{Relative length of chromosome} = \frac{\text{chromosome length} \times 100}{\text{Total chromosome length}}$$

The longest chromosome of each ideogram was assigned on arbitrary value of 100 percent and the other chromosomes in the ideogram were assigned percentage values relative to the longest chromosome. The centromeric ratio or arm length ratio is determined from the ratio of the length of the long arm to the length of the short arm and is given by,

$$\text{Centromeric ratio} = \frac{\text{length of long arm}}{\text{length of short arm}}$$

The chromosome pairs from photomicrography prints were cut and arranged according to size in parallel rows and in order of decreasing mean length. The terminology for chromosome morphology follows that of Green and Sessions (1991) (appendix 3).

CHAPTER V

RESULTS

The one hundred and seventy-eight specimens of 19 species of *Litoria* tree frogs were caught and prepared mitotic chromosomes by Prof. Dr. Michael Schmid in 1988 (Figure 1 and Table 1). For this study the karyotype of 19 species of *Litoria* were analyzed following conventional staining, C-banding, Ag-NOR staining, DAPI/Distamycin A, DAPI/Mithramycin, Q-banding and Telomere FISH. Therefore, in some species of *Litoria* was not able to study and report on all banding techniques due to the limited amount of sample and can not add any more, so it makes some species has been reported only a few techniques. This study on the 19 species of *Litoria* tree frogs includes the 4 new species (*L. barringtonensis*, *L. genimaculata*, *L. nyakalensis* and *L. personata*) here studied and the other 15 species previously studied by Menzies and Tippett (1976) [13] and King et al. (1990) [14], showed a karyotype of 2n=26 except in *L. infrafrenata* which has 2n=24. In determining the arm ratios of the chromosomes in the present study the secondary constrictions had not been included in the measurements because of marked despiralization in some cases. Standard karyotypes, sizes and shapes of chromosomes and chromosome markers of each species were shown in Table 2 and were described below.

Mountain stream tree frog

Litoria barringtonensis (Copland, 1957)

Locality: Krombit and Cannondale, Qld.

Bar represents 1 cm.

Northern dwarf tree frog

Litoria bicolor (Gray, 1842)

Locality: Finnis River, Jabiru, N.T.

White's tree frog

Litoria caerulea (White, 1790)

Locality: Fannie Bay, Darwin, N.T.

Copland's rock frog

Litoria coplandi (Tyler, 1968)

Locality: Bowerbird, N.T.

Dahl's aquatic frog

Litoria dahlii (Boulenger, 1896)

Locality: Adelaide river flood plain, Aruhem Highway

Figure 1 Photographs of all 19 species of *Litoria* species collected for this study.

Fringed tree frog

Litoria eucnemis (Lõnnberg, 1900)

Locality: Mt. Lewis, Qld.

Eastern dwarf tree frog

Litoria fallax (Peters, 1880)

Locality: Cannondale, Qld.

New Guinea tree frog

Litoria genimaculata (Horst, 1883)

Locality: Mt. Lewis, Qld.

Giant tree frog

Litoria infrafrenata (Günther, 1867)

Locality: Tully, Qld.

Lesueur's frog

Litoria lesueurii (Dumeril and Bibron, 1841)

Locality: Millstream, Qld.

Figure 1 Photographs of all 19 species of *Litoria* species collected for this study (continued).

Rockhole frog

Litoria meiriana (Tyler, 1969)

Locality: Bowerbird and Ja Ja, N.T.

Waterfall frog

Litoria nannotis (Anderson, 1916)

Locality: Paluma, Qld.

Striped rocket frog

Litoria nasuta (Gray, 1842)

Locality: Jabiru, N.T.

Nyakala frog

Litoria nyakalensis Liem, 1974

Locality: Paluma, Qld.

Pale frog

Litoria pallida Davies, Martin, and Watson, 1983

Locality: Millstream, Qld.

Figure 1 Photographs of all 19 species of *Litoria* species collected for this study (continued).

Peron's tree frog

Litoria peronii (Tschudi, 1838)

Locality: Balranald, N.S.W. and Krombit, Qld.

Masked frog

Litoria personata Tyler, Davies, and Martin, 1978

Locality: Bowerbird, N.T.

Verreaux's tree frog

Litoria verreauxii (Dmeril, 1853)

Locality: Paluma, Qld.

Watjulum frog

Litoria watjulumensis (Copland, 1957)

Locality: Scotts Creek, N.T.

Figure 1 Photographs of all 19 species of *Litoria* species collected for this study (continued).

Table 1 Names, number of specimens and places of sample collection of all 19 species of *Litoria* species.

Species	No. of collected specimen		Localities
	Males	**Females**	
1. Moutain stream tree frog (*Litoria barringtonensis)*	14	2	Krombit and Cannondale, Qld.
2. Northerm dwarf tree frog (*L. bicolor)*	8	10	Finnis River, Jabiru, N.T.
3. White's tree frog (*L. caerulea*)	3	9	Fannie Bay, Darwin, N.T.
4. Copland's rock frog (*L. coplandi*)	6	4	Bowerbird, N.T.
5. Dahl's aquatic frog (*L. dahlia*)	-	2	Adelaide river flood plain, Aruhem Highway
6. Fringed tree frog (*Litoria eucnemis*)	2	-	Mt. Lewis, Qld.
7. Eastern dwarf tree frog (*L. fallax*)	12	7	Cannondale, Qld.
8. New Guinea tree frog (*L. genimaculata*)	19	1	Mt. Lewis, Qld.
9. Giant tree frog (*L. infrafrenata*)	2	2	Tully, Qld.
10. Lesueur's frog (*L. lesueurii*)	11	1	Millstream, Qld.
11. Rockhole frog (*L. meiriana*)	8	2	Bowerbird and Ja Ja, N.T.

Table 1 Names, number of specimens and places of sample collection of all 19 species of *Litoria* species (continued).

Species	No. of collected specimen		Localities
	Males	**Females**	
12. Waterfall frog (*L. nannotis*)	5	2	Paluma, Qld.
13. Striped rocket frog (*L. nasuta*)	1	5	Jabiru, N.T.
14. Nyakala frog (*L. nyakalensis*)	4	-	Paluma, Qld.
15. Pale frog (*L. pallida*)	5	1	Millstream, Qld.
16. Person's tree frog (*L. peronii*)	5	4	Balranald, N.S. W. and Krombit, Qld.
17. Masked frog (*L. personata*)	-	2	Bowerbird, N.T.
18. Verreaux's tree frog (*L. verreauxii*)	-	1	Paluma, Qld.
19. Watjulum frog (*L. watjulumensis*)	8	7	Scotts Creek, N.T.

Table 2 Diploid chromosome numbers, chromosome markers and mitotic karyotypes of the 19 species of *Litoria* species.

Species	2n	NOR	m	sm	st
1. *L. barringtonensis*	26	11q	1, 3, 4, 9, 10, 13	2, 6, 7, 11, 12	5, 8
2. *L. bicolor*	26	11q	1, 4, 8, 10, 11, 12, 13	2, 6, 7, 9,	3, 5
3. *L. caerulea*	26	11q	1, 4, 10, 11, 12, 13	2, 6, 7, 8, 9	3, 5
4. *L. coplandi*	26	12q	1, 4, 9, 10, 13	2, 6, 7, 8, 11, 12	3, 5
5. *L. dahlii*	26	11q	1, 4, 7, 13	2, 6, 8, 9, 10, 12	3, 5
6. *L. eucnemis*	26	7p	1, 4, 7, 12, 13	2, 6, 8, 9, 10, 11	3, 5
7. *L. fallax*	26	11q	1, 4, 8, 9, 10, 12, 13	2, 6, 7, 11	3, 5
8. *L. genimaculata*	26	7p	1, 4, 12, 13	2, 6, 7, 8, 9, 10, 11	3, 5
9. *L. infrafrenata*	24	5p	1, 4, 10, 12	2, 6, 7, 9, 11, 13	3, 5, 8
10. *L. lesueurii*	26	11q	1, 4, 8, 10, 13	2, 6, 7, 9, 11, 12	3, 5
11. *L. meiriana*	26	12q	1, 4, 8, 9, 11, 13	2, 6, 10, 12	3, 5, 7
12. *L. nannotis*	26	11q	1, 4, 9, 10, 11, 12, 13	2, 6, 7, 8	3, 5
13. *L. nasuta*	26	11q	1, 4, 8, 10, 11, 13	2, 6, 7, 9, 12	3, 5
14. *L. nyakalensis*	26	11q	1, 4, 11, 12, 13	2, 6, 7, 8, 9, 10	3, 5
15. *L. pallida*	26	12q	1, 4, 8, 9, 10, 11, 13	2, 6, 7, 8, 12	3, 5
16. *L. peronii*	26	11q	1, 4, 12, 13	2, 6, 7, 9, 10, 11	3, 5, 8
17. *L. personata*	26	8q	1, 4, 10, 11, 12, 13	2, 6, 7, 8	3, 5
18. *L. verreauxii*	26	1p	1, 4, 9, 11, 12, 13	2, 6, 7, 8, 10	3, 5
19. *L. watjulumensis*	26	11q	1, 4, 8, 9, 11, 12, 13	2, 6, 7, 10	3, 5

Remarks: m=metacentric; sm=submetacentric; st=subtelocentric; p=short arm; q=long arm.

Table 3 Chromosome number (1-13), relative length (RL) and centromeric ratio (CR) with standard variation (±SD) of 19 species of *Litoria* species.

m = metacentric (CR= 1.00-1.67); sm=submetacentric (1.68-3.00) and st=acrocentric (3.01-7.00). * Chromosome showing a secondary constriction.

Name		1	2	3	4	5	6	7	8	9	10	11	12	13
1. *L. barringtonensis*	RL	14.20±0.10	13.85±0.12	12.38±0.04	11.55±0.14	9.48±0.17	7.94±0.10	6.34±0.13	5.44±0.05	4.76±0.07	4.34±0.02	3.35±0.08	3.29±0.10	3.21±0.04
	CR	1.60±0.26	1.69±0.06	1.60±0.21	1.08±0.10	3.36±0.10	2.36±0.14	2.51±0.33	3.97±0.13	1.31±0.06	1.58±0.09	1.13±0.21	1.71±0.13	1.89±0.20
	Type	m	sm	m	m	st	sm	sm	st	m	m	sm	sm	m
2. *L. bicolor*	RL	13.80±0.23	12.53±0.43	11.86±0.01	9.62±0.10	9.16±0.27	7.84±0.32	6.59±0.38	5.84±0.38	5.67±0.07	5.28±0.06	4.32±0.08	4.02±0.18	3.91±0.15
	CR	1.53±0.29	1.93±0.14	3.74±0.31	1.17±0.18	3.42±0.35	1.80±0.20	2.07±0.41	1.62±0.10	2.30±0.15	1.49±0.02	1.38±0.03	1.56±0.03	1.63±0.05
	Type	m	sm	st	m	st	sm	sm	m	sm	m	m	m	m
3. *L. caerulea*	RL	14.24±0.06	12.79±0.05	11.31±0.08	9.57±0.53	7.98±0.04	7.61±0.18	7.37±0.34	6.78±0.10	5.36±0.19	5.00±0.12	4.39±0.12	4.00±0.25	3.54±0.19
	CR	1.53±0.27	1.71±0.02	3.29±0.15	1.10±0.12	3.03±0.10	2.21±0.30	2.14±0.25	2.07±0.01	1.43±0.27	1.58±0.03	1.78±0.03	2.44±0.06	1.62±0.14
	Type	m	sm	st	m	st	sm	sm	sm	m	m	sm	sm	m
4. *L. coplandi*	RL	13.60±0.14	12.53±0.07	11.75±0.01	10.63±0.06	9.45±0.28	8.09±0.43	7.23±0.38	6.68±0.06	5.87±0.07	5.48±0.02	4.51±0.17	4.22±0.02	3.79±0.06
	CR	1.38±0.08	1.90±0.05	3.77±0.12	1.30±0.03	3.57±0.08	1.78±0.06	2.07±0.07	1.99±0.05	1.31±0.05	1.57±0.15	1.95±0.05	2.53±0.04	1.60±0.02
	Type	m	sm	st	m	st	sm	sm	sm	m	sm	sm	sm	m
5. *L. dahlii*	RL	13.17±0.14	12.43±0.43	11.18±0.10	10.81±0.26	9.78±0.06	9.11±0.12	7.48±0.06	5.66±0.05	4.98±0.04	4.23±0.05	3.94±0.13	3.57±0.14	3.34±0.21
	CR	1.58±0.05	1.94±0.08	3.55±0.07	1.51±0.05	3.15±0.23	2.71±0.03	1.66±0.08	1.97±0.04	2.4±0.07	2.51±0.17	1.85±0.19	2.63±0.06	1.58±0.13
	Type	m	sm	st	m	st	sm	m	sm	sm	sm	sm	sm	m
6. *L. eucnemis*	RL	13.37±0.21	12.42±0.10	11.16±0.11	10.47±0.06	9.50±0.05	8.03±0.30	7.05±0.35	6.36±0.08	5.45±0.16	5.07±0.05	3.73±0.11	3.32±0.12	3.05±0.28
	CR	1.42±0.04	2.55±0.08	3.55±0.07	1.33±0.17	3.00±0.03	2.01±0.10	1.33±0.06	1.79±0.04	1.68±0.03	1.70±0.04	2.44±0.10	1.62±0.09	1.67±0.02
	Type	m	sm	st	m	st	sm	m	sm	sm	sm	sm	m	m
7. *L. fallax*	RL	13.96±0.04	13.04±0.37	12.60±0.09	11.75±0.10	9.68±0.13	8.40±0.24	6.12±0.14	5.87±0.16	5.11±0.07	4.15±0.05	3.88±0.16	3.35±0.30	13.85±0.12
	CR	1.56±0.25	1.86±0.04	3.44±0.10	1.17±0.08	3.84±0.04	2.11±0.12	2.03±0.06	1.64±0.02	1.48±0.04	1.24±0.19	1.89±0.22	1.42±0.13	1.67±0.06
	Type	m	sm	st	m	st	sm	sm	m	m	m	sm	m	m

Table 3 Chromosome number (1-13), relative length (RL) and centromeric ratio (CR) with standard variation (±SD) of 19 species of *Litoria* species. m = metacentric (CR= 1.00-1.67); sm=submetacentric (1.68-3.00) and st=acrocentric (3.01-7.00). * Chromosome showing a secondary constriction (continued).

Name		1	2	3	4	5	6	7	8	9	10	11	12	13
8.	RL	14.15±0.78	11.73±0.15	11.23±0.12	9.77±0.57	8.57±0.42	7.92±0.28	7.46±0.53	6.15±0.07	5.79±0.13	5.20±0.02	4.28±0.04	3.81±0.06	3.34±0.09
L. genimaculata	CR	1.44±0.04	1.69±0.06	3.37±0.42	1.32±0.12	3.61±0.12	1.98±0.14	1.95±0.05	2.11±0.05	1.88±0.02	2.52±0.01	2.25±0.14	1.33±0.35	1.40±0.71
	Type	m	sm	st	m	st	sm	sm	sm	sm	sm	sm	m	m
9.	RL	14.92±0.02	11.88±0.55	10.78±0.32	10.62±0.09	9.41±0.23	8.72±0.25	7.80±0.31	6.57±0.05	5.89±0.04	5.40±0.22	4.12±0.07	3.92±0.18	
L. infrafrenata	CR	1.64±0.56	2.62±0.14	3.34±0.09	1.18±0.06	3.67±0.03	2.08±0.30	2.19±0.10	3.85±0.03	2.42±0.05	1.22±0.06	2.17±0.28	1.57±0.24	
	Type	m	sm	st	m	st	sm	sm	st	sm	m	sm	m	
10.	RL	13.30±0.03	11.95±0.65	10.80±0.34	10.44±0.07	8.98±0.24	8.11±0.15	7.17±0.43	6.77±0.04	5.68±0.05	5.44±0.02	4.27±0.07	3.83±0.26	3.35±0.12
L. lesueurii	CR	1.59±0.05	2.54±0.11	3.34±0.09	1.25±0.03	3.64±0.04	2.11±0.06	1.97±0.08	1.51±0.02	2.47±0.04	1.58±0.06	2.36±0.03	2.41±0.19	1.48±0.13
	Type	m	sm	st	m	st	sm	sm	m	sm	m	sm	sm	m
11.	RL	13.84±0.13	12.98±0.08	12.00±0.11	11.06±0.32	9.79±0.19	8.83±0.32	7.01±0.17	5.91±0.07	4.97±0.05	4.36±0.02	3.71±0.13	3.32±0.30	2.78±0.27
L. meiriana	CR	1.45±0.27	2.59±0.05	3.94±0.02	1.21±0.12	3.73±0.10	1.97±0.08	2.43±0.06	1.42±0.15	1.46±0.03	2.58±0.09	1.48±0.10	2.35±0.12	1.48±0.08
	Type	m	sm	st	m	st	sm	sm	m	m	sm	m	sm	m
12.	RL	14.93±0.11	12.13±0.26	10.81±0.57	10.15±0.19	8.39±0.18	8.00±0.14	7.10±0.07	6.00±0.14	5.62±0.12	5.04±0.05	4.40±0.22	4.06±0.04	3.53±0.08
L. nannotis	CR	1.62±0.05	2.24±0.10	3.74±0.06	1.64±0.08	3.67±0.07	1.73±0.11	2.40±0.06	2.66±0.02	1.62±0.04	1.49±0.14	1.64±0.04	1.56±0.03	1.62±0.14
	Type	m	sm	st	m	st	sm	sm	sm	m	m	m	m	m
13. *L. nasuta*	RL	15.33±0.04	12.23±0.40	11.61±0.15	10.72±0.09	9.04±0.14	8.19±0.06	7.16±0.17	6.02±0.22	5.38±0.19	4.58±0.02	3.77±0.12	3.40±0.27	2.89±0.25
	CR	1.65±0.07	1.87±0.09	3.70±0.04	1.18±0.05	3.81±0.04	2.46±0.04	2.38±0.06	1.53±0.20	1.75±0.29	1.65±0.16	1.46±0.15	2.41±0.08	1.52±0.03
	Type	m	sm	st	m	st	sm	sm	m	sm	m	m	sm	m

Table 3 Chromosome number (1-13), relative length (RL) and centromeric ratio (CR) with standard variation (±SD) of 19 species of *Litoria* species. m = metacentric (CR= 1.00-1.67); sm=submetacentric (1.68-3.00) and st=acrocentric (3.01-7.00). * Chromosome showing a secondary constriction (continued).

Name		1	2	3	4	5	6	7	8	9	10	11	12	13
14.	RL	14.26±0.82	12.10±0.16	10.67±0.02	10.44±0.10	8.71±0.15	7.87±0.15	6.97±0.18	6.17±0.08	5.28±0.11	5.15±0.12	4.91±0.01	4.15±0.16	3.73±0.17
L. nyakalensis	CR	1.55±0.12	2.00±0.08	3.06±0.01	1.53±0.05	3.08±0.05	2.05±0.05	2.04±0.04	2.01±0.01	1.69±0.01	2.43±0.16	1.41±0.04	1.65±0.08	1.47±0.01
	Type	m	sm	st	m	st	sm	sm	sm	sm	sm	m	m	m
15. *L. pallida*	RL	13.92±0.07	12.20±0.31	11.01±0.17	10.40±0.06	9.18±0.27	8.09±0.15	7.05±0.16	6.13±0.15	5.55±0.08	4.94±0.06	4.10±0.21	3.90±0.22	3.47±0.10
	CR	1.65±0.26	2.15±0.09	3.01±0.25	1.30±0.38	3.53±0.03	1.71±0.07	1.89±0.06	1.65±0.27	1.28±0.38	1.45±0.35	1.62±0.27	1.90±0.02	1.41±0.13
	Type	m	sm	st	m	st	sm	sm	m	m	m	m	sm	m
16. *L. peronii*	RL	14.90±0.08	12.68±0.34	12.40±0.49	11.65±0.08	9.22±0.15	7.76±0.14	6.57±0.17	5.95±0.12	5.45±0.03	4.90±0.06	4.26±0.04	3.79±0.20	3.24±0.22
	CR	1.60±0.05	2.09±0.68	3.59±0.12	1.42±0.03	3.15±0.03	1.75±0.02	2.46±0.04	3.64±0.03	2.62±0.30	2.55±0.16	1.83±0.09	1.53±0.16	1.56±0.12
	Type	m	sm	st	m	st	sm	sm	st	sm	sm	sm	m	m
17.	RL	15.18±0.28	12.47±0.03	11.61±0.02	10.18±0.07	8.31±0.02	7.39±0.02	6.20±0.01	5.84±0.06	5.49±0.01	5.16±0.04	4.80±0.02	4.49±0.12	3.57±0.07
L. personata	CR	1.46±0.02	2.12±0.01	3.15±0.02	1.45±0.07	3.39±0.08	2.14±0.01	1.88±0.01	1.85±0.01	2.55±0.10	1.48±0.01	1.36±0.01	1.16±0.01	1.13±0.01
	Type	m	sm	st	m	st	sm	sm	sm	sm	m	m	m	m
18.	RL	14.50±0.20	11.43±0.48	9.97±0.21	9.76±0.09	9,14±0.45	7.61±0.59	7.34±0.12	6.11±0.14	5.88±0.11	5.25±0.18	4.34±0.05	3.94±0.18	3.77±0.14
L. verreauxii	CR	1.27±0.30	1.84±0.14	3.11±0.14	1.19±0.11	3.27±0.36	1.80±0.33	2.65±0.07	2.22±0.26	1.26±0.05	2.29±0.04	1.32±0.04	1.14±0.02	1.18±0.20
	Type	m	sm	st	m	st	sm	sm	sm	m	sm	m	m	m
19.	RL	14.71±0.13	11.89±0.30	10.88±0.53	10.13±0.06	8.79±0.07	8,02±0.28	7.05±0.07	6.24±0.13	5.58±0.09	5.15±0.01	4.48±0.05	4.04±0.33	3.49±0.20
L. watjulumensis	CR	1.66±0.04	2.11±0.14	3.67±0.08	1.29±0.08	3.75±0.06	2.04±0.10	2.35±0.26	1.60±0.30	1.56±0.14	2.43±0.04	1.30±0.12	1.29±0.30	1.45±0.13
	Type	m	sm	st	m	st	sm	sm	m	m	sm	m	m	m

5.1 Mitotic karyotypes and chromosomes banding of 19 species of *Litoria* tree frogs

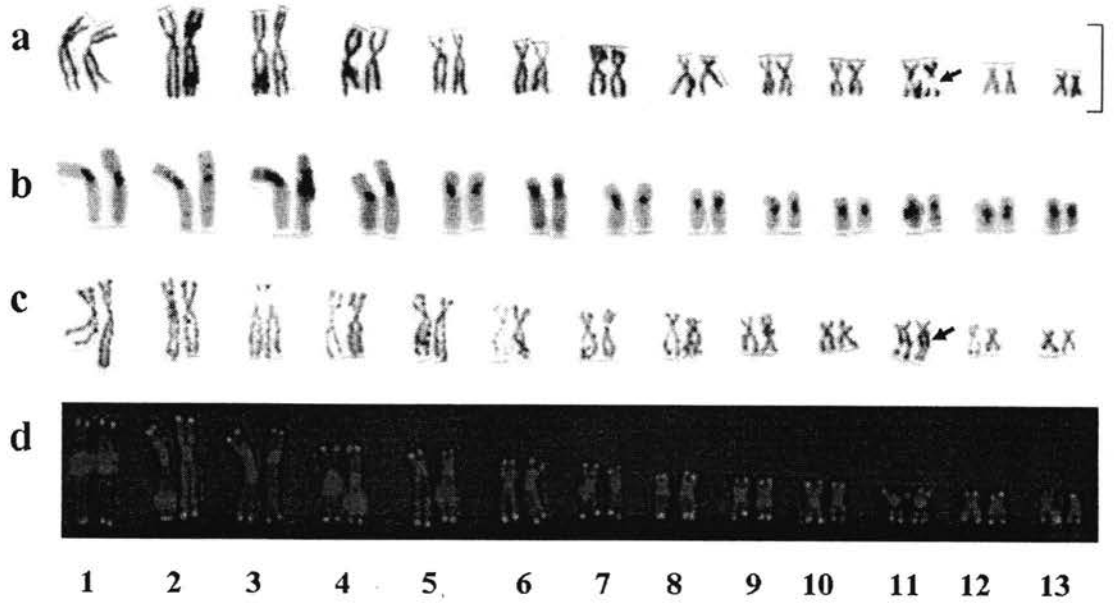
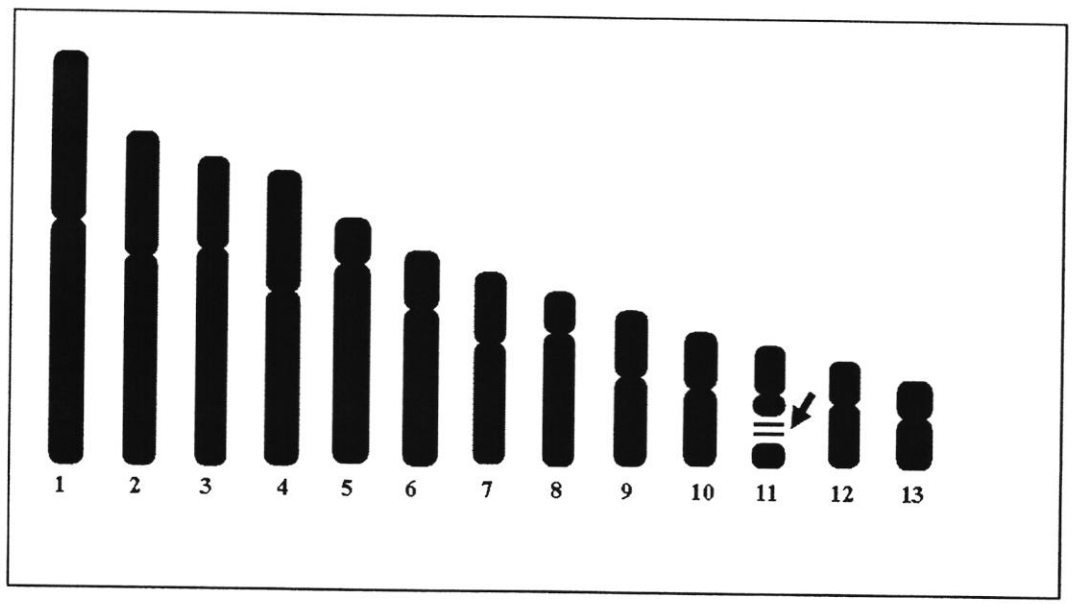


Figure 2 Karyotypes of *L. barringtonensis* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions and (d) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.

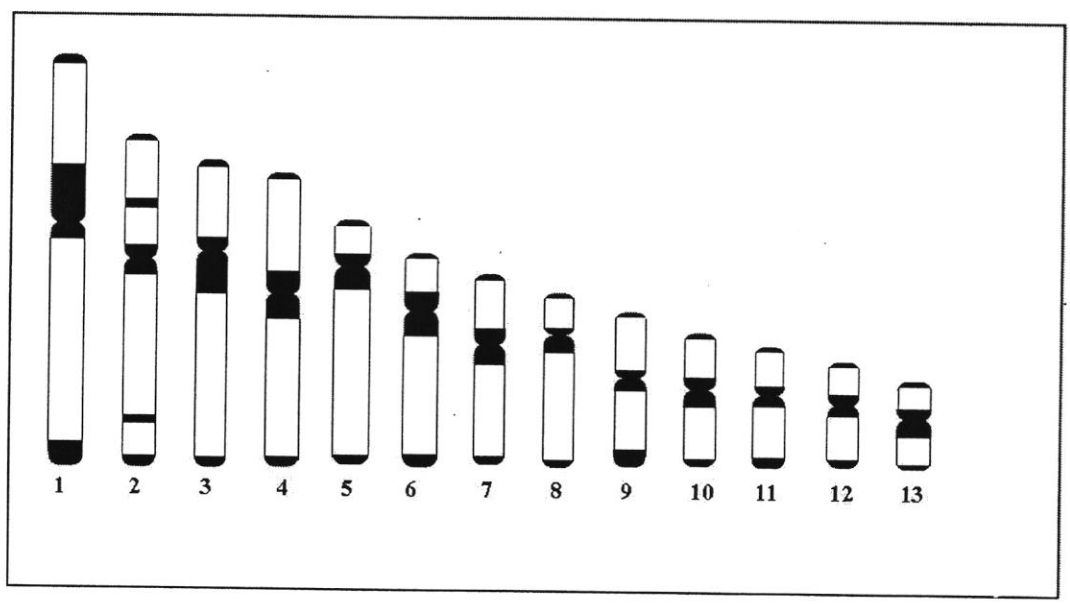
5.1.1 *L. barringtonensis*

All specimens of *L. barringtonensis* showed 13 pairs of chromosomes. Pairs 1, 3, 4, 9, 10 and 13 were metacentric, pairs 2, 6, 7, 11 and 12 were submetacentric and pairs 5 and 8 were subtelocentric. Chromosome pair 11 (11q) showed a secondary constriction near the centromeric region (Figure 2a). Figure 3 (a) showed the idiogram by conventional staining.

In the C-banded karyotype of *L. barringtonensis,* constitutive heterochromatin can be discerned mainly in the centromeric and telomeric regions of all chromosomes. Interstitial C-band was demonstrated close to the centromeric regions in the short arm of pairs 2 and 3 and the telomeric region of the long arm of the chromosome pair 2 shown large double barred grey C-band (Figure 2b). Figure 3 (b) showed the idiogram from C-banding.



(a)



(b)

Figure 3 Idiogram of *L. barringtonensis* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

Silver staining showed that in all *L. barringtonensis* analyzed the nucleolus organizer region was in the subcentromeric region of chromosome pair 11 (Figure 2c). No positive silver labeling was visible in the other chromosomes.

In the fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$,

oligomers demonstrated distinct telomeric labeling signals in all chromosomes of *L. barringtonensis* (Figure 2d). No interstitial hybridization signals were detected.

5.1.2 *L. bicolor*

The karyotype of *L. bicolor* consisted of 2n=26 chromosomes which can be arranged in 13 homologous pairs. Pairs 1, 4, 8, 10, 11, 12 and 13 were metacentric, pairs 2, 6, 7 and 9 were submetacentric and pairs 3 and 5 were subtelocentric (Figure 4a). Figure 5 (a) showed the idiogram of *L. bicolor* from conventional staining.

The C-banded karyotypes of *L. bicolor* exhibited constitutive heterochromatin in the centromeric. A large amount of constitutive heterochromatin was located on the long arm close the centromeric region of pair 11 where the nucleolus organizer region was located. Interstitial C-band was demonstrated on the chromosome pair 4 and shown the procentric bands on the chromosome pairs 1, 6, 8 and 9. The telomeric region of the long arm of the chromosome pair 2 presented a large double barred grey C-band (Figure 4b). The idiogram of *L. bicolor* by C-banding shown on Figure 5 (b).

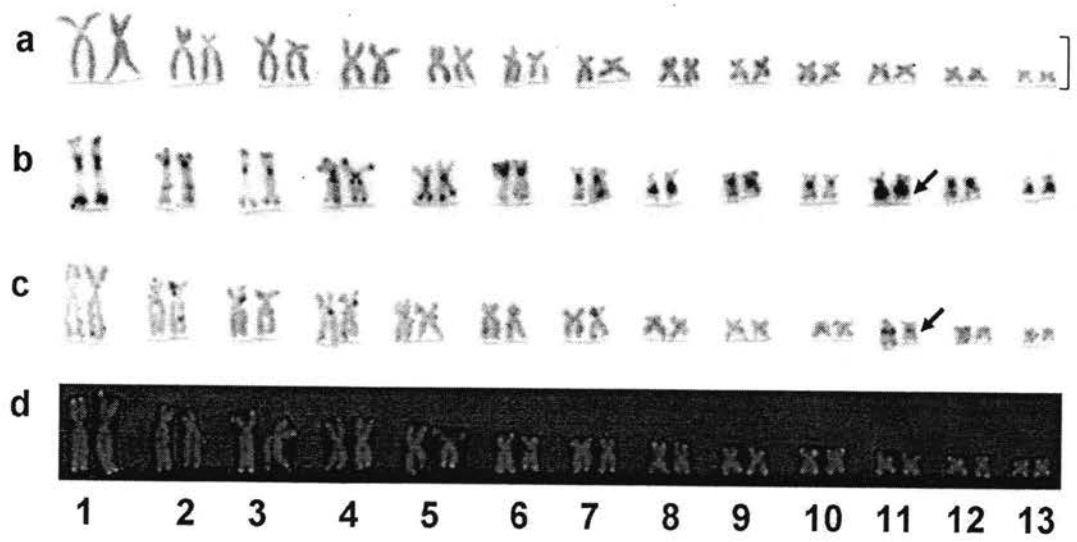
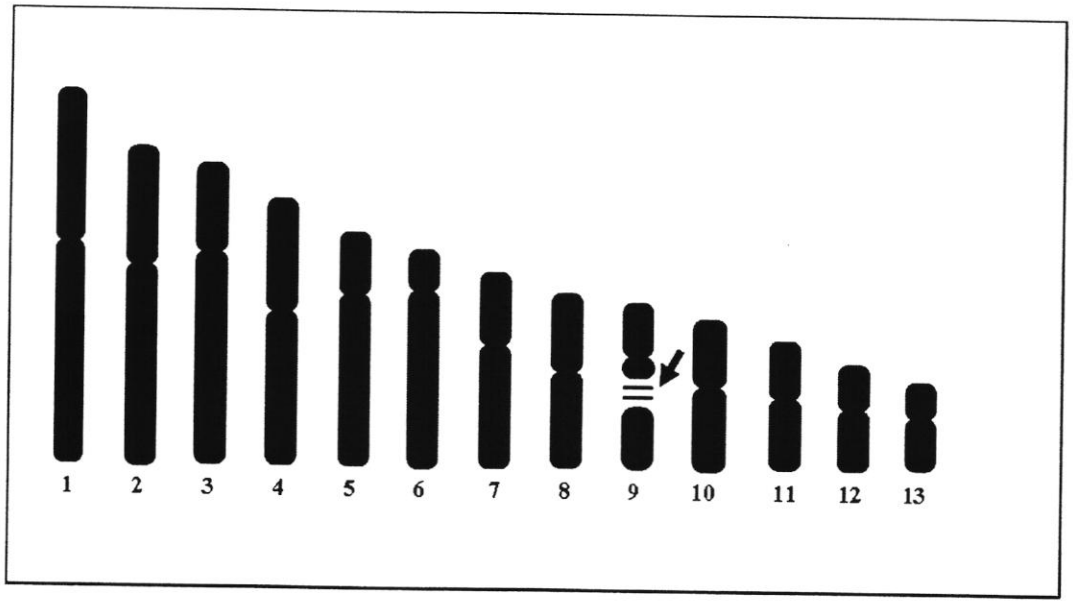
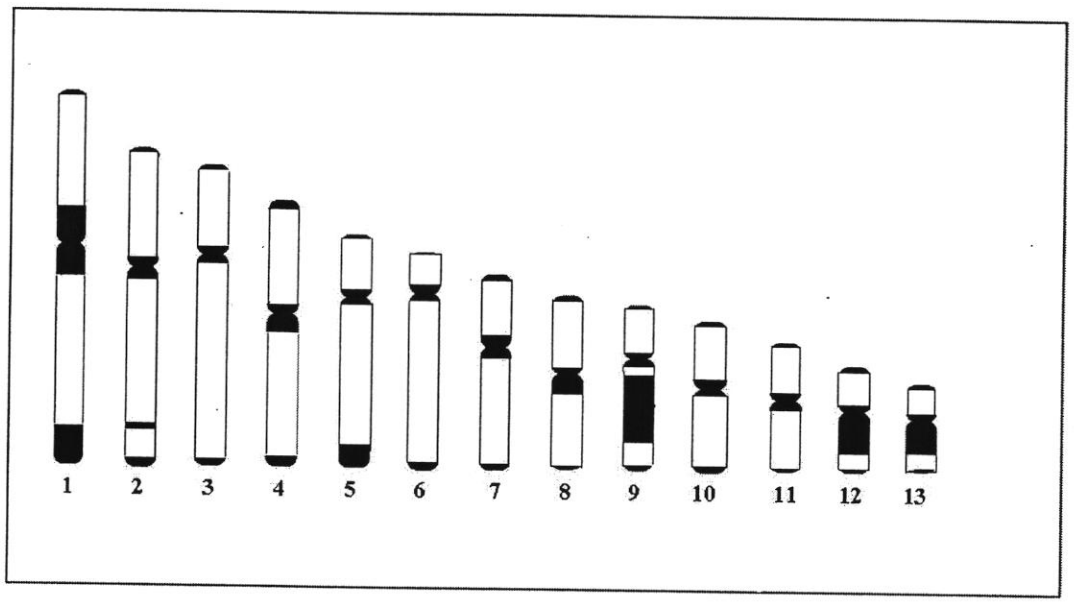


Figure 4 Karyotypes of *L. bicolor* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions and (d) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.



(a)



(b)

Figure 5 Idiogram of *L. bicolor* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

Silver staining showed that in all *L. bicolor* analyzed, the nucleolus organizer region was in the centromeric region of the chromosome pair 11 (Figure 4c). No positive silver labeling was visible in the other chromosomes.

As expected, fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers demonstrated distinct telomeric labeling signals in all chromosomes of *L. bicolor* (Figure 4d). No interstitial hybridization signals were detected.

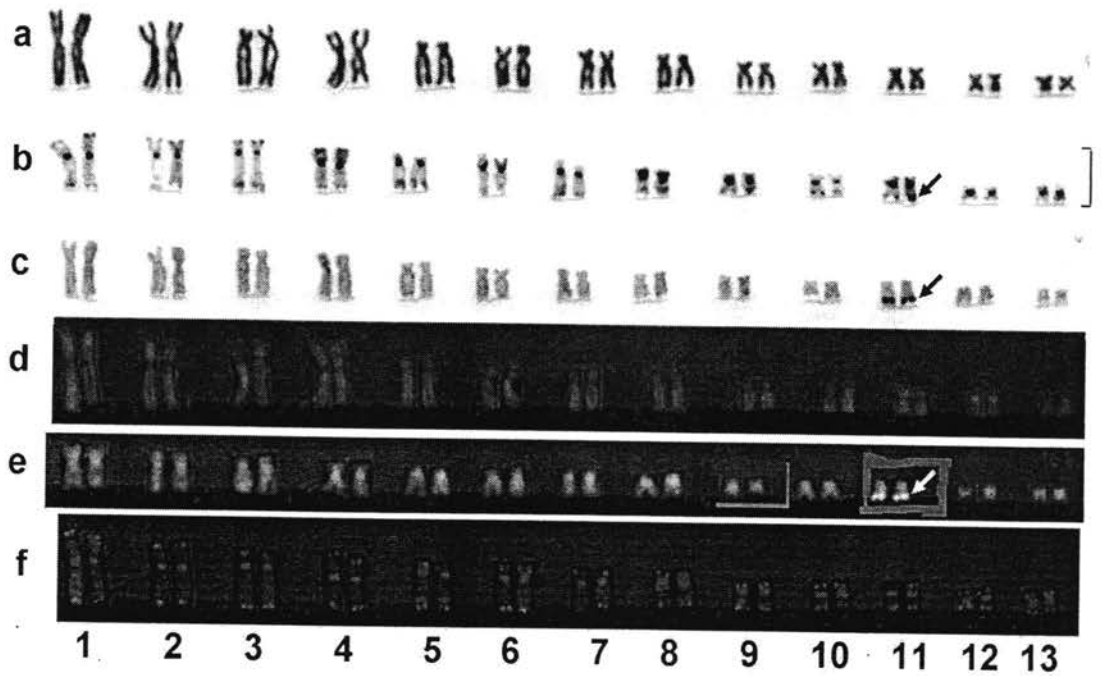


Figure 6 Karyotypes of *L. caerulea* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions, (d) distamycin A/DAPI counterstaining, (e) distamycin A/mithramycin counterstaining and (f) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.

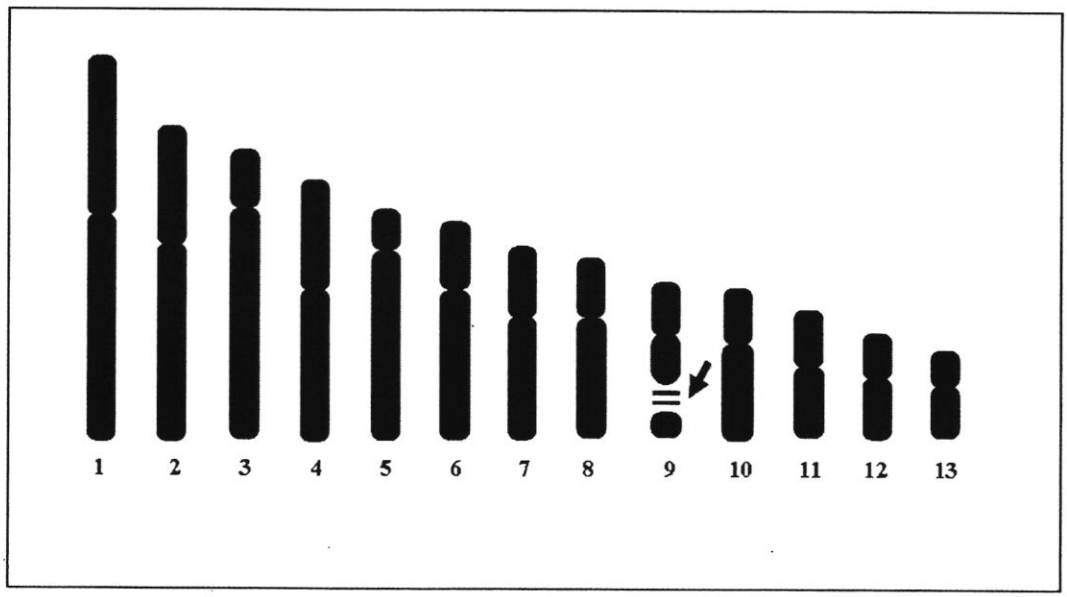
5.1.3 *L. caerulea*

All specimens of *L. caerulea* examined have 2n=26 chromosomes which can be arranged in 13 pairs. Pairs 1, 4, 10, 11, 12 and 13 were metacentric, pairs 2, 6, 7, 8 and 9 were submetacentric and pairs 3 and 5 were subtelocentric (Figure 6a). Figure 7 (a) showed the idiogram of *L. caerulea* from conventional staining.

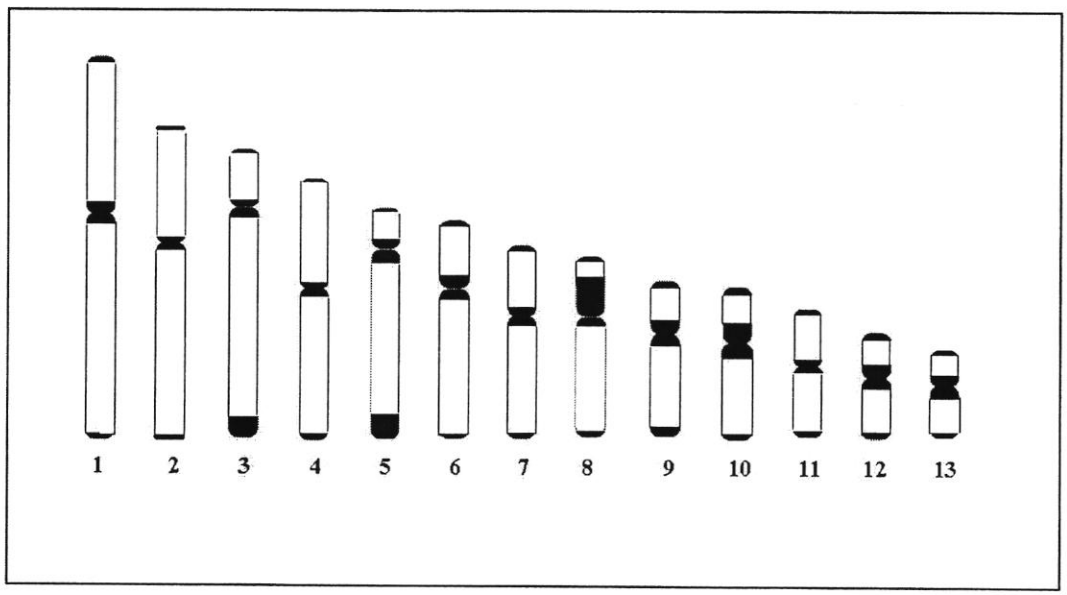
The C-banded karyotypes of *L. caerulea* constitutive heterochromatin can be discerned mainly in the centromeric and telomeric regions of all chromosomes. Procentric C-bands shown on the chromosome pairs 8, 9, 10, 12 and 13 and the large double barred grey

C-band were presented on the long arm of pair 3 (Figure 6b). Figure 7 (b) showed the idiogram of *L. caerulea* by C-banding.

Silver staining showed that in all *L. caerulea* analyzed the nucleolus organizer region was in the subtelomeric region of the chromosome pair 11 (Figure 4c). No positive silver labeling was visible in the other chromosomes.



(a)



(b)

Figure 7 Idiogram of *L. caerulea* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

In the distamycin A/DAPI counter stained metaphase of *L. caerulea*, the karyotypes showed a uniform fluorescence (Figure 6d).

In the mithramycin-stained metaphase, mithramycin induces banding patterns in the *L. caerulea* karyotype which the opposite was obtained by quinacrine mustard. Thus, the centromeric and telomeric heterochromatin in chromosomes 1-13 were mithramycin-positive.

The NOR, located close to the telomere in the long arm of chromosome pair 9 presented the brightest mithramycin fluorescence in the karyotype (Figure 6e).

Fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers demonstrated distinct telomeric labeling signals in all chromosomes of *L. caerulea* (Figure 6f). No interstitial hybridization signals were detected.

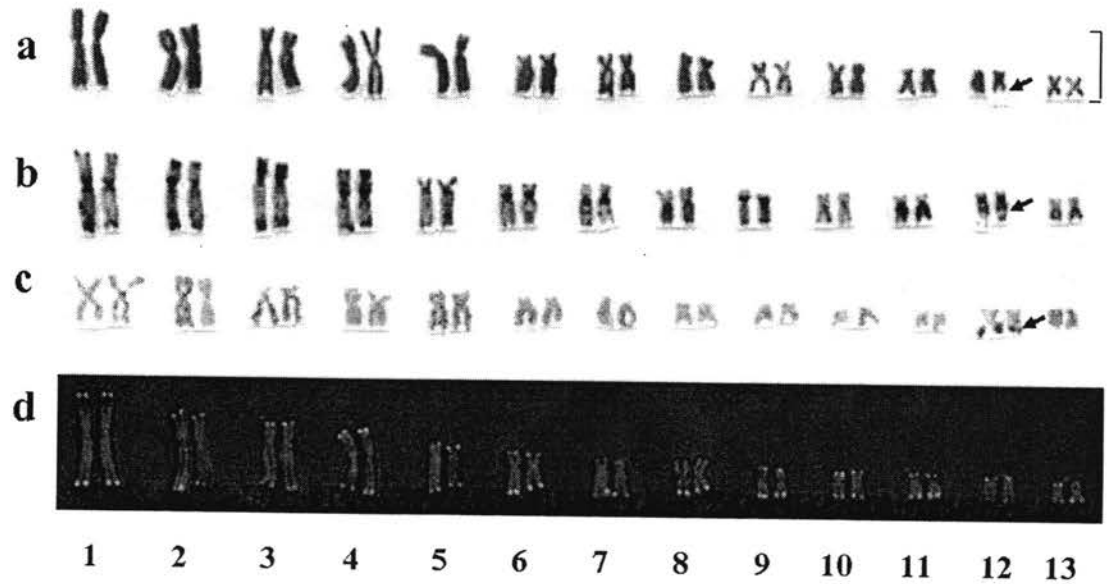
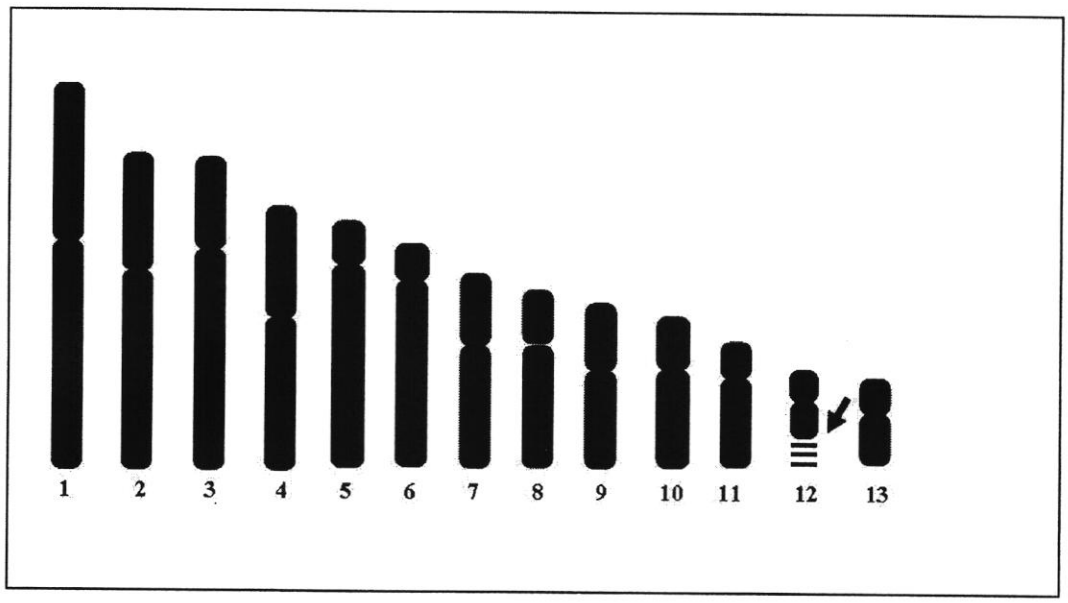


Figure 8 Karyotypes of *L. coplandi* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions and (d) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.

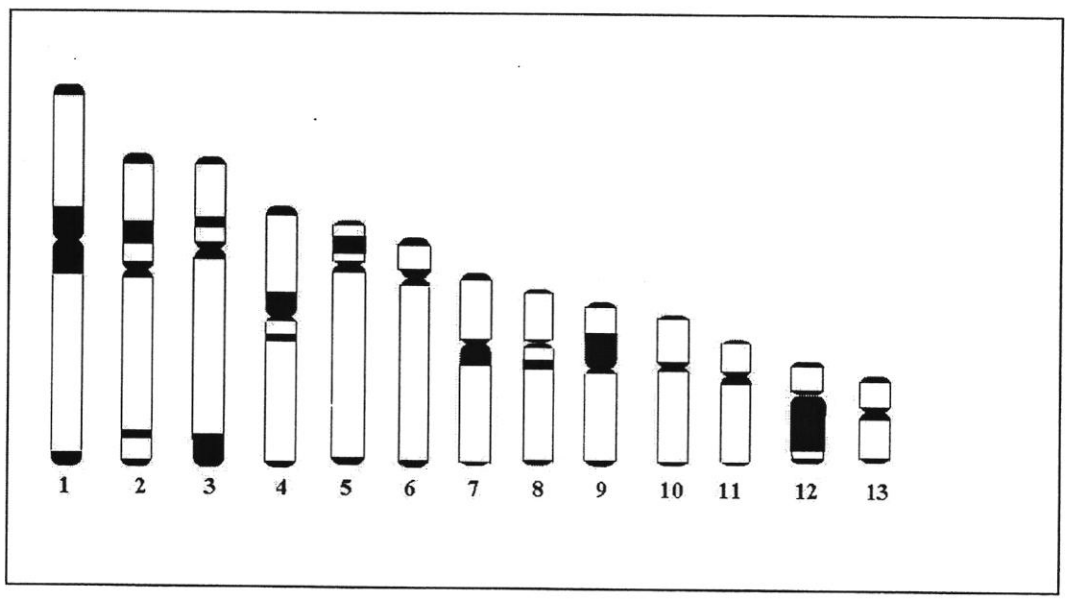
5.1.4 *L. coplandi*

All specimens of *L. coplandi* showed 2n=26 chromosomes which can be arranged in 13 pairs. Pairs 1, 4, 9, 10 and 13 were metacentric, pairs 2, 6, 7, 8, 11 and 12 were submetacentric and pairs 3 and 5 were subtelocentric. Chromosome pair number 12 (12q) showed

a secondary constriction (Figure 8a). Figure 9 (a) showed the idiogram of *L. coplandi* from conventional staining.



(a)



(b)

Figure 9 Idiogram of *L. coplandi* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

The C-banded karyotypes of *L. coplandi*, constitutive heterochromatin can be discerned mainly in the centromeric and telomeric regions of all chromosomes. Interstitial

C-bands were visible in the pericentromeric regions of chromosomes pairs 1 and 4 and the large double barred grey C-band were presented on the chromosome pairs 2, 3 and 5. Large amounts of constitutive heterochromatin was located in the long arm close the centromeric region of pair 12 where the nucleolus organizer region was located (Figure 8b).

Silver staining showed that in all *L. coplandi* analyzed, the nucleolus organizer region was in the the subtelomeric region of pair 12 (Figure 8c). No positive silver labeling was visible in the other chromosomes.

As expected, fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers demonstrates distinct telomeric labeling signals in all chromosomes of *L. coplandi* (Figure 8d). No interstitial hybridization signals were detected.

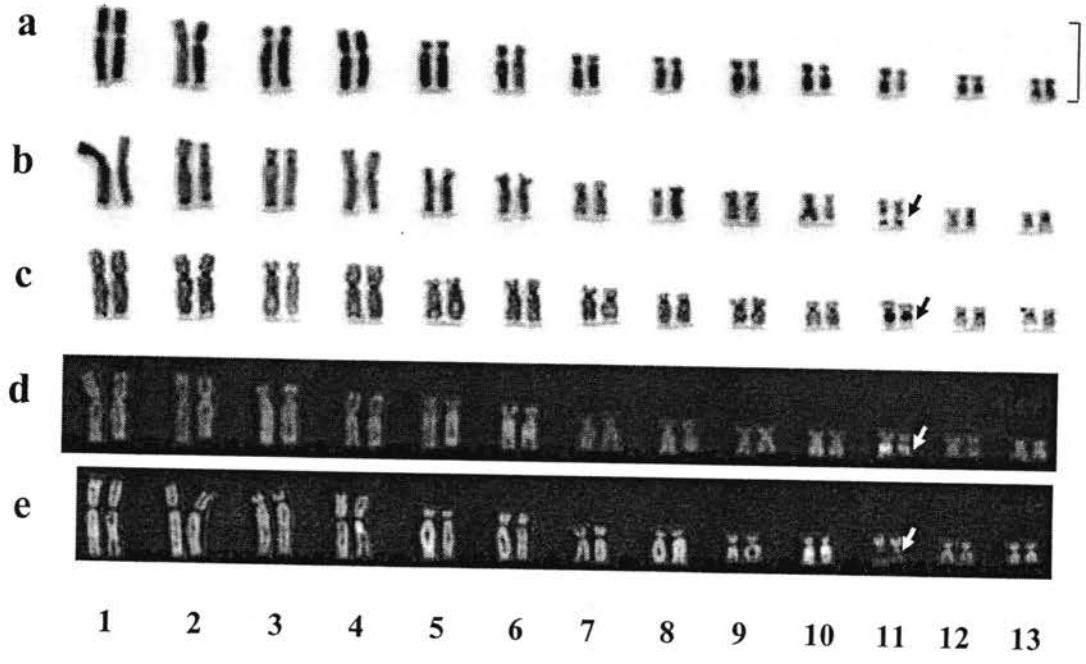


Figure 10 Karyotypes of *L. dahlii* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions, (d) distamycin A/mithramycin counterstaining and (e) quinacrine banding. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.

5.1.5 *L. dahlii*

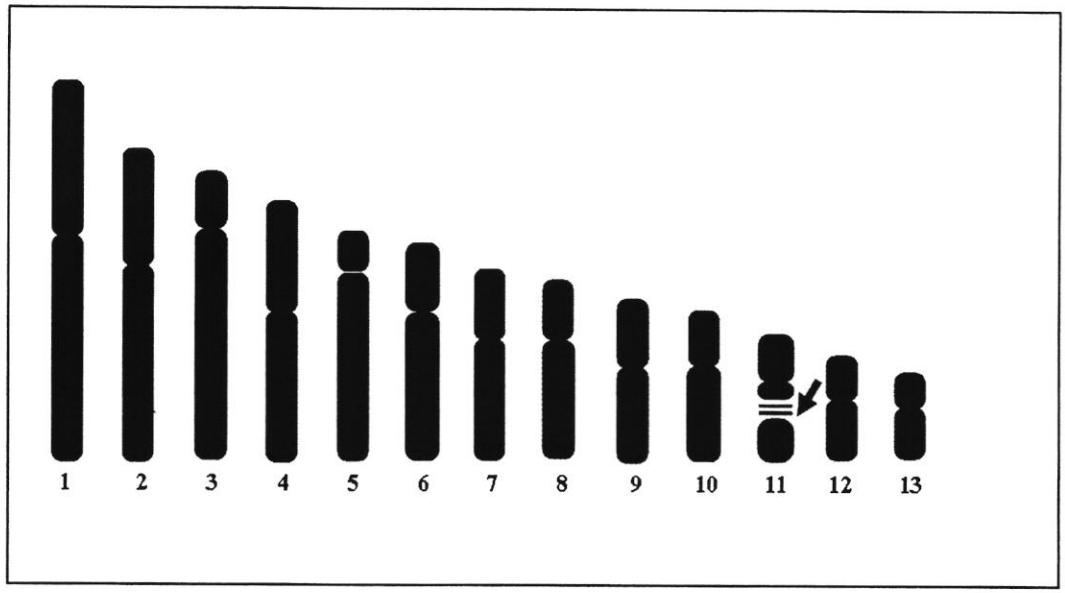
The karyotype of *L. dahlii* can be arranged into 13 pairs. Pairs 1, 4, 7 and 13 were metacentric, pairs 2, 6, 8, 9, 10 and 12 were submetacentric and pairs 3 and 5 were acrocentric (Figure 10a). Figure 11 (a) showed the idiogram of *L. dahlii* from conventional staining.

In the C-banded karyotypes of *L. dahlii*, constitutive heterochromatin showed in the centromeric regions of all chromosomes. Smaller heterochromatic bands were present in the telomeric regions of most chromosomes, best visible in the long arm of pair 11 where the nucleolus organizer region was located (Figure 10b). Figure 11 (b) showed the idiogram of *L. dahlii* from C-banding.

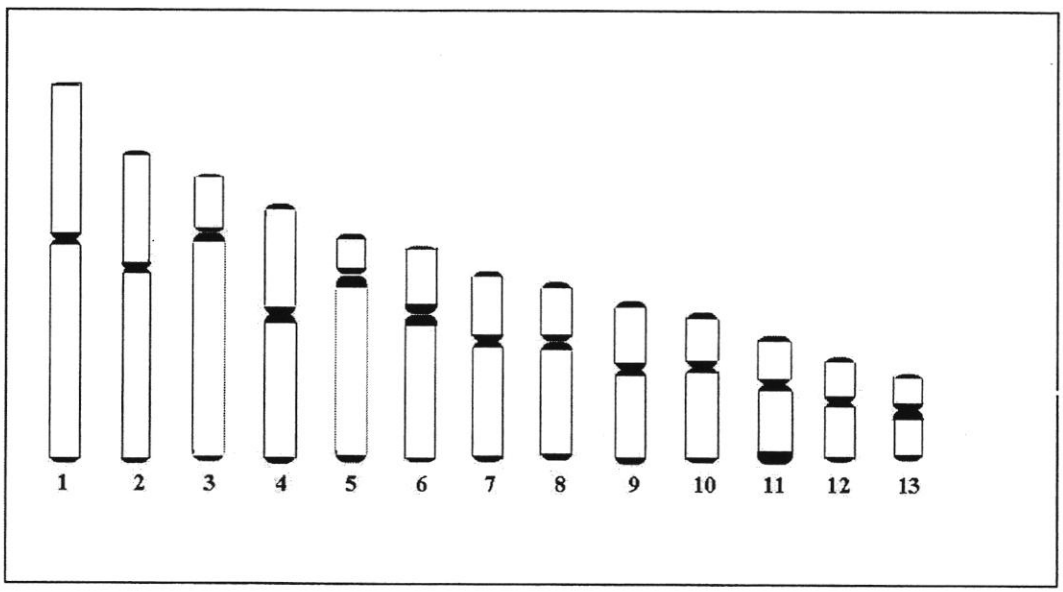
Silver staining showed that in all *L. dahlii* analyzed, the nucleolus organizer region was in the subcentromeric region of pair 11 (Figure 10c). No positive silver labeling was visible in the other chromosomes.

In the mithramycin-stained metaphase, mithramycin induces banding patterns in the *L. dahlii* karyotype which is the opposite of what was obtained by quinacrine mustard. Thus, the centromeric and telomeric heterochromatin in chromosomes 1-13 were mithramycin-positive. The NOR, located close to the telomere in the long arm of chromosome pair 11 presented the brightest mithramycin fluorescence in the karyotype (Figure 10d).

In quinacrine-stained preparations, the fluorescence intensity of most centromeric and telomeric C-bands in chromosomes 1-13 were weaker than that of the euchromatic chromosome segments. No quinacrine fluorescence at all exhibited in the NOR on the long arm of chromosome pair 11 (Figure 10e).



(a)



(b)

Figure 11 Idiogram of *L. dahlii* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

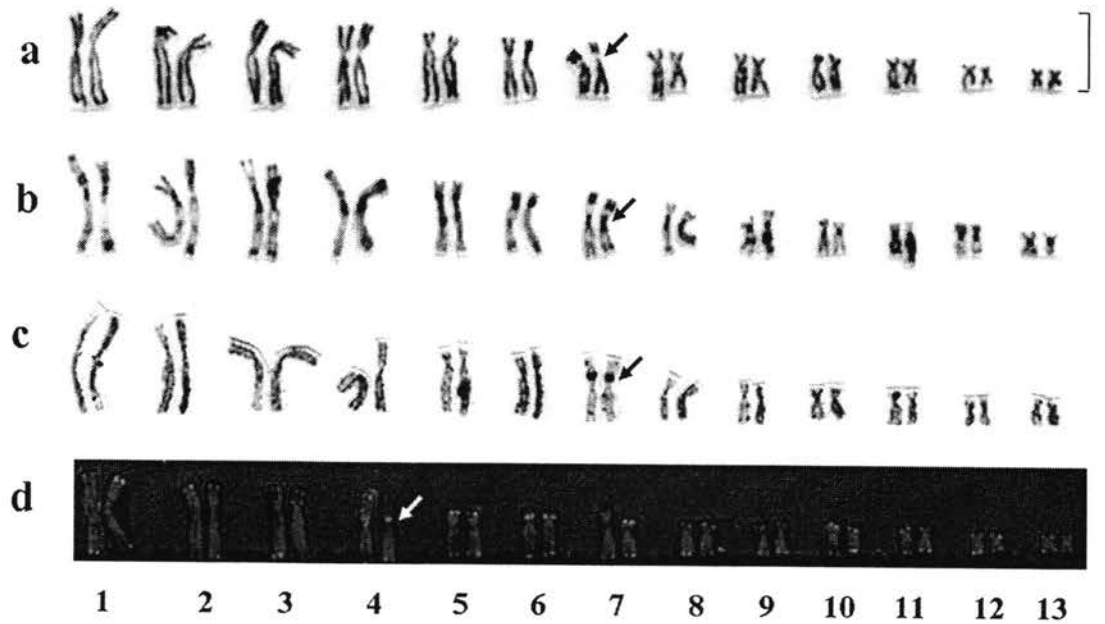


Figure 12 Karyotypes of *L. eucnemis* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions and (d) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions and the centromeric region of hybridization signals (5000x). Bar represents 10 μm.

5.1.6 *L. eucnemis*

The diploid chromosome number of *L. eucnemis* was 2n=26 and the chromosomes can be arranged into 13 pairs. Pairs 1, 4, 7 and 13 were metacentric, pairs 2, 6, 8, 9, 10 and 11 were submetacentric and pairs 3 and 5 were subtelocentric. Chromosome pair number 7 (7p) showed a secondary constriction near the centromeric region (Figure 12a). Figure 13 (a) showed the idiogram of *L. eucnemis* from conventional staining.

The C-banded karyotype of *L. eucnemis* constitutive heterochromatin was located in the centromeric and telomeric regions of all chromosomes. Procentric bands shown on the chromosome pairs 4, 6, 7 and 8 and interstitial C-bands were visible in the pericentromeric regions of chromosomes pairs 1, 2, 3 and 5. Terminal grey bands exhibited large double barred grey C-bands were presented on the chromosome pairs 1, 2, 4 and 5 (Figure 12b). Figure 13 (b) showed the idiogram of *L. eucnemis* from C-banding.

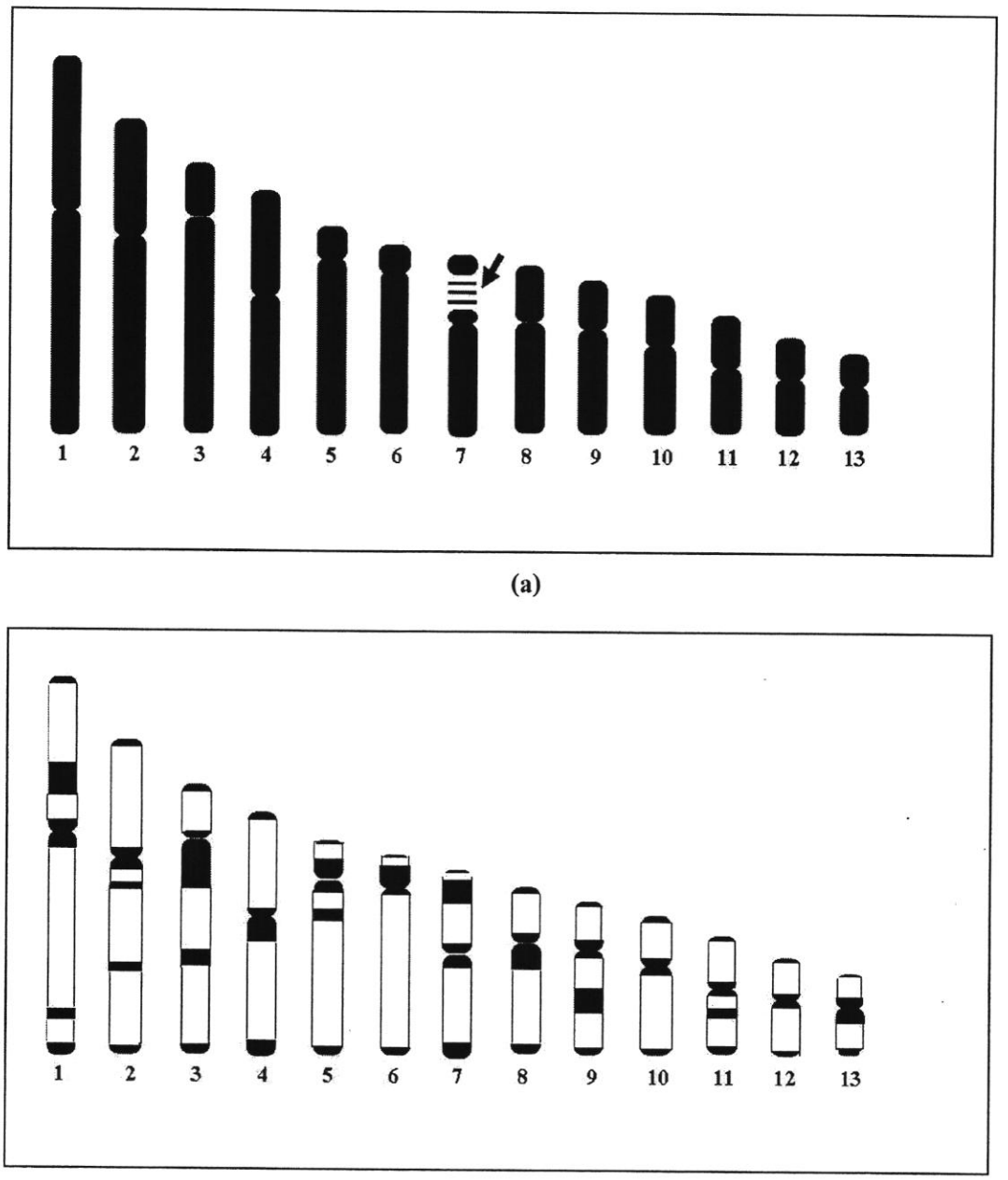


Figure 13 Idiogram of *L. eucnemis* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

Silver staining showed that in all *L. eucnemis* analyzed the nucleolus organizer region was located in the subcentromeric region of chromosome pair 7 (Figure 12c). No positive labeling was visible in the other chromosomes.

In situ hybridization with the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers revealed, as expected, distinct hybridization signals at the telomeres of all chromosomes. Furthermore, all

specimens of *L. eucnemis* presented strong centromeric regions hybridization signals in chromosome pair 4 (Figure 12d).

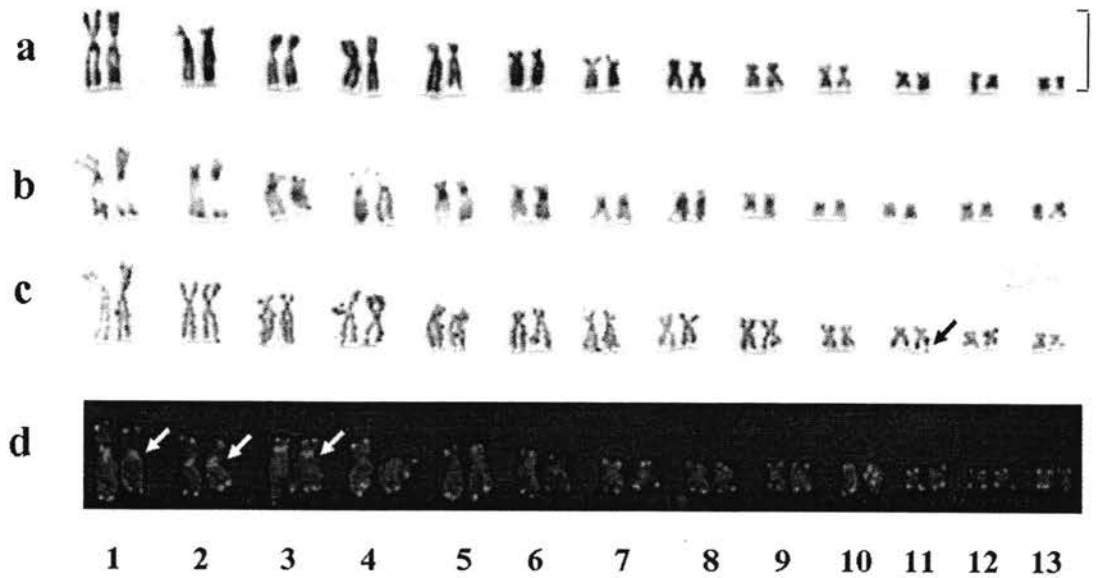


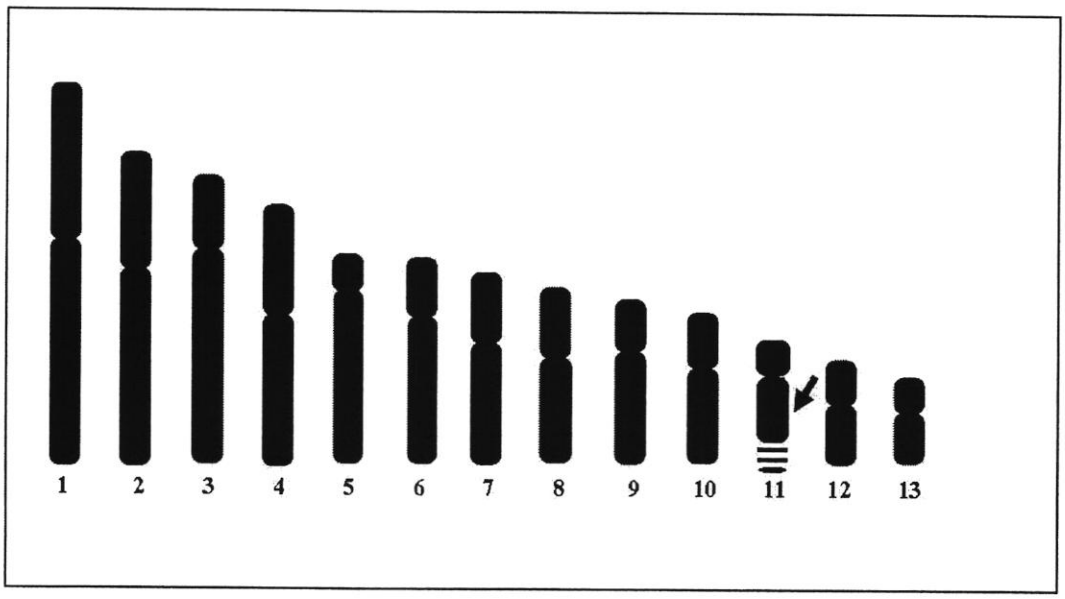
Figure 14 Karyotypes of *L. fallax* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions and (d) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions and the centromeric region of hybridization signals (5000x). Bar represents 10 μm.

5.1.7 *L. fallax*

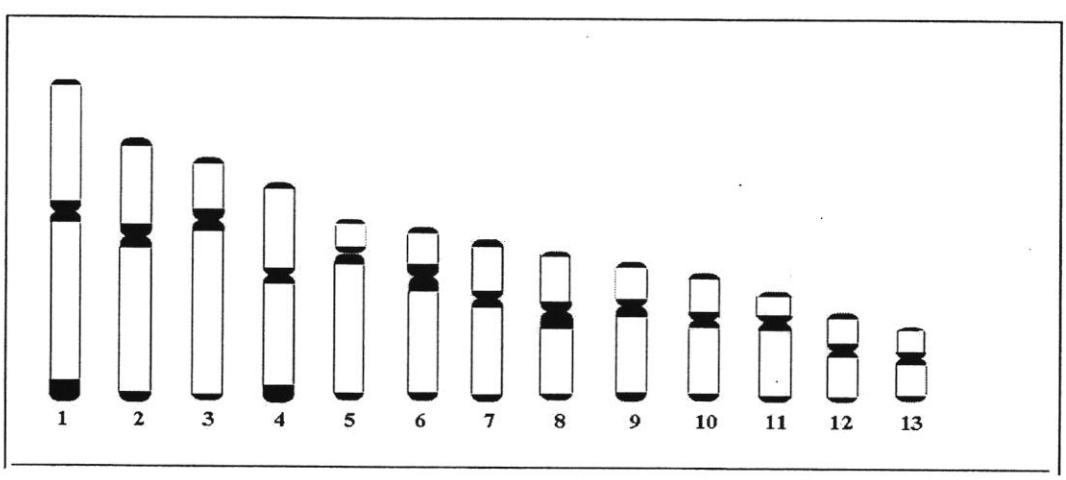
All specimens of *L. fallax* showed 2n=26 chromosomes which can be arranged in 13 pairs (Figure 14a). Chromosome pairs 1, 4, 8, 9, 10, 12 and 13 were metacentric, pairs 2, 6, 7 and 11 were submetacentric and pairs 3 and 5 were subtelocentric. Figure 15 (a) showed the idiogram of *L. fallax* by conventional staining.

The C-banded karyotype of *L. fallax* showed heterochromatin bands at the centromere and telomere of all chromosomes (Figure 14b). The large c-band presented on the long arm of pairs 4 and 8. Figure 15 (b) showed the idiogram of *L. fallax* from C-banding.

Silver staining showed that in all *L. fallax* analyzed, the nucleolus organizer region was in the telomeric region of chromosome pair 11 (Figure 14c). No positive labeling was visible in the other chromosomes.



(a)



(b)

Figure 15 Idiogram of *L. fallax* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

In situ hybridization with the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers reveals, as expected, distinct hybridization signals at the telomeres of all chromosomes. Furthermore, all specimens showed strong centromeric regions hybridization signals in chromosomes pairs 1, 2 and 3 (Figure 14d).

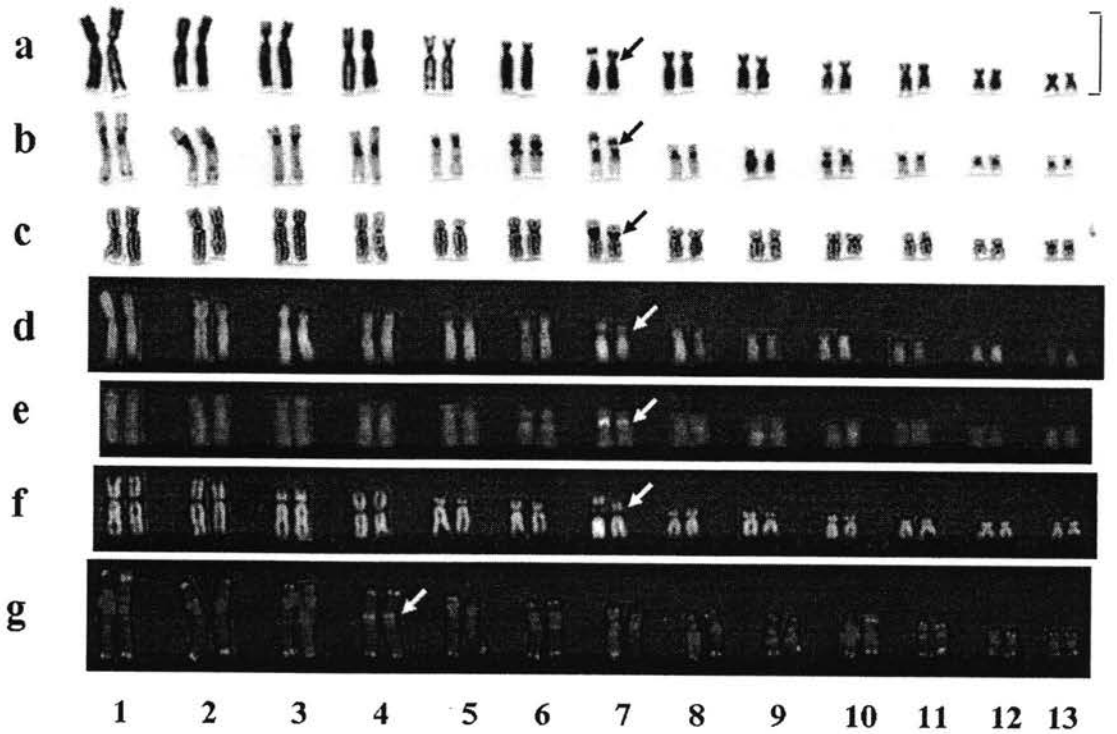


Figure 16 Karyotypes of *L. genimaculata* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions, (d) distamycin A/DAPI counterstaining, (e) distamycin A/mithramycin counterstaining, (f) quinacrine mustard staining and (g) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions and the centromeric region of hybridization signals (5000x). Bar represents 10 μm.

5.1.8 *L. genimaculata*

All specimens of *L. genimaculata* showed 13 pairs of chromosomes (Figure 9a). Pairs 1, 4, 12 and 13 were metacentric, pairs 2, 6, 7, 8, 9, 10 and 11 were submetacentric and pairs 3 and 5 was subtelocentric. Chromosome pair 7 (7p) showed a secondary constriction near the centromere position (Figure 16a). Figure 17 (a) showed the idiogram of *L. genimaculata* from conventional staining.

The C-banded karyotypes of *L. genimaculata*, constitutive heterochromatin can be discerned mainly in the centromeric regions of all chromosomes (Figure 16b). The long arms of chromosomes pair 9 showed entirely of heterochromatin. Interstitial C-bands were visible in the

pericentromeric regions of chromosomes 1, 2, 6 and 10 (Figure 16b). Figure 17 (b) showed the idiogram of *L. genimaculata* from C-banding.

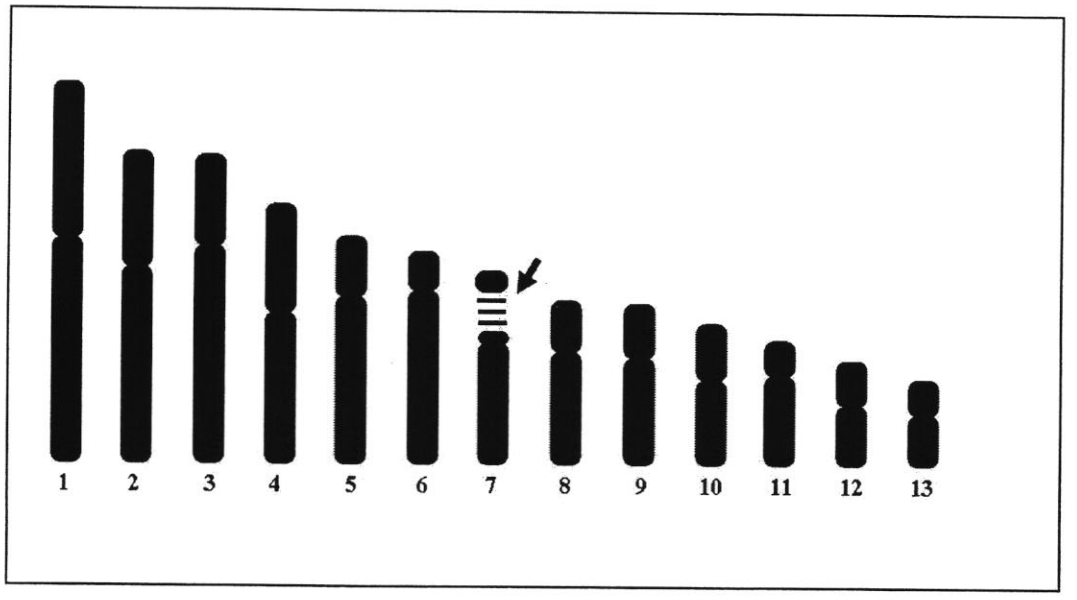
Silver staining showed that in all *L. genimaculata* analyzed, the nucleolus organizer region was located in the subtelomeric region of pair 7 (Figure 16c). No positive silver labeling was visible in the other chromosomes.

Distamycin A/DAPI counterstaining showed uniform fluorescence intensities in all chromosomes (Figure 16d). In the short arm of chromosome pair 7 showed reduce distamycin A/DAPI fluorescence.

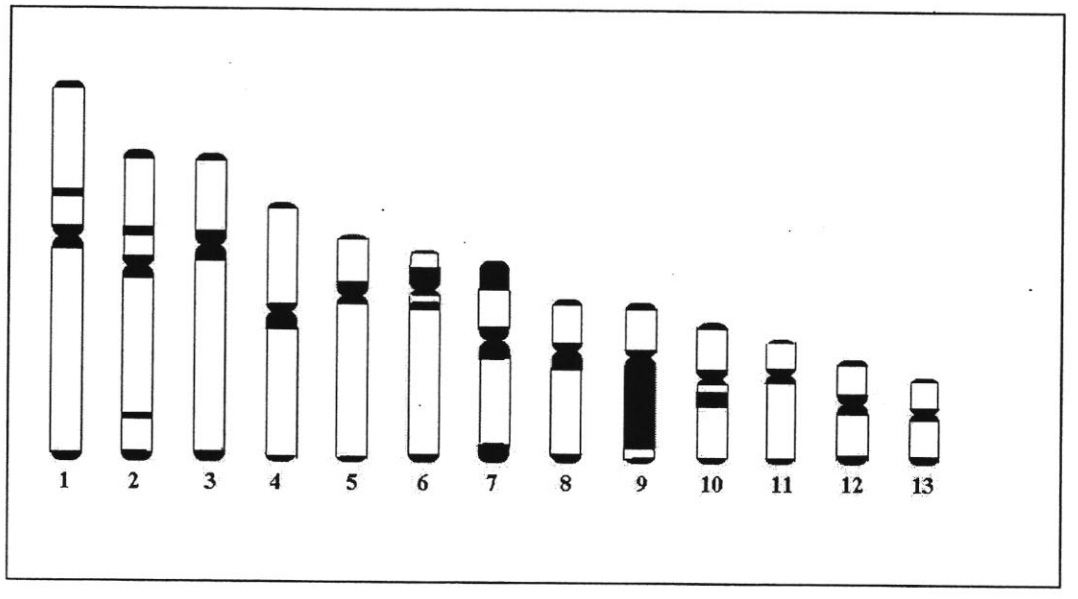
In the mithramycin-stained metaphase, mithramycin induces banding patterns in the *L. genimaculata* karyotype showed mithramycin-positive in all chromosomes (Figure 9e). The NOR, located close the telomere in the short arm of chromosome pair 7 showed the brightest mithramycin fluorescence in the karyotype (Figure 16e).

In quinacrine-stained preparations, the fluorescence intensity of most centromeric and telomeric C-bands in chromosomes 1-13 were weaker than that of the euchromatic chromosome segments. No quinacrine fluorescence at all exhibited in the NOR in the short arm close to the centomeric region of chromosome pair 7 (Figure 16f).

Fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers exhibited distinct telomeric labeling signals in all chromosomes of *L. genimaculata*. Furthermore, all specimens showed strong centromeric regions hybridization signals in chromosome pair 4 (Fig 16g).



(a)



(b)

Figure 17 Idiogram of *L. genimaculata* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

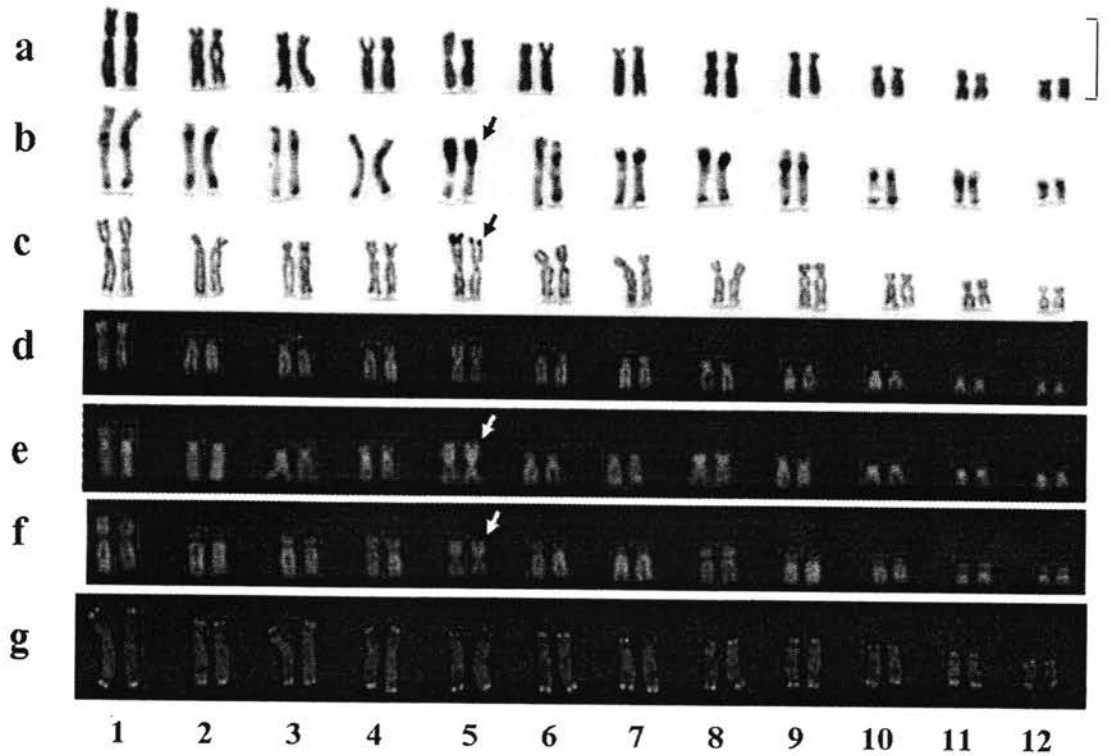
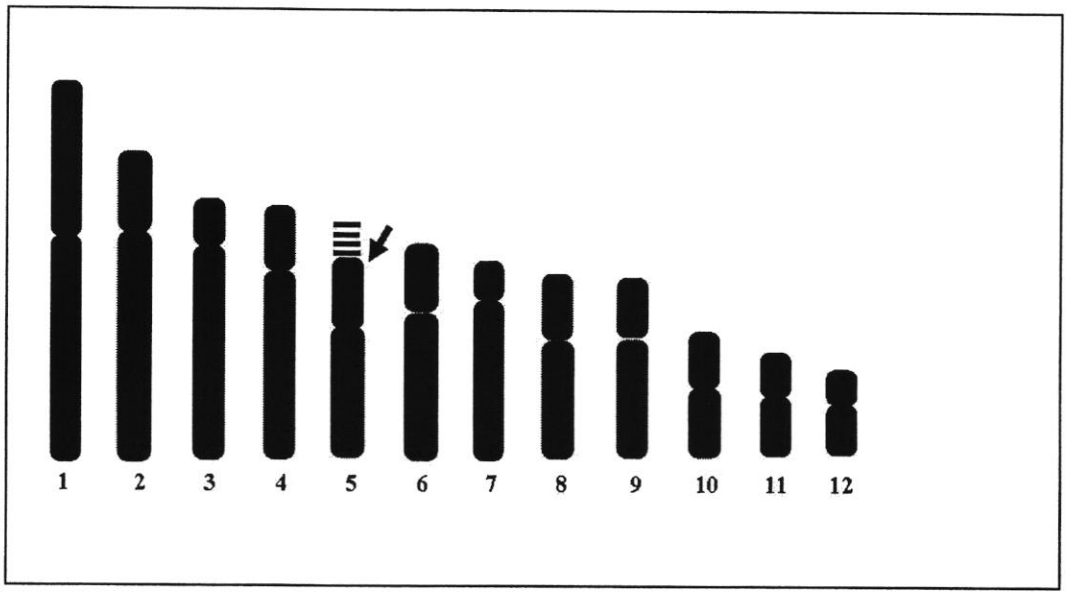


Figure 18 Karyotypes of *L. infrafrenata* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions, (d) distamycin A/DAPI counterstaining, (e) distamycin · A/mithramycin counterstaining, (f) quinacrine mustard staining and (g) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.

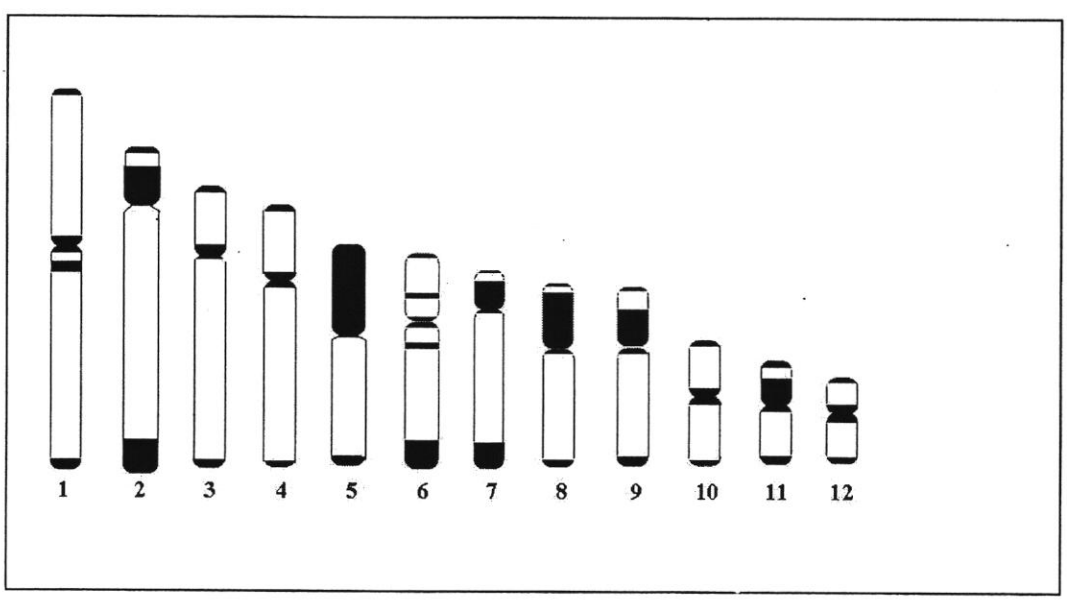
5.1.9 *L. infrafrenata*

The karyotype of *L. infrafrenata* exhibited 24 biarmed chromosomes which were consisted of 12 chromosome pairs (Figure 18a). Pairs 1, 4, 10 and 12 were metacentric, pairs 2, 6, 7, 9, 11 and 13 were submetacentric and pairs 3, 5 and 8 were subtelocentric. Figure 19 showed the idiogram of *L. infrafrenata* from conventional staining and C-banding.

The C-banded karyotypes of *L. infrafrenata*, constitutive heterochromatin can be discerned mainly in the centromeric and telomeric regions of all chromosomes (Figure 10b). The chromosomes pairs 2, 7, 9 and 11 showed procentric bands. Interstitial bands presented on the chromosome pairs 1 and 6. The short arm of chromosome pairs 5 and 8 had shown a very large additional heterochromatin segment (Figure 18b).



(a)



(b)

Figure 19 Idiogram of *L. infrafrenata* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

Silver staining showed that in all *L. infrafrenata* analyzed the nucleolus organizer region was located in the telomeric region of chromosome pair 5 (Figure 18c). No positive silver labeling was visible in the other chromosomes.

Distamycin A/DAPI counterstaining showed uniform fluorescence intensities in all chromosomes (Figure 18d).

In the mithramycin-stained metaphase of *L. genimaculata* karyotype showed mithramycin-positive in all chromosomes (Figure 18e). The NOR, located in the short arm of chromosome pair 5 exhibited the brightest mithramycin fluorescence in the karyotype (Figure 18e).

In quinacrine-stained preparations, the fluorescence intensity of most centromeric and telomeric C-bands in chromosomes 1-13 were weaker than that of the euchromatic chromosome segments. No quinacrine fluorescence exhibited in the NOR in the short arm close to the telomeric region of chromosome pair 5 (Figure 18f).

Fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers showed distinct telomeric labeling signals in all chromosomes of *L. infrafrenata* (Figure 18g).

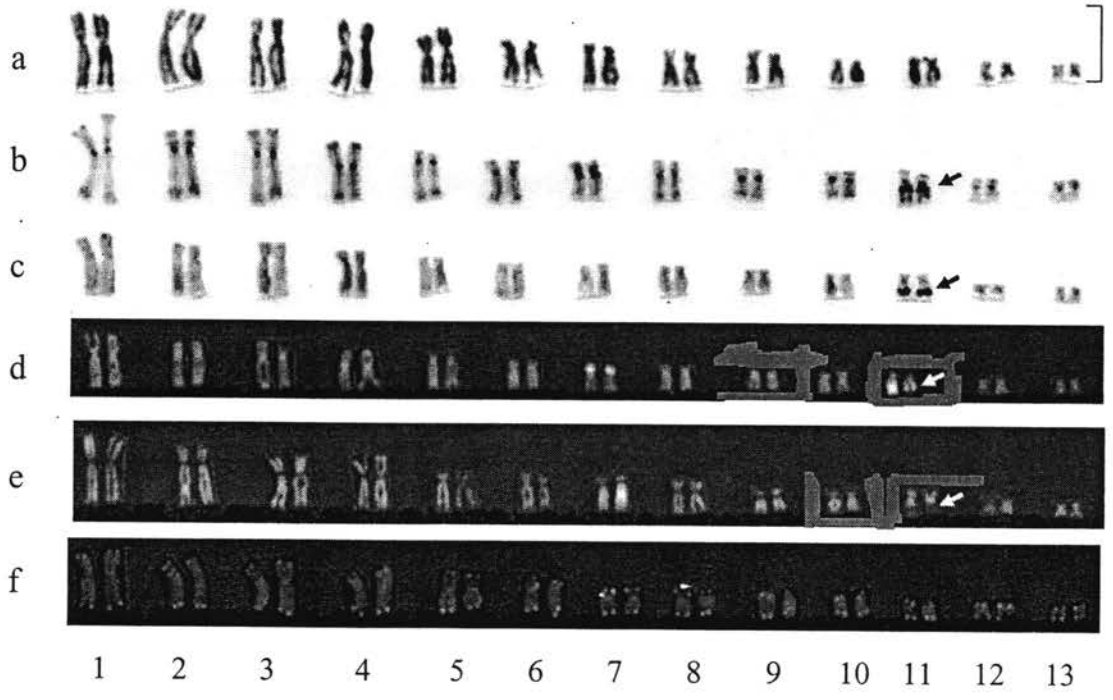
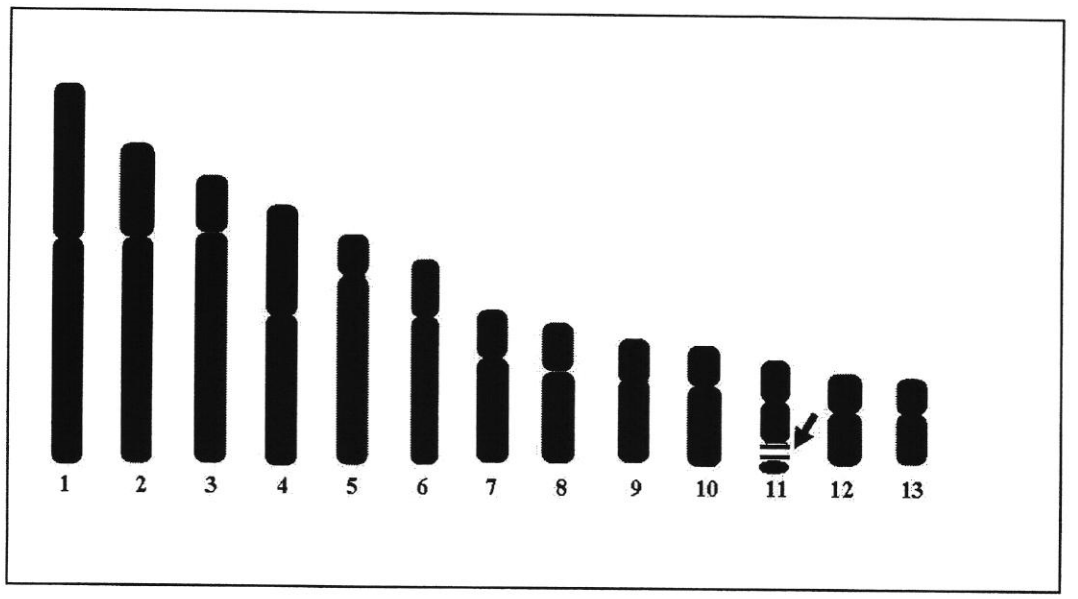
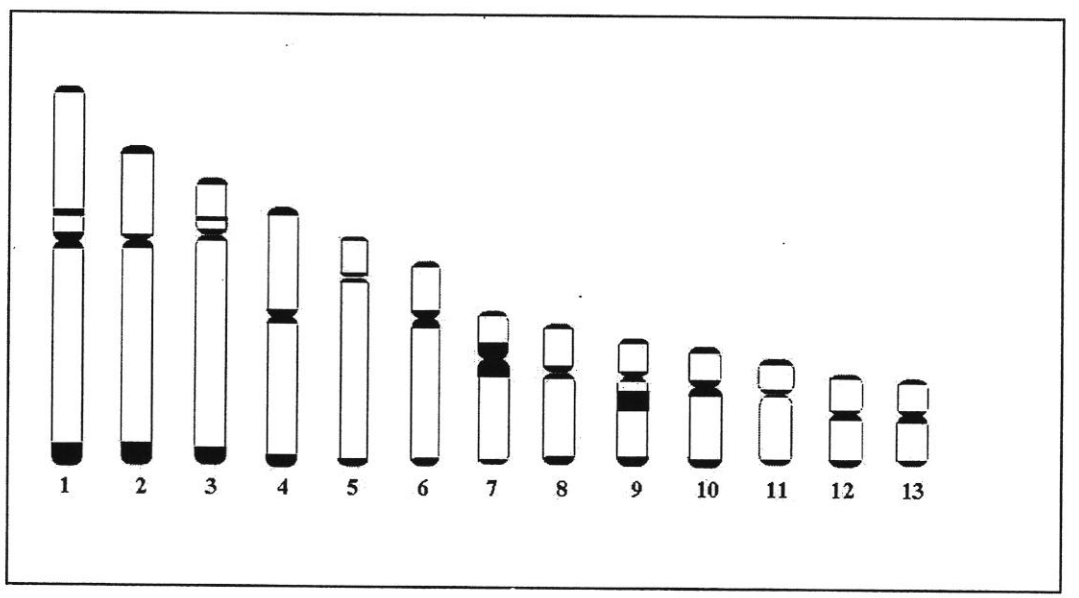


Figure 20 Karyotypes of *L. lesueurii* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions, (d) distamycin A/mithramycin counterstaining, (e) quinacrine mustard staining and (f) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.



(a)



(b)

Figure 21 Idiogram of *L. lesueurii* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

5.1.10 *L. lesueurii*

All specimens of *L. lesueurii* showed 13 pairs of chromosomes (Figure 20a). Pairs 1, 4, 8, 10 and 13 were metacentric, pairs 2, 6, 7, 9, 11 and 12 were submetacentric and pairs 3 and 5 were subtelocentric. Figure 21 (a) showed the idiogram of *L. lesueurii* from conventional staining.

In the C-banded karyotypes of *L. lesueurii*, constitutive heterochromatin can be discerned mainly in the centromeric and telomeric regions of all chromosomes (Figure 20b). Interstitial C-band was demonstrated close to the centromeric regions in the chromosomes of pairs 2 and 7 and a large c-block in the long arm of pair 11 where the nucleolus organizer region was located. Figure 21 (b) showed the idiogram of *L. lesueurii* from C-banding.

Silver staining showed that in all *L. lesueurii* analyzed the nucleolus organizer region was located in the subcentromeric region of chromosome pair 11 (Figure 20c). No positive silver labeling was visible in the other chromosomes.

In the mithramycin-stained metaphase of *L. lesueurii* karyotype which were the reverse to obtain by quinacrine mustard. Thus, the centromeric and telomeric heterochromatin in chromosomes 1-13 were mithramycin-positive. The centromeric region of chromosome pair 7 showed brighter than the other chromosomes. The NOR, located close the telomere in the long arm of chromosome pair 9 exhibited the brightest mithramycin fluorescence in the karyotype (Figure 20d).

In quinacrine-stained preparations, the fluorescence intensity of most centromeric and telomeric C-bands in chromosomes 1-13 were weaker than that of the euchromatic chromosome segments. No quinacrine fluorescence at all showed the NOR in the long arm of chromosome pair 9 (Figure 20e).

In the fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers exhibited distinct telomeric labeling signals in all chromosomes of *L. lesueurii* (Figure 20f). No interstitial hybridization signals were detected.

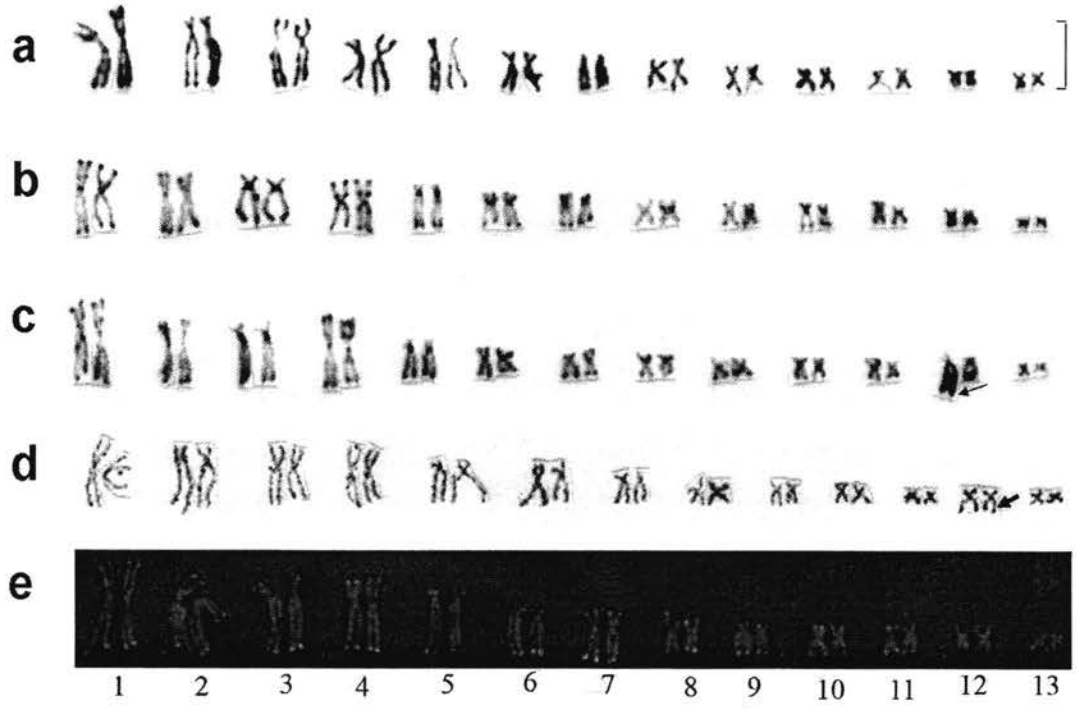


Figure 22 Karyotypes of *Litoria meiriana* showing (a) Giemsa staining, (b) silver staining of the nucleolus organizer regions and (c) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.

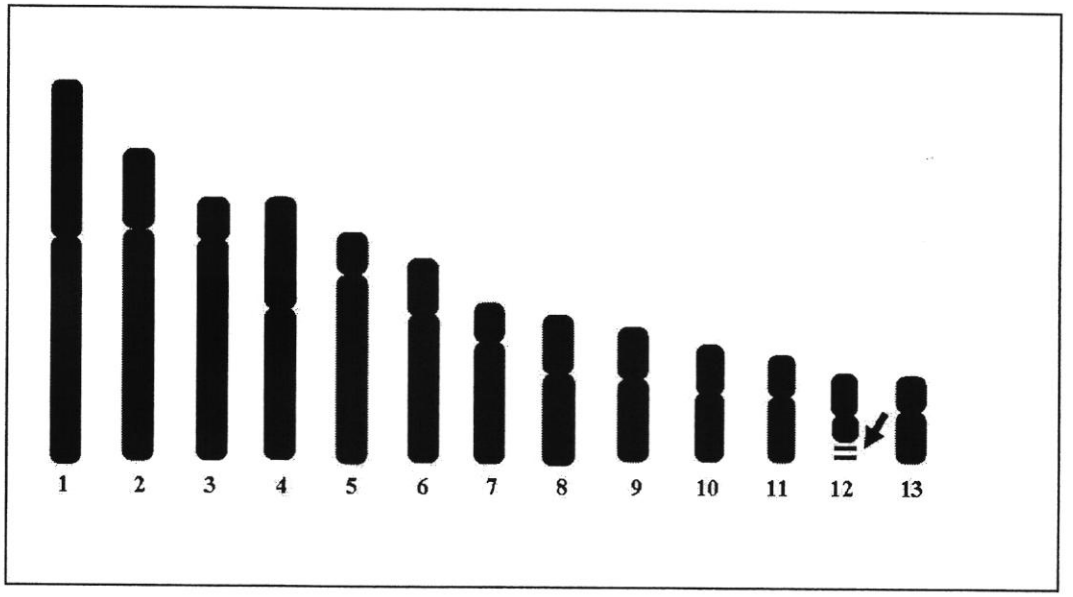
5.1.11 *L. meiriana*

All specimens of *L. meiriana* showed a diploid chromosome number 2n=26 was determined (Figure 22a). Pairs 1, 4, 8, 9, 11 and 13 were metacentric, pairs 2, 6, 10 and 12 were submetacentric and pairs 3, 5 and 7 were subtelocentric. Figure 23 (a) showed the idiogram of *L. meiriana* from conventional staining.

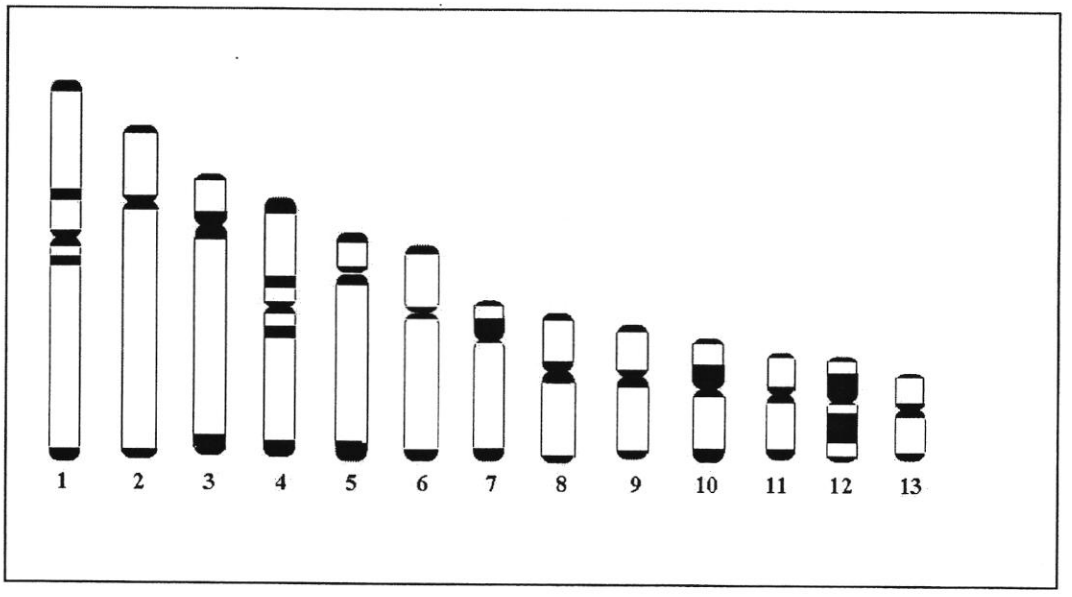
In the C-banded karyotypes of *L. meiriana*, constitutive heterochromatin can be discerned mainly in the centromeric and telomeric regions of all chromosomes (Figure 22b). Interstitial C-band was demonstrated close to the centromeric regions in the chromosomes of pairs 1 and 4 and a very large additional and polymorphic segment was present on pair 12. Figure 23 (b) showed the idiogram of *L. meiriana* from C-banding.

Silver staining showed that in all *L. meiriana* analyzed the nucleolus organizer region was located in the telomeric of chromosome pair 12 (Figure 22c). No positive silver labeling was visible in the other chromosomes.

In the fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers exhibited distinct telomeric labeling signals in all chromosomes of *L. meiriana* (Figure 22d). No interstitial hybridization signals were detected.



(a)



(b)

Figure 23 Idiogram of *L. meiriana* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

Figure 24 Karyotypes of *L. nannotis* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions and (d) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.

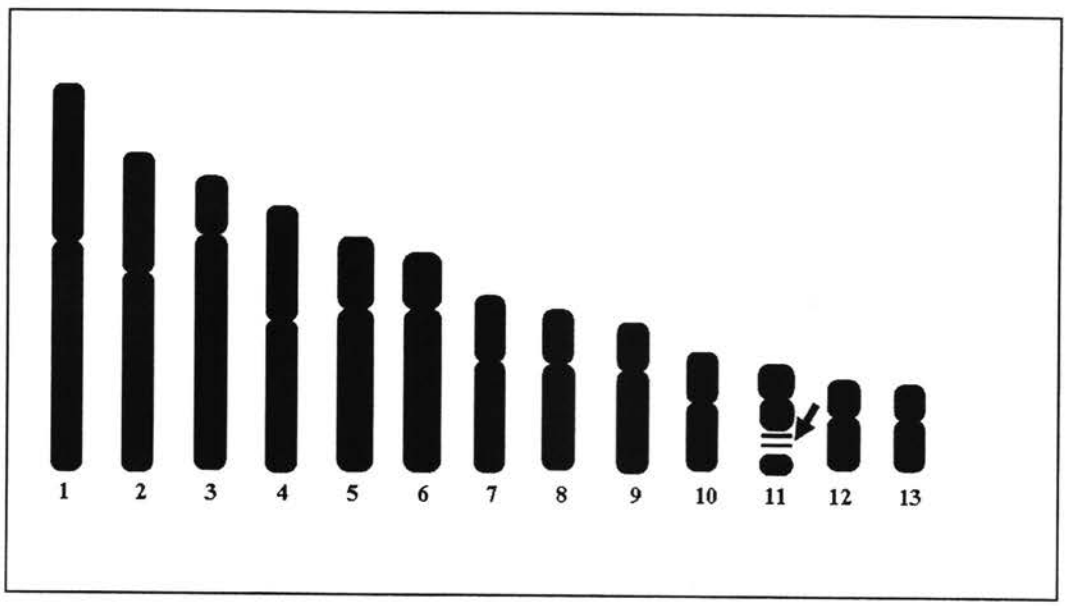
5.1.12 *L. nannotis*

The karyotype of *L. nannotis* consisted of 2n=26 chromosomes which can be arranged in 13 homologous pairs (Figure 24a). Chromosome pairs 1, 4, 9, 10, 11, 12 and 13 were metacentric, pairs 2, 6, 7 and 8 were submetacentric and pairs 3 and 5 were subtelocentric. Figure 25 (a) showed the idiogram of *L. nannotis* from conventional staining.

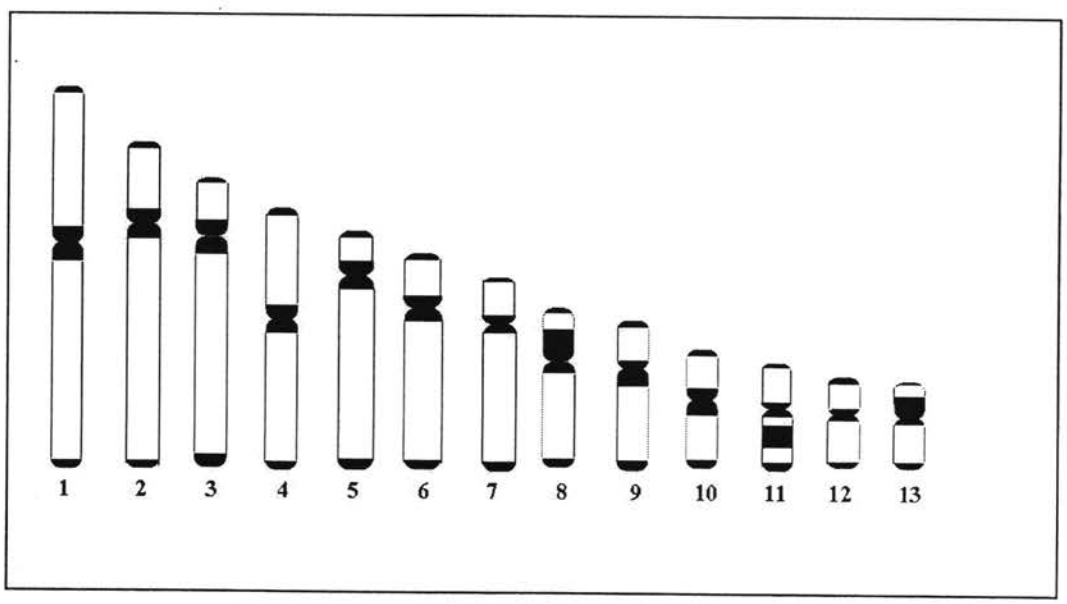
In the C-banded karyotypes of *L. nannotis*, constitutive heterochromatin can be discerned mainly in the centromeric and telomeric regions of all chromosomes (Figure 24b). Large amounts of constitutive heterochromatin were located in the short arm close the centromeric region of pair 8 and in the long arm of pair 11 where the nucleolus organizer region was located. Procentric bands shown uniformly of all chromosomes. Figure 25 (b) showed the idiogram of *L. nannotis* from C-banding.

Silver staining showed that in all *L. nannotis* analyzed the nucleolus organizer region was located in the subcentromeric region of chromosome pair 11 (11q) (Figure 24c). No positive silver labeling was visible in the other chromosomes.

In the fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers demonstrated distinct telomeric labeling signals in all chromosomes of *L. nannotis* (Figure 24d). No interstitial hybridization signals were detected.



(a)



(b)

Figure 25 Idiogram of *L. nannotis* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

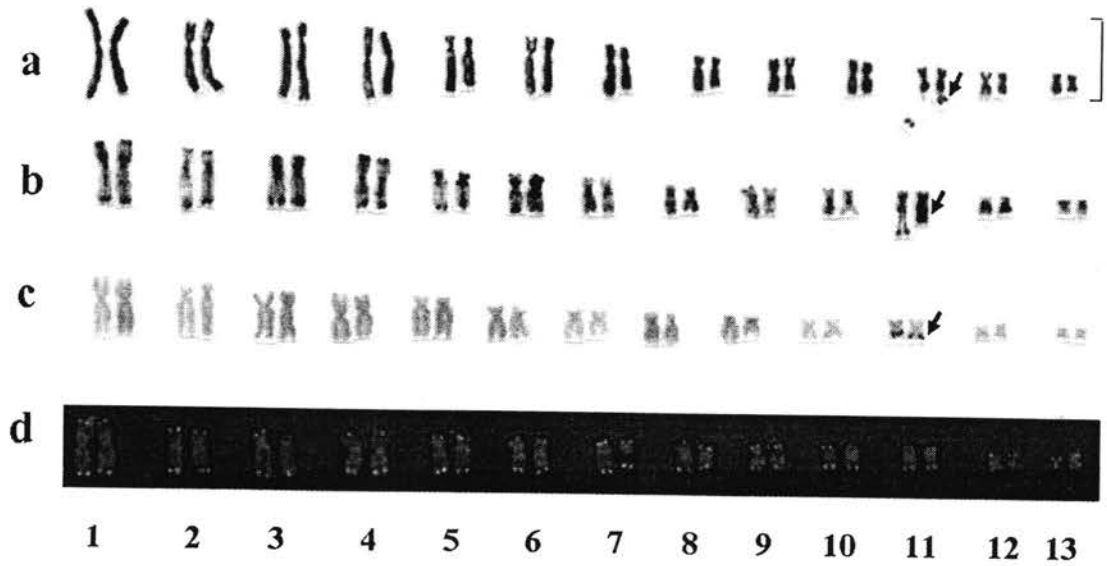


Figure 26 Karyotypes of *L. nasuta* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions and (d) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.

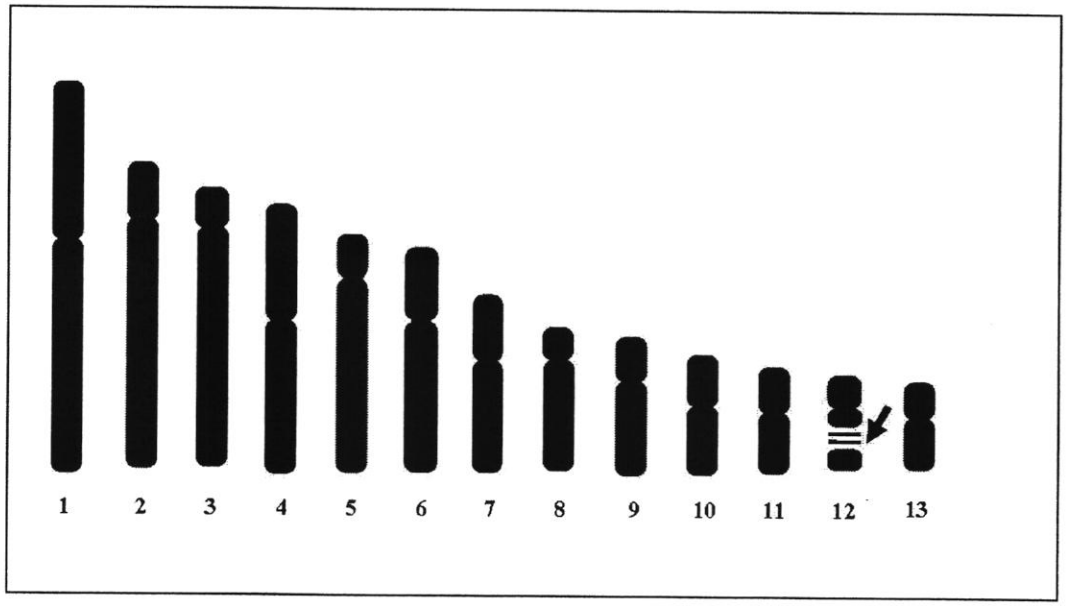
5.1.13 *L. nasuta*

The karyotype of *L. nasuta* exhibited 26 biarmed chromosomes which were consisted of 6 large and 7 small chromosome pairs (Figure 26a). Pairs 1, 4, 8, 10, 11 and 13 were metacentric, pairs 2, 6, 7, 9 and 12 were submetacentric and pairs 3 and 5 were subtelocentric. Chromosome pair 11 (11q) showed polymorphic of a secondary constriction. Figure 27 (a) showed the idiogram of *L. nasuta* from conventional staining.

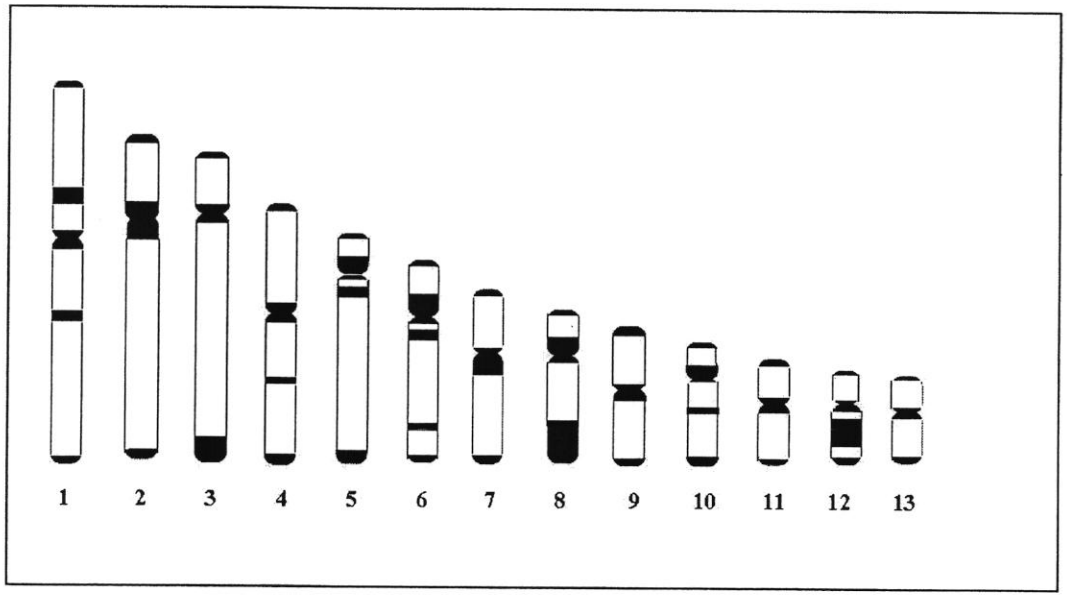
The C-banded karyotypes of *L. nasuta*, constitutive heterochromatin can be discerned mainly in the centromeric and telomeric regions of all chromosomes (Figure 26b). The long arms of chromosomes pair 12 consisted a large additional and polymorphic segment. Interstitial C-bands were visible in the pericentromeric regions of chromosomes 1, 2, 5 and 6. Figure 27 (b) showed the idiogram of *L. nasuta* from C-banding.

Silver staining showed that in all *L. nasuta* analyzed the nucleolus organizer region was in the subtelomeric region of chromosome pair 11 (Figure 26c). No positive silver labeling was visible in the other chromosomes.

In the fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers demonstrated distinct telomeric labeling signals in all chromosomes of *L. nasuta* (Figure 26d). No interstitial hybridization signals were detected.

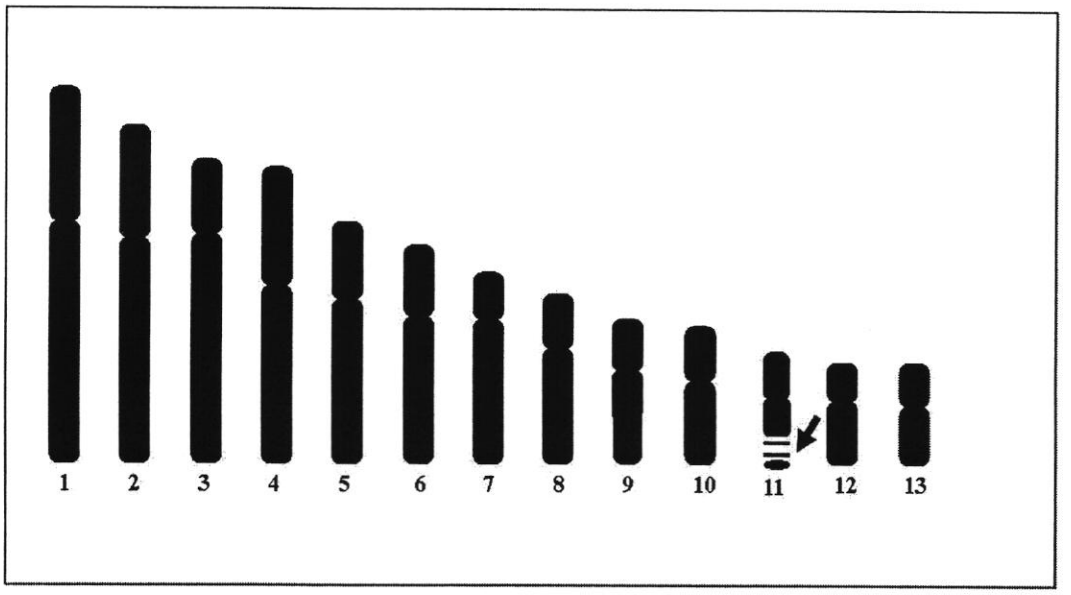


(a)

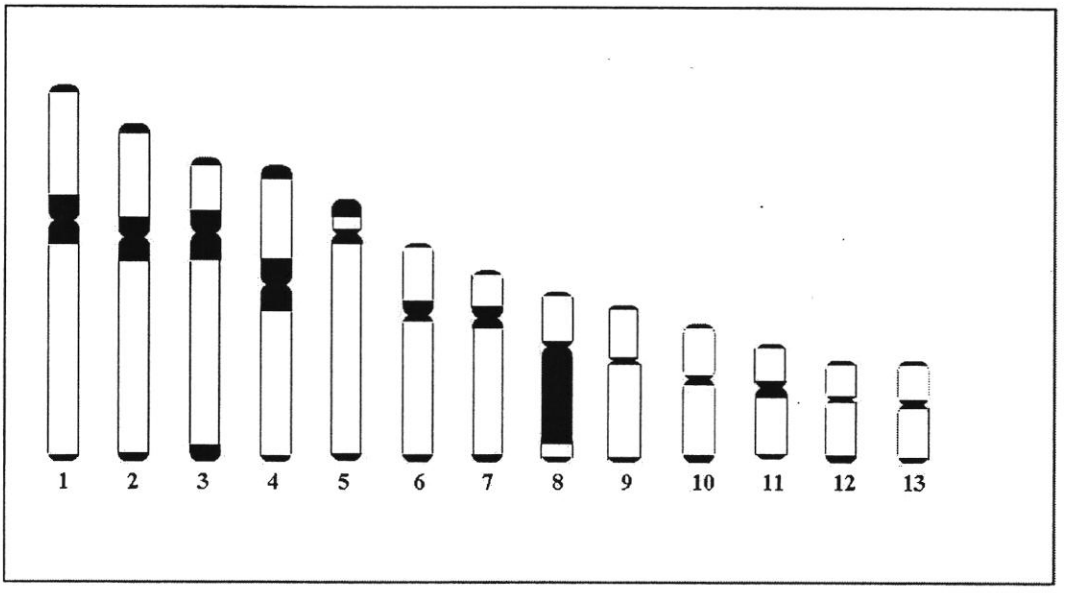


(b)

Figure 27 Idiogram of *L. nasuta* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.



(a)



(b)

Figure 29 Idiogram of *L. nyakalensis* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

Following quinacrine staining, the fluorescence intensity of most centromeric and telomeric C-bands in chromosomes 1-13 were weaker than that of the euchromatic chromosome segments. In contrast to this, the pericentromeric regions of chromosomes pairs 1-8 were characterized by very bright quinacrine fluorescence (Figure 28d). No quinacrine fluorescence at all showed in the NOR in the long arm of chromosome pair 9.

In the fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers demonstrated distinct telomeric labeling signals in all chromosomes of *L. nyakalensis* (Figure 28e). No interstitial hybridization signals were detected.

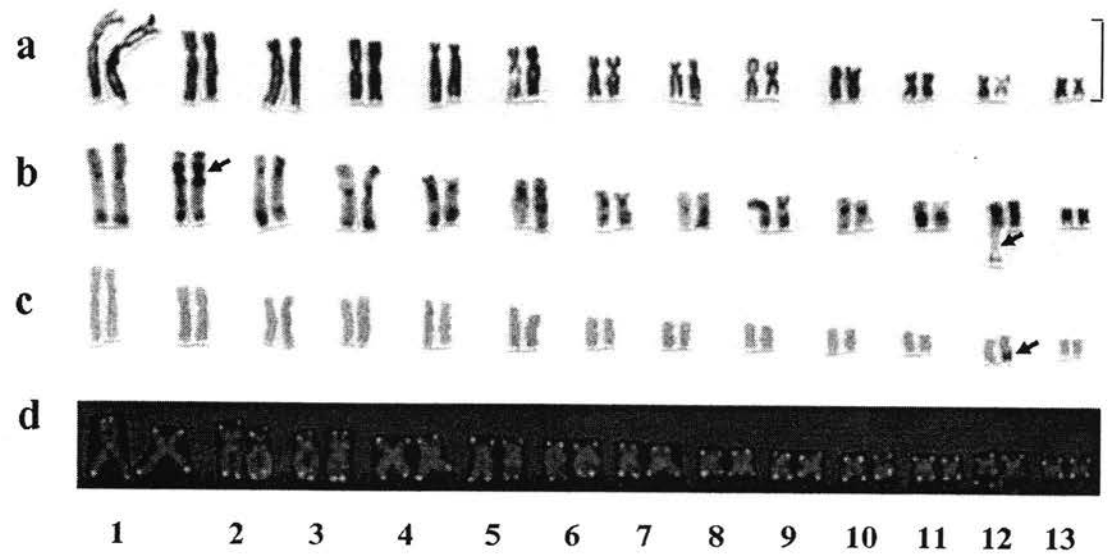
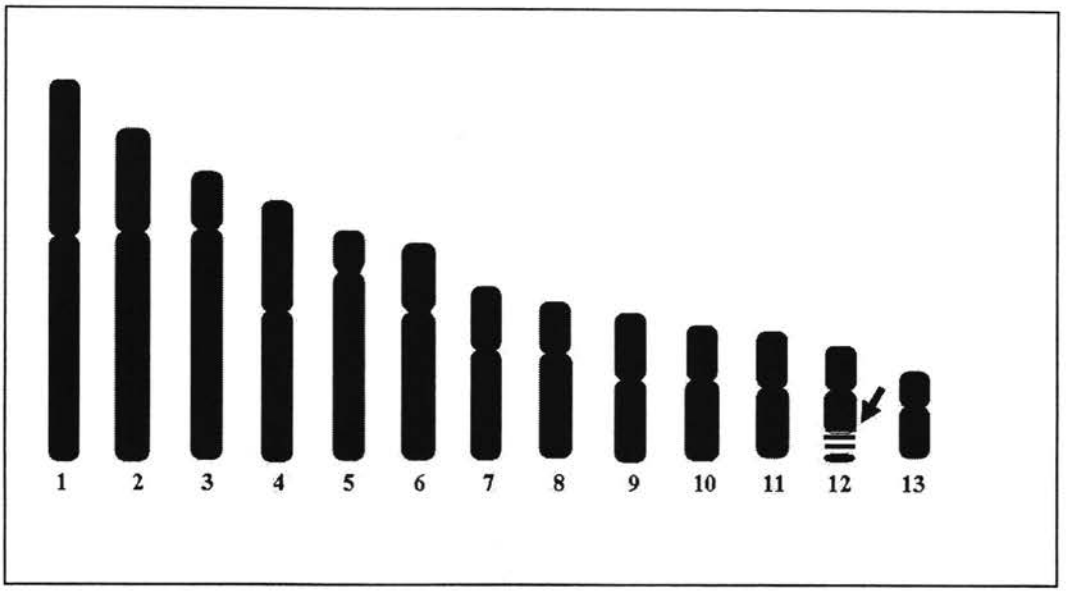


Figure 30 Karyotypes of *L. pallida* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin and (d) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.

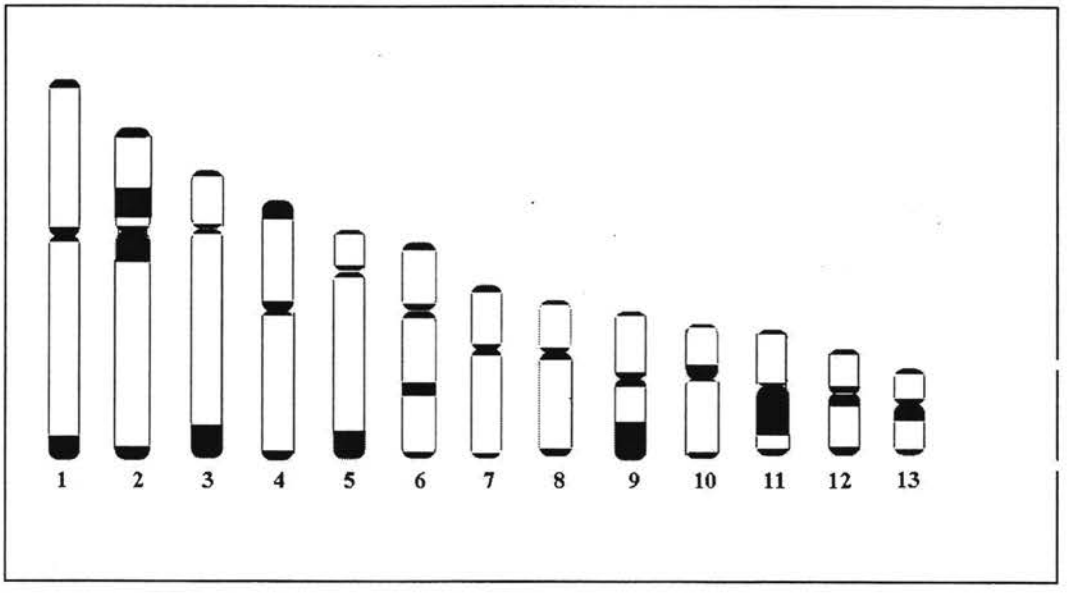
5.1.15 *L. pallida*

In all individuals of *L. pallida* showed a diploid chromosome number of 2n=26 were determined (Figure 30a). Chromosome pairs 1, 4, 8, 9, 10, 11 and 13 were metacentric, pairs 2, 6, 7, 8 and 12 were submetacentric and pairs 3 and 5 were acrocentric. Figure 31 (a) showed the idiogram of *L. pallida* from conventional staining.

In the C-banded karyotypes of *L. pallida*, constitutive heterochromatin can be discerned mainly in the centromeric and telomeric regions of all chromosomes (Figure 30b). A large amount of constitutive heterochromatin was located in the whole long arm of chromosome pair 11. Interstitial C-bands was located on chromosome pair 2. The long arm of chromosome pair 12 showed a heteromorphic for the constriction where the nucleolus organizer region was located. Figure 31 (b) showed the idiogram of *L. pallida* from C-banding.



(a)



(b)

Figure 31 Idiogram of *L. pallida* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

Silver staining showed that in all *L. pallida* analyzed the nucleolus organizer region was in the subtelomeric region of chromosome pair 12 (Figure 30c). No positive silver labeling was visible in the other chromosomes.

In the fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers demonstrated distinct telomeric labeling signals in all chromosomes of *L. pallida* (Figure 30d). No interstitial hybridization signals were detected.

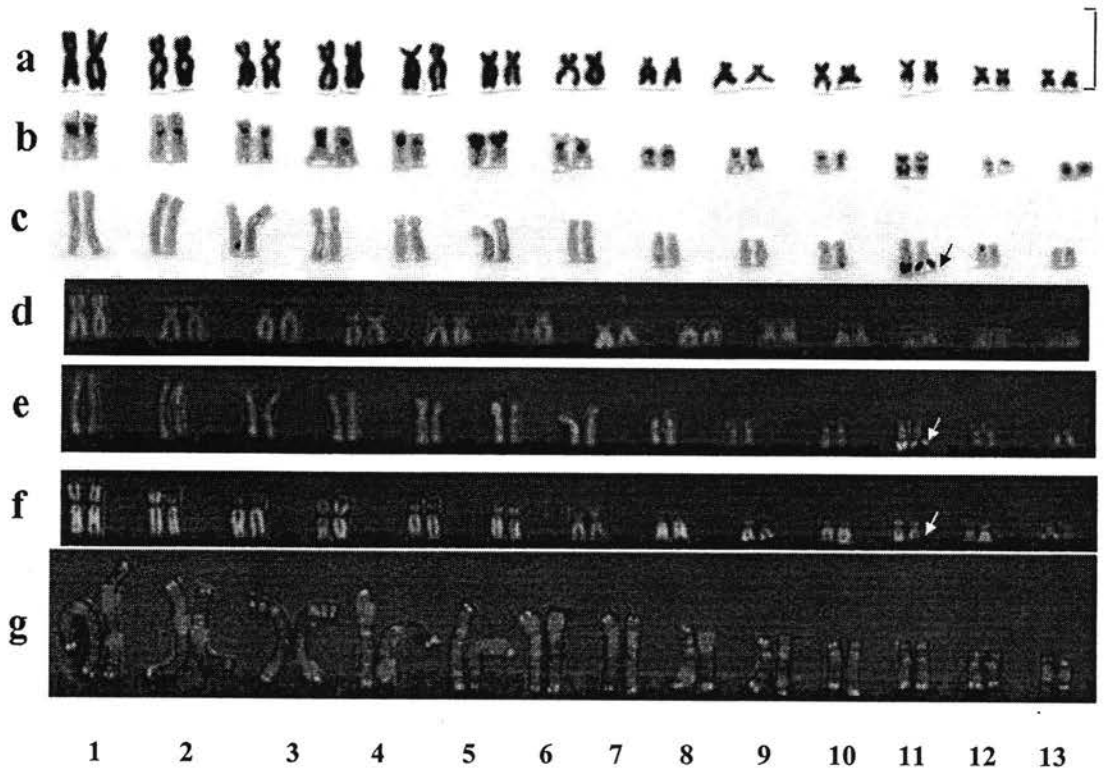
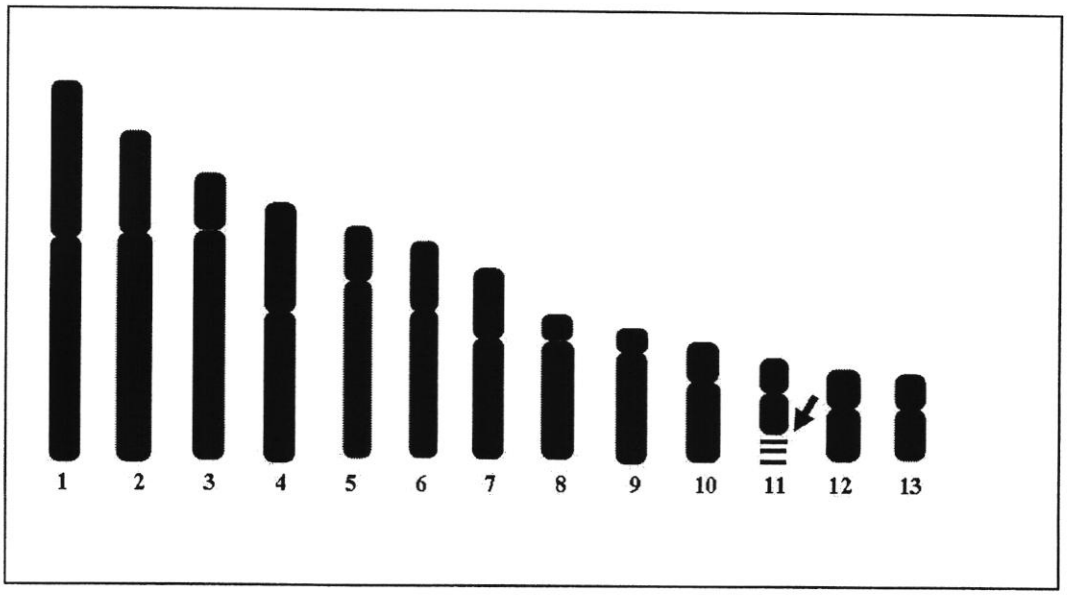
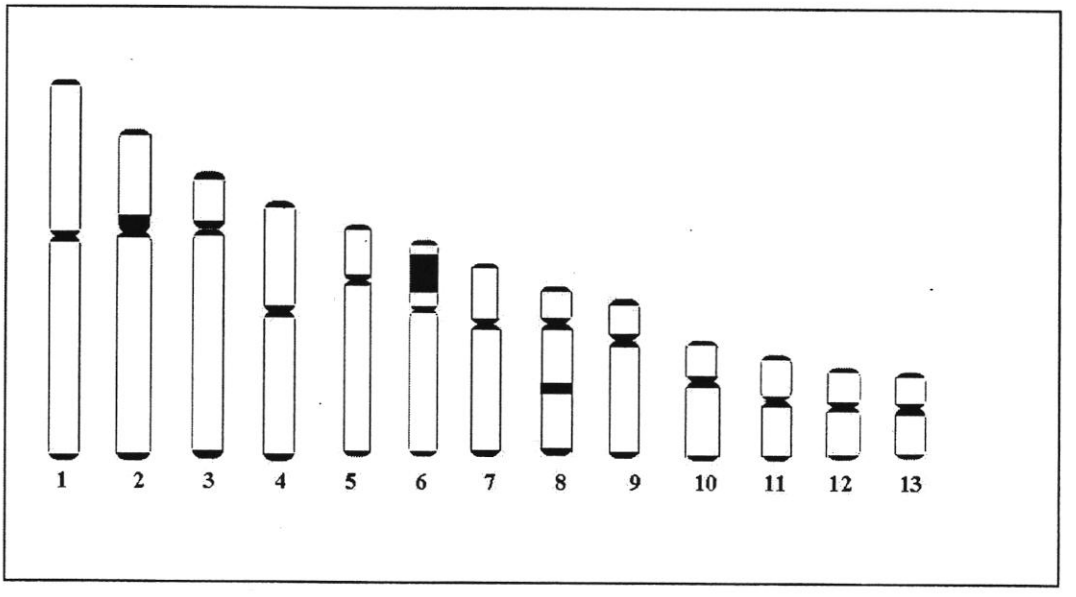


Figure 32 Karyotypes of *L. peronii* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions, (d) distamycin A/DAPI counterstaining, (e) distamycin A/mithramycin counterstaining, (f) quinacrine mustard staining and (g) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.



(a)



(b)

Figure 33 Idiogram of *L. peronii* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

5.1.16 *L. peronii*

All specimens of *L. peronii* showed 13 pairs of chromosomes (Figure 32a). Pairs 1, 4, 12 and 13 were metacentric, pairs 2, 6, 7, 9, 10 and 11 were submetacentric and pairs 3, 5 and 8 were subtelocentric. Figure 33 (a) showed the idiogram of *L. peronii* from conventional staining.

In the C-banded karyotypes of *L. peronii*, constitutive heterochromatin showed in the centromeric regions of all chromosomes (Figure 32b). Smaller heterochromatic bands were present in the telomeric regions of most chromosomes, best visible in the long arm of pairs 11 where the nucleolus organizer region was located. The short arm of chromosome pair 6 was completely heterochromatic. Figure 33 (b) showed the idiogram of *L. peronii* from C-banding.

Silver staining showed that in all *L. peronii* analyzed the nucleolus organizer region was located in the telomeric region of chromosome pair 11 (Figure 32c). No positive silver labeling was visible in the other chromosomes.

In the distamycin A/DAPI counterstained metaphase of *L. peronii* the karyotypes showed a uniform fluorescence (Figure 32d).

In the mithramycin-stained metaphase, mithramycin induces banding patterns in the *L. peronii* karyotype which were the reverse to obtain by quinacrine mustard. Thus, the centromeric and telomeric heterochromatin in chromosomes 1-13 were mithramycin-positive. The NOR, located close the telomere in the long arm of chromosome pair 11 exhibited the brightest mithramycin fluorescence in the karyotype (Figure 32e).

In quinacrine-stained preparations, the fluorescence intensity of most centromeric and telomeric C-bands in chromosomes 1-13 were weaker than that of the euchromatic chromosome segments. No quinacrine fluorescence at all exhibited in the NOR on the long arm of chromosome pair 11 (Figure 32f).

In the fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers showed distinct telomeric labeling signals in all chromosomes of *L. peronii* (Figure 32g). No interstitial hybridization signals were detected.

Figure 34 Karyotypes of *L. personata* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions and (d) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.

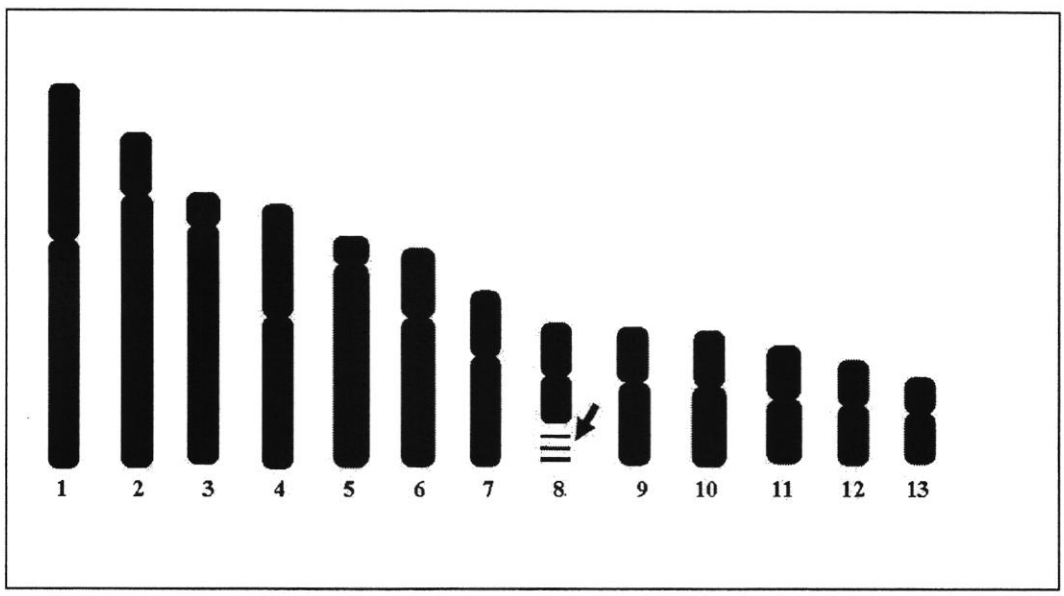
5.1.17 *L. personata*

All specimens of *L. personata* showed 2n=26 chromosomes which can be arranged in 13 pairs (Figure 34a). Chromosome pairs 1, 4, 10, 11, 12 and 13 were metacentric, pairs 2, 6, 7, 8 and 9 were submetacentric and pairs 3 and 5 were subtelocentric. Chromosome pair 8 (8q) showed a secondary constriction. Figure 35 (a) showed the idiogram of *L. personata* from conventional staining.

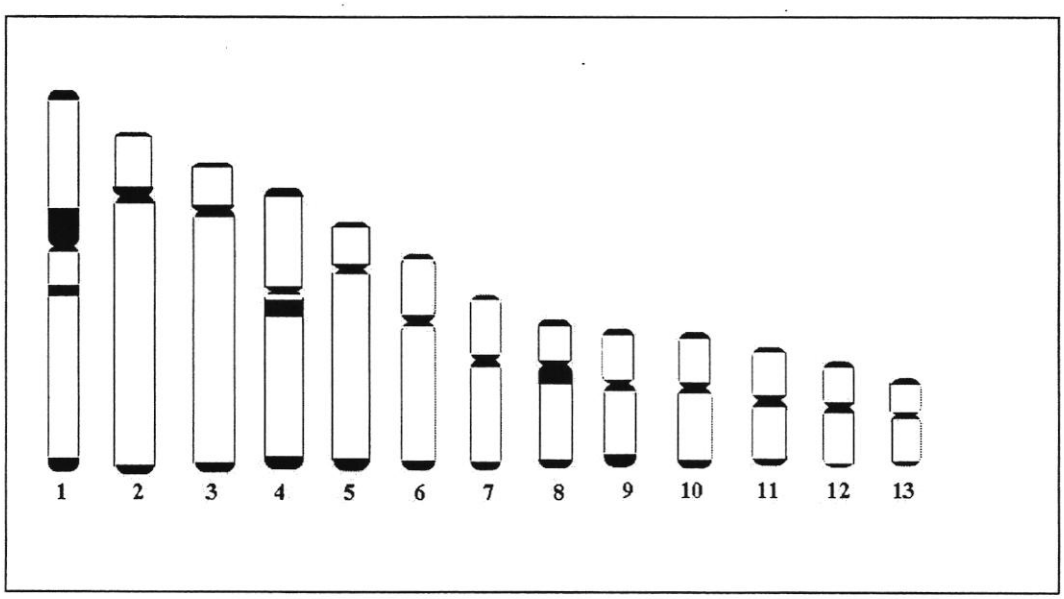
In the C-banded karyotypes of *L. personata*, constitutive heterochromatin can be discerned mainly in the centromeric and telomeric regions of all chromosomes (Figure 34b). Procentric C-bands were visible on the chromosome pairs 1, 4 and 8 and interstitial bands on chromosome pair 1. Figure 35 (b) showed the idiogram of *L. personata* from C-banding.

Silver staining showed that in all *L. personata* analyzed the nucleolus organizer region was in the telomeric region of chromosome pair 8 (Figure 34c). No positive silver labeling was visible in the other chromosomes.

In the fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers exhibited distinct telomeric labeling signals in all chromosomes of *L. personata* (Figure 34d). No interstitial hybridization signals were detected.



(a)



(b)

Figure 35 Idiogram of *L. personata* 2n (diploid) = 26, by (a) conventional staining, (b) C- banding. The arrows point to secondary constrictions.

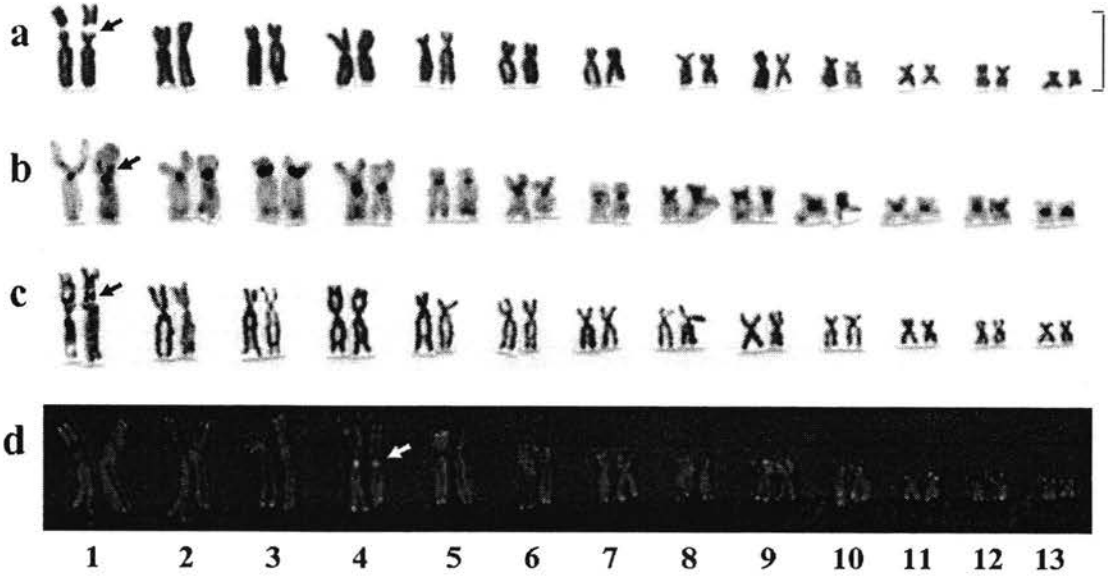
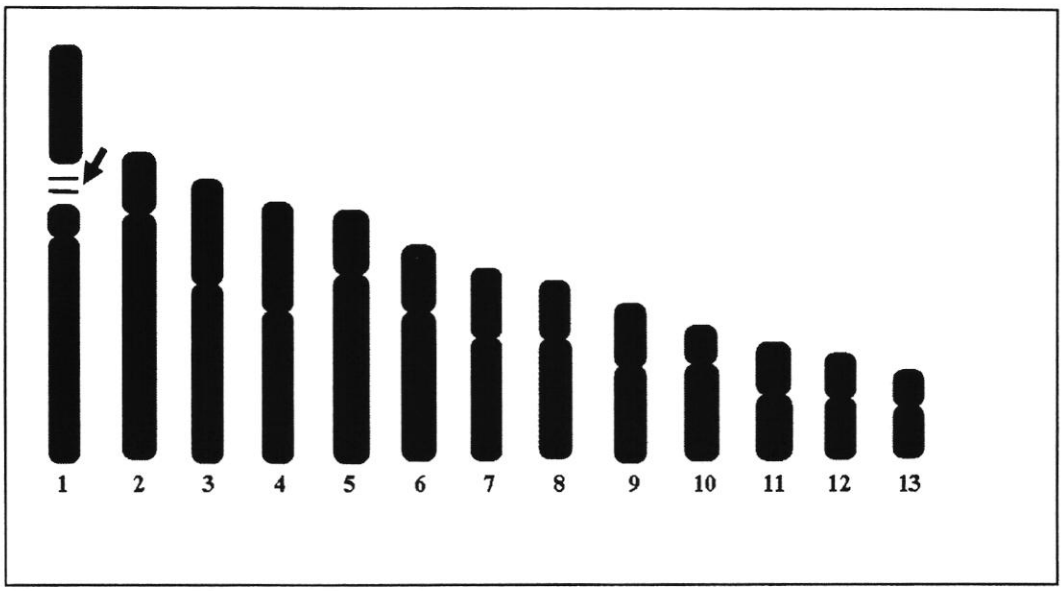


Figure 36 Karyotypes of *L. verreauxii* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions and (d) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions and the centromeric region of hybridization signals (5000x). Bar represents 10 μm.

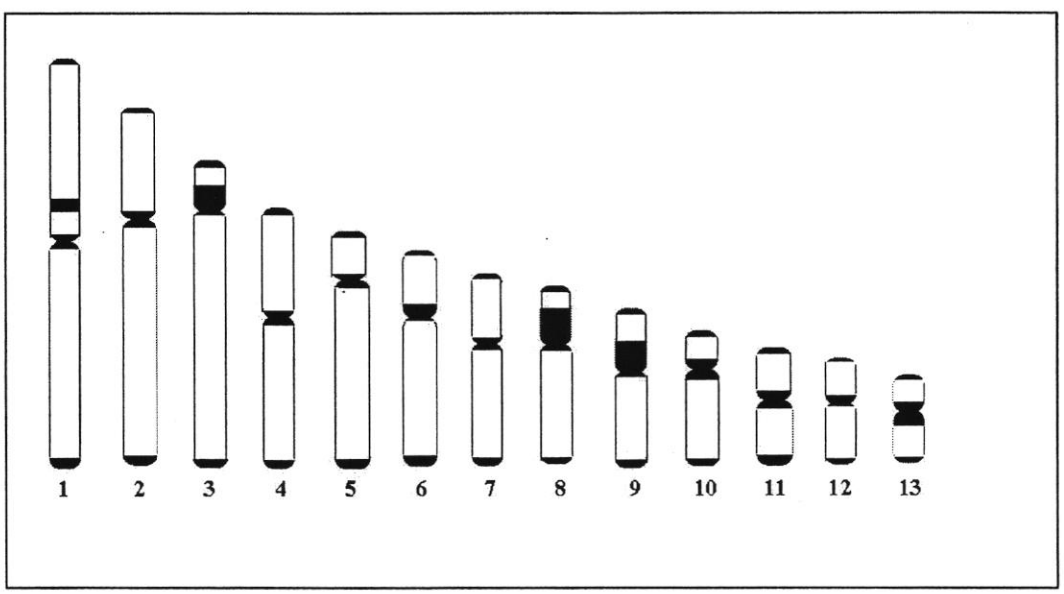
5.1.18 *L. verreauxii*

The karyotype of *L. verreauxii* exhibited 26 biarmed chromosomes which were consisted of 13 chromosome pairs (Figure 36a). Pairs 1, 4, 9, 11, 12 and 13 were metacentric, pairs 2, 6, 7, 8 and 10 were submetacentric and pairs 3 and 5 were subtelocentric. Chromosome no. 1 (1p) showed a secondary constriction. Figure 37 (a) showed the idiogram of *L. verreauxii* from conventional staining.

The C-banded karyotypes of *L. verreauxii*, constitutive heterochromatin can be discerned mainly in the centromeric and telomeric regions of all chromosomes (Figure 36b). Interstitial C-bands were visible in the pericentromeric regions of chromosomes pair 1 (1p) where the nucleolus organizer region was located. The chromosome pair 3 showed the large C-bands. Figure 37 (b) showed the idiogram of *L. verreauxii* from C-banding.



(a)



(b)

Figure 37 Idiogram of *L. verreauxii* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

Silver staining showed that in all *L. verreauxii* analyzed the nucleolus organizer region was located in the subcentromeric region of chromosome pair 1 (1p) (Figure 36c). No positive silver labeling was visible in the other chromosomes.

In the fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers demonstrated distinct telomeric labeling signals in all chromosomes of *L. verreauxii*

(Figure 36d). No interstitial hybridization signals were detected. Furthermore, all specimens showed centromeric regions hybridization signals in chromosome pair 4.

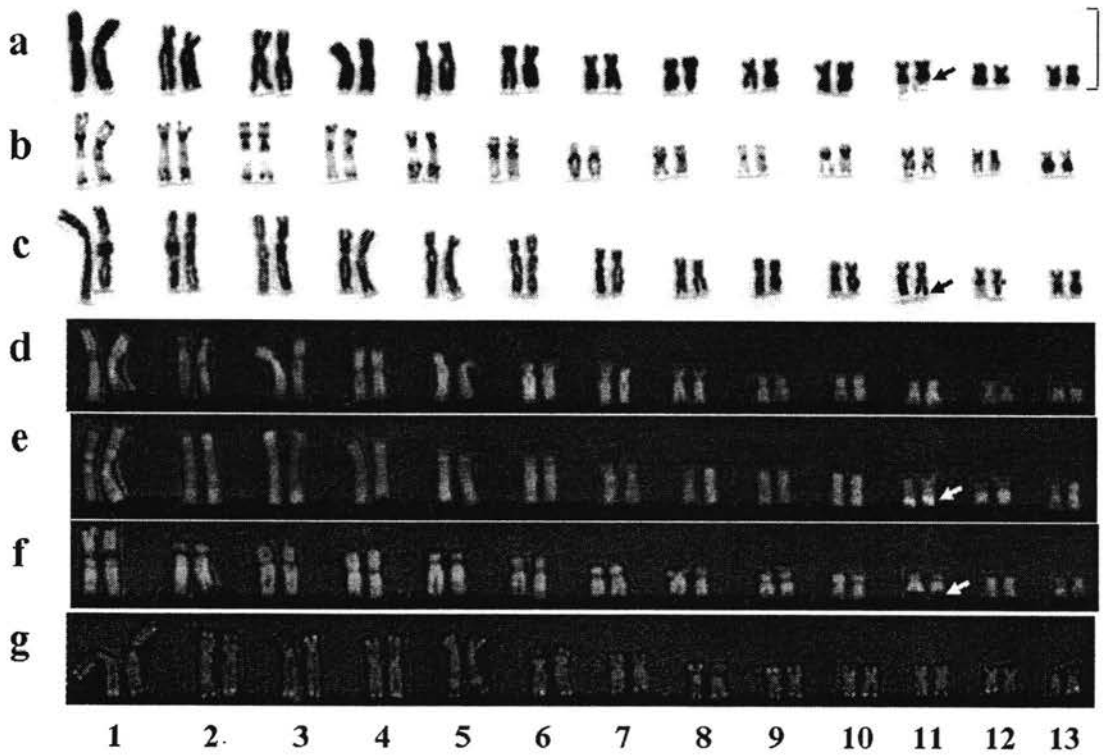


Figure 38 Karyotypes of *L. watjulumensis* showing (a) Giemsa staining, (b) C-banding of the constitutive heterochromatin, (c) silver staining of the nucleolus organizer regions, (d) distamycin A/DAPI counterstaining, (e) distamycin A/mithramycin counterstaining, (f) quinacrine mustard staining and (g) hybridization with $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers. The arrows point to secondary constrictions (5000x). Bar represents 10 μm.

5.1.19 *L. watjulumensis*

All specimens of *L. watjulumensis* showed 13 pairs of chromosomes (Figure 38a). Pairs 1, 4, 8, 9, 11, 12 and 13 were metacentric, pairs 2, 6, 7 and 10 were submetacentric and pairs 3 and 5 were subtelocentric. Figure 39 (a) showed the idiogram of *L. watjulumensis* from conventional staining.

The C-banded karyotypes of *L. watjulumensis*, constitutive heterochromatin was located in the centromeric and telomeric regions of all chromosomes (Figure 38b). Interstitial C-bands was demonstrated on the chromosome pairs 1, 2, 3, 5, 6 and the long arms of chromosome pair 13 consisted entirely of heterochromatin. The telomeric region showed a large double barred grey C-band on the long arm of chromosome pairs 2 and 5. Figure 39 (b) showed the idiogram of *L. watjulumensis* from C-banding.

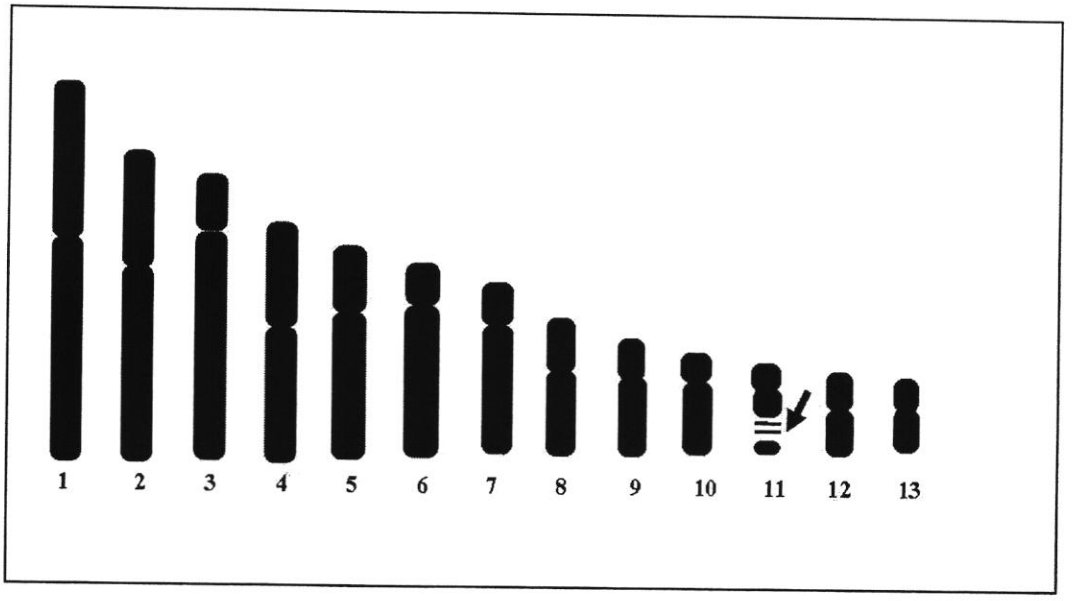
Silver staining showed that in all *L. watjulumensis* analyzed, the nucleolus organizer region was in the telomeric region of chromosome pair 11 (Figure 38c). No positive silver labeling was visible in the other chromosomes. All of specimens showed homologous silver-stained NORs of unequally-sized.

In the distamycin A/DAPI counter stained metaphase of *L. watjulumensis*, the karyotypes showed a uniform fluorescence (Figure 38d).

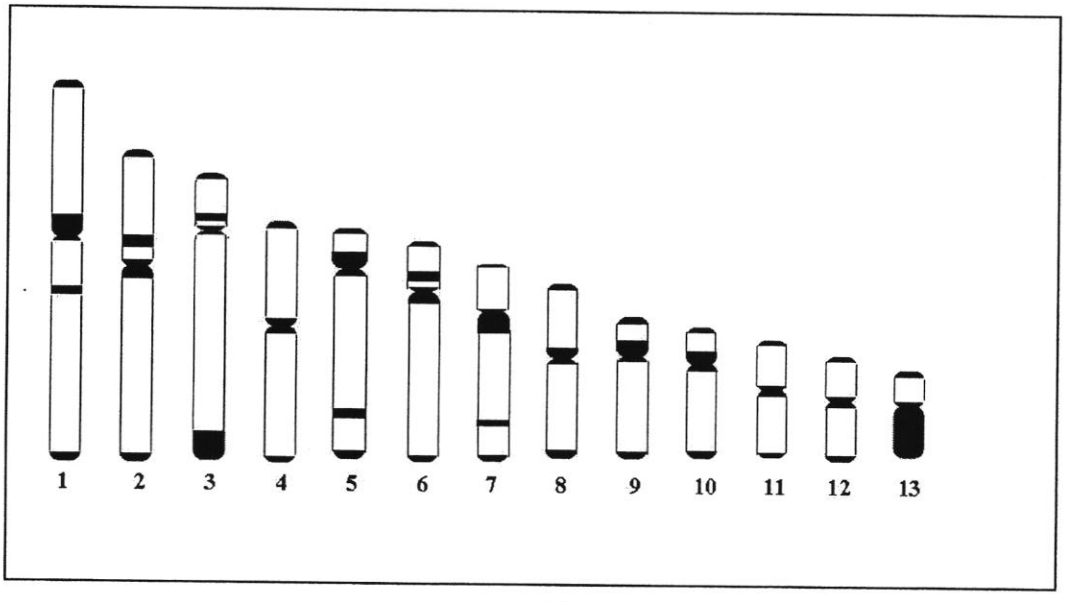
In the mithramycin-stained metaphase, mithramycin induces banding patterns in the *L. watjulumensis* karyotype which were the reverse to obtain by quinacrine mustard. Thus, the centromeric and telomeric heterochromatin in chromosomes 1-13 were mithramycin-positive. The NOR, located close the telomere in the long arm of chromosome pair 11 presented the brightest mithramycin fluorescence in the karyotype (Figure 38e).

In quinacrine-stained preparations, the fluorescence intensity of most centromeric and telomeric C-bands in chromosomes 1-13 were weaker than that of the euchromatic chromosome segments. No quinacrine fluorescence at all exhibited in the NOR on the long arm of chromosome pair 11 (Figure 38f).

As expected, fluorescence *in situ* hybridization of the $(GGGTTA)_7$ and $(TAACCC)_7$ oligomers demonstrated distinct telomeric labeling signals in all chromosomes of *L. watjulumensis* (Figure 38g). No interstitial hybridization signals were detected.



(a)



(b)

Figure 39 Idiogram of *L. watjulumensis* 2n (diploid) = 26, by (a) conventional staining, (b) C-banding. The arrows point to secondary constrictions.

The 19 species of *Litoria* showed no sexual dimorphism in its sex chromsomes.

The chromosomal data from 19 species of *Litoria* can be analyzed following banding techniques and described in karyotype morphology, secondary constriction morphology and heterochromatin distribution.

(1) Karyotype morphology

Almost all species exhibited chromosome morphology such as pairs 1 and 4 that were metacentric, pairs 2 and 6 were submetacentric, pairs 3 and 5 were subtelocentric and pairs 7 to 13 were mainly metacentric or submetacentric except in *L. barringtonensis* and *L. coplandi* that showed pair 3 was metacentric and in *L. infrafrenata* and *L. bicolor* showed similar chromosome morphology; pair 2 was subtelocentric, pair 3 was submetacentric, pair 5 was metacentric and pair 6 was submetacentric, except pair 4 in *L. infrafrenata* which was submetacentric but *L. bicolor* was metacentric. The chromosomes of pairs 1-6 were distinctly larger than those of pairs 7-13. In *L. bicolor* and *L. fallax* chromosome pairs 7 to 13 were more regularly metacentric than other *Litoria* species (Figure 4 and 14). In determining the arm ratios of the chromosome in the present study the secondary constrictions have not been included in the measurements.

(2) Secondary constriction morphology

Most of *Litoria* species in this study expressed secondary constriction. These constant constrictions may show either major or minor degrees of despiralization, which is directly reflected by the size of the achromatic gap. Some species have variable constriction, which are expressed in some, but not all cells and varies both within and between individuals. The following description is of secondary constrictions in three groups based on a combination of conventional staining, C-banding and silver stains.

Type 1. These constant constrictions may occur terminally, subterminally or interstitially on long arm and have been observed in *L. coplandi* pair 12 (Figure 8), *L. nasuta* pair 11 (Figure 24), *L. nyakalensis* pair 9 (Figure 28), *L. peronii* pair 11 (Figure 32), *L. personata* pair 8 (Figure 34) and *L. watjulumensis* pair 11 (Figure 38). These constrictions have a large and often variable achromatic gap, which in some species may be heteromorphic in size between homologues. When C-banded, the gap itself stains darkly and silver staining showed nucleolus organizer region.

Type 2. This constant constriction exhibited close to the centromeric region on the long arm of the chromosome, which was only observed in *L. barringtonensis* pair 11 (Figure 2). When C-banded, the achromatic gap itself stained darkly and silver staining showed in the nucleolus organizer region.

Type 3. These constant constrictions expressed on the interstitial of the short arm and have been observed in *L. eucnemis* pair 7 (Figure 12), *L. genimaculata* pair 7 (Figure 16) and *L. verreauxii* pair 1 (Figure 36). These constrictions have variable achromatic gaps, which in all species are heteromorphic in size between homologues. These constrictions have light grey C-banding, except in *L. verreauxii, which* has dark banding.

(3) Heterochromatin distribution

Although the 2n=26 karyotypes of *Litoria* species are remarkably uniform in their morphology, the considerable variation in the structure of the secondary constriction implies extensive heterochromatic reorganization as shown by C-banding. When examining the C-banding patterns between species the most striking feature was that no two of them share the same pattern. In addition to C-bands associated with secondary constrictions there are four arbitrary classes of C-heterochromatin. These are (1) procentric bands, (2) interstitial bands, (3) terminal grey bands and (4) major C-blocks that occupy most, if not all, of a chromosome arm.

(1) Procentric bands

There is remarkable variation in the quantity and distribution of these C-bands, both between chromosomes and between karyotypes in *Litoria* species; *L. barringtonensis*, *L. bicolor*, *L. genimaculata* and *L. nannotis* have shown uniformly procentric bands, whereas *L. nyakalensis* had relatively large bands extending from the centromere into both arms (Figure 28). Other species exhibited small procentric C-bands in some chromosomes; *L. eucnemis* pairs 4, 6, 7 and 8 (Figure 12), *L. caerulea* pairs 8, 9, 10, 12 and 13 (Figure 6), *L. infrafrenata* pairs 2, 7, 9 and 11 (Figure 18), *L. pallida* pair 10 (Figure 30), *L. personata* pair 1 (Figure 34), *L. verreauxii* pairs 3, 8, 9, 10 and 13 (Figure 36) and *L. watjulumensis* pair 10 (Figure 38).

(2) Interstitial bands

Relatively few interstitial C-bands were encountered and when present, they occurred only as fine bands. They were found in *L. barringtonensis* pairs 2 (Figure 2),

L. bicolor pair 1 (Figure 4), *L. coplandi* pairs 1 and 4 (Figure 8), *L. eucnemis* pairs 1, 2, 3 and 5 (Figure 12), and in *L. genimaculata* pairs 1, 2 and 6 (Figure 16), *L. infrafrenata* pairs 1 and 6 (Figure 18), *L. lesueurii* pairs 2 and 7 (Figure 20), *L. meiriana* pairs 1 and 4 (Figure 22), *L. nasuta* pairs 1, 2, 5 and 6 (Figure 24), *L. nyakalensis* pairs 1, 2, 3 and 4 (Figure 28), *L. pallida* pairs 2 and 6 (Figure 30), *L. personata* pairs 1, 4 and 8 (Figure 34), *L. verreauxii* pair 1 (Figure 36) and *L. watjulumensis* pairs 1, 2, 3, 5, 6 and 7 (Figure 39).

(3) Terminal grey bands

Lighter grey C-bands were presented in most chromosomes of all species and appear in the telomeric regions. Large double barred grey C-bands were presented on the long arm of pair 2 in *L. barringtonensis*, *L. bicolor*, *L. genimaculata*, *L. lesueurii* and *L. nyakalensis*; on the long arm of pair 3 in *L. caerulea*, *L. meiriana*, *L. nasuta* and *L. personata*; on the long arm of pairs 2 and 3 in *L. coplandi*; on the long arm of pairs 2 and 5 in *L. pallida* and *L. watjulumensis*; on the long arm of pairs 4 and 5 in *L. verreauxii* and on the long arm of pairs 1, 2, 4 and 5 in *L. eucnemis*.

(4) Major C-blocks

One of the most striking features of this study is the high incidence of large darkly stained C-band blocks, which often occupy entire chromosome arms. These blocks were expressed in two forms:

(1) In *L. meiriana* a very large additional and polymorphic segment was presented on pair 12(Figure 22). The situation appears to be polymorphic since both heterterozygotes and homozytotes for the absence of the block have been observed naturally in the same populations and are not sex correlated.

(2) The most common form of C-block occupies either a large proportion or else the whole of a chromosome arm and does not appear to have modified the external chromosome dimensions. In species; *L. bicolor* pair 9 (Figure 4), *L. infrafrenata* pair5 (Figure 18), *L. lesueurii* pair 9 (Figure 20), *L. nasuta* pair 11 (Figure 24) and *L. nyakalensis* pair 9 (Figure 28) these major C-blocks were associated with secondary constrictions. By contrast, in *L. coplandi* pair 11 (Figure 8), *L. eucnemis* pair 11 (Figure 12), *L. infrafrenata* pairs 7 and 8 (Figure 18), *L. nannotis* pair 11 (Figure 24), *L. nasuta* pair 12 (Figure 26), *L. peronii* pair 6 (Figure 32) and *L. watjulumensis* pair 13 (Figure 39), these blocks were not associated with constriction.

5.2 The construction of phylogenetic tree of *Litoria* species in Australia

For my study, morphological and cytogenetic data were combined to construct a phylogenetic tree of *Litoria* tree frog investigated in Australia (Figure 40). Phylogenetic relationships of *Litoria* tree frogs from 19 species based on the chromosome number, fundamental number and the NOR position and plotted on the tree. In *Litoria* tree frog show that a karyotype of 2n=26 is accepted in *L. infrafrenata* 2n=24 with metacentric, submetacentric and subtelocentric chromosomes, and the chromosomes pairs 1-6 larger than the chromosome pairs 7-13. *L. infrafrenata* is the advance species of *Litoria* tree frog. It seem possible that karyotype of *L. infrafrenata* can be derived from some species by chromosome translocation (fission and fusion) leading to increasing the number of large-sized subtelocentric chromosome, and reducing the number of small chromosome.

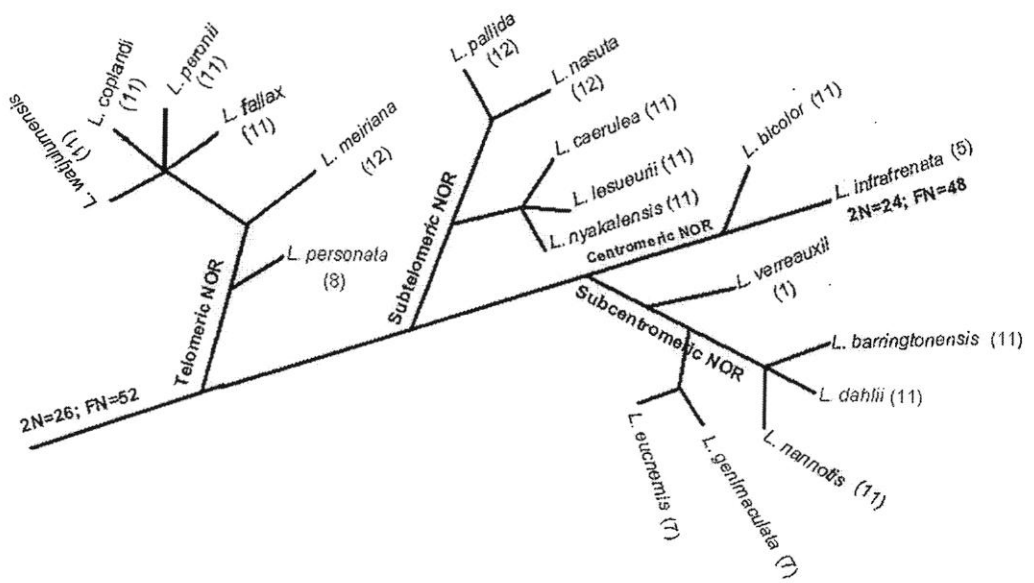


Figure 40 Hypothesized phylogenetic relationships within species groups of *Litoria* tree frog base mainly on results from chromosomal and karyotypic analyses. The branches show relationship among the species. The diploid number, fundamental number and the NOR position were plotted on the tree.

CHAPTER VI

DISCUSSION

6.1 Chromosome morphology of *Litoria* species

This study on the 19 species of *Litoria* tree frogs includes the 4 new species (*L. barringtonensis*, *L. genimaculata*, *L. nyakalensis* and *L. personata*) studied here and the other 15 species previously studied by Menzies and Tippett (1976) [13] and King et al. (1990) [14], showed a karyotype of 2n=26 except in *L. infrafrenata* which has 2n=24, agreed with the reports of Menzies and Tippett (1976) [13] and King et al. (1990) [14]. The chromosomes of the *Litoria* tree frogs are metacentric, submetacentric and subtelocentric morphology. King et al. (1990) [14] suggested that in term of arm ratios and centromere positions the chromosome morphology of *Litoria* species was very characteristic; pairs 1 and 4 were metacentric, pairs 2 and 6 were submetacentric and pairs 3 and 5 were subtelocentric and in terms of overall size the members of the karyotype fall naturally into two cluster; pairs 1-6 and pairs 7-13. For this study the chromosome morphology of 15 species of *Litoria* tree frogs supported for the report of King et al. (1990) [14] except in *L. barringtonensis* and *L. coplandi* showed pair 3 was metacentric and in *L. infrafrenata* and *L. bicolor* showed similar chromosome morphology; pair 2 was subtelocentric, pair 3 was submetacentric, pair 5 was metacentric and pair 6 was submetacentric by contrast in pair 4 of *L. infrafrenata* was submetacentric but *L. bicolor* was metacentric. Moreover, the morphology of the pairs 1-6 was highly conserved. In *Litoria* species pericentromeric inversions very probably are the rearrangements responsible for metacentric/submetacentric transitions that occurred along the chromosome. These may either be the result of a small pericentric inversion or else stem from the presence of a small constriction in the short arm of these chromosomes [6, 56]. This cytogenetic evidence suggests the conservative nature of karyotypes of the genus *Litoria* in Australia.

6.2 Secondary constriction structure in *Litoria* tree frogs

A series of studies on the products and probable functions of secondary constriction has been made on a number of amphibian species [57, 58, 59, 24]. However, there is a sizeable gap in our knowledge between the molecular RNA/DNA hybridization studies carried out by these workers and our basic understanding of the structure of secondary constrictions at the chromosome level. This stems largely from the fact that in past studies structural classes of secondary constrictions have not been adequately defined, nor has the distribution of heterochromatin in relation to these constrictions been described [5].

In 1980, King was organizing a group of *Litoria* species based on the C-banding pattern, which can be divided into five types. The 19 species of *Litoria* studied were divided into three types based on a combination of conventional staining, C-banding and silver stains, all types of secondary constrictions are NORs. The result showed that types 1 and 2 are consistent with types 2 and 5 in King's report (1980) [5]. For type 3 studied here it displayed the nucleolus organizer regions on the short arm of a large chromosome pair. These may be the result of structural rearrangements (insertions or inversions) that have moved the secondary constrictions throughout the karyotype. It is probable that subsequent to, or during the course of this functional amplification of a site, there are a series of internal structural modifications involving the production and redistribution of heterochromatin. This reorganization of heterochromatin may produce the characteristic secondary constriction types in *Litoria*. Blocks of heterochromatin may be simply associated with these major secondary constrictions to prevent crossing over in them.

6.3 Modes of heterochromatin evolution in the genus *Litoria*

King (1980) [5] has shown the major classes of C-banding material observed in *Litoria* (dark procentric C-bands, interstitial C-bands, telomeric light grey C-bands and major dark C-banding blocks), and it is the procentric C-bands and major blocks that give him an insight into the mode of heterochromatin evolution of *Litoria* species. For this study the results support the report of King (1980) [5], and there are two processes occurring; the addition of heterochromatin and the transformation of euchromatin. With the addition of heterochromatin; three species of *Litoria* in this studied have shown the addition of procentric blocks on chromosome including

L. infrafrenata pairs 7, 9 and 11, short arm, *L. pallida* on both arms of pair 2 and *L. verreauxii* on the short arm of pair 3. Transformation of euchromatin; in fact many of the karyotypic differences between species of *Litoria* involve such major C-blocks and these are clearly grounds for arguing that the process involved in their production is one of euchromatin transformation. The genus *Litoria* is a closely related species, which shares the same basic karyotype in terms of external chromosome morphology. This feature is emphasized by the retention of certain C-bands common to all species, for example the presence of a large grey telomeric block on the long arm of chromosome pairs 2 or 3. When major block differences of the transformation type are present they appear as derived forms. This suggests that the transformation process is an evolutionary derived state, i.e. it involves a change from euchromatin into heterochromatin. The fact that completely different chromosomes in different species exhibit such a contrast in form supports the concept of transformation. Thus, on short arms of pairs 5 and 8 in *L. infrafrenata* and *L. nyakalensis* pair 9q fall into this category. Many species showed a large C-banded block on the long arm including pair 9 in *L. bicolor*, *L. eucnemis*, *L. genimaculata* and *L. lesueurii*, pair 11 in *L. coplandi*, *L. meiriana* and *L. pallida*, pair 12 in *L. meiriana* and pair 13 in *L. watjulumensis*. In all these species relative chromosome dimensions are unaltered in the karyotype. Apart from the above-mentioned major blocks, most of which appear to have a procentric origin, there are numerous minor procentric blocks, which vary in size between species and are also probable transformation products.

A mechanism which permits the transformation of euchromatic areas to heterochromatin necessarily provides a means of stabilizing or 'locking up' major gene complexes by preventing recombination in their vicinity. This would be particularly useful in those cases where secondary constrictions are newly amplified with heterochromatinization occurring around these sites. Similarly heterologous sex regions can also be effectively isolated by such a transformation process [60, 5, 6].

6.4 NOR localization and heteromorphism in *Litoria* species

In agreement with the previous report of King (1990) [6], the 19 species of *Litoria* species have shown a single NORs site on the karyotypes. Most species displayed the nucleolus organizer regions on the long arms of the small chromosome pairs, except in *L. eucnemis* and

L. genimaculata observed on the short arms of chromosome pair 7 and *L. verreauxii* showed on the short arm of chromosome pair 1. Multiple NOR-bearing chromosome pairs have been considered a derived state in the Anura [14] and have been found in species of several families [61, 62, 19, 63, 64]. Possible mechanisms involved in NOR dispersion in anuran genomes include inversions and translocations involving chromosomal segments containing NORs, transpositions by mobile genetic elements, amplifications of rDNA cistrons and reinsertion error during extrachromosomal amplification of ribosomal cistrons [65, 66, 14, 61, 64]. Most species of the genus *Litoria* in this study displayed a heteromorphism in NOR size. The vast majority of anuran amphibians, although being characterized by having a single NOR site, display a remarkably high level of heteromorphism in NOR size [6]. Schmid (1982) [22] found that 67% of the 260 specimens he analysed have fixed heteromorphisms occurring between homologues. That is, heteromorphisms are found in all cells within that specimen. While deletion of one of the NORs in a pair of homologues has been documented in certain anuran species [22], the vast majority of heteromorphism observed involve amplification of the ribosomal DNA in one of a pair of homologues. The possibility that deletion has also been involved is supported by the presence of a single silver stained NOR in one of two homologues [22]. When amplification is present it may take two forms, first, the amplification of certain ribosomal sequences producing subtle size variation between homologues; second, the amplification of the entire NOR. In this latter case examination of the homologues shows a duplication, or in some case triplication of the NOR [5, 67, 22]. Explanations offered for the high frequency of fixed heteromorphic differences between homologues appear to operate at two levels, First, the differences found between homologues may reflect functional variation of the NOR in the preceding interphase. Second, Macgregor et al. (1977) [68] suggested that since NORs of different size are segregating randomly within each population, and knowing the diversity in size which exists, it is not surprising that heterozygosity is the rule and homozygosity the exception.

6.5 Fluorescence banding of *Litoria* species

Although this study cannot be reported in all species, the results of fluorescence banding have shown that not only quantitative, but also qualitative, differences in constitutive heterochromatin have evolved in the karyotype of most species. Previously, only those reported by

Schmid et al. (2003) [41] in *L. infrafrenata* showed centromeric heterochromatin in all chromosomes and the secondary constriction exhibiting the brightest fluorescence by using DAPI/Mithramycin-stained. Quinacrine mustard demonstrates quenched fluorescence of the centromeric and most of the interstitial heterochromatic regions. This study on *Litoria* species; in the distamycin A/DAPI counter stained the karyotypes and showed a uniform fluorescence. In DAPI/Mithramycin reverse to obtain by quinacrine mustard. Silver staining of *Litoria* species chromosomes confirms the result obtained by mithramycin fluorescence in locating the nucleolus organizer region, agreed with the report of Schmid et al. (2003) [41]. The hybridization of the telomeric probe outside of the ends of all chromosomes in all species and pair 4 in *L. eucnemis*, *L. genimaculata* and *L. verreauxii* and pairs 1, 2 and 3 in *L. fallax*, indicated the presence of repeats similar to $(TTAGGG)_n$ in the repetitive centromeric region. Another possible type of repetitive centromeric region corresponded to that of the chromosomes of four species, since neither the base-specific fluorochromes nor the telomeric probe yielded a fluorescent labeling. Occasionally, interstitial hybridization of the telomeric probe may represent true vestiges of telomeres, corroborating structural rearrangements occurred during chromosome evolution, as described in rodents [69]. Nevertheless, this possibility was excluded in the other frogs presenting interstitial telomeric sequence [70]. Regardless, the presence of repetitive DNA bearing telomere-like sequences outside the telomeres might represent an additional cytological marker for species or even species groups [71].

6.6 Sex chromosomes in *Litoria* species

In agreement with earlier investigations [6, 25, 26, 9], the 19 species of *Litoria* showed the sex chromosomes are morphologically undifferentiated (homomorphic). Nevertheless, the sex chromosomes of Amphibia are attractive for several reasons: (1) Due to the large size of most amphibian chromosomes, the sex chromosomes are conveniently amenable to analyses with cytogenetic techniques, (2) The pairing arrangements of the sex chromosomes can be studied not only in the male stages of meiosis, but also in the fine-structured lampbrush chromosomes in the oocytes of females. (3) Both types of common chromosomal sex-determining mechanisms (XX/XY and ZW (female/male)) and even a very rare WO/OO (female/male) have evolved in Anura. (4) Various morphs of Y or W chromosomes can coexist within the same population of some species.

(5) Several of the Y and W chromosomes that have been found are still in an initial stage of morphological differentiation [28]. Advances in cytogenetically techniques have enabled proof that both XY/XX and ZW/ZZ sex chromosomes exist among Anura and Urodela. Whereas most amphibians present homomorphic sex chromosomes, species have also been found in which an increasing structural complexity of the Y and W chromosomes has been demonstrated. In many cases, the morphological differentiation of the sex chromosomes occurred as a result of changes affecting the quantity of repetitive DNA sequences in the constitutive heterochromatin of the Y and the W chromosome. The greater the structural differences are between the sex chromosomes, the less is the extent of their meiotic pairing. Some species of amphibians possess unusual forms of XY/XX, ZW/ZZ, or even an OW/OO system of sex determination, not existing in the other classes of vertebrate [26, 72].

6.7 Chromosome evolution in the genus *Litoria*

The present results led me to the general conclusion that the karyotype of most species in the *Litoria* species is quite stable, characterized by a similar macrostructure (2n = 26 mostly meta and submetacentric chromosomes). This seems to be evidence for a low chromosome evolution rate. Chromosome evolution rates have been identified in several organisms, ranging from quite reduced values, as is the case for amphibians, to high values, as is the case for mammals [37]. All species examined had 2n=26, with the exception of *L. infrafrenata* 2n=24. Apart from *L. infrafrenata* with 2n=24, the diploid chromosome number larger than all other species 2n=26. This implies that chromosome fissions must have occurred a number of times during *Litoria* chromosome evolution. The karyotypes of most species of *Litoria* that exhibited a single pair of NORs were close to the telomeric region, by means of translocations restricted to the very terminal chromosome segments, the original NORs could have been shifted to the telomeric regions of other chromosomes. King (1980) [5] observed the number, site and location of NORs in Australian tree frogs of the genus *Litoria* and suggests that the apparently erratic distribution of the NORs is probably related with the existence of a series of latent nucleolar organizer sites throughout a karyotype and that during the evolution of a species, particular sites take over the primary nucleolar function.

6.8 The phylogeny of the genus *Litoria*

Previous studies of the genus *Litoria* were based on limited sampling of species and banding patterns of chromosomes [73], for the present study the results of NOR position and major chromosome morphology strongly supported the phylogenetic tree based on nuclear and mitochondrial genes constructed by Wiens et al. (2010) [50]. The phylogenetic analysis suggests that the 4 new species (*L. barringtonensis*, *L. genimaculata*, *L. nyakalensis* and *L. personata*) studied here were a monophyletic group. This phylogenetic arrangement supports previously published phylogenies [74, 75, 49, 50]. Additional comparative high-resolution molecular cytogenetic studies will be necessary to precisely define the rearrangement in the genus *Litoria* to clarify their phylogenetic relationships.

CHAPTER VII

CONCLUSION

The mitotic chromosome of 19 species of the genus *Litoria* and the 4 new species (*L. barringtonensis*, *L. genimaculata*, *L. nyakalensis* and *L. personata*) studied here were directly prepared from bone marrow cells after *in vivo* colchicine treatment using conventional method and chromosome banding techniques. The diploid chromosome number of 19 species of the genus *Litoria* was successfully examined. All species have 2n=26, except in the *L. infrafrenata* which has 2n=24. The 19 species of the genus *Litoria* have shown remarkable karyological uniformity. This refers to general chromosome morphology, to heterochromatin distribution and to NOR locations. Pairs 1 and 4 were metacentric, pairs 2 and 6 were submetacentric and pairs 3 and 5 were subtelocentric. Many species expressed major secondary constriction including *L. barringtonensis* (pair 11), *L. nasuta* (pair 12), *L. nyakalensis* (pair 9), *L. personata* (pair 8) and *L. verreauxii* (pair 1). These constrictions silver stain and are regarded as nucleolar organizers. In *L. meiriana* results showed a very large additional and polymorphic segment of C-block on the chromosome pair 12. In 19 species of the genus *Litoria*, no two species are karyotypically identical in terms of their C-banding pattern. All species of *Litoria* in this study showed a single NORs site; the 4 species have shown the location of NORs on the short arm of the large chromosome pair and the other species are shown on the long arm of a small chromosome pair. For fluorescence banding in this study it can not be reported in all species, but the results show that there are not only quantitative, but also qualitative differences in constitutive heterochromatin that have evolved in the karyotype of most species. The hybridization of the telomeric probe outside of the ends of all chromosomes in all species, and the chromosome of pair 4 in *L. eucnemis*, *L. genimaculata* and *L. verreauxii* and pairs 1, 2 and 3 in *L. fallax*, indicated the presence of repeats similar to $(TTAGGG)_n$ in the repetitive centromeric region. No heteromorphic pairs of chromosomes have been identified in all *Litoria* species examined. The phylogenetic analysis suggests these species in this study were a monophyletic group except in the *L. infrafrenata* and *L. meiriana*, which were a paraphyletic group and where it was observed that

each monophyletic clade had the NORs position in a specific chromosome pair. Additional comparative high-resolution molecular cytogenetic studies will be necessary to precisely define the rearrangement in *Litoria* to clarify their phylogenetic relationships. The results presented here will be useful in directing future studies of the genus *Litoria* and phylogenetic relationships within Pelodryadinae more generally.

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APPENDIX

Appendix 1. Chemical agents for chromosome preparation.

1.1 0.3% colchicine solution. Dissolve 30 mg colchicines powder (Sigma) in 10 ml of distilled water. This solution can be stored in the refrigerator (2-8 °C). It can be stable for approximately 1 month.

1.2 0.075 M. KCl hypotonic solution. Dissolve 0.5588 g of potassium chloride crystal (Merck) in 100 ml of distilled water. This solution is stable for approximately 1 month.

1.3 Carnoy's fixative. Mix 3 parts of absolute Methanol (Merck) with 1 part of absolute glacial acetic acid (Merck) in a ratio 3:1. This solution can be stored in the refrigerator (-4°C) (made day of use).

Appendix 2. Chemical agents for chromosome staining

2.1 Giemsa stain

2.1.1 Giemsa stock solution. Dissolve 7.5 g of Giemsa powder (Sigma) in 250 ml of glycerol and 750 ml of absolute methanol. Giemsa stain should be prepared at least 2 weeks before use and stored in a dark bottle in a 37°C incubator.

2.1.2 Phosphate buffer solution (PBS) (pH 6.8)

2.1.2.1 0.01 M Potassium dihydrogen phosphate (KH_2PO_4) solution (Stock A). Dissolve 9.1 g of KH_2PO_4 (Fluka AG) in 1 L of distilled water.

2.1.2.2 0.01 M di-Sodium hydrogen phosphate (Na_2HPO_4) solution (Stock B). Dissolve 9.5 g of Na_2HPO_4 (Fluka AG) in 1 L of distilled water.

2.1.2.3 Mix 50.2 ml of Stock A solution with 49.8 ml of Stock B solution.

2.1.3 5% Giemsa solution. Mix 5 ml of Giemsa stock solution with 95 ml of PBS (made day of use). The mixed solution was filtrated before use.

2.2 Nucleolar organizer region stain

2.2.1 50% Silver nitrate ($AgNO_3$). Dissolve 5 g of Silver nitrate ($AgNO_3$) in 10 ml of distilled water.

2.2.2 Gelatin Dissolve 1.5 g of gelatin in 50 ml distilled water and add 500 µl formic acid.

2.2.3 2% Giemsa solution. Mix 2 ml of Giemsa stock solution with 98 ml of PBS (made day of use). The mixed solution was filtrated before use.

2.3 Constitutive heterochromatin banding

2.3.1 Barium hydroxide [$Ba(OH)_2$] (Merck) solution. Dissolve 5 g [$Ba(OH)_2$] x 8 H_2O in 100 ml distilled water and store in an air-tight bottle until immediately prior to use.

2.3.2 0.2 N HCl Dissolve 10 ml of 2 N HCl in 100 ml distilled water.

2.3.3 2 x SSC Solution. Mix 0.03 M Sodium citrate (17.6 g/l) with 1 part of 0.03 M NaCl (8.82 g/l).

2.4 Fluorescence banding

2.4.1 McIlvaine's Buffer. Dissolve 7.98 g (Citric acid monohydrate) and 57.68 g (Na_2HPO_4 x $2H_2O$) in 2L distilled water.

2.4.2 Distamycin A. Dissolve 300 µg Distamycin A in 1 ml McIlvaine's buffer.

2.4.3 DAPI stain. Dissolve 2 mg DAPI in 10 ml McIlvaine's buffer.

2.4.4 Mithramycin stain. Dissolve 1 mg Mithramycin and 20.33 mg $MgCl_2$ in 10 ml McIlvaine's buffer.

2.4.5 Quinacrine-Mustard. Dissolve 5 mg of Quinacrine-Mustard in 100 ml distilled water.

2.4.6 Telomere FISH

2.4.6.1 TBS buffer. 1 TBS foil package in 1 L distilled water (1/2 year at 4 °C)

2.4.6.2 3.7% formaldehyde in TBS (working solution). Add 8 ml 37% formaldehyde to 72 ml TBS buffer (no more than 4 weeks after preparation).

2.4.6.3 Pre-treatment solution (working solution). Add 40 µl Pre-treatment solution to 80 ml TBS buffer (prepare fresh for each experiment).

2.4.6.4 Rinse Solution (working solution). Add 2 ml Rinse solution in 98 ml distilled water (1 year at 4 °C) for experiment use at room temperature.

2.4.6.5 Wash solution (working solution). Add 2 ml Wash solution in 98 ml distilled water pre-heating of Wash solution to 65 °C 1.5 hr. before use.

Appendix 3 Nomenclature for chromosome

Table 4 Nomenclature for centromere position on mitotic chromosomes based on the centromeric ratio.

Chromosome types	Abbreviation	Centromeric ratio
Metacentric	m	1.00-1.67
Submetacentric	sm	1.68-3.00
Subtelocentric	st	3.01-7.00
Telocentric	t	7.01-œ