SYSTEMATICS AND BIOGEOGRAPHY OF FLYING SQUIRRELS IN THE EASTERN AND THE WESTERN TRANS-HIMALAYAS

By

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TABLE OF CONTENTS

page

ACKNOWLEDGMENTS iii

LIST OF TABLES ix

LIST OF FIGURES xii

ABSTRACT xvii

CHAPTERS

1 INTRODUCTION 1

1.1 Evolution of Flying Squirrels 2
1.2 Evolution of Chinese Flying Squirrels 6
1.3 Objectives of This Study 10

2 METHODS AND TECHNIQUES OF PHYLOGENETIC STUDY 13

2.1 Molecular Study 14
2.1.1 Mitochondrial Cytochrome b Gene 14
2.1.2 Phylogenetic Analysis 16
2.1.2.1 Parsimony method 17
2.1.2.2 Likelihood method 17
2.1.2.3 Distance method 18
2.2 Morphometric Study 19

3 PHYLOGENY AND BIOGEOGRAPHY OF *EUPETAURUS* 22

3.1 Introduction 22
3.2 Materials and Methods 25
3.2.1 Samples 25
3.2.2 Methods 27
3.2.2.1 Mitochondrial DNA isolation 27
3.2.2.2 PCR amplification 27
3.2.2.3 Sequence analysis 28
3.3 Results 30
3.3.1 Phylogenetic Relationship of *Eupetaurus* between the Eastern and the Western Trans-Himalayas 30

3.3.2 Phylogenetic Analysis between *Eupetaurus* and *Petaurista* 31
3.4 Discussion 33
3.4.1 Phylogenetic Status of the Population of *Eupetaurus* in the Eastern Trans-Himalayas 33
3.4.2 Phylogenetic Relationship between *Eupetaurus* and *Petaurista* 35
3.5 Summary 39

4 PHYLOGENY OF GIANT FLYING SQUIRREL (*PETAURISTA*) IN SW CHINA AND PAKISTAN: IMPLICATIONS FOR DEVELOPMENT OF MOLECULAR AND MORPHOLOGICAL ANALYSIS 49

4.1 Introduction 49
4.2 Materials and Methods 54
4.2.1 Specimens in Morphometric Study 54
4.2.2 Species for Molecular Analysis 56
4.2.3 Morphometric Analysis 58
4.2.4 Molecular Analysis 59
4.3 Results 60
4.3.1 Phylogenetic Relationships of Chinese *P. philippensis* 60
4.3.1.1 Morphological data 60
4.3.1.2 Molecular data 63
4.3.2 Phylogenetic Relationship between *P. xanthotis* and *P. leucogenys* 65
4.3.3 Phylogenetic Relationship of *P. petaurista* 66
4.3.4 Phylogenetic Relationships of Chinese *Petaurista* 70
4.4 Discussion 76
4.4.1 Phylogeny of the Trans-Himalayan *P. petaurista* (*albiventer*) 76
4.4.2 Taxonomic Status of *P. philippensis*, *P. yunanensis*, and *P. hainana* 80
4.4.3 Phylogenetic Relationship between *P. xanthotis* and *P. leucogenys* 83
4.4.4 Systematics of Chinese *Petaurista* 85
4.5 Summary 89

5 PHYLOGENY OF *EOGLAUCOMYS* AND *HYLOPETES* IN THE EASTERN AND THE WESTERN TRANS-HIMALAYAS AS INFERRED FROM MOLECULAR AND MORPHOMETRIC STUDY 114

5.1 Introduction 114
5.2 Materials and Methods 117
5.2.1 Materials 117
5.2.2 Morphometric Analysis 119
5.2.3 Biochemical Study 120
5.3 Results 121
5.3.1 Comparison between *Eoglaucomys* and the Chinese *Hylopetes* 121
5.3.1.1 Morphological data 121
5.3.1.2 Molecular data 123
5.3.2 Phylogenetic Relationships of *Hylopetes* 124

5.4 Discussion 128
5.4.1 Taxonomic Status of *Eoglaucomys* 128
5.4.2 Phylogenetic Status of *H. electilis* 130
5.4.3 Phylogenetics and Biogeography of *Eoglaucomys* and *Hylopetes* 132
5.5 Conclusion 135

6 PYLOGENY OF FLYING SQUIRRELS IN THE TRANS-HIMALAYAS AND OTHER PARTS OF CHINA 152

6.1 Introduction 152
6.2 Materials and Methods 155
6.2.1 Specimens 155
6.2.2 Methods of Phylogenetic Analyses 158
6.3 Results 158
6.3.1 Comparative Study of the Eastern and the Western Trans-Himalayan Flying Squirrels 158
6.3.2 Phylogenetic Relationships among Flying Squirrels in China 161
6.4 Discussion 165
6.4.1 Phylogeny of the Trans-Himalayan Flying Squirrels 165
6.4.2 Systematics of Chinese Flying Squirrels 170
6.5 Summary 176

7 SUMMARY AND FUTURE WORK 192

7.1 Summary 192
7.2 Future Work 193

APPENDIX

GEOLOGICAL EPOCHS 196

LIST OF REFERENCES 197

BIOGRAPHICAL SKETCH 210

LIST OF TABLES

Table page

1.1 McKenna's classification of flying squirrels in Petauristinae 3

1.2 Mein's classification, determined from dental characters 4

1.3 The latest estimate of flying squirrels in *Pteromystinae* 4

1.4 Comparison of different classifications of flying squirrels 5

1.5 Flying squirrels and their distributions 6

1.6 Chinese flying squirrels 8

3.1 Thirteen historical and 2 recent specimens of *Eupetaurus* 24

3.2 Specimens of *Eupetaurus* and other flying squirrels examined in this study* 26

3.3 Cycling program of PCR amplification 28

3.4 Percentage differences of *Eupetaurus* and *Petaurista* based on the pairwise comparisons of cytochrome b gene (390 bp) 31

3.5 Transversional substitutions at the third codon positions of cytochrome b gene between *Eupetaurus* and *Petaurista* (based on 390 bp) 32

3.6 Estimated divergent times among *Eupetaurus* and *Petaurista* based on a rate of divergence for the third codon positions of mammalian cytochrome b gene of ca. 0.5% *10^6 years 33

4.1 Chinese *Petaurista* forms 50

4.2 Forms of *Petaurista* 52

4.3 Major forms of *P. philippensis* 52

4.4 Species and localities of *Petaurista* populations examined in morphometric analysis 55

4.5 Variables in morphometric study 56

4.6 Samples of *Petaurista* examined in molecular study 57

4.7 Sequence data of *Petaurista* and *Pteromys* used in this study 58

4.8 Discriminant function analysis of five *P. philippensis* forms 62

4.9 Principal components analysis of five *P. philippensis* forms..... 63

4.10 Pairwise comparison based on the partial sequences (409 bp) of cytochrome b gene between five *P. philippensis* forms. Data below the diagonal are the numbers of nucleotide substitutions, transitions vs. transversions. Data above the diagonal represent the genetic differences between samples. The samples were defined in Table 4.6 and 4.7. 64

4.11 Percentage of genetic differences between *P. xanthotis* and other giant flying squirrels based on pairwise comparison of the partial cytochrome b sequences (409 bp). See Table 4.6 and Table 4.7 for sample information..... 65

4.12 Discriminant function analysis of *P. petaurista*..... 67

4.13 Principal components analysis of *P. petaurista* 68

4.14 Percentage of differences and the numbers of transversional and transitional substitutions between *P. petaurista* (*albiventer*) populations based on pairwise comparison of the partial sequence (375 bp) of cytochrome b gene..... 69

4.15 Discriminant function analysis of Chinese *Petaurista*..... 71

4.16 Principal components analysis of Chinese *Petaurista* 72

4.17 Pairwise comparison of Chinese *Petaurista* based on the partial sequences (380 bp) of cytochrome b gene. Data above the diagonal were the percentage of genetic differences between samples, and data below the diagonal were the numbers of transitions vs. transversions between samples. 74

4.18 Transversional substitutions at the third codon positions of the partial sequences (375 bp) of cytochrome b gene in *Petaurista*..... 75

4.19 The estimated divergence time between species based on a divergence rate for the third codon positions of mammalian cytochrome b gene of ca. 0.5% *10^6 years 75

5.1 Species of *Hylopetes* and *Eoglaucomys*..... 116

5.2 Species and localities of *Hylopetes* and *Eoglaucomys* examined in morphometric analysis..... 118

5.3 Samples of *Eoglaucomys* and *Hylopetes* examined in molecular study 119

5.4 Discriminant function analysis between the Chinese *Hylopetes* and *Eoglaucomys* . 122

5.5 Principal components analysis between Chinese *Hylopetes* and *Eoglaucomys*..... 123

5.6 Pairwise comparison of cytochrome b nucleotide sequences (400 bp) between Chinese *Hylopetes* and *Eoglaucomys* 124

5.7 Pairwise comparison of *Hylopetes* and *Eoglaucomsy* based on the partial cytochrome b sequences (375 bp) 126

5.8 Transversional substitution rates at the third codon positions of the partial sequences (375 bp) of cytochrome b gene between species 127

5.9 Estimated divergence time between species using the rate of the transversional substitutions at the third codon positions of mammalian cytochrome b gene of ca. 0.5% *10^6 years 127

6.1 Classification of the extant flying squirrels determined from 44 dental characters .. 153

6.2 Chinese flying squirrels other than *Petaurista* 153

6.3 Species and localities of flying squirrels used in morphometric analysis 156

6.4 Samples of flying squirrels used in molecular study 157

6.5 Discriminant function analysis on the eastern and the western trans-Himalayan flying squirrels 159

6.6 Principal components analysis of trans-Himalayan flying squirrels on the first three factors 161

6.7 Pairwise comparison based on the partial cytochrome b sequences (375 bp) between species. See Table 6.4 for the sample abbreviations. 162

6.8 Pairwise comparison of the transversional substitutions at the third codon positions of the partial cytochrome b sequences (366 bp) between samples. Data below the diagonal are the numbers of transversions at the third codon positions. Data above the diagonal represent the transversional percentage difference between samples. Table 6.4 shows the information of samples. 163

6.9 Estimated divergence time between samples based on the rate of the transversional substitutions at the third codon of cytochrome b sequences proposed by Irwine et al. (1991). See Table 6.4 for sample abbreviations. 164

6.10 Multivariate analyses of Chinese flying squirrels 165

6.11 Key to the genera of Chinese flying squirrels 176

LIST OF FIGURES

Figure page

3.1 Historical records of *Eupetaurus* specimens in the world. The numbers in the map stand for the collecting localities of specimens, which are corresponding to the numbers of Table 3.1. 40

3.2 Phylogenetic tree of *Eupetaurus* reconstructed by the maximum parsimony (MP) method. Numbers above branches indicate the bootstrap values (%). Sample abbreviations are coded in Table 3.2. 41

3.3 Phylogenetic tree of *Eupetaurus* reconstructed by the UPGMA method. Sample abbreviations are coded in Table 3.2. 42

3.4 Phylogenetic tree of *Eupetaurus* and *Petaurita* constructed with UPGMA method. Sample abbreviations are defined in Table 3.2. 43

3.5 Phylogenetic relationships of *Eupetaurus*, *Petaurita*, and *G. volans* constructed with the parsimony maximum (MP) method. Numbers above branches indicate the bootstrap values (%). Sample abbreviations are defined in Table 3.2. 44

3.6 Phylogenetic relationships of *Eupetaurus*, *Petaurita*, and *G. volans* constructed with the neighbor-joining (NJ) method. Scales in the tree represent branch length in terms of nucleotide substitutions per site. Sample abbreviations are coded in Table 3.2. 45

3.7 *Eupetaurus cinereus* in Pakistan and SW China 46

3.8 Ventral views of the skulls of *E. cinereus*, *P. petaurista*, and *P. xanthotis* 47

3.9 *E. cinereus*, *P. petaurista*, and *P. xanthotis* 48

4.1 Chinese giant flying squirrels (*Petaurista*) 90

4.2 Plot of five *P. philippensis* forms onto discriminant function 1 (CAN I) and function 2 (CAN II) 91

4.3 Plot of five *P. philippensis* forms onto discriminant function 1 (CAN I) and function 3 (CAN III) 92

4.4 Principal components analysis of five *P. philippensis* forms onto factor 1 (PRIN I) and factor 2 (PRIN II) 93

4.5 Principal components analysis of five *P. philippensis* forms onto factor 1 (PRIN I) and factor 3 (PRIN III) 94

4.6 Phylogenetic relationships of *P. philippensis* forms based on the cytochrome b gene using maximum parsimony method (MP). Numbers above branches indicate the bootstrap values (%). Sample abbreviations are defined in Table 4.6 and Table 4.7. 95

4.7 Phylogenetic relationships of *P. philippensis* forms based on the cytochrome b gene using neighbor-joining method (NJ). Scales in the tree represent branch length in terms of nucleotide substitutions per site. See sample abbreviations in Table 4.6 and Table 4.7. 96

4.8 Phylogenetic tree of *P. xanthotis* and other giant flying squirrels constructed using maximum parsimony method (MP). Numbers above branches indicate the bootstrap values (%). Sample abbreviations are defined in Table 4.6 and Table 4.7. 97

4.9 Phylogenetic tree of *P. xanthotis* and other giant flying squirrels constructed using neighbor -joining method (NJ). Scales in the tree represent branch length in terms of nucleotide substitutions per site. See sample codes in Table 4.6 and Table 4.7. 98

4.10 Plot of *P. petaurista* populations of discriminant function analysis onto the first and the second discrimiant function (CAN I to CAN II) 99

4.11 Plot of *P. petaurista* populations of discriminant function analysis onto the first and the third discrimiant function (CAN I to CAN III) 100

4.12 Principal components analysis of *P. petaurista* populations onto factor 1 and factor 2 (PRIN I to PRIN II) 101

4.13 Principal components analysis of *P. petaurista* populations onto factor 1 and factor 3 (PRIN I to PRIN III) 102

4.14 Phylogenetic relationships within the populations of *P. petaurista* reconstructed by maximum parsimony (MP) method with *Pteromys volans* as the outgroup. Numbers above branches are the bootstrap values (%). The abbreviations of taxa are defined in Table 4.6 and Table 4.7. 103

4.15 Phylogenetic relationships within the populations of *P. petaurista* reconstructed by neighbor-joining (NJ) method with *Pteromys volans* as the outgroup. Scales in the tree represent branch length in terms of nucleotide substitutions per site. The abbreviations of taxa are defined in Table 4.6 and Table 4.7. 104

4.16 Discriminant function analysis of Chinese *Petaurista* on discriminant function 1 (CAN I) and function 2 (CAN II)..... 105

4.17 Discriminant function analysis of Chinese *Petaurista* on discriminant function 1 (CAN I) to function 3 (CAN III)..... 106

4.18 Principal components analysis of Chinese *Petaurista* on factor 1 (PRIN I) and factor 2 (PRIN II) 107

4.19 Principal components analysis of Chinese *Petaurista* on factor 1 (PRIN I) and factor 3 (PRIN III) 108

4.20 Phylogenetic topology of *Petaurista* based on the maximum parsimony method. The number of bootstrap value (%) is given above each branch. The abbreviations of taxa are defined in Table 4.6 and Table 4.7. 109

4.21 Phylogenetic topology of *Petaurista* based on the neighbor-joining method. Scales in the tree represent branch length in terms of nucleotide substitutions per site. The abbreviations of taxa are defined in Table 4.6 and Table 4.7. 110

4.22 Phylogenetic topology of *Petaurista* based on the UPGMA method. The abbreviations of taxa are defined in Table 4.6 and Table 4.7. 111

4.23 *P. petaurista* in Pakistan and W Yunnan, China..... 112

4.24 *P. philippensis* and *P. yunanensis* in Yunnan, China..... 113

5.1 Distribution of Chinese *Hylopetes* and *Eoglaucomys*..... 136

5.2 Discriminant function analysis between Chinese *Hylopetes* and *Eoglaucomys* onto function 1 and function 2 137

5.3 Discriminant function analysis between Chinese *Hylopetes* and *Eoglaucomys* onto function 1 and function 3 138

5.4 Principal components analysis of Chinese *Hylopetes* and *Eoglaucomys* onto factor 1 and factor 2..... 139

5.5 Principal components analysis of Chinese *Hylopetes* and *Eoglaucomys* onto factor 1 and factor 3..... 140

5.6 Phylogenetic topology of *Eoglaucomys* and Chinese *Hylopetes* constructed using the neighbor-joining method based on the partial sequences (400 bp) of cytochrome b gene. Scales in the tree represent branch length in terms of nucleotide substitutions per site. Sample abbreviations are coded in Table 5.3..... 141

5.7 Scatter-plot of *Hylopetes* and *Eoglaucomys* along the first two discriminant functions..... 142

5.8 Scatter-plot of *Hylopetes* and *Eoglaucomys* onto the first and the third discriminant function..... 143

5.9 Plot of principal components analysis of *Hylopetes* and *Eoglaucomys* onto factor 1 and factor 2..... 144

5.10 Plot of principal components analysis of *Hylopetes* and *Eoglaucomys* onto factor 1 and factor 3..... 145

5.11 Phylogenetic tree of *Hylopetes* and *Eoglaucomys* generated by MP method on partial sequences (375 bp) of cytochrome b gene. Numbers above branches are the bootstrap values (%). Sample abbreviations are coded in Table 5.3..... 146

5.12 Phylogenetic tree of *Hylopetes* and *Eoglaucomys* generated by NJ method on partial sequences (375 bp) of cytochrome b gene. Scales in the tree represent branch length in terms of nucleotide substitutions per site. See sample abbreviations in Table 5.3..... 147

5.13 *Eoglaucomys fimbriatus* and *Hylopetes alboniger*..... 148

5.14 *Hylopetes electilis* and *Hylopetes phayrei* 149

5.15 *Hylopetes electilis* and *Hylopetes nigripes*..... 150

5.16 *Hylopetes lepidus* and *Hylopetes spadiceus*..... 151

6. 1 Phylogenetic reconstruction of flying squirrels, tree squirrels, and fossil squirrels based on 44 dental characteristics..... 178

6.2 Chinese flying squirrels..... 179

6.3 Trans-Himalayan flying squirrels..... 180

6.4 Scatter-plot of discriminant function analysis of the trans-Himalayan flying squirrels onto the first two functions (CAN I and CAN II) 181

6.5 Scatter-plot of discriminant function analysis of the trans-Himalayan flying squirrels onto function 1 and function 3 (CAN I and CAN III)..... 182

6.6 Principal components analysis of the trans-Himalayan flying squirrels onto the first two factors (PRIN I and PRIN II) 183

6.7 Principal components analysis of the trans-Himalayan flying squirrels onto the first and the third factors (PRIN I and PRIN III)..... 184

6.8 Phylogenetic relationships of all Chinese flying squirrels constructed using neighbor-joining (NJ) method. Scales in the tree represent branch length in terms of nucleotide substitutions per site. Sample abbreviations are defined in Table 6.4..... 185

6.9 Phylogenetic relationships of all Chinese flying squirrels constructed via the maximum parsimony method using heuristic search algorithm. Scales in the tree represent branch length in terms of nucleotide substitutions per site. Sample abbreviations are defined in Table 6.4. 186

6.10 Plot of Chinese flying squirrels based on discriminant function analysis onto the function 1 and function 2 (CAN I and CAN II) 187

6.11 Plot of Chinese flying squirrels based on principal components analysis onto the factor 1 and factor 2 (PRIN I and PRIN II) 188

6.12 *Trogopterus xanthipes* and *Pteromys volans* 189

6.13 *Eoglaucomys*, *Hylopetes*, and *Glaucomys* 190

6.14 *Aeretes mlanopterus* and *Belomys personii* 191

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SYSTEMATICS AND BIOGEOGRAPHY OF FLYING SQUIRRELS IN THE EASTERN AND THE WESTERN TRANS-HIMALAYAS

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The areas of the Himalayas where high mountain ranges meet the lowlands of Asia in a series of deep, narrow, and often xeric gorges are described as the "trans-Himalayas," including the eastern extreme (SW China, Burma) and the western extreme (Pakistan and Afghanistan). The systematics and biogeography of many flying squirrels in this region, however, are poorly understood and remain uncertain. This is especially true for Chinese flying squirrels. Analyses of the partial sequences of mitochondrial cytochrome b gene and morphological data were performed for investigating the phylogenetic relationships of forms or populations of *Eupetaurus*, *Petaurista*, and *Hylopetes* (*Eoglaucomys*) along the trans-Himalayas.

First, the molecular data revealed that the two specimens in SW China are *Eupetaurus*, which differs significantly from the population in Pakistan, suggesting two distinct species. They diverged at the end of the Miocene. The glaciations and the uplift

of the Himalayas during the Pliocene-Pleistocene period are the major factors that affected the present distribution of *Eupetaurus*.

Second, morphological and molecular data suggested that the population of *P. petaurista* in Pakistan apparently differentiated from the population in W Yunnan of China. *P. yunanensis* is distinctive from *P. philippensis* and is a valid species. There is no basis for retaining *P. hainana* as a recognizable species, however. *P. xanthotis* is a valid Chinese endemic species and has a close phylogenetic relationship with *P. leucogenys* in Japan and China.

Third, the comparative study of *Eoglaucomys* and *Hylopetes* in the eastern and the western trans-Himalayas showed that *Eoglaucomys* is morphologically and genetically distinct from *Hylopetes* and is a valid genus. *H. electilis* on the island of Hainan, China, is a valid species of *Hylopetes*, although it shares similar morphological characters with *H. phayrei* in skull.

Last, all Chinese flying squirrels can be divided into five groups: 1) *Petaurista*; 2) *Pteromys*; 3) *Eupetaurus*; 4) *Hylopetes* and *Petinomys*; and 5) *Trogopterus* and *Belomys*. The morphological and molecular data of this study support that the current distributions of Chinese flying squirrels owe much to both major climatic changes in the late Pleistocene and the physical barriers to migration.

CHAPTER 1
INTRODUCTION

The areas of the Himalayas where high mountain ranges meet the lowlands of Asia in a series of deep, narrow, and often xeric gorges are described as the "trans-Himalayas." Its eastern and western extremes are considered as the eastern and the western trans-Himalayas. The former includes southwestern Yunnan, eastern Tibet, southern Yunnan, northern Burma and India, while the latter consists of northwestern India, Pakistan, and Afghanistan. They form, in effect, the left and right sides of an open book, with the Tibet Plateau as the center.

The eastern trans-Himalayas consists of several distinct topographic regions defined by drainage patterns associated with the parallel mountain chains. These deep gorges have only eastern and southern outlets. The collections of plants and animals made by Pere Armand David, a French missionary-naturalist, were made in the eastern trans-Himalayas, an area also known as the western Chinese highland (Allen, 1940). In this great area the forests are largely of fir and spruce with hardwoods at middle elevations. The high ridges (2,800 to 3,000m) are characterized by thickets of small bamboo and rhododendron. These old north-south orientated ranges are isolated by deep ravines and characterized by diversified habitats which afford asylum for many peculiar, often primitive, types of mammals unknown elsewhere in the world today. However, many of these species are now threatened by logging, over-hunting, trapping, fuel collecting, mushroom rearing, and overgrazing. Deforestation is now widespread in this region where critical habitats for many species are becoming fragmental. The plant and

animal species associated with the once broadly interconnected inter-mountain ecosystems are now fragmented into patterns of disjunctive distributions. More work in western and southern China, as well as adjacent Myanmar, and in Assam, Bhutan, Nepal, northern India and Pakistan, to shed more light on species distributions in this area of great geographic complexity and high relief is clearly desirable (Hoffmann, 2001). The systematics, geographical distributions, and conservation status of many species in these rugged and remote regions are poorly understood. Flying squirrels are especially poorly understood because they occur in deep forest habitats and are nocturnal in habits.

1.1 Evolution of Flying Squirrels

Since Cuvier (1798) separated flying squirrels (=Volant) from the non-volant squirrels, placing them in the single genus *Pteromys*, several different revisions of the classification and the distribution of flying squirrels have been proposed based on geographical distributions and external structures (Anderson, 1878; Allen, 1940; Ellerman, 1940; Zahler and Woods, 1997), morphological features (Shaub, 1958; McKenna, 1962; Mein, 1970; Johnson-Murray, 1977; Thorington, 1984; Thorington and Heaney, 1981; Thorington and Darrow, 1996; Thorington et al., 1996, 1997, 1998; Thorington and Stafford, 2001), and biochemical and molecular analyses (Arbogast, 1999; Oshida and Masuda, 2000; Oshida et al., 2000a, 2000b, 2001). By studying the skull morphology and color variation of the flying squirrels in western Yunnan, Anderson (1878) put all 29 species of flying squirrels into the same genus, *Pteromys*, of the family Sciuridae. Allen (1940) classified flying squirrels as an independent family, Petauristidae, based on their unique parachute membrane extending from the ankles to the wrists and characteristics of the skull, such as the short, triangular and slightly raised postorbital processes, and the distinct depression between the orbits, a result of the large

crepuscular eyes. Ellerman (1940) comprehensively summarized the early evolutionary history of the flying squirrels in the book *The Families and Genera of Living Rodents*. McKenna (1962) categorized the living petauristine sciurids into five major tribes, *Glaucomys*, *Iomys*, *Petinomys*, *Trogopterus*, and *Petaurisa*, on the basis of the differences of the dentitions, auditory regions, and bacula, paralleling the classification of the subfamily Sciurinae (Table 1.1). Later on, McKenna et al. (1977) put all flying squirrels in Pteromyinae instead of Petauristinae because of the membrane.

Table 1.1 McKenna's classification of flying squirrels in Petauristinae

Group	Genus
Glaucomys	*Eoglaucomys*, *Glaucomys*, *Pteromys*, *Petaurillus*
Iomys	*Iomys*
Petinomys	*Aeromys*, *Petinomys*, *Hylopetes*
Trogopterus	*Pteromyscus*, *Belomys*, *Trogopterus*
Petaurista	*Aeretes*, *Petaurista*, *Eupetaurus*

Mein (1970) made comparisons between the dentition of fossil forms and of modern forms of flying squirrels. Based on his study and description of dental characters, he categorized flying squirrels into three different groups (Table 1.2). The distinct anatomical structures of the wrist support the hypothesis that flying squirrels and non-flying squirrels have different phylogenetic histories (Oshida et al., 2000c, 2000d; Thorington and Darrow, 2001). Corbet and Hill (1992) promoted flying squirrels as a separate family, Pteromyidae. More recently, at the mammal meeting in Seattle, Washington, based on the muscular and skeletal features, Thorington (personal comm.,

1998) suggested the possibility that all flying squirrels belong to two groups: *Glaucomys*-like forms and *Petaurista*-like forms, each consisting of different subgroups (Table 1.3).

Table 1.2 Mein's classification, determined from dental characters

Group	Genus
I	*Glaucomys*, *Eoglaucomys*, and *Iomys*
II	*Pteromys*, *Trogopterus*, *Pteromyscus*, *Belomys*, *Aeretes*, *Petaurista*, and *Eupetaurus*
III	*Petinomys*, *Hylopetes*, *Aeromys*

Table 1.3 The latest estimate of flying squirrels in *Pteromystinae*

Group name	Subgroup and genus
Glaucomys	Subgroup I: *Glaucomys*, *Eoglaucomys* Subgroup II: *Hylopetes*, *Petinomys*, *Petaurillus*, *Iomys*
Petaurista	Subgroup I: *Petaurista*, *Aeretes* Subgroup II: *Trogopterus*, *Belomys*, *Pteromyscus* Subgroup III: *Eupetaurus* Subgroup IV: *Aeromys* Subgroup V: *Pteromys*

All extant flying squirrels whether in Petauristinae, a subfamily of Sciuridae (Nowak, 1991, 1999; Wilson and Reeder, 1992), or in Pteromyidae (Corbet and Hill, 1992) belong to 14 or 15 genera and 37-52 species (Table 1.4) found in both the Old and New World. Of them, one genus, *Glaucomys*, is confined to North American evergreen and deciduous forests, and the others are centered chiefly in the subtropical forests of the oriental region (Table 1.5). With few exceptions, such as Chakraborty (1981) and Thorington et al. (1996), who considered *Eoglaucomys* as a valid genus distinct from *Hylopetes*, the current classification of flying squirrels at the generic level is widely accepted (Bruijin and Uney, 1989; Corbet and Hill, 1992; Wilson and Reeder, 1992).

The taxonomic controversies of flying squirrels at the species level are mainly within the genera of *Hylopetes* and *Petaurista*.

Table 1.4 Comparison of different classifications of flying squirrels

Genus	Number of Species			
	Ellerman 1940	Nowak 1991	Wilson and Reeder 1992	Corbet and Hill 1991
Petaurista	11	6	8	8
Aeromys	2	2	2	2
Pteromys	4	2	2	2
Glaucomys	2	2	2	2
Hyloptetes	13	8	10	11
Eoglaucomys	1	-	synonymy of *Hylopetes*	synonymy of *Hylopetes*
Petinomys	11	7	8	8
Petaurillus	3	3	3	2
Aeretes		1	1	1
Trogopterus	1	1	1	1
Belomys	1	1	1	1
Pteromyscus	1	1	1	1
Iomys	1	1	2	3
Biswanmoy-opterus	-	1	1	1
Eupetaurus	1	1	1	1
Total	52	37	43	44

However, these classifications are mainly based on the shared primitive features (= plesiomorphic characters) and as a result there is great confusion. Many species or forms are frequently referenced as different species, subspecies or synonyms (Allen, 1940; Corbet and Hill, 1991, 1992; Nowak, 1991; Wilson and Reeder, 1992). Despite the high level of taxonomic, ecological, and morphological information available for some species of flying squirrels, the phylogenetic relationships of many taxa remain uncertain. This taxonomic uncertainty is especially true for Chinese flying squirrels.

Table 1.5 Flying squirrels and their distributions

Genus	Common name	Distribution
Petaurista	Giant flying squirrel	Kashmir, northern Indochina, Malay peninsula, Sumatra, Java, Borneo, Japan, Korea, Manchuria, Taiwan
Aeromys	Large black flying squirrel	Malaya, Sumatra, and Borneo
Pteromys	Old World flying squirrel	Coniferous forest zone of Eurasia (Japan, Finland to Korea)
Glaucomys	New World flying squirrel	Canada, W and E USA, Honduras
Hyloptetes	Arrow-tailed flying squirrel	Northern India, Thailand, south China, Indochina, Sumatra, Java, Borneo, and Naruna Islands
Eoglaucomys	Small Kashmir flying squirrel	Afghanistan, Pakistan, Kashmir
Petinomys	Dwarf flying squirrel	Java, Malaya, Sumatra, Borneo, Philippines, S India, Sri Lanka
Petaurillus	Pygmy flying squirrel	Borneo and Malaya
Aeretes	Groove-toothed flying squirrel	NE China and Sichuan, China
Trogopterus	Complex-toothed flying squirrel	China, Himalayas and Indochina
Belomys	Hairy-footed flying squirrel	E Nepal - Indochina, Taiwan
Pteromyscus	Smoky flying squirrel	S Thailand - Sumatra, Borneo
Iomys	Horsfield's flying squirrel	Malaya - Java, Borneo, Sumatra
Biswanmoyo pterus	Namdapha flying squirrel	NE India, Southeast Asia
Eupetaurus	Woolly flying squirrel	Pakistan, SW China, North India, Sikkim

1.2 Evolution of Chinese Flying Squirrels

The flying squirrels distributed in China belong to seven genera and 14 or 15 recognized species (Allen, 1940; Corbet and Hill, 1991, 1992; Wilson and Reeder, 1992; Nowak, 1999). The most comprehensive discussions of Chinese flying squirrels are found in Allen's (1940) *The Mammals of China and Mongolia*, Ellerman and Morrison-Scott's (1966) *Checklist of Palaearctic and Indian Mammals*, and Corbet and Hill's

(1992) *The Mammals of the Indomalayan Region*. Chinese flying squirrels belong to four geographical regions: 1) southwestern China (Tibet, Yunnan, and Sichuan); 2) southern China including the island of Hainan; 3) northern China; and 4) central China (Table 1.6). The region favored by most flying squirrels is southwestern China where there are more than 10 species.

Southwestern China is the main part of the eastern trans-Himalayas. It comprises a total area of 767,000 km^2, stretching from the southeast corner of Tibet through central and northern Yunnan, western Sichuan, and the hills of the eastern Tibet plateau, particularly the Hengduan and Min mountain systems. Elevation in this region varies from below 1,000 m on the valley floors to over 6,000m on the highest snow covered ridges. The topographical features of this region are very complicated. The limestone bedrock forms diverse landforms including karst landscapes, sharp peaks, intermountane basins, rocky gorges, grottos, and underground rivers. This area can be divided into three great steps with increasing altitudes from low hills at 1,200m in the southeast to high mountain peaks at 3,000-4,000m in the northwest. The vegetation is highly diversified and changes progressively from southeast to northwest. The transition of plant communities in this zone is more altitudinal than latitudinal. The three parallel rivers are the Yangtze, Mekong, and Salaween and are separated by snow-capped mountains. Mountains are vertically stratified with distinct vegetation and typically show a complete spectrum from subtropical evergreen broadleaf forests at lower altitudes to deciduous temperate broadleaf forests, mixed broadleaf coniferous forests at middle levels, and coniferous subalpine forests with dense bamboo and rhododendron associated with alpine meadows at higher altitudes. As a result of the influence of the continental monsoon

from the north and the maritime monsoons from the southwest and southeast, winters are generally cool and dry while summers are warm and wet.

Table 1.6 Chinese flying squirrels

Genus	Species	Distribution
Aeretes	*A. melanopterus*	NE Hebei and Sichuan
Belomys	*B. pearsonii*	Hunan, SW China, Hainan, and Guizhou
Trogopterus	*T. xanthipes*	Heber, Huber, Yunnan, Sichuan
Eupetaurus	*E. cinereus*	N Pakistan, Kashmir to Sikkim (India), Yunnan and Tibet
Hylopetes	*H. alboniger*	Sichuan, Yunnan, and Hainan
	H. phayrei	Fukien and Hainan
Petaurista	*P. alborufus*	Sichuan, and S and C China, Taiwan
	P. elegans	Sichuan and Yunnan
	P. leucogenys	Gansu, Sichuan, and Yunnan
	P. xanthotis	Sichuan, Tibet, Gansu, and Yunnan
	P. petaurista	Sichuan, Yunnan and Fukien
	P. philippensis	Taiwan, South China and Hainan
	P. magnificus	Tibet
Pteromys	*P. volans*	North China and West China

Southwestern China is the most interesting and remarkable of the Chinese faunal and floral divisions and has the highest level of biodiversity among Chinese provinces. More than half of the country's protected or endangered mammals, including 25 species under the first class list and 29 of the second, and 50% of the country's total flora, including four first class protected species and 60 of the second class, are found here (Mackinnon et al., 1996). This region provides an ideal habitat for flying squirrels and it is possible that the region is the center of the radiation of flying squirrels. However,

because the region is remote and economic conditions are very poor, the local people and government officially (or unofficially) harvest Yunnan pine, firs, and spruce trees at a very high rate. As a consequence of the rapidly disappearing pine and spruce trees upon which flying squirrels feed and which provide important habitats for flying squirrels, most species are threatened. *Eupetaurus*, *Petaurista* and *Hylopetes* are the taxa most threatened, and are the genera where most of the species-level taxonomic controversies exist.

Eupetaurus was previously thought to occur only in northern Pakistan and to be very rare or even extinct. However, a review of museum specimens suggests a historical distribution that also includes India, Tibet, Sikkim, and SW China. Zahler (1996) and Zahler and Woods (1997) documented the continued existence of *E. cinereus* in northern Pakistan. Two "skins" (only skins, no skulls) represented in the collection at the Kunming Institute of Zoology (KIZ) of the Chinese Academy of Sciences have been identified as *Eupetaurus* based on the pelage color and external features (Wang and Yang, 1986; Corbet and Hill, 1991, 1992). I have found no further evidence of its presence or distribution in southwestern China. Comparisons between *Eupetaurus cinereus* from Pakistan and the "skins" collected in SW China are important in order to understand the radiation and taxonomy of the genus *Eupetaurus* and to clarify the taxonomic and phylogenetic status of *Eupetaurus* within flying squirrels.

The separation of *Eoglaucomys* from *Hylopetes* in the western trans-Himalayas is based on dental differences (Ellerman, 1947, 1963; Chakraborty, 1981; Thorington et al., 1996). Some authors do not accept *Eoglaucomys* as a valid genus (Wilson and Reeder, 1992; Corbet and Hill, 1992) and consider that *H. alboniger* and *H. phayrei* are the

species of *Hylopetes* distributed in China. I believe that an examination of the validity of *Eoglaucomys* based on molecular analysis rather than morphometric is critical for the clarification of the taxonomy of *Hylopetes* group. As part of this analysis I will examine *Hylopetes* populations distributed in the eastern trans-Himalayas. The validity of *H. electilis* in Hainan, China, and the phylogenetic relationships among *H. electilis*, *H. alboniger* and *H. phayrei* are as well interesting topics to elucidate the phylogenetic relationship of Chinese flying squirrels.

Petaurista is a polymorphic genus with considerable variation in pelage coloring. More than 10 species of *Petaurista* have been recognized (Corbet and Hill, 1991, 1992; Nowak, 1991, 1999; Wilson and Reeder, 1992; Zhang et al. 1997; Wang, 2002). Of various species included within this genus, it is difficult to resolve the numerous intraspecific and interspecific taxonomic and phylogenetic problems. Various authors still express serious doubts concerning the validity of *P. xanthotis*, *P. philipensis*, *P. hainana*, and *P. yunanensis* (Nowak, 1999; Wilson and Reeder, 1992; Corbet and Hill, 1992, Wang, 2002). To resolve the affinities of these complex taxa, a comprehensive revision to clarify the relationships among these forms is essential because the available morphometric and molecular data are too scanty to throw any light on the problems.

1.3 Objectives of This Study

Most recent phylogenetic studies have concentrated on the analysis of molecular data, particularly DNA sequences. But the combination of both molecular and morphological analyses in systematics has been attracting more and more attention from both systematists and evolutionary biologists (Minelli, 1998; Schierwater and Kuhn, 1998; Benton, 1998; Hugot, 1998; Flynn and Nedbal, 1998; Baker et al., 1998; Smith, 1998; Smith and Patton, 1991; Staongnhope et al., 1998; Barome et al., 1998; Ruedi et

al., 1998; Shoshani and McKenna, 1998; Goodman et al., 1998; Huelsnebeck et al., 1996). Despite the increasing use of DNA sequence data, morphometric analysis still remains one of the most useful techniques available to investigate phylogenetic relationships between taxa (Sanderson et al., 1993).

Flying squirrels exhibit a high level of geographic variation in morphological characters such as pelage color, cranium morphology and dental structures. Opportunities for studying flying squirrels traditionally have been limited to studying specimens represented in museums and institutes. In part, this is reflected in the continuing problems of flying squirrel classification. Despite the agreements or disagreements on the taxonomic status of different nominate genera and species in the literature, flying squirrels have not been the main subject of any comprehensive systematic revision. The phylogenetic analysis of mtDNA in rodents has centered largely on murids (Ferris et al., 1983; Smith and Patton, 1991). The overall phylogenetic relationship of flying squirrels remains poorly understood because they are nocturnal, elusive, and difficult to capture. No attempt has been made to investigate the systematics of Chinese flying squirrels.

There appear to be similar patterns of speciation and distribution of flying squirrels in the eastern and the western trans-Himalayas. A comprehensive and comparative analysis of the phylogeography and systematic relationships of flying squirrels in the eastern and the western trans-Himalayas will be extremely valuable. In this study, molecular analyses of partial sequences of mitochondrial cytochrome b gene and morphometric study of skull data were performed for the flying squirrels distributed in various areas of China and Pakistan. I also discuss the biogeographic history and

phylogenetic relationships among them as well as the taxonomic status of the groups or forms in *Eupetaurus*, *Petaurista*, and *Hylopetes* (*Eoglaucomys*) from SW China and Pakistan. The objectives of this study are to seek the answers for the following questions:

1. Search for confirmation of the continued presence of the "lost species" -- *Eupetaurus cinereus*, in SW China and investigate the taxonomic status of genus *Eupetaurus*.

2. Examine the taxonomic and phylogenetic relationship between *Hylopetes* and *Eoglaucomys*, and the taxonomic validity of *H. electilis.*

3. Determine if the *Petaurista petaurista* (sensu lato) groups in different localities along the eastern and western trans-Himalayas form a single species *P. petaurista* (*albiventer*), or a complex of species. Confirm the validity of *P. xanthotis*, *P. philipensis*, *P. hainana*, and *P. yunanensis* and reconstruct the phylogenetic relationships among *Petaurista* groups distributed in SW China and Pakistan.

4. Investigate what the systematic relationships are among Chinese flying squirrels, including *Petaurista*, *Eupetaurus*, *Trogopterus*, *Hylopetes*, *Eoglaucomys*, *Belomys*, and *Pteromys*.

In the remaining chapters, I will attempt to answer the above questions. In Chapter 2, I will briefly discuss the techniques and methods of phylogenetic analysis used in the present study. Chapter 3 is about the phylogeny and zoogeography of *Eupetaurus* inferred from an analysis of the cytochrome b gene. Chapter 4 will focus on the phylogenetic relationships of *Peaturista* distributed in SW China and Pakistan based on a molecular analysis and morphometric study. The phylogenetic relationship between/within *Hylopetes* and *Eoglaucomys* is discussed in Chapter 5. Chapter 6 is a discussion of the systematics and biogeography of the trans-Himalayan flying squirrels. The results and the tentative future work are summarized in Chapter 7.

CHAPTER 2
METHODS AND TECHNIQUES OF PHYLOGENETIC STUDY

There is little doubt that the introduction of molecular techniques has already significantly enhanced the capacity to address fundamental questions in phylogenetic relationships and will make an even greater contribution in future. Theoretically the phylogenetic analysis of molecular level can provide different and well-corroborated estimates of phylogenetic relationships. It is possible to compare and contrast phylogenies based on morphological data with biochemical data such as genome data, globins, or cytochrome b (Benton, 1998). Messenger and McGuire's (1998) study of cetaceans showed that combined analyses of the morphological and molecular data provide a well-supported phylogenetic estimate consistent with that based on the morphological data alone. But, intraspecific variation is ubiquitous in systematic characters, including morphology, allozymes, and DNA sequences. Sometimes the analysis of one data set (i.e., molecular) provides one highly corroborated phylogeny; whereas analysis of another data set (i.e., morphological) provides a different highly corroborated phylogeny. The phylogenetic inferences based on characters derived from morphology are corroborated by molecular evidence in some mammal groups (Flynn and Ndebal, 1998; Shoshani and McKenna, 1998). However the integration of distinct data sets, such as the molecular data and morphological data in phylogenetic analysis, has caused considerable debate among evolutionary biologists in recent years (William and Ballard, 1996; Wiens, 1998a, 1998b; Wiens and Servedio, 1998).

2.1 Molecular Study

The molecular biological revolution of the last several decades has reached into every conceivable corner of biological investigation (McKenzie and Batterham, 1994; Poinar, 1999). Molecular sequences are information-rich and there are many different ways of extracting useful information from them. Molecular phylogenetic analysis has been developed to infer the branching pattern of different taxa from their common ancestor and the sequential dates when such branching events or cladogenic speciation events occurred. The results of DNA sequence data have been successfully used to reconstruct the phylogenetic relationship in rodents, which provides strong support for the monophyly of rodents, a conclusion that has considerable support from morphology (Honeycutt and Adkins, 1993; Luckeet and Hartenberger, 1993; Frye and Hedges, 1995).

2.1.1 Mitochondrial Cytochrome b Gene

The molecular methods used to detect genetic variation within and between species have led to exciting advances in studies of historical biogeography. Molecular survey of DNA sequence data is one of the popular techniques used to quantitatively measure genetic variations among taxa (Storfer, 1996). In principle, any part of the genome can be used for DNA studies. Over the past few years, a variety of different observations have challenged some notions of mitochondrial biology, such as the variable rates of mitochondrial DNA (mtDNA) sequence evolution among taxa (Rand, 1994). However mtDNA is by far the most commonly used methodology in phylogenetic analysis and evolutionary biology since it features several advantages that make it the usual choice for population-level questions (Meyer, 1994). For example, mtDNA sequence data can provide the perspective of a maternally inherited marker on patterns and levels of geographic structuring and the analyses of polymorphic restriction sites. On

the basis of the gene sequences of mtDNA, researchers obtained valuable information on evolutionary relationships and divergence times for mammalian subspecies, species, and higher level taxa (Ferris et al., 1983; Brown et al., 1979; Zhang and Ryder, 1993; Avise,1987, 1994; Janke et al., 1994; Miyamoto and Fitch, 1995; Wettstein et al., 1995; Miyamoto, 1996).

To extract historical information from molecular data, it is important to understand the dynamic nature of the sequences and know how molecular sequences change over geological time. Variation of evolutionary rates occurs at several levels in DNA sequences: among the sites (e.g., the second position vs. the third position), among the kinds of substitutions (e. g., transition vs. transversion, or silent vs. replacement), and among regions of the molecule (Ferris et al., 1983; Avise, 1994). At the nucleotide level, which is the most fundamental level for any mutation, there are 12 possible changes, with four being transitional changes and eight being transversional changes. In general, recent divergences are related to rapidly evolving changes and older divergences are related to slowly evolving changes (Graybeal, 1993). These patterns hold for cytochrome b gene, with studies at the population and species level using all informative characters (Smith and Patton, 1991; Moritz, 1994).

The mitochondrial cytochrome b gene is one valuable molecule for evolutionary relationship reconstruction among populations, species, and higher taxa in animals and has been used extensively in molecular phylogenetic studies. Because it is slow in terms of amino acid substitutions and the rate of evolution for silent substitutions at the third codon positions is similar to that of other mitochondrial genes, the cytochrome b gene that facilitates the alignment of sequences permits comparisons among widely divergent

taxa (Kocher et al., 1989; Irwin et al., 1991). Using the partial or complete sequences of the mitochondrial cytochrome b gene, researchers and scientists successfully determined the interspecific phylogenies in a wide range of mammalian taxa, such as the phylogenetic analysis of relationships in bats and some murids (Wright et al., 1999; Iudica, 2000, Barome et al., 1998), and genetic analysis of intrageneric relationships of some squirrels and flying squirrels (i.e., *Petaurista*, *Glaucomys)* (Hafner, et al., 1994; Oshida and Obara, 1992; Oshida and Yoshida, 1999; Oshida et al., 1996, 2000a, 2000b, 2001; Arbogast, 1999).

2.1.2 Phylogenetic Analysis

As far as phylogeny is concerned, finding the best trees and using the best tree to reconstruct the phylogenetic relationship are main objectives. With the development of the polymerase chain reaction (PCR), it is possible to recover genetic information from even severely degraded tissues, such as hair, old skin, and excrement. The widespread successful application of molecular analysis in animal phylogenetic reconstruction and evolutionary biology can be attributed partly to the discovery of this versatile PCR technique. The use of DNA from museum skins can reconstruct phylogenetic trees among organisms and develop conservation policies for endangered species.

A typical phylogenetic analysis involves a bewildering array of decisions, including what type of data to sample (molecular or morphological), what phylogenetic methods to apply (distance, likelihood, and parsimony), whether or not to order or weight characters, and which taxa and characters to include or exclude. The common methods used for molecular phylogenetic reconstruction include distance method (e.g., the unweighted pair-group method with arithmetic mean (UPGMA), the neighbor-joining, or

the more complicated Fitch-Margoliash method), parsimony method, and likelihood method.

2.1.2.1 Parsimony method

Parsimony is a character-based analysis. Each character is considered independent of its neighbor, but only informative sites are considered during the calculation. The parsimony method calculates only the order of the branches of the tree and does not give branch-length estimates. Maximum parsimony is the easiest and most practical to implement, and when evolutionary times are short, maximum parsimony, maximum likelihood, and compatibility tend to yield the same estimated phylogeny (Crandall et al., 1994). The accuracy of parsimony depends largely on how polymorphic characters are coded and the sample size (individuals per species), which is usually an important component of phylogenetic accuracy (Wiens and Servedio, 1998). The advantage of this method is that it uses a logical model and the calculations are rapid. However, a major shortcoming of the method is that a large amount of data that are not informative are discarded.

2.1.2.2 Likelihood method

In likelihood method every site of sequences is considered and the likelihood of the replacement of a particular nucleotide from pools of nucleotides is calculated. It is based on random similarity rather than on common descent, and increases with increasing divergence between the outgroup and the ingroup taxa (Milinkovitch and Lyons-Weiler, 1998). Likelihood method considers every site including unchanged sites and gives an accurate estimate of branch lengths. The maximum parsimony method can be viewed as an approximation to the maximum likelihood method, which has been used extensively

for parameter estimation in molecular data analysis. The disadvantage is that this method is very time-consuming.

2.1.2.3 Distance method

Distance methods calculate the total number of changes, scored according to the type of change, between every pair of sequences in the alignment. The method is based on distance to calculate branch length that visually represents the number of changes between sequences with consideration of unchanged characters and ambiguous alignments.

In practice, the choice among all methods is a serious concern because the intraspecific and interspecific variation is so widespread that the application of different methods can give radically different trees for the same data set. Even subtle differences in how polymorphism is treated can have a significant impact on tree topology (Wiens, 1995; Wiens and Servedio, 1998). UPGMA (unweighted pair group method using arithmetic averages) is the most commonly used clustering method, in which the averaging of the distances is based on the total number of taxa in the cluster analysis (Swofford et al., 1996; Swofford, 2000). Like the likelihood method, it generally gives the most accurate results compared to other techniques (Wiens and Servedio, 1998; Rohlf and Wooten, 1988).

In this study, the partial mitochondrial cytochrome b genes (315 - 420 bp) were amplified with PCR technique. Some sequence data are retrieved from the GenBank of NCBI (National Center for Biotechnology Information). Maximum parsimony (MP), neighbor-joining (NJ), and UPGMA methods were used to reconstruct the molecular phylogeny of Chinese flying squirrels to determine whether the specific genetic structures

and geographic patterns are correlative within each group and to reveal their systematic relationships.

2.2 Morphometric Study

Most recent phylogenetic studies focus mainly on the analysis of molecular data, but tracing changes in morphological characters is also an important way to evaluate the distribution of the characters on which those taxonomic units are based. The quantitative description, analysis, and interpretation of shape and shape variation in biology are a fundamental area of research. Taxonomic modifications and reinterpretation of morphological characters in the context of the molecular tree requires further scrutiny.

Studies of morphology contribute in different ways to the understanding of evolutionary patterns and processes. Ideally a functional morphological study provides information about the interdependency of characters, and also a transformation scheme of characters that is biomechanically feasible (Galis, 1996). Morphometric studies have applied univariate analyses to differentiate morphotypes (Lee and Cheng., 1996), and multivariate analyses were used to produce an overview of the associations between variables and species patterns (Gauch, 1982).

The common multivariate analyses include principal components analysis (PCA), discriminant function analysis, and cluster analysis (Manly, 1994). In multivariate analysis, the selection and the number of the characters used are critical since the interpretation of the results is based on them. Sometimes, raw data probably are transformed by logarithm function to reduce the skewness of original data, make their variances homogeneous, and correct the heterogeneity in magnitude of variables. If there are adequate sample sizes, multivariate analyses allow one to make overall tests as well as a proper posterior test of sets of variables.

Discriminant function analysis is to portray the relationships based on the canonical variables, and to develop linear models of variables to maximize the separation of groups. The discriminant functions are extracted from the between-group covariance matrix standardized by the within-group covariance matrix. Mahalanobis distances are used to measure phenetic distances, which are the indices to evaluate the overlap between pairs of populations, and to transform original distances to maximize power of differentiations between individual specimens to describe the relationship among species.

Principal components analysis (PCA) is based upon the variance-covariance matrix of the log-transformed variables. It is performed to identify variables that account for maximum variation in data and to produce a smaller number of uncorrelated factors that are linear combinations of original variables. The first axis lies in the direction of the greatest variability between the sample means, and each succeeding axis lies in the direction of the next greatest variability. Factor loadings, describing the relative contribution of each variable to the principal components, are used to compare the morphological structures between samples.

The discrepancy between cluster analysis and other analysis becomes understandable and less important when characteristics of various statistical techniques are considered (Sneath and Sokal, 1973). Cluster accurately represents distance between adjacent groups. Euclidean distance between centroids and an unweighted pair-group method using arithmetic average (UPGMA) clustering algorithm is usually applied to generate a phenogram, depicting morphological relationships among taxa.

In this study, all related specimens were pooled together for univariate analysis, which is restricted to one-way analysis of variance (ANOVA) and F-test to calculate the

mean, standard deviation (SD), and the significant test among variables for species. The multivariate analyses including discriminant function analysis, principal components analysis and cluster analysis are used to determine how the groups are related when all the characters are considered simultaneously. Because these multivariate methods are unable to deal with missing data, or else they deal with missing information in a rather arbitrary manner (Rohlf and Marcus, 1990), in this study, only those variables that are available in all sampled groups are selected for further analysis.

CHAPTER 3
PHYLOGENY AND BIOGEOGRAPHY OF *EUPETAURUS*

3.1 Introduction

Eupetaurus cinereus, the woolly flying squirrel, is one of the most unusual and least known species in the world (Chakraborty and Agrawal, 1977; Zahler, 1996; Zahler and Woods, 1997). Because observations in the wild have been precluded by the rarity of specimens, virtually, nothing is known of its food habits, reproduction, distribution, habitat preference, behavior, anatomy, or systematics. It is considered among the most endangered mammals (IUCN No.: EN 8 A2ce, B1+2cd[1]) (Baillie and Groombridge, 1996), probably the most threatened of all flying squirrels.

Eupetaurus is a crucial genus in the phylogenetic study of flying squirrels. The cheekteeth of *E. cinereus* are hypsodont and share many characteristics with rodents that have high-crowned teeth with flat surfaces, such as capromyids (hutias) from the West Indies, thryonomyids (cane rats) from Africa, and New World echimyids (spiny rats), rather than other members of Sciuridae (McKenna, 1962). The highly specialized grinding teeth feature many advantages that allow it to live in relatively treeless rocky areas and possibly supplement its diet during the winter months by eating some abrasive material. Since the structure of teeth is so divergent from other flying squirrels, Schaub (1958), and Grasse and Dekeyser (1955) placed *Eupetaurus* in its own rodent family,

[1] EN: the category of threat is endangered; A2ce: Population decline projected in the future; B1+2cd: small distribution and decline because of the severely fragmented population and habitat; C2a: Small population size and continuing decline by fragmented habitat.

Eupetauridae. By comparing the dentition with the giant flying squirrel (*Petaurista xanthotis*), McKenna (1962) proposed that *Eupetaurus* is a very high-crown flying squirrel and demonstrably a petauristine sciurid on the basis of a large number of characters other than the dentition, and returned it to the sciurid subfamily Petauristinae. Its present taxonomical status is as follows:

Order Rodentia Bowdich, 1821

Suborder Sciuromorph Brandt, 1855

Superfamily Sciuroidea Gill, 1872

Family Sciuridae Gray, 1821

Subfamily Petauristinae Simpson, 1945

Genus Eupetaurus Thomas, 1888

Species Eupetaurus cinereus Thomas, 1888

Eupetaurus had been considered to be very rare or even extinct until a live specimen was captured in northern Pakistan in 1994, which confirmed the existence of woolly flying squirrel. Zahler and Woods (1997) summarized are of the available information on the ecology and conservation of *Eupetaurus* in Pakistan. *Eupetaurus* in Pakistan is limited to the region of the Sai Valley in the central Indus River Valley near Nanga Parbat, the most westerly main massif in the Himalayan Range. It lives in caves of high alpine zones that are characterized as high, cold desert dominated by *Artemisia* and *Juniperus* above 2,000 m, and apparently shows quite unique ecological adaptations for surviving in regions that are inhospitable to any other flying squirrels. The present estimate of the number living in Pakistan is between 1,000 and 3,000 (Zahler and Woods, 1997).

Eupetaurus was historically found from Pakistan, to India, Tibet, Sikkim and SW China, based on 13 available museum specimens in London (British Museum of Natural History), Netherlands (Laiden Museum), Bombay (=Mumbai, Bombay Natural History Society), Calcutta (Indian Museum), and China (Kunming Institute of Zoology, China, KIZ), which confirms presence of *Eupetaurus* from Pakistan to China (Figure 3.1 and Table 3.1).

Table 3.1 Thirteen historical and 2 recent specimens of *Eupetaurus*

	Locality	Museum ID	Collector and date	Additional information
1	Tibet, China	LM: 19524	Anderson, J. 1878	Skin and skull
2	Yunnan, China	KIZ: 73372	Wang, Y. X. 1984	Skin only
3	Yunnan, China	KIZ: 73921	Wang, Y. X. 1984	Skin only
4	Sikkim	IM: 19103	Gill, J. S.	Skin only
5	Gilgit, Pakistan	IM: 9492	M. Miles, 1887	Skin and skull
6	Gilgit, Pakistan	BNHS: 7107	MacPherson, M. A. 1916	Skin only
7	Sai Nalah, Pakistan	BNHS: 7108	Lorimer, Lt. C. D. 1924	The collection site is not associated with the specimen. Skin is in very poor shape.
8	Chitral, Pakistan	BNHS: 7109	Fulton, H. T.	Skin only
9	Sai Valley, Pakistan	BNHS: 7110	Maj. L. MacKenzie, 1924	Skin only
10	Astor, Pakistan	BMNH: 88.9.29.1	Purchased by Lydekker, R. 1879	Co-type with a skin and fragmentary snout. The location is said to be from the Astor District.
11	Gyantse Bazar, Tibet	BMNH: 23.11.10.2	No record	Purchased from Tibet
12	Gilgit, Pakistan	No record	No record	Partial skin
13	Chitral, Pakistan	No record	No record	Partial skin
14	Gilgit, Pakistan	UF: 26583	Woods, C. 1996	Partial skin
15	Gilgit, Pakistan	UF: 28620	Woods, C. 1996	Skin and skull

Note: BNHS: Bombay Natural History Society, India; BMNH: British Museum of Natural History, England; IM: Indian Museum in Calcutta, India; LM: Leiden Museum, the Netherlands; KIZ: Kunming Institute of Zoology, China.

However, it is not possible to establish with certainty that all specimens are the same species, since the majority of the specimens available were collected at the beginning of the last century and some specimens are incomplete and lack skulls. The collecting sites of some specimens are not conclusively associated with individual specimens since the data were recorded solely according to the description of dealers. Therefore taxonomic and phylogenetic studies have been hampered by questionable records and the paucity of *Eupetaurus* specimens in collections. For example, questions still remain concerning the exact collecting site of the two skins from China represented in the KIZ and the specimen from Sikkim. The phylogentic status of *Eupetaurus* and the phylogenetic relationships between *Eupetaurus* and other flying squirrels, such as *Petaurista*, are not yet well understood.

In this study, *Eupetaurus* specimens from different localities were compared with new specimens obtained from Pakistan by analyzing the sequence data from mitochondrial cytochrome b gene using parsimony and distance methods. Here I compare the taxonomic status of *Eupetaurus* populations in Pakistan and SW China, determine how much variety exists within the genus *Eupetaurus* along its extensive distribution, and reconstruct the phylogenetic relationship between *Eupetaurus* and *Petaurista*.

3.2 Materials and Methods

3.2.1 Samples

Among those 15 known *Eupetaurus* specimens represented in museums and institutes, some skins were in very poor shape and without the corresponding skulls; some records were not precisely associated with the specimens; and some specimens were only purchased or shipped by the dealers or a third party. Of 10 available skin

samples obtained from museums and institutes, eight specimens have reliable data associated with them. The rest were described mainly based on comments from either the medicinal dealers or the intermediate persons who shipped or purchased the specimens from different locations.

Table 3.2 Specimens of *Eupetaurus* and other flying squirrels examined in this study*

Species	Code	Collecting locality	Museum ID
E. cinereus	ECL	Tibet, China	LM: 19524
	ECK1	YUNNAN, CHINA	KIZ: 73372
	ECK2	Yunnan, China	KIZ: 73921
	ECI	Sikkim	IM: 19103
	ECB1	Gilgit, Pakistan	BNHS: 7107
	ECB2	Chitral, Pakistan	BNHS: 7109
	ECB3	Sai Valley, Pakistan	BNHS: 7108
	ECF1	Gilgit, Pakistan	UF: 26583
	ECF2	Gilgit, Pakistan	UF: 28620
	ECD	Pakistan	Sequence provided by Dr. Roth
Other flying squirrels used in this study			
P. petaurista	PPF	Gilgit, Pakistan	UF ID: 28236
	PPY	Yunnan, China	KIZ: 353209
P. xanthotis	PTK	Gansu, China	NBI ID: 85063
G. volans	GV	Tennessee, US	Sequence #: AF063066

Note: The abbreviations of the museums and Institutes see Table 3.1.
NIB: Northwestern Biological Institute, China

In this study, two samples (ECB3 and ECI) from Pakistan and Sikkim (Table 3.1) were not included because of their highly degraded sequences. The samples that are in good condition and recorded by collectors with certainty were used for molecular

analysis. The detailed information of *Eupetaurus* and other flying squirrels examined is given in Table 3.2. Dr. Louise Roth, who is an associate professor of biology at Duke University, provided the sequence data for the sample ECD from Pakistan.

3.2.2 Methods

3.2.2.1 Mitochondrial DNA isolation

Total DNAs of all samples used were extracted from dry skins using the DNeasy tissue kit (QIAGEN Inc., Valencia, CA91355-1106) and the protocol for animal tissue recommended by the manufacturer. Initially, 180 μl of buffer ATL and 20 μl of proteinase K (20 mg/ml) were added to the 2 ml tube containing the decalcified material. The sample was mixed and placed into a 55^0C H_2O bath for 48 hours. After vortexed for 15 seconds, 200 μl of buffer AL was added. Then it was heated at 70^0C for 10 minutes. After added 200 μl of 100% ethanol, the sample was applied to a Dneasy tissue kit-mini column. During the elution step, 100 μl of buffer AE was added. After incubated at room temperature for 1 minute, it was centrifuged and stored at -4^0C for PCR.

3.2.2.2 PCR amplification

The following primers were used to amplify the partial nucleotide sequence (315-402 bp) of the mitochondrial DNA (mtDNA) with polymerase chain reaction (PCR) at the interdisciplinary center for biotechnology research (ICBR), University of Florida (UF), and Kunming Institute of Zoology, Kunming, China:

L14725 5’-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3’

L14841 5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3'

H15149 5’- AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3’.

Primer names correspond to the light (L) and heavy (H) strand and the 3' end-position of the primers in the human mtDNA sequence (Anderson et al., 1981). All primers were synthesized at either ICBR of UF or Shanghai of China.

The 25 μl of PCR reaction mixture contained 2 μl 10ng of genomic DNA, 2.5 μl of each primer (H15149 and L14725 or L14841), 3.0 μl 10x PCR buffer (100 mM Tris-HCl, pH 8.3 at 25^0C, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), 4.0 μl of dNTPs (10 mmol), 3.0 μl $MgCl_2$ (25 mmol), and 0.2 μl Tag (Sigma tag DNA polymerase (Sigma Chemical CO., St Louis, MO 63178)). The cycling program in Table 3.3 was used for PCR amplification.

Table 3.3 Cycling program of PCR amplification

Step	Temperature (^{0}C)	Time	Cycles
Initial denaturing	94	5 minute	1
Denaturing	94	1 minute	38
Annealing	55	1 minute	
Extension	72	1 minute 5 second	
Final extension	72	5 minute	1

PCR products were purified with the Qia-quick PCR purification kit protocol (QIAGEN Inc., Valencia, CA91355-1106). Automatic sequencing was performed with an automated DNA sequencer at University of Florida. The variant sites of sequences were rechecked by comparing the four-color electromorph of sequencing data against the computer results.

3.2.2.3 Sequence analysis

Cladistic analysis was performed using the phylogenetic analysis using parsimony (PAUP version 4.0) (Swofford, 2000). Both distance and parsimony methods were applied for phylogenetic analysis to infer gene genealogies of mtDNA. The maximum

parsimony (MP) method using the branch and bound search algorithm (Penny and Hendy, 1986) with the 50% majority-rule consensus, the neighbor-joining (NJ) method (Nei, 1987; Saitou and Nei, 1987), and the UPGMA method (Swofford et al., 1996) were used to reconstruct the phylogenetic trees. In NJ method, numbers of nucleotide substitutions per site were estimated for multiple substitutions by the Kimura's two-parameter method (Kimura, 1980). Branch-and-bound search was performed to ensure that all minimum-length trees were identified (Zhang and Ryder, 1994). If all characters change sufficiently slowly, they may be equally weighted during phylogenetic inference, even though they do not change actually with equal probability (Felsenstein, 1981,1983, Graham et al., 1998). The MP trees were generated by equal weighted parsimony and the bootstrap values (Felsenstein, 1985) were derived from 1000 heuristic replicates.

Quantitative pairwise comparisons between all taxa under the two-parameter model of Kimura (1980) were made for the partial cytochrome b gene sequences. The proportions of nucleotide substitutions between all pairs of sequences were calculated, including the percentage of sequence divergence within and between taxa, the ratio between the transitions and transversions, and the transverstional substitutions at the third codon positions between taxa. Relying on dates of divergence estimated from fossil material for a number of mammalian taxa, Irwin et al. (1991) thought that the average rate of sequence divergence at third positions of the cytochromoe-b gene of mammals is about 10% per million years. But the relative rate of molecular evolution in rodents has been estimated to be ca. 1.5-2 times faster than that of other mammalian lineages (Britten, 1986; Dewalt et al., 1993; Li et al., 1990). In this study, the divergence time between taxa was estimated according to the transverstional substitution rate at the third

codon positions of mammalian cytochrome b gene proposed by Irwin et al. (1991), which was 0.5% per million years.

Petaurista petaurista (*albiventer*) from Pakistan and NW China, and *P. xanthotis* from NW China, which had been considered as the close relatives of *Eupetaurus*, were used for phylogenetic analyses. *Glaucomys volans* from North America was used as the out-group for phylogenetic reconstruction. The DNA sequence data of *G. volans* were quoted from GeneBank that is submitted by Arbogast (1999).

3.3 Results

The specimens of woolly flying squirrels collected around the turn of the century were mostly from the general region of Gilgit in the area of the confluence of the Himalayan, Karakoram, and Hindu Kush mountain ranges in northern Pakistan. Because some samples were collected more than 100 years ago and some are in very poor condition, the recovered sequences were short and fragmental, usually between 300 to 400 base-pair longs.

3.3.1 Phylogenetic Relationship of *Eupetaurus* between the Eastern and the Western Trans-Himalayas

The partial sequences (389 bp) of cytochrome b gene were successfully determined for seven *Eupetaurus* samples. The genetic differences obtained from the pairwise comparison (Table 3.4) separated the woolly flying squirrels as two distinct groups. The first group was the eastern tans-Himalayan group (*Eupetaurus* I) including samples from Tibet (ECL) and Yunnan (ECK1 and ECK2) in China. The second group was the western trans-Himalayan group (*Eupetaurus* II) including samples from Chitral (ECB2) and Gilgit (ECB1, ECD, ECF1, and ECF2) in Pakistan. The genetic difference between these two groups was about 12%. The maximum parsimony (MP) and UPGMA

analysis based on all *Eupetaurus* individuals generated the similar branching in trees, all of which contained the same two major clades (Figure 3.2 and Figure 3.3). In MP analysis, only one most parsimonious phylogenetic tree was produced with high bootstrap value (99 - 100%).

Table 3.4 Percentage differences of *Eupetaurus* and *Petaurista* based on the pairwise comparisons of cytochrome b gene (390 bp)

	ECL	ECK 1	ECK 2	ECB 1	ECB 2	ECF 1	ECF 2	ECD	PPY	PPF	PTK
ECL		3.8	3.8	8.4	11.36	11.0	11.0	11.0	16.7	15.9	17.0
ECK1	9/6		0	13.4	14.0	13.3	13.3	13.3	17.9	17.2	19.4
ECK2	9/6	0		13.4	14.0	13.3	13.3	13.3	17.9	17.2	19.4
ECB1	25/6	37/13	37/13		3.6	3.8	3.8	3.8	17.1	16.2	17.5
ECB2	32/11	42/16	42/16	7/4		0	0	0	18.1	17.0	18.7
ECF1	32/11	41/11	41/11	8/6	0/1		0	0	16.9	16.7	19.1
ECF2	32/11	41/11	41/11	8/6	0/1	0		0	16.9	16.7	19.1
ECD	32/11	41/11	41/11	8/6	0/1	0	0		16.9	16.7	19.2
PPY	39/26	44/26	44/26	36/30	42/27	41/25	41/25	41/25		5.2	14.0
PPF	38/24	43/24	43/24	33/30	38/27	40/25	40/25	40/25	18/2		12.1
PTK	41/26	48/28	48/28	35/32	40/32	46/29	46/29	46/29	43/12	37/10	

Note: Data below the diagonal are the numbers of nucleotide substitutions, transitions vs. transversions. Data above the diagonal represent the genetic differences between samples.

3.3.2 Phylogenetic Analysis between *Eupetaurus* and *Petaurista*

Although *P. petaurista* had been thought as the closest relative of *Eupetaurus* for long time, Mckenna (1962) regarded *P. xanthotis* as the closest living relative of *E. cinereus* for their similar dental structures. The genetic discrepancies between *Eupetaurus* and *Petaurista* based on the pairwise comparisons of cytochrome b gene were not consistent with Mckenna's hypothesis (Table 3.4). The genetic difference between *P. xanthotis* and *Eupetaurus* was 17.0 ~ 19.4%, which was higher than that

between *P. petaurista* and *Eupetaurus* (16.7 ~ 18.1%). The phylogenetic tree did not support his conclusion either, in which all samples were clustered into two genetic clades (Figure 3.4).

The estimated dates of divergence among mtDNA clades of *Eupetaurus* and *Petaurista* were calculated based on their rates of divergence for the third codon positions of cytochrome b gene (Table 3.5 and Table 3.6). The approximate divergent time between the eastern and the western trans-Himalayan *Eupetaurus* was about 10 million years ago, and approximately 29.2 ~ 32.2 million years ago between *Eupetaurus* and *Petaurista*.

Table 3.5 Transversional substitutions at the third codon positions of cytochrome b gene between *Eupetaurus* and *Petaurista* (based on 390 bp)

	Eupetaurus I	*Eupetaurus* II	PPF	PPY	PTK
Eupetaurus I		5.4	14.6	14.6	14.6
Eupetaurus II	7		15.4	15.4	16.1
PPF	19	20		1.0	6.1
PPY	19	20	1		5.4
PTK	19	21	8	7	

Note: Data below the diagonal are the numbers of transversions at the third codon positions. Data above the diagonal represent the transversional percentage difference between samples.

With *Glaucomys volans* as the outgroup taxon, the phylogenetic relationships among *Eupetaurus*, *P. petaurista* and *P. xanthotis* were reconstructed using maximum parsimony and neighbor-joining methods. Three major clades were formed in both MP and NJ trees (Figure 3.5 and Figure 3.6). The first dichotomy isolated *Petaurista* including PPY, PPF, and PTK from *Eupetaurus* group. Then all individuals of

Eupetaurus were clustered as two groups: one was the Pakistan *Eupetaurus* (ECF1, ECF2, ECB1, ECB2, and ECD); another was the Chinese *Eupetaurus* (ECK1, ECK2, and ECL).

Table 3.6 Estimated divergent times among *Eupetaurus* and *Petaurista* based on a rate of divergence for the third codon positions of mammalian cytochrome b gene of ca. 0.5% $*10^6$ years

mtDNA clades		Estimated date of divergence ($1.0*10^6$ years)
Eupetaurus I vs.	*P. petaurista* (*albiventer*)	29.2
	P. xanthotis	29.2
	Eupetaurus II	10.8
Eupetaurus II vs.	*P. petaurista* (*albiventer*)	30.8
	P. xanthotis	32.2
P. petaurista vs.	*P. xanthotis*	10.8 ~ 12.2

3.4 Discussion

3.4.1 Phylogenetic Status of the Population of *Eupetaurus* in the Eastern Trans-Himalayas

The available morphological comparisons and taxonomic studies of *Eupetaurus* were solely based on the pelage characteristics and the dental forms of the specimens represented in museums and institutes. The two specimens collected in the deep gorge country of SW Yunnan near the Thailand, Burma, and Tibet border by KIZ were recognized as *E. cinereus* on the basis of the pelage color and the feet (Wang and Yang, 1986; Corbert and Hill, 1992; Zahler and Woods, 1997), which are externally similar to those in Pakistan (Figure 3.7). The molecular data of this study strongly support their identification. These two Chinese *Eupetaurus* show the similar molecular features with the population of *Eupetaurus* distributed in Tibet. The genetic difference is 3.8% (Table 3.4), less than the intraspecific cytochrome b differences of squirrels (Oshida and

Yoshida, 1999). This confirms the presence of *Eupetaurus* in southwestern China, although lack the morphological data from these specimens.

Eupetaurus was commonly considered as a monotypic genus, consisting of a single species, *E. cinereus*. However, the genetic distances in NJ tree and the polycotomy in MP tree of the present study suggest that the populations in the eastern and the western trans-Himalayas can be divided into two major mtDNA clades (Figure 3.2 and Figure 3.3). The genetic distance between these two clades was 11.0 – 13.3% (Table 3.4). Since the reported intraspecific cytochrome b differences of squirrels was < 3.0%, and was applied to other flying squirrels, such as *Glaucomys* (Arbogast, 1999) and *Petaurista* (Oshida and Yoshida, 1999), the genetic difference between *Eupetaurus* I and *Eupetaurus* II can be referred as the interspecific variation. This implies that the *Eupetaurus* populations in the western (Pakistan) and the eastern (China) trans-Himalayas might be two distinct species.

Using the substitution rate at the third codon positions estimated from the mammalian cytochrome b genes (Irwin et al., 1991), the divergence between the two groups could have occurred approximately 10.8 Myr (million years) ago (Table 3.8), suggesting that the two populations diverged early before the glacial period and the uplift of the Himalayas and Qinghai-Tibet plateau during Pliocene – Pleistocene (see APPENDIX for time scales). It is inferred that the ancestor stock of *Eupetaurus* originated somewhere along the Himalayas mountain chain. Before the glacial eustacy, the eastern and the western trans-Himalayan *Eupetaurus* diverged from the ancestral *Eupetaurus* and separately migrated to their present locations after the closure of the Tethys Sea at the end of Miocene. During the glacial period of the Pliocene, they became

adapted to the cold environments of mountain rallies. During inter-glacial times, they migrated to higher elevations to avoid warmer conditions. The subsequent glaciations and the uplift of the Himalayas and Qinghai-Tibet plateau in Pleistocene (Xu, 1981; Zheng et al., 2000) caused a great change of climate and ecological system along the Himalayas, which consisted of several distinct topographic regions determined by drainage patterns and the parallel mountain chains in both the western and the eastern trans-Himalayan regions, such as the Yangtze river system, Salaween river system, and Mekong river system. These tectonic events led to southwestern China, also possibly northern Pakistan, becoming refuges for some special mammals, such as *Eupetaurus*. It is possible that the present distribution of *Eupetaurus* in the trans-Himalayas is secondarily related to the tectonic activities of the Cenozoic that caused dramatic changes of environment in the Eurasian continent (Wang, 1984).

In Pakistan, the distributions of *E. cinereus, P. petaurista,* and *Eoglaucomys fimbriatus* are sympatic, which correspond to different ecological habitats, indicating their different feeding habits. A similar pattern of sympatric distribution of *E. cinereus, P. peturista*, and *Hylopetes alboniger* was also found in SW China where the two *Eupetaurus* skins were collected. But, without sufficient morphological evidence, it is premature to raise these two groups as distinct species, although it is noteworthy that the genetic characteristics between these two groups are corresponding to their geographic distances of sampling localities.

3.4.2 Phylogenetic Relationship between *Eupetaurus* and *Petaurista*

Petaurista and *Eupetaurus* have been considered as the closely related species for a long time because of their similar external structures and sympatric distribution (Figure 4.8). Their common ancestor was likely to have originated in the eastern Himalayas and

Indo-China (Zahler and Woods, 1997). According to McKenna (1962), the differentiation of the recent genus *Eupetaurus* from a *Petaurista*-like sciurid provided a significant parallel to the derivation of various dentally high-crowned rodents from sciurid and paramyid stock in the early and middle Tertiary (Wood, 1962). The differences between *Petaurista* and *Eupetaurus* are primarily the result of the high-crowned teeth (Figure 3.8).

Eupetaurus has high-crowned teeth and survives in more restricted areas that appear to meet their unique habitat requirements. Thomas (1888) believed that woolly flying squirrels fed mainly on lichens, mosses, and other plants associated with rocky areas. Local people in Pakistan believe that *Eupetaurus* feeds on seeds, needles, bark of conifers, spruce buds, and some abrasive materials (Zahler and Woods, 1997). In eastern Tibet and NW Yunnan, forests grow up to an elevation of 3,500-4,500 m, where there is a mixed coniferous and broad-leaved forest that composes predominantly of spruce, fir, and oak. It is the optimum habitat for *Eupetaurus*. The adaptive shift of the feeding mechanism is analogous to the shifts that led to the distinctive morphology of the dentition of beavers, mylagaulids, eutypomyids, and numerous other high-crown rodents (McKenna, 1962). But, the pattern of the dentition in *Eupetaurus* is unlike that of any other known rodent, either fossil or recent. A good ecomorph of *E. cinereus* is *Plagiodontia aedium* from Hispaniola, which also lives in rock crevices and caves, located in rocky areas at high elevation (3,000m). Both forms probably feed upon the similar abrasive materials for their similar modifications to the masticator apparatus (Woods and Howland, 1979).

Since *Eupetaurus* is so distinct from other flying squirrels, it appears that *E. cinereus* is morphologically convergent with capromyids as well as cane rats and New World spiny rats, rather than with other petauristines. The unique dental form of *Eupetaurus* might be due to isolation in marginal habitats or the strong competitve pressure from *P. petaurista* and *Eoglaucomys fimbriatus* or *H. alboniger*. The present molecular findings indicate that *Eupetaurus* had diverged from *Petaurista* before the Oligocene-Miocene radiation of giant flying squirrels in Europe (Oshida, et al., 2000a), and that *Eupetaurus* is a specialized species that genetically differs from *Petaurista* (Figure 3.5 and Figure 3.6).

Petaurista xanthotis and *P. petaurista* were considered as the closest living relative of *Eupetaurus* (Mckenna, 1962; Zahler and Woods, 1996) (Figure 3.9), but the genetic data of the present study reveals that the isolation between the ancestors of *Eupetaurus* and *Petaurista* occurred approximately at the end of Oligocene, about 30.8 – 32.2 Myr ago (Table 3.4 and Table 3.5). The phylogenetic reconstruction also demonstrates that *Eupetaurus* and *Petaurista* are different clades with significant genetic distances, 15.9 – 19.4% (Table 3.3). Considering their divergence time, *Eupetaurus* diverged from *Petaurista*-stock much earlier than the formation of *P. xanthotis*, which diverged from *Petaurista* about 11 Myr ago. The present distribution of *P. petaurista* (*albiventer*) in western trans-Himalayas is a recent dispersal.

Petaurista xanthotis is a Chinese endemic species and is found living from Tsing Hai and Kansu, southeastward to Hubei and southward to Sichuna and Yunnan, China. The elevation ranges from 2,000m in Gansu to 3,300m in Yunnan where the eastern extremes of the Himalayas and the Tibetan Plateau separate the range of *P. xanthotis*

from *Eupetaurus*. *P. xanthotis* has semi-hypsodont molariform teeth, which is significantly different from *P. petaurista* (Figure 3.8). The principal differences between *P. xanthotis* and *Eupetaurus* are the result either of the increased height of crown of its molar teeth of *Eupetaurus* or of a few minor changes in molar crown pattern acquired by *P. xanthotis*. Mckenna (1962) proposed that the lineage leading to *P. xanthotis* had changed little and *E. cinereus* had modified the dentition and the masticatory musculature to a considerable degree. The similar dental structure between *Eupetaurus* and *P. xanthotis* might be the convergent adaptation to the similar feeding resources.

According to the paleontological records, by the late Miocene the geography of the trans-Himalayas was similar to that of today (Wang, 1984). The presence of the Himalayas helped cause the diversification of climate, and it became an important regulator of the Asian environment. There were either three or four major glacial periods in Europe and Asia, separated by warmer, interglacial periods in Pliocene-Pleistocene. *Eupetaurus* migrated to its current geographical distribution in the early Miocene when the radiation of *Petaurista* occurred in the Eurasian continent (Oshida et al., 2000a). After diverging from the ancestral *Petaurista* in the middle Miocene, *P. xanthotis* was restricted to the southern parts of the Eurasian continent. During glacial spisodes, it adapted to cold environments and high elevations, and its feeding habits became specialized. With the retreating of glaciers and the uprising of the Himalayas, it subsequently expanded into northward, where it inhabited in the temperate forest. The present distribution of *P. xanthotis* is mainly due to the geographic events of the Pliocene-Pleistocene. The molecular data here indicate that the phylogenetic relationship

between *Eupetaurus* and *P. xanthotis* or *P. petaurista* is not as close as indicated from morphology.

3.5 Summary

In this study, the partial cytochrome b gene sequences (390 bp) of *Eupetaurus* were analyzed to elucidate the phylogenetic status of *Eupetaurus* in the eastern and the western trans-Himalayas. I also discussed the phylogenetic relationship between *Eupetaurus* and *Petaurista*. The phylogenetic trees were reconstructed using neighboring-joining and maximum parsimony methods. The following results were concluded in the present study:

1. The two specimens that were collected in northwestern Yunnan, China, are *Eupetaurus*.

2. The *Eupetaurus* populations in the eastern and the western trans-Himalayas are significantly different (>13%). The genetic characters between these two populations are corresponding to their geographic distribution. They are two distinct species.

3. The divergence time of the two *Eupetaurus* populations was at the end of Miocene. The glacial period and the uplift of the Himalayas and Qinghai-Tibet plateau during the Pliocene-Pleistocene period are the major factors that secondarily affected on the present distribution of *Eupetaurus* in trans-Himalayas.

4. There is not a very close phylogenetic relationship between *Eupetaurus* and *P. xanthotis*. The similar dental characters might be of the convergent adaptation to the similar food resource.

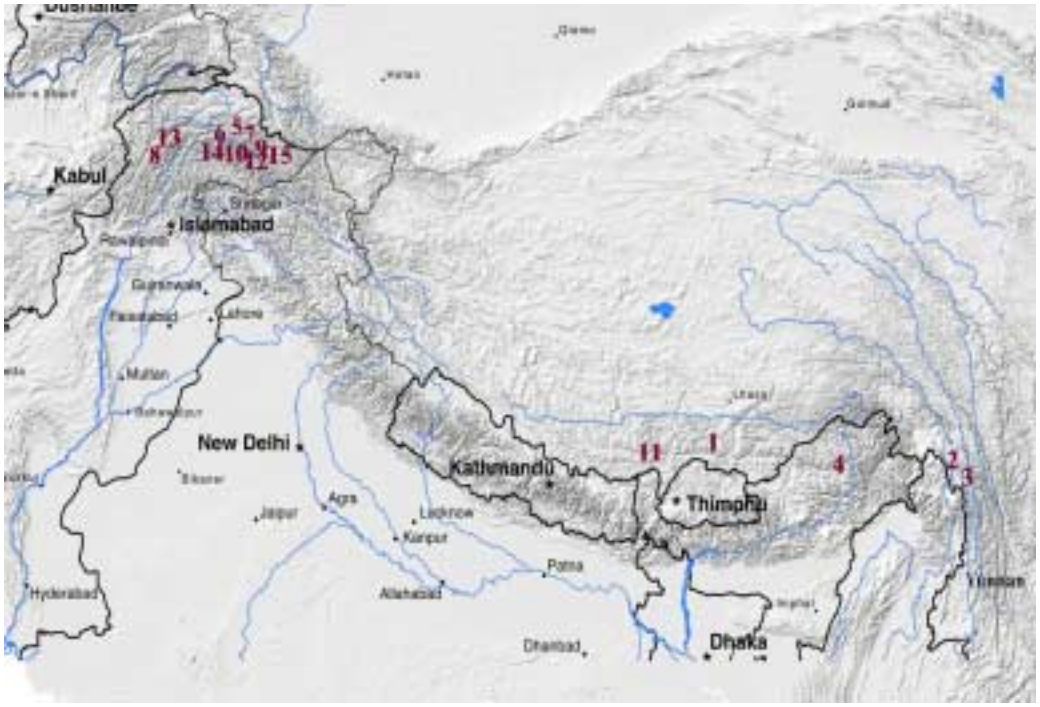

Figure 3.1 Historical records of *Eupetaurus* specimens in the world. The numbers in the map stand for the collecting localities of specimens, which are corresponding to the numbers of Table 3.1.

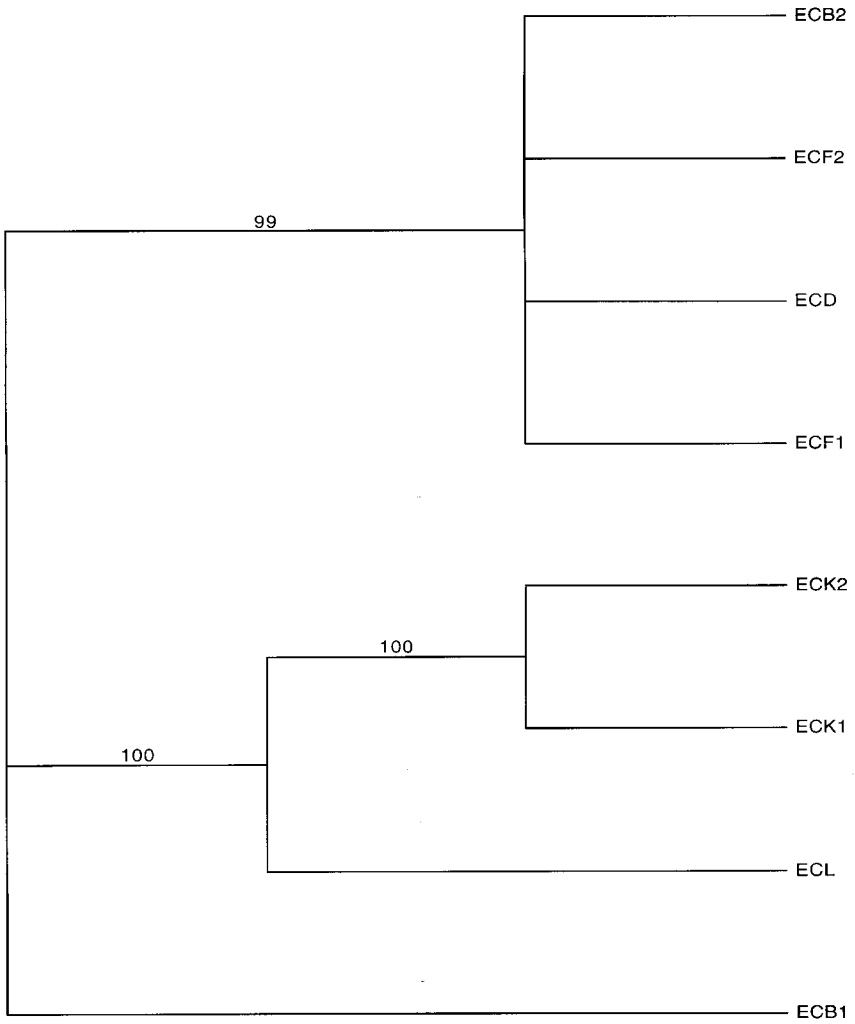

Figure 3.2 Phylogenetic tree of *Eupetaurus* reconstructed by the maximum parsimony (MP) method. Numbers above branches indicate the bootstrap values (%). Sample abbreviations are coded in Table 3.2.

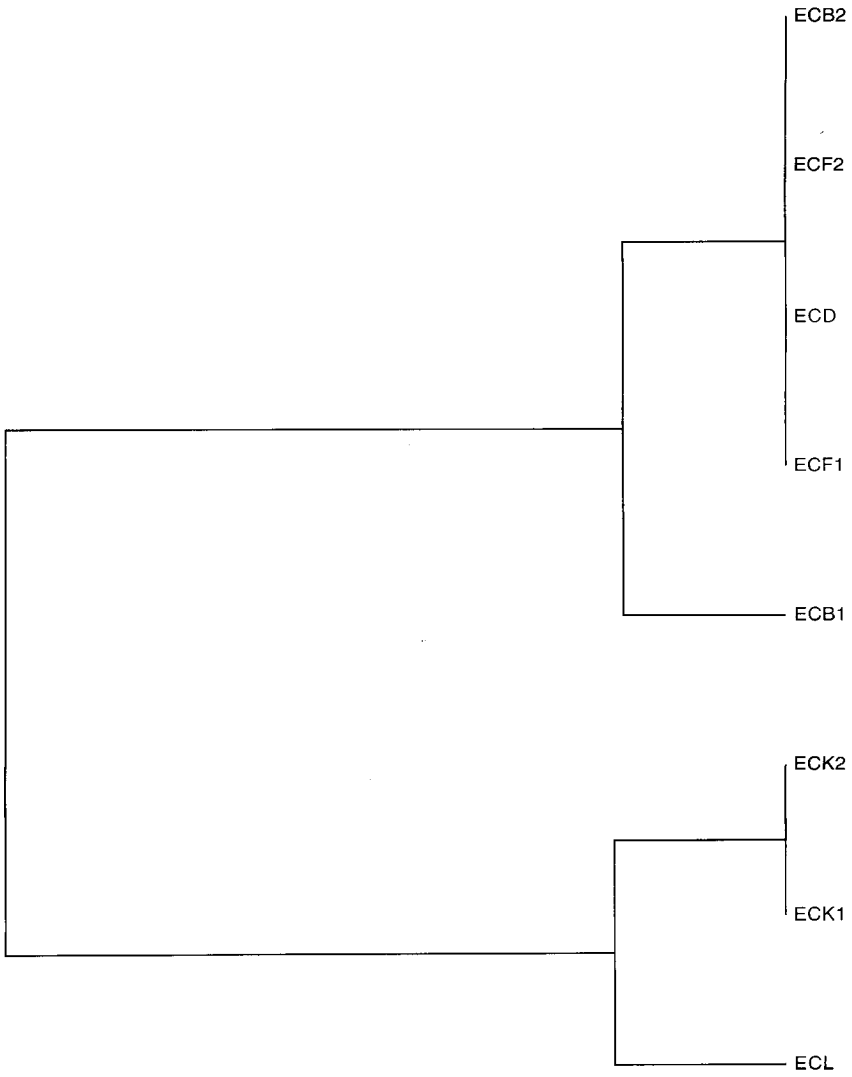

Figure 3.3 Phylogenetic tree of *Eupetaurus* reconstructed by the UPGMA method. Sample abbreviations are coded in Table 3.2.

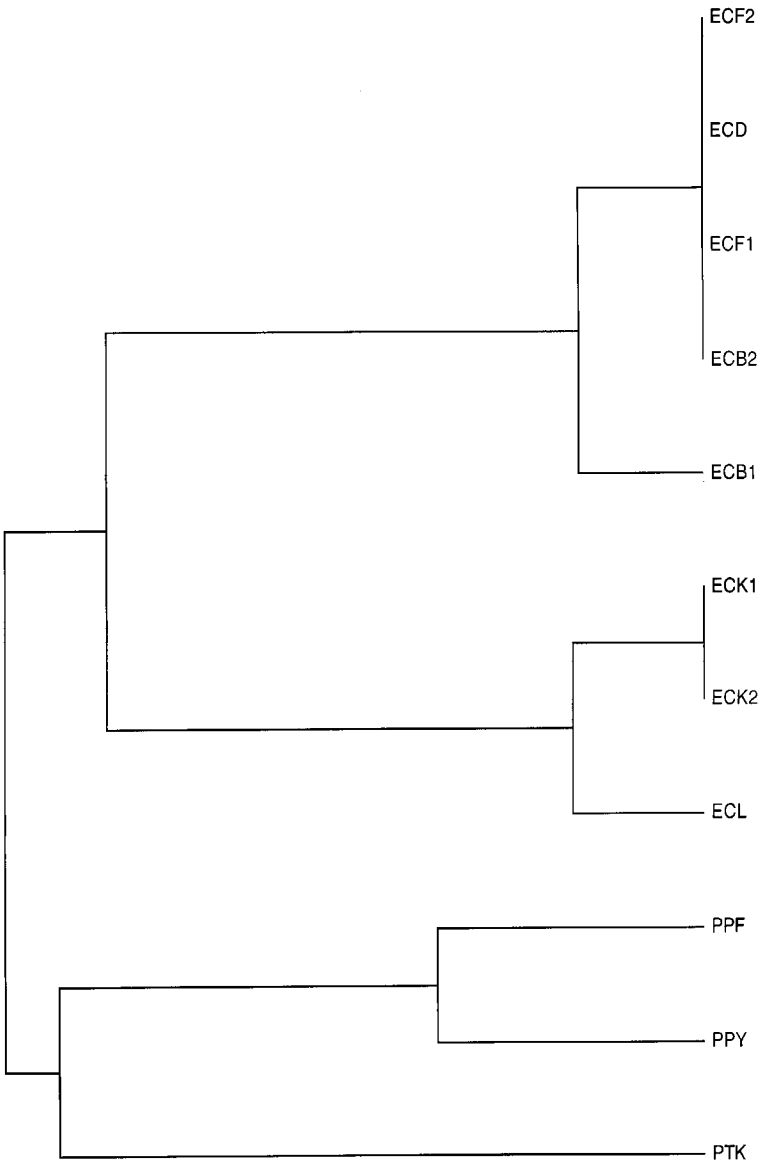

Figure 3.4 Phylogenetic tree of *Eupetaurus* and *Petaurita* constructed with UPGMA method. Sample abbreviations are defined in Table 3.2.

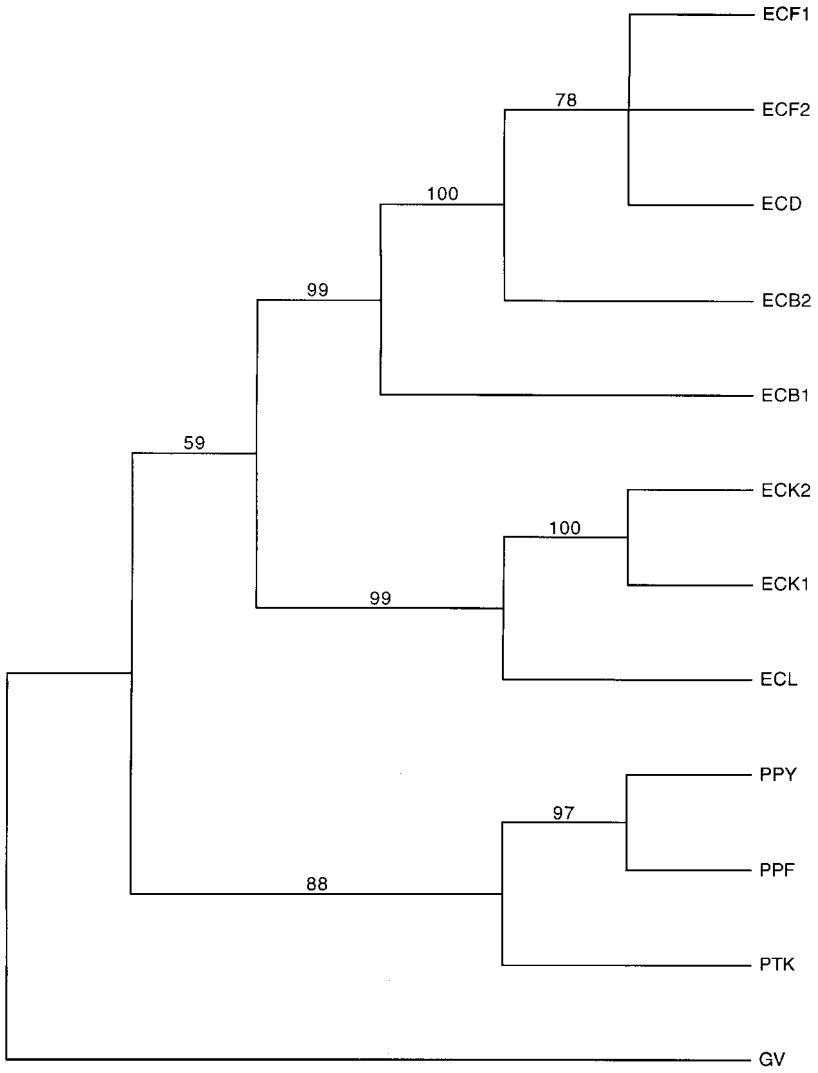

Figure 3.5 Phylogenetic relationships of *Eupetaurus*, *Petaurita*, and *G. volans* constructed with the parsimony maximum (MP) method. Numbers above branches indicate the bootstrap values (%). Sample abbreviations are defined in Table 3.2.

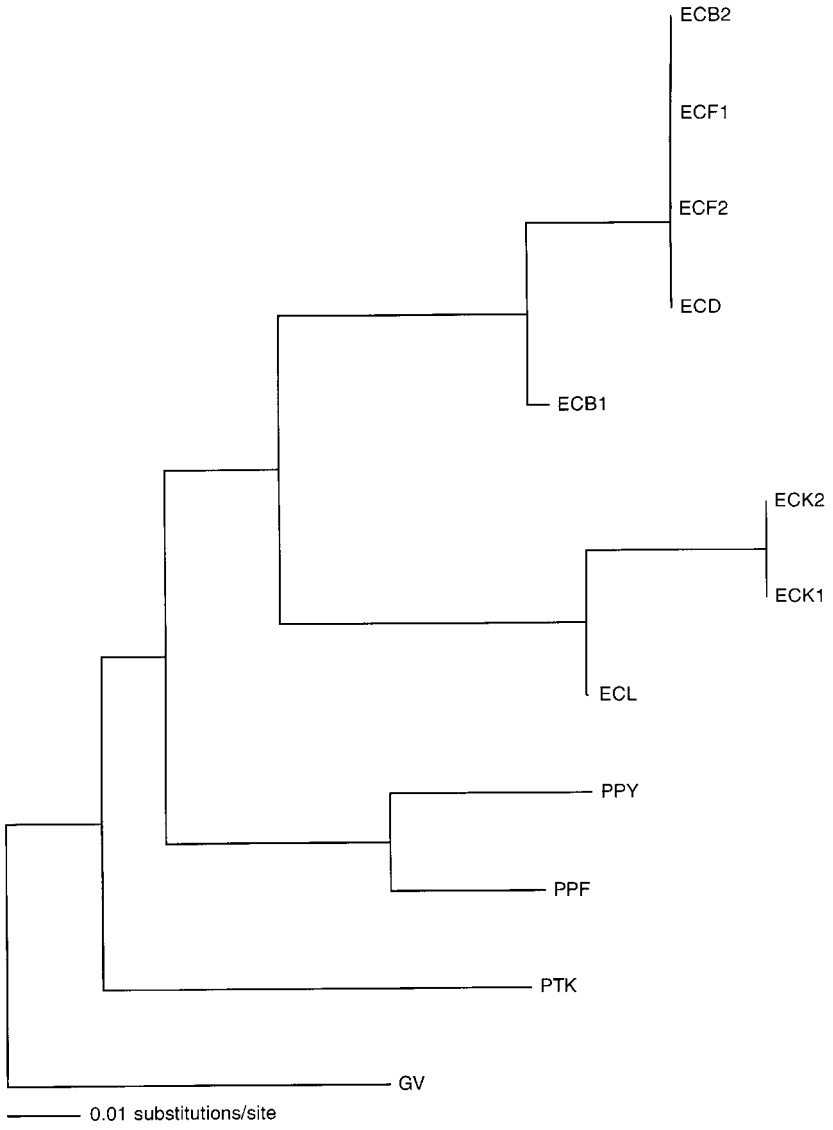

Figure 3.6 Phylogenetic relationships of *Eupetaurus*, *Petaurita,* and *G. volans* constructed with the neighbor-joining (NJ) method. Scales in the tree represent branch length in terms of nucleotide substitutions per site. Sample abbreviations are coded in Table 3.2.

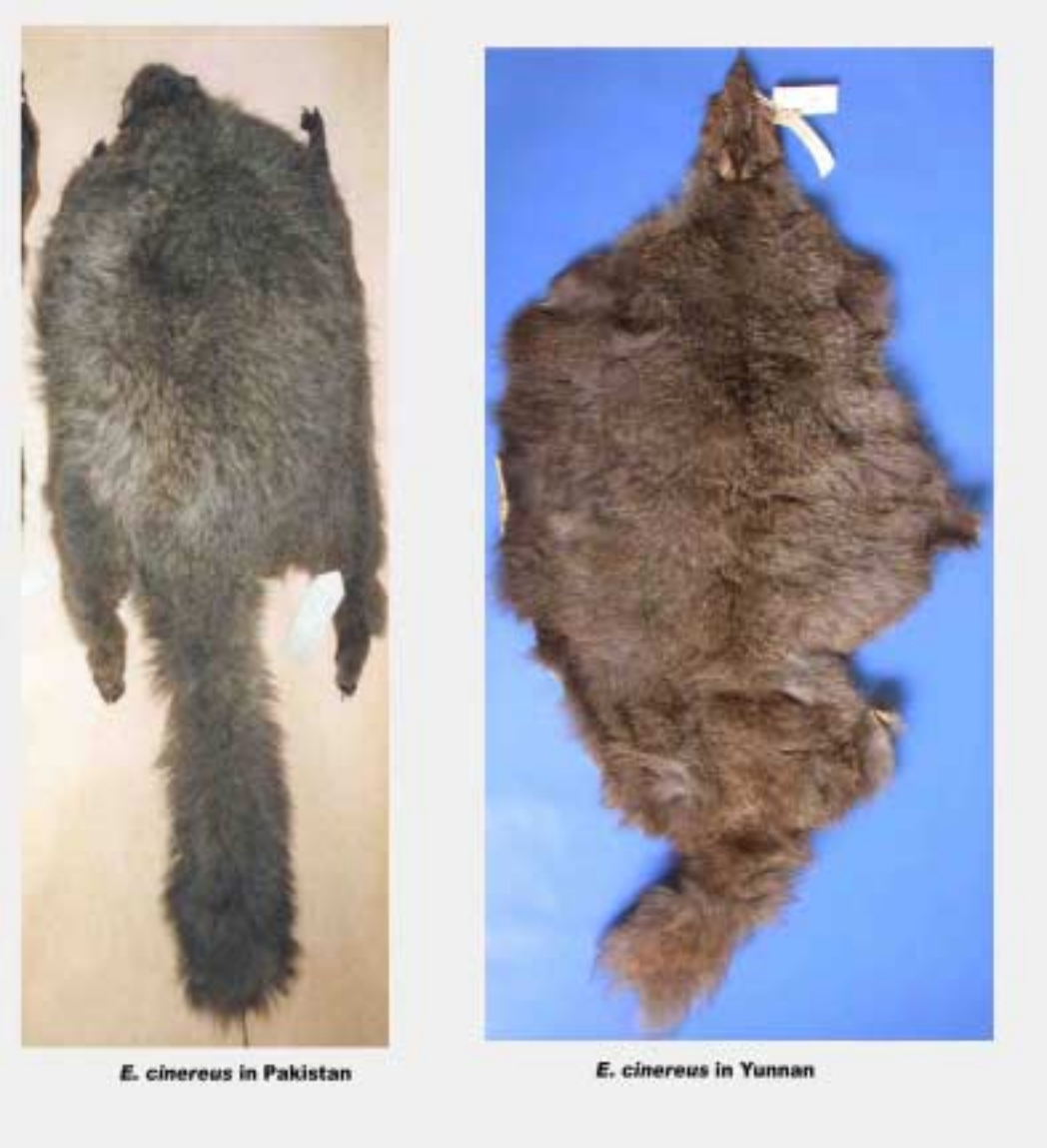

Figure 3.7 *Eupetaurus cinereus* in Pakistan and SW China

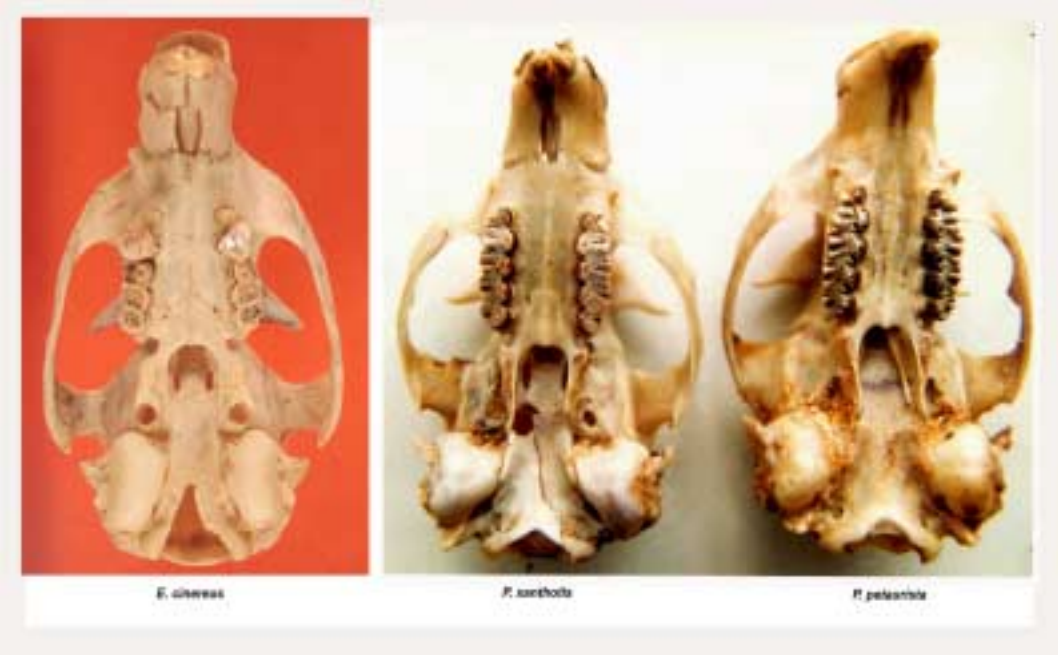

Figure 3.8 Ventral views of the skulls of *E. cinereus*, *P. petaurista*, and *P. xanthotis*

Figure 3.9 *E. cinereus*, *P. petaurista*, and *P. xanthotis*

CHAPTER 4
PHYLOGENY OF GIANT FLYING SQUIRREL (*PETAURISTA*) IN SW CHINA AND PAKISTAN: IMPLICATIONS FOR DEVELOPMENT OF MOLECULAR AND MORPHOLOGICAL ANALYSIS

4.1 Introduction

Giant flying squirrel, *Petaurista,* contains many recognized distinct species occupying different habitats. They inhabit various kinds of forests either in lowlands or in mountains up to 4,000 meters in elevation from Pakistan, Kashmir to China, northern Indochina, the Malayan Peninsula, Sumatra, Java, Borneo, Japan, Korea, and Manchuria. Several taxonomic revisions have been proposed on the basis of dental and cranial characteristics and external structures (Allen, 1940; Ellerman, 1940; Corbet and Hill, 1991, 1992; Nowak, 1991; Wilson and Reeder, 1992). More than 18 forms in *Petaurista* have been described as valid species, but some of them are only referenced with very few specimens; some are based solely on skins with no corresponding skulls (Allen, 1940; Ellerman, 1940); and some actually are the synonyms or subspecies of other valid species (Corbet and Hill, 1992). The populations of *Petuarista* that are distributed in China and occupy different habitats are recognized as ten distinct species by recent authorities (Corbet and Hill, 1992; Zhang et al., 1997) (Table 4.1 and Table 4.2). Figure 4.1 depicted the geographic distributions of Chinese *Petaurista*. In *Petaurista*, the species *P. elegans, P. petaurista, P. alborufus, P. magnificus, P. philippensis,* and *P. leucogeny* are commonly accepted as valid species, with each intricately divided into various forms or subspecies (Corbet and Hill, 1991, 1992; Nowak, 1991, 1999; Wilson and Reeder, 1992; Zhang et al., 1977; Wang, 2002).

However, various species with significantly geographical variations are included within this genus, the taxonomy and the intra- and the inter-specific phylogenetic relationships are confusing and inconclusive, especially the giant flying squirrels that are distributed in the eastern and western trans-Himalayas.

Table 4.1 Chinese *Petaurista* forms

Species	Subspecies	Distribution	Habitat
P. petaurista	*P. p. grandis*	Taiwan	Tropical and subtropical forest
	P. p. miloni	Guangxi	
	P. p. rufipes	Fujian, Guangdong	
	P. p. rubicundus	Sichuan, Gansu	
	P. p. nigra	W Yunnan	
P. alborufus	*P. a. alborufus*	Shanxi, W Sichuan	Tropical and subtropical forest
	P. a. lena	Taiwan	
	P. a. castaneus	Hubei, Sichuan, Guizhou	
	P. a. ochraspis	Yunnan, Guangxi	
P. elegans	*P. e. clarkei*	Yunnan, Guizhou	Forest
	P. e. gorkhali	S Xizang	
P. yunanensis		Yunnan, Guangxi, Tibet	Tropical forest
P. hainana		Hainan	Tropical forest
P. pectoralis		Taiwan	Tropical forest
P. xanthotis		Gansu, Qinghai, Yunnan, Xizang	Mountain coniferous
P. philippensis		Yunnan, Guizhou	Forest
P. magnificus		Xizang	Mountain forest
P. marica		Yunnan, Guangxi	Tropical forest

NOTE: THE ASSIGNMENTS ARE DESCRIBED BY ZHANG ET AL. (1997)

Ellerman (1940), Corbet and Hill (1992), and Wang (2002) had comprehensively studied Chinese *Petaurista* based on the morphological and external characteristics, but there is not sufficient evidence from either morphometric study or molecular analysis to ascertain conclusively these specific conclusions. The major problems of Chinese *Petaurista* are about the conspecific relations within *P. petaurista* (sensu stricto), the taxonomic statuses of *P. philippensis, P. xanthotis, P. hainana,* and *P. yunanensis*, and the phylogenetic relationships of *Petaurista* at the specific level.

Petaurista petaurista is a polymorphic species with considerable variation in pelage coloring (Allen, 1940). This species mainly occurs in plantations as well as in forest in southern China. Its broad distribution is beyond China including northern India, Bhutan, Nepal, Pakistan, northern Afghanistan, and the Greater Sunda Islands. A dozen of nominal subspecies have been described (Allen, 1940; Ellerman, 1940; Ellerman and Morrison-Scott, 1966). Corbet and Hill (1992) put all *Petaurista* populations into seven major forms (Table 4.2). However, from our comparative study of *Eupetaurus* (Chapter 3) and observations of some other rodents distributed in the western and eastern trans-Himalayas, it seems extreme to allocate the populations of *Petaurista* in Pakistan and W Yunnan to the same form, *P. p. albiventer*. Further study needs to be done to clarify the inter-group relations for their various geographical distributions.

With few exceptions (Nowak, 1991), *P. philippensis* and *P. xanthotis* have been merited as valid species based on their distinct external and dental structures (Corbet and Hill, 1991, 1992; Wilson and Reeder, 1992). *P. philippensis* is a polymorphic species with extensive geographical variations and used to be included in *P. petaurista*

(Ellerman, 1940; Ellerman and Morrison-Scott, 1966). Because this species is clearly separable from *P. petaurista* by the external structures, Corbet and Hill (1991, 1992) ranked *P. philippensis* as a valid species and classified its populations as seven major forms (Table 4.3). All populations distributed in China including the island of Hainan were recognized as *P. philippensis yunanensis*. Without further evidence, much remains to be done to clarify their taxonomic relationships. Some forms included by Corbet and Hill (1992) may warrant separate specific rank.

Table 4.2 Forms of *Petaurista*

Form (subspecies)	Distribution
P. p. albiventer	W Pakistan, W Yunnan, China
P. p. petaurista	Java
P. p. marchio	Borneo and Malayan Peninsula
P. p. batuana	W Sumatra
P. p. terutaus	S Thailand
P. p. taylori	S Burma and W Thailand
P. p. candidula	Burma and Thailand

Table 4.3 Major forms of *P. philippensis*

Subspecies	Distribution
P. p. philippensis	Peninsular India and Sri Lanka
P. p. cineraceus	W Burma
P. p. mergulus	Mergui Is, Burma
P. p. lylei	E Burma, Thailand
P. p. annamensis	Vietnam, S China
P. p. yunnanensis	SW CHINA, HAINAN, N ASSAM
P. p. grandis	Taiwan

Petaurista xanthotis is a Chinese endemic species with an extensive range, from the spruce forests of northwestern Kansu southward in the highlands of western China to the Likiang region. *P. xanthotis* was considered as a form of Japanese giant flying

squirrel, *P. leucogenys*, by Ellerman and Morrison-Scott (1966), but it was elevated as a distinct species for its much complex cheek-teeth (Corbet and Hill, 1991, 1992, Nowak, 1999). Except for a morphological illustration of teeth by McKenna (1962), almost nothing is known about this species.

Petaurista yunanensis and *P. hainana* are generally treated either as the subspecies (Corbet and Hill, 1992) or as the synonyms (Wilson and Reeder, 1992) of *P. philippensis* based on the dental structures and pelage coloration, although both were merited as two valid species of *Petaurista* early by Anderson (1878) and Allen (1940). The former occurs from extreme southwestern Yunnan probably into Burma and Indochina, and the latter is only distributed in Hamfong, Hainan, China (Figure 4.1). Very little seems to be known as to their taxonomic statuses and the inter- or intraspecific relationships with *P. philippensis*. A comprehensive study on the basis of morphometric and molecular analysis is much needed.

Mitochondrial DNA (mtDNA) is one of those valuable molecules for evolutionary relationship reconstruction among populations, subspecies, and species. Using the polymerase chain reaction (PCR), it is possible to recover genetic information from severely degraded tissues. With the mtDNA information it is able to infer the inter- and intra-specific relationships, to investigate the genetic differentiation, and to reconstruct the phylogenetic topology of the controversial taxa. Although some molecular data and geographical and morphological variations involving morphology, myology, and karyology have been intensively used for studying the species, subspecies or forms of *Petaurista* (Cuvier, 1856; Bryant, 1945; Harrison, 1960; Johnson-Murray, 1977; Throington and Heaney, 1981; Oshida et al., 1992; Oshida et al., 1996; Oshida

and Masuda, 2000; Oshida et al., 2000a, 2000b), it is too scanty to throw any light on the problems. There are still arguments on the validity of a number of species or forms in *Petaurista*, and very little is known on the phylogenetic relationships within the genus *Petaurista*.

In attempting to resolve the affinities of the complex taxa, a comprehensive study based on both morphological data including 14 measurements of skull and molecular data using partial sequences (375 – 400 bp) of mitochondrial cytochrome b gene were conducted in this study. The objectives were to answer the following questions:

1. Do the populations of *P. petaurista* (*albiventer*) along the eastern and the western trans-Himalayas form a single species -- *Petaurista albiventer*, or a complex of species?
2. Are *P. philippensis*, *P. xanthotis*, *P. hainana*, and *P. yunanensis* valid species, subspecies, or synonyms*?*
3. What are the phylogenetic relationships among the populations of Chinese *Petaurista* and the populations of *Petaurista* in Pakistan and SE Asia?

4.2 Materials and Methods

4.2.1 Specimens in Morphometric Study

A total of 193 recent specimens of giant flying squirrels (*Petaurista*) were used in morphometric analysis (Table 4.4). Specimens were conventional museum specimens preserved as skulls, skeletons, and fluid. These specimens are deposited in the following collections: American Museum of Natural History, New York (AMNH), Florida Museum of Natural History, University of Florida, Gainesville (FLMNH), National Museum of Natural History, Smithsonian Institution, Washington, DC (USNM), Chinese Institute of Zoology, Beijing (China, BIZ), Northwestern Institute of Biology, Qinhai (China, NIB), and Kunming Institute of Zoology, Kunming (China, KIZ).

Fourteen cranial measurements were taken with digital caliper to the nearest 0.01 mm. The definitions and abbreviations of measurements were given in Table 4.5.

Table 4.4 Species and localities of *Petaurista* populations examined in morphometric analysis

Species	Subspecies	Specimens	Locality	Museum
P. petaurista	*P. p. albiventer*	10 (5F, 5M)	NW Yunnan, China	KIZ, AMNH
	P. p. albiventer	31 (14F, 7M)	NWFP, Pakistan, Kashmir	KIZ, USMNH
	P. p. candidula	4 (2F, 2M)	Lakhuni, India, Chiengmail, Thailand	AMNH
	P. p. melanotus	24 (12F, 12 M)	E Sumatra, Borneo, Malayan Peninsula	USMNH, AMNH
	P. p. petaurista	10 (6F, 4 M)	Java	AMNH
	P. p. batuana	11 (6F, 5M)	W Sumatra	AMNH
P. philippensis	*P. philippensis*	9 (3F, 6 M)	Yunnan, Guangxi, China	KIZ, BIZ, AMNH
	P. p. lyei	10 (6F, 4M)	Indochina, Thailand	USMNH
	P. p. grandis	14 (3F, 11U)	Taipei, Taiwan	AMNH, FLMNH
P. yunanensis		15 (5F, 3M)	Burma, Yunnan, China	BIZ, AMNH
P. hainana		9 (5F, 4M)	Namfong, Hainan	AMNH
P. elegans		12 (6F, 6 M)	Yunnan, Guangxi, China	KIZ
P. alborufus		29 (5M, 24U)	Taiwan, Yunnan, Burma	KIZ, AMNH
P. xanthotis		5 (3F, 2 M)	Yunnan, Qinhai, China	KIZ, NIB
Total		193	F = Female, M = Male, U=Unknown sex	

Note: Assignments of species and subspecies were based upon the classification of Corbet and Hill (1992) and Zhang et al. (1997).

Table 4.5 Variables in morphometric study

Variable	Definition
CRANL	Cranial length from the tip of the occipital protuberance to the alveolar
BCASEL	Braincase length from the tip of the occipital protuberance to orbit
CRANW	Cranial width, distance between the points on the left and right superametal crests above the external acousticmeatus
BPORW	Width between the left and right zygomatic arch
POCL	Postorbital constriction, the least distance across the top to skull posterior to the postorbital process
PGA	Distance from the base of the anterior surface of the postglenoid process to the M3
NAL	Nasal length
TBL	Tympanic bullae length
DSL	Diastema length between the second incisor and the first premolar
MTRL	Length of the maxillary tooth row
MTRW	Width of the maxillary tooth row at M_2
LMDL	Maximum mandible length
LMDH	Maximum height of low jaw
LMTL	Length of the maxillary tooth row of lower jaw

4.2.2 Species for Molecular Analysis

Species of giant flying squirrels (*Petaurista*) used in molecular analysis were listed in Table 4.6. All skin tissues were collected from AMNH, USNM, BIZ, NIB, FLMNH, and KIZ. Fresh tissues were either obtained from the Cellbank of Kunming Institute of Zoology, the Chinese Academy of Sciences, or collected from the locality in where the species is distributed. To reconstruct the phylogenetic relationships, the

sequence data of *P. petaurista* and *Pteromys volans* were quoted from the GenBank of NCBI. The detailed information was given in Table 4.7.

Table 4.6 Samples of *Petaurista* examined in molecular study

Species	Code	Museum ID	Collecting locality
P. p. albiventer (PPF and PPY)	PPF1	FLMNH: 28236	Pakistan
	PPF2	USMNH: 353209	Pakistan
	PPY1	KIZ: 640229	Yunnan, China
	PPY2	KIZ: 73382	Yunnan, China
P. xanthotis (PTK)	PTK1	QIZ: 85063	Gansu, China
	PTK2	QIZ: 984	Gansu, China
P. philippensis (PPH)	PPH1	KIZ: Fresh tissue	Pianma,Yunnan, China
	PPH2	KIZ: 620028	Yunnan, China
	PPH3	KIZ: 74540	Yunnan, China
	PPH4	KIZ	Yunnan, China
P. yunanensis (PYK)	PYK1	KIZ: Fresh tissue	W Yunnan, China
	PYK2	KIZ: Fresh tissue	Gongshan, Yunnan, China
	PYK3	KIZ: Fresh tissue	Gongshan, Yunnan, China
	PYK4	KIZ: 73270	Gongshan, Yunnan, China
	PYK5	KIZ:	Gongshan, Yunnan, China
P. hainana (PHK)	PHK1	KIZ: 22686	Hainan, China
	PHK2	KIZ: 259442	Namfong, Hainan, China
P. alborufus (PAK)	PAK1	KIZ: 006679	Sichuan, China
	PAK2	KIZ: 43178	Likiang, China
	PAK3	KIZ: 73369	Yunnan, China
	PAK4	KIZ: 64042	Luodian, Guizhou, China
P. elegans (PEK)	PEK1	KIZ: 84354	Mile, Yunnan, China
	PEK2	KIZ: 73375	Lijiang, Yunnan, China
	PEK3	KIZ: 73369	Bijiang, Yunnan, China

Table 4.7 Sequence data of *Petaurista* and *Pteromys* used in this study

Species	Code	Locality	Accession No
*P. p. petaurista***	PPB	Borneo, E Malaysia	AF063067
P. p. melanotus	PPM1	Laos	AB023908
	PPM2	S China	AB023909
P. philippensis grandis	PPG	Natou, Taiwan	AB023907
P. leucogenys leucogenys	PLL1	Japan	AB023903
	PLL2	Japan	AB023904
P. leucogenys nikkonis	PLN1	Japan	AB023905
	PLN12	Japan	AB023906
P. alborufus castaneus	PAC1	S China	AB023898
	PAC2	S China	AB023899
	PAC3	S China	AB023900
P. alborufus lena	PAL1	Nantou, Taiwan	AB023901
	PAL2	Hualien, Taiwan	AB023902
Pteromys volan	PVO	Japan	AB023910

Note: Most sequence data were quoted from GenBank of NCBI, which were published by Oshida et al. (2000a).
** The sequence was quoted from GenBank of NCBI, which was pub by Arbogast (1999).

4.2.3 Morphometric Analysis

Statistical analyses in morphometric study were performed using the SAS (SAS Institute, 1982) or Statmost program (StatMost, 1995). The associations of cranial characters and species were assessed by discriminant function analysis and principal component analysis (PCA).

Discriminant function analysis carried out a multiple discriminant analysis in a stepwise manner, selecting the variable entered by finding the variable with the greatest F-value. This method was used to determine which of two or more groups a given individual should be assigned to and placed them with the group to which they were nearest on the discriminant functions. The relationships between groups onto the first three discriminant functions were plotted. Principal components analysis was performed to identify variables that account for maximum variation in data set and to accurately represent distances between major groups, in assessing the specific relationships among and between pooled individuals. The loadings or the eigenvector scores describing the relative significance of each variable to principal components were used to compare the morphological similarity and difference of skull. The projections of the row-points on the first three factors were depicted to describe the morphological relationships among samples examined.

4.2.4 Molecular Analysis

Because some samples were collected more than 50 years ago, the recovered sequences were usually between 300 to 400 base-pair longs. The following primers were used to amplify the partial nucleotide sequence (300 - 425 bp) of mitochondrial DNA (mtDNA) with polymerase chain reaction (PCR):

L14725 5’- CGA AGC TTG ATA TGA AAA ACC ATC GTT G -3’

L14841 5'- AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA -3'

H15149 5’- AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTCA -3’.

ICBR and KIZ synthesized all primers. The techniques and protocols used for DNA isolation, PCR amplification and purification, sequencing analysis, and phylogenetic reconstruction were the same as those in Chapter 3. Most molecular work was done at

the interdisciplinary center for biotechnology research (ICBR), University of Florida, and Kunming Institute of Zoology (KIZ), the Chinese Academy of Sciences, China. Some PCR products were sequenced at the University of Vermont, US.

One sample of *Pteromys volans* from NE China was used as the outgroup taxon for constructing phylogenetic trees.

4.3 Results

All measurements of skull were taken by author at museums and institutes in China and US, thereby avoiding the statistical complications and other associated problems of inter-observer error in a multivariate data analysis. A percent difference analysis was performed on 15 randomly chosen individuals to obtain an estimate of the amount of intra-observer error involved in measuring the specimens. In molecular study, because the recovered sequences of some old skins were highly degraded, the sequence sizes of individuals used for building phylogenetic trees were different.

4.3.1 Phylogenetic Relationships of Chinese *P. philippensis*

Following the taxonomic assignments of Corbet and Hill (1992), five forms of *P. philippensis*, including 57 specimens of *P. p. philippensis* (SW China), *P. p. lyei* (Thailand), *P. p. grandis* (Taiwan), *P. yunanensis*, and *P. hainana*, were selected in morphormetric analysis. The molecular analyses were based on the partial sequences (409 bp) of cytochrome b gene. One individual of *P. petaurista* from Borneo was used as an out-group.

4.3.1.1 Morphological data

Multivariate analyses revealed highly significant differences in five forms of *P. philippensis*. The results of discriminant analysis were given in Table 4.8. The first three axes accounted for 97% of the total variance. The forms in Thailand (*P. p. lyei*),

Taiwan (*P. p. grandis*), and Hainan (China, *P. hainana*) were distinguished as distinct groups without overlapping with other forms on discriminant function 1 (CAN I) (Figure 4.2). The populations from Yunnan, China, including *P. philippensis* and *P. yunanensis* shared similar morphological structures in skull and were clustered together. All characters except for the variable BCASEL (braincase length), PORCL (postorbital constriction), and LMTL (length of the maxillary tooth row of lower jaw) gave high contributions to CAN I. Inspection of the plot based on the discriminant function 1 and 3 revealed that *P. p. grandis* and *P. hainana* were still separated as distinct groups (Figure 4.3). BCASEL and MTRWL (Width of the maxillary tooth row) were the variables having the highest canonical scores on CAN II and CAN III, respectively.

The PCA results in the first three factors and the eigenvector scores of variables were presented in Table 4.9. Along the first principal component (PRIN I) that accounted for 80% of the original total sample variance, all eigenvector coefficients of variables were positive. The primary separation of taxa along this axis was among *P. p. lylei*, *P. p. grandis*, and *P. hainana*. The population of *P. philippensis* distributed in Yunnan was overlapped with *P. yunanensis* (Figure 4.4). The morphological variables mainly responsible for this segregation were CRANL and LMDL. In the scatter-plot of PRIN I against PRIN III, *P. p. grandis*, and *P. hainana* were separated as distinct groups once again, but *P. p. lylei*, *P. yunanensis* and *P. philippensis* in Yunnan shared similar cranial structures and were greatly overlapped (Figure 4.5). The variable BCASEL and LMDL were strongly correlated with PRIN II and PRIN III, respectively. The results of PCA were consistent with those of disrciminant function analysis.

Table 4.8 Discriminant function analysis of five *P. philippensis* forms

CAN	Eigenvalue	Proportion	Cumulative
I	15.94	0.72	0.72
II	4.37	0.20	0.92
III	1.14	0.05	0.97
Canonical score			
Variable	CAN I	CAN II	CAN III
CRANL	0.95	0.02	-0.11
BCASEL	0.54	0.59	0.03
CRANW	0.94	0.05	-0.17
BPORW	0.94	-0.05	0.03
PORCL	0.66	-0.08	-0.19
PGA	0.94	0.12	0.02
NAL	0.78	0.03	-0.07
TBL	0.70	0.40	0.07
DSL	0.85	-0.09	-0.01
MTRL	0.84	-0.18	0.18
MTRW	0.76	-0.22	0.31
LMDL	0.94	0.08	0.20
LMDH	0.84	-0.03	0.30
LMTL	0.65	-0.03	-0.02

Table 4.9 Principal components analysis of five *P. philippensis* forms

PRIN	Eigenvalue	Proportion	Cumulative
I	87.96	0.80	0.80
II	9.14	0.08	0.89
III	3.24	0.03	0.92
Eigenvector score			
Variables	PRIN I	PRIN II	PRIN II
CRANL	0.57	-0.06	-0.51
BCASEL	0.23	0.24	0.13
CRANW	0.26	-0.01	-0.13
BPORW	0.38	-0.10	-0.17
PORCL	0.08	-0.14	-0.05
PGA	0.24	-0.02	-0.05
NAL	0.17	-0.02	-0.22
TBL	0.10	0.00	0.03
DSL	0.13	-0.07	-0.06
MTRL	0.10	-0.04	0.09
MTRW	0.10	-0.02	0.01
LMDL	0.48	-0.26	0.75
LMDH	0.17	0.07	0.15
LMTL	0.11	0.00	0.14

4.3.1.2 Molecular data

Totally 12 samples of five forms of *P. philippensis* were examined to reconstruct the phylogenetic relationships. *P. petaurista* from southeastern Asia was used as an outgroup. Table 4.10 showed the sequences differences that were corrected by Kimura's

two-parameter model (1980), and the numbers of transversions and transitions obtained from pairwise comparison between samples.

Table 4.10 Pairwise comparison based on the partial sequences (409 bp) of cytochrome b gene between five *P. philippensis* forms. Data below the diagonal are the numbers of nucleotide substitutions, transitions vs. transversions. Data above the diagonal represent the genetic differences between samples. The samples were defined in Table 4.6 and 4.7.

	PPH1	PPH2	PPH3	PPH4	PHK1	PHK2	PYK1	PYK2	PYK3	PYK4	PYK5	PPG	PPB
PPH1		1.3	1.7	1.5	5.6	4.7	8.3	8.6	8.3	8.3	8.3	12.5	5.7
PPH2	4/1		1.0	1.2	5.9	3.9	9.3	9.4	9.0	9.0	9.0	11.4	6.4
PPH3	5/1	4/0		0	7.1	5.1	9.7	9.7	9.3	9.3	9.3	11.7	6.8
PPH4	4/0	4/0	0		6.6	4.8	9.4	9.4	9.1	9.1	9.1	11.3	6.8
PHK1	21/1	20/2	25/2	25/2		2.2	10.0	9.6	9.9	10.0	10.0	12.9	8.0
PHK2	19/0	15/0	20/0	19/0	5/1		9.4	8.6	9.0	9.0	9.0	12.9	6.3
PYK1	31/3	33/4	35/4	35/3	35/4	35/3		0.5	0.5	0.5	0.5	13.8	8.6
PYK2	31/4	34/4	35/4	35/3	33/6	35/3	1/1		0.2	0.3	0.2	14.1	8.9
PYK3	30/4	32/4	33/4	33/3	34/6	33/3	1/1	1/0		0	0	13.8	8.3
PYK4	30/4	32/4	33/4	33/3	34/6	33/3	1/1	1/0	0		0	13.8	8.6
PYK5	30/4	32/4	33/4	33/3	34/6	33/3	1/1	1/0	0	0		13.8	8.3
PPG	42/8	37/7	37/8	37/8	43/8	43/8	46/9	46/10	45/10	46/10	45/10		12.1
PPB	23/0	21/2	25/2	27/0	30/2	24/0	32/2	32/2	31/3	30/3	31/3	41/8	

The considerable sequence variations existed among all forms. The form from Taiwan (*P. p. grandis*) was significantly different from the forms in mainland with high genetic differences (11.3 – 14.1%). The form *P. yunanensis* apparently differed from *P. philippensis* and *P. hainana* with 8.3 – 10% differences in sequence. The genetic difference between *P. philippensis* and *P. hainana* was about 5%. The phylogenetic reconstructions using maximum parsimony (MP) and neighbor-joining distance (NJ)

methods resulted in essentially the same branching patterns. Each of five forms was formed as distinct clade (Figure 4.6 and Figure 4.7). The bootstrap values to support these branching orders were high, ranging from 87% to 100%.

4.3.2 Phylogenetic Relationship between *P. xanthotis* and *P. leucogenys*

A partial sequence (409 bp) of cytochrome b gene of *P. xanthotis* was successfully sequenced from two museum specimens. The sequence data of Japanese *P. leucogenys* were retrieved from GenBank of NCBI.

Table 4.11 Percentage of genetic differences between *P. xanthotis* and other giant flying squirrels based on pairwise comparison of the partial cytochrome b sequences (409 bp). See Table 4.6 and Table 4.7 for sample information.

	PTK1	PTK2	PLL1	PLL2	PLN1	PLN2	PYK	PPH	PHK	PPB
PTK1		3.8	14.1	12.9	13.2	13.2	14.7	15.5	13.4	14.3
PTK2	11/3		11.7	10.2	9.2	9.2	11.94	11.57	12.7	12.5
PLL1	41/16	34/12		1.3	2.8	2.1	15.4	14.4	15.1	16.0
PLL2	37/15	27/12	4/1		2.6	1.8	14.9	13.6	14.4	15.0
PLN1	37/16	24/12	11/0	9/1		1.0	14.6	13.1	13.5	14.3
PLN2	37/16	24/12	8/0	6/1	3/0		14.3	13.3	14.1	14.3
PYK	46/13	40/8	51/11	47/13	47/11	46/11		8.6	9.6	8.9
PPH	52/10	38/8	49/9	46/8	43/9	44/9	31/4		5.6	5.7
PHK	43/9	41/8	50/11	48/10	43/11	46/11	33/6	21/1		8.1
PPB	47/9	43/7	52/8	50/7	46/8	46/8	32/3	23/0	26/1	

Note: Data below the diagonal are the numbers of nucleotide substitutions, transitions vs. transversions. Data above the diagonal represent the genetic differences between samples.

For the sake of comparison, *P. philippensis*, *P. yunanensis*, and *P. hainana* were used to build their phylogenetic relationships with *P. petaurista* distributed in southeastern Asia as an outgroup. Table 4.11 presented the percentage differences that

were corrected by Kimura's two-parameter model (1980), and the transversional and transitional numbers between samples using pairwise comparison. *P. xanthotis* significantly differentiated from other *Petaurista* forms for their highly genetic differences, varying from 9.2% to 15.5%. The phylogenetic trees generated with MP and NJ methods were concordant with the results of pairwise comparison. Compared to *P. philippensis*, *P. yunanensis*, and *P. hainana*, there was a closed relationship between *P. xanthotis* and *P. leucogenys*, although each of them formed as a distinct clade. (Figure 4.8 and Figure 4.9). The bootstrap value in MP tree for branching was 99%.

4.3.3 Phylogenetic Relationship of *P. petaurista*

The morphological study of *P. p. albiventer* between the eastern and the western trans-Himalayan populations was based on 41 specimens from N Pakistan and W Yunnan, China (Table 4.4). The populations from India, E and W Sumatra, Borneo, Java, and Malayan Peninsula (49 specimens) were included to investigate the morphological relationships among populations of *P. petaurista.* Table 4.12 showed the results of discriminant function analysis on the first three functions, which were outlined graphically in Figure 4.10 and Figure 4.11. The first discriminant function that accounted for 68% of the total variance separated the population in W Yunnan from the rest groups (Figure 4.10). CRANW, BPORW, and LMDL were the major variables contributing to CAN I. The morphological variables contributing to CAN II included LMTL, LMDH, and BCASEL. In the plot of CAN I to CAN III (Figure 4.11), the populations distributed in Yunnan (SW China) and Burma were distinguished as different assemblages. The rest populations were overlapped as a complicated assemblage, although the overlap was little between the populations in Java and in Pakistan. BCASEL was the dominant variable on CAN III.

Table 4.12 Discriminant function analysis of *P. petaurista*

CAN	Eigenvalue	Proportion	Cumulative
I	20.43	0.68	0.68
II	5.42	0.18	0.86
III	4.16	0.14	1.00
CANONICAL SCORE			
Variable	CAN I	CAN II	CAN III
CRANL	0.76	-0.44	0.38
BCASEL	0.40	-0.57	0.64
CRANW	0.81	-0.03	0.31
BPORW	0.85	-0.21	0.37
PORCL	0.32	0.45	0.25
PGA	0.68	-0.29	0.48
NAL	0.71	-0.54	0.09
TBL	0.02	-0.28	0.14
DSL	0.73	-0.04	0.51
MTRL	0.72	-0.38	0.17
MTRW	0.70	-0.59	0.06
LMDL	0.81	-0.13	-0.26
LMDH	0.68	-0.57	0.06
LMTL	0.64	-0.60	0.07

The results of principal components analysis of *P. p. albiventer* were presented in Table 4.13. The first principal component factor that accounted for 76% of the total variance isolated the W Yunnan and Burma populations from others, forming two distinct clusters (Figure 4.12). The main determinants were CRANL on PRIN I, and

BCASEL and LMDL on PRIN II. All specimens in the plot of the fist principal factor and the third principal factor were clustered as two groups (Figure 4.13). The first group consisted of the populations in Yunnan and Burma, and the second group included the remaining populations. The main variable contributing to the observed association was LMDL.

Table 4.13 Principal components analysis of *P. petaurista*

PRIN	Eigenvalue	Proportion	Cumulative
I	50.07	0.76	0.76
II	6.52	0.10	0.85
III	3.46	0.05	0.91
Eigenvector score			
Variables	PRIN I	PRIN II	PRIN III
CRANL	0.63	0.17	0.04
BCASEL	0.20	0.75	-0.17
CRANW	0.24	-0.16	0.37
BPORW	0.37	-0.16	0.38
PORCL	0.04	-0.06	0.38
PGA	0.24	0.15	0.15
NAL	0.24	-0.03	-0.22
TBL	0.03	0.09	-0.06
DSL	0.18	-0.02	0.33
MTRL	0.14	-0.03	-0.08
MTRW	0.14	-0.03	-0.12
LMDL	0.27	-0.56	-0.35
LMDH	0.28	-0.09	-0.43
LMTL	0.16	-0.01	-0.17

The molecular study of the populations of *P. p. albiventer* was conducted based on the partial sequences (375 bp) that were obtained from 4 museum skin samples. The sequence data of *P. p. petaurista* from Java and *P. p. melantous* from Malaya Peninsular were used to build the phylogenetic topology.

Table 4.14 Percentage of differences and the numbers of transversional and transitional substitutions between *P. petaurista* (*albiventer*) populations based on pairwise comparison of the partial sequence (375 bp) of cytochrome b gene.

	PPF1	FPPF2	PPY1	PPY2	PPB	PPM1	PPM2	PVO
PPF1		0.5	8.4	8.8	8.9	13.4	13.8	17.1
PPF2	2/0		8.3	8.6	8.9	13.3	13.8	17.7
PPY1	29/2	29/2		0.5	6.3	11.6	12.2	16.8
PPY2	32/2	32/2	3/0		6.3	11.6	12.2	16.8
PPB	32/1	32/2	24/0	24/1		11.5	11.5	15.9
PPM1	41/7	41/7	37/7	37/7	34/7		0.5	15.9
PPM2	44/7	44/7	39/8	39/8	34/7	2/0		15.9
PVO	43/19	46/19	37/21	39/20	37/20	37/20	37/20	

Note: Data above the diagonal were the percentage of genetic differences between samples and data below the diagonal were the numbers of transitions vs. transversions between samples. See Table 4.4 for sample abbreviations.

Table 4.14 was the percentage differences and the numbers of transversional and transitional substitutions on the pairwise comparison between samples. The population of *P. p. albiventer* in Pakistan was apparently different from that in Yunnan with the genetic distance, 8.6 - 8.9%. The population in Java showed similar genetic characters with the population in Yunnan, China. Both the MP and NJ trees generated the similar branching patterns (Figure 4.14 and Figure 4.15). The populations in Yunnan and in

Pakistan were separated as distinct branches with a high bootstrap value (98%). The population in Malaysia, *P. p. melantosus*, had an early divergence from other groups.

4.3.4 Phylogenetic Relationships of Chinese *Petaurista*

The morphometric analyses were performed on 65 specimens including 5 species, *P. alborufus*, *P. elegans*, *P. xanthotis*, *P. philippensis*, and *P. petaurista* (*albiventer*). The results of discriminant function analysis and principal components analysis were displayed in Table 4.15 and Table 4.16, respectively. The first discriminant function (CAN I) accounted for 60% of the original sample variance. Along CAN I, five *Petaurista* species were assembled as three clusters: *P. alborufus* was one group, *P. elegans* and *P. xanthotis* formed one group, and *P. philippensis* and *P. p. albiventer* formed the third group (Figure 4.16). This separation was attributed to the morphological variable PORCL, DSL, and LMDL on CAN I, and BCASEL on CAN II, which accounted for 32% of the total variance. On the graph plotted on CAN I to CANIII, *P. alborufus*, *P. elegans*, and *P. xanthotis* were successfully distinguished as distinct groups. The individuals of *P. philippensis* and *P. p. albiventer* were still widely overlapped, showing similar morphological structures (Figure 4.17). The major variables that contributed to CAN III were TBL and MTRW.

Table 4.15 Discriminant function analysis of Chinese *Petaurista*

CAN	Eigenvalue	Proportion	Cumulative
I	18.39	0.60	0.60
II	9.67	0.32	0.92
III	1.85	0.06	0.98
Canonical score			
Variable	CAN I	CAN II	CAN III
CRANL	0.54	0.78	0.01
BCASEL	-0.16	0.95	0.04
CRANW	0.58	0.74	-0.24
BPORW	0.47	0.83	0.06
PORCL	0.68	0.42	-0.07
PGA	0.55	0.70	-0.06
NAL	0.39	0.71	0.19
TBL	0.24	0.13	0.57
DSL	0.68	0.51	0.01
MTRL	0.42	0.77	0.24
MTRW	0.57	0.58	0.35
LMDL	0.68	0.60	0.14
LMDH	0.39	0.76	0.29
LMTL	0.29	0.55	0.26

In PCA, the first principal factor was the most important, which accounted for 80% of the total variable variance, with the cranial length (CRANL) having the highest eigenvector value (Table 4.16). Along PRIN I, all specimens of *Petaurista* were identified as three different groups (Figure 4.18), which were consistent with the result

of discriminant function analysis (see Figure 4.16). *P. alborufus* was firstly separated as a group, and the rest four made up other two groups: one group comprising *P. elegans* and *P. xanthotis*, and another group including *P. philippensis* and *P. petaurista*. BCASEL with negative eigenvector score was the dominant variable that contributed to PRIN II.

Table 4.16 Principal components analysis of Chinese *Petaurista*

PRIN	Eigenvalue	Proportion	Cumulative
I	85.29	0.80	0.80
II	9.63	0.09	0.89
III	2.73	0.03	0.91
Eigenvector score			
Variables	PRIN I	PRIN II	PRIN II
CRANL	0.52	0.13	-0.05
BCASEL	0.37	-0.87	-0.07
CRANW	0.27	0.14	-0.56
BPORW	0.41	0.05	-0.07
PORCL	0.10	0.18	-0.32
PGA	0.22	0.06	-0.19
NAL	0.17	-0.01	0.19
TBL	0.03	0.03	0.17
DSL	0.11	0.15	-0.07
MTRL	0.13	0.00	0.19
MTRW	0.15	0.12	0.21
LMDL	0.38	0.37	0.23
LMDH	0.22	-0.03	0.37
LMTL	0.12	0.00	0.44

When all specimens were plotted onto PRIN I and PRIN III, except for *P. petaurista* and *P. philippensis* that extensively overlapped, *P. alborufus, P. xanthotis,* and *P. elegans* could be identified as distinct groups, although there existed a few overlaps between each other (Figure 4.19). The most contributions to PRIN III were from the morphological variables CRANW and LMTL.

In molecular analyses, the partial sequences (380 bp) were isolated from 8 species (senso lato), including *P. alborufus, P. elegans, P. yunanensis, P. hainana, P. philippensis, P. xanthtis,* and *P. petaurista* (Zhang et al., 1997; Wang, 2002). The sequence data of *P. a. castaneus, P. a. lena,* and *P. l. leucogenys* (Oshida et al., 2000) were quoted from GenBank of NCBI. *Pteromys volans* was assigned as an outgroup to reconstruct the phylogeny. Table 4.17 showed the genetic results from pairwise comparisons between samples. *P. elegans* and *P. xanthotis* were the two most diverse species in *Petaurista*. The genetic differences between *P. elegans* and other *Petaurista* groups were 9.2% - 15.0%. *P. xanthtis* showed 12.8% - 15.4% differences from other groups. The population of *P. alborufus* in China (PAC) was apparently different from that in Japan (PAL) with sequence difference at 12.5% level. The phylogenetic reconstructions using UPGMA, MP and NJ methods yielded the similar topological patterns (Figure 4.20, Figure 4.21, and Figure 4.22). *P. xanthotis, P. elegans,* and the population of *P. alborufus* in Japan formed one group. The population of *P. petaurista* in Pakistan and *P. yunanensis* formed another group. And the rest were clustered together as the third group, including the population of *P. alborufus* in Yunna, *P. philippensis,* the population of *P. petaurista* in Yunnan, and *P. hainana*.

Table 4.17 Pairwise comparison of Chinese *Petaurista* based on the partial sequences (380 bp) of cytochrome b gene. Data above the diagonal were the percentage of genetic differences between samples, and data below the diagonal were the numbers of transitions vs. transversions between samples.

	PAK1	PAK2	PAK3	PAC	PHK	PPH	PPF	PYK	PPY	PEK1	PEK2	PEK3	PAL	PTK	PVO
PAK1		0.5	0.6	0.6	8.5	6.3	9.7	8.4	6.4	13.7	13.1	13.7	12.5	14.4	14.8
PAK2	2/0		0	0	8.2	5.7	9.7	8.4	6.1	13.2	12.8	13.1	12.5	13.8	14.7
PAK3	2/0	0		0	8.2	5.4	9.3	8.4	5.9	13.3	12.8	13.1	12.7	13.7	14.5
PAC	2/0	0	0		8.4	5.9	10.0	8.7	6.0	12.9	12.8	13.1	12.5	14.2	14.7
PHK	30/1	29/1	29/1	30/1		5.6	9.7	9.6	5.3	12.8	12.8	12.9	15.1	13.4	17.0
PPH	24/0	22/0	20/0	22/0	19/1		9.1	8.9	1.8	10.8	9.2	9.9	14.7	15.4	16.4
PPF	35/2	35/2	33/2	36/2	33/3	33/2		6.1	8.3	13.4	13.1	14.0	14.5	12.8	17.0
PYK	28/4	28/4	27/4	28/4	31/5	30/4	21/2		8.9	14.2	14.5	15.0	15.6	14.6	17.6
PPY	24/0	23/0	23/0	22/0	19/1	5/0	30/2	30/4		9.8	9.9	9.9	12.9	14.4	15.8
PEK1	44/8	42/8	42/8	40/8	40/8	33/8	41/10	42/12	30/8		4.5	4.4	16.1	14.1	18.1
PEK2	41/8	40/8	40/8	39/8	38/10	26/8	41/8	47/8	29/8	16/1		1.1	13.9	13.5	17.0
PEK3	44/7	41/8	41/8	39/8	37/9	30/7	45/7	49/7	29/8	14/2	4/0		14.5	15.1	17.2
PAL	38/8	38/8	39/9	37/8	46/10	47/8	43/11	48/10	44/5	48/13	38/12	42/12		14.1	15.8
PTK	44/10	42/10	41/9	43/10	40/9	48/10	38/10	43/12	47/8	41/12	41/9	46/10	38/15		19.3
PVO	33/22	33/22	32/22	33/22	40/24	39/22	43/20	46/20	38/23	43/25	42/21	42/21	38/20	50/23	

To estimate the divergence time between species and populations, the transversional substitutions at the third codon positions were obtained from pairwise comparison (Table 4.18). The divergence times were calculated using the rate of divergence for the third codon positions of mammalian cytochrome b gene of ca. 0.5% $*10^6$ (Table 4.19). *P. xanthotis* and *P. elegans* were the earliest species that diverged from *Petaurista*, approximately 11.2 to 13 million years ago. In *P. petaurista*, the divergence time between the population in Pakistan and the population in W Yunnan was about 1.2 – 3.2 million years ago.

Table 4.18 Transversional substitutions at the third codon positions of the partial sequences (375 bp) of cytochrome b gene in *Petaurista.*

	PAK	PYK	PPH	PHK	PEK	PTK	PPF	PPB	PPY	PVO
PAK		2.4	0	0	4.8	5.6	1.6	0	0	15.4
PYK	3		2.4	2.4	6.5	5.6	0.8	1.6	2.4	13.8
PPH	0	3		0	4.8	5.6	0.8	0	0	15.4
PHK	0	3	0		5.6	5.6	1.6	0	0	15.4
PEK	6	8	6	7		6.5	7.3	5.6	4.8	16.3
PTK	7	7	7	7	8		5.6	6.5	5.6	13.8
PPF	2	1	1	2	9	7		0.8	0.8	13
PPB	0	2	0	0	7	8	1		0	15.4
PPY	0	3	0	0	6	7	1	0		15.4
PVO	19	17	19	19	20	17	16	17	19	

Note: Data below the diagonal are the transversional numbers at the third codon positions. Data above the diagonal represent the transversional percentage difference between samples. The sample abbreviations were defined in Table 4.6.

Table 4.19 The estimated divergence time between species based on a divergence rate for the third codon positions of mammalian cytochrome b gene of ca. 0.5% $*10^6$ years

	PAK	PYK	PPH	PHK	PEK	PTK	PPF	PPB	PPY
PYK	4.8								
PPH	0	4.8							
PHK	0	4.8	0						
PEK	9.6	13	9.6	11.2					
PTK	11.2	11.2	11.2	11.2	13				
PPF	3.2	1.6	1.6	3.2	14.6	11.2			
PPB	0	3.2	0	0	11.2	13	1.6		
PPY	0	4.8	0	0	9.6	11.2	1.6	0	
PVO	30.8	27.6	30.8	30.8	32.6	27.6	26	30.8	30.8

4.4 Discussion

4.4.1 Phylogeny of the Trans-Himalayan *P. petaurista* (*albiventer*)

Petaurista petaurista is extensively distributed in southeastern China, Sichuan, Yunnan and Fukien. This species has a broad distribution beyond China including northern India, Bhutan, Nepal, Pakistan, northern Afghanistan, and southeastern Asia. The populations of *P. petaurista* in Pakistan and W Yunnan, China, are named as the same subspecies, *P. p. albiventer* (Corbet and Hill, 1992; Zhang et al., 1997) since their similar external and dental structures (Figure 4.23). Wang (2002) elevated the populations of *P. petaurista* in Pakistan and W Yunnan (China) as a valid species, *P. albiventer*. The present study based on the morphological and molecular analyses reveal that these two populations are significantly different. The principal components analysis indicates that their main differences are in skull size (The eigenvector scores are all positive on the first principal component factor) and the morphological structure of lower jaw, which apparently separate it from the rest populations (Figure 4.10 and Figure 4.12). When compared with other populations, the population in Pakistan shares more cranial characteristics with the populations from SE Asian rather than with *P. albiventer* in Yunnan, China. This can be inferred that these two populations may occupy different habitats that result in different adaptations. This inference agrees with Ellerman's (1940) study, which shows that the forms *candidula, barroni,* and *taylori* of *P. petaurista* in SE Asia are much similar to the Himalayan forms (*P. p. albiventer*).

The only species or subspecies of *Petaurista* distributed in Pakistan is the Himalayan giant flying squirrel, *P.* (*p.*) *albiventer*. The area of suitable forest where this giant flying squirrel can be found is comparatively limited. In Pakistan, it mainly occurs in Himalayan moist temperate forest, extending in the northwest of Pakistan into Deodar

(*Cedrus deodara*) forest or subtropical pine (*Pinus roxburghii*) zone, elevation from about 1,350 m to upper limit of the tree line at about 3,000 m (Roberts, 1997). *P. petaurista* is sympatrically distributed in N Pakistan with *E. cinereus* and *Eoglaucomys fimbriatus*. The competition for food resources among them is unavoidable. Since they have different morphological dental structures, the feeding strategies and food selection are different. The giant flying squirrel selectively feeds upon the young green leaves, the fir and pine cones, the nuts, even the young twigs and tree buds (*Quercus dilatata*), which are partially different from *Eupetaurus* and *Eoglaucomys* (Roberts, 1997). Himalayan moist temperate forest commonly consisting of the hill oak (*Quercus dilatata*), horse chestnuts (*Aesculus indica*), and walnuts (*Juglans regia*) supplies this species with sufficient food resource. Since partitioning of microhabitats among competing species is thought to contribute to coexistence among rodent species (Price, 1978), the different habitat selections of these three flying squirrels are a major contributor to this coexistence.

The similar sympatric distribution of *E. cinereus, P. petaurista,* and *Hylopetes alboniger* are also found in W Yunnan, China. *P. p. albiventer* in W Yunnan is widely overlapped with *H. alboniger* from NW Yunnan toward southern Yunnan at different elevations (500m to 3500m) (Zhang et al., 1997). These areas have typically high diversity of plant species that correspond with the mixed dietary habits of these flying squirrels. According to local people, both flying squirrels mainly inhabit in coniferous forest. *P. petaurista* frequently feeds on walnuts, acorns, and corns, and occasionally descends into farm to feed on corn at dawn. This is a little different from the population of Pakistan, indicating that the Pakistan giant flying squirrel is more adaptable to harsh

or less mesic conditions than the giant flying squirrel in W Yunnan. Their morphological differentiations, such as skin (Figure 4.23), are apparently associated with their different living conditions.

The molecular data are partially consistent with the morphological data. The difference in cytochrome b gene between the eastern and the western trans-Himalayan giant flying squirrel (*P. petaurista*) is significant (Figure 4.13 and Figure 4.14). The genetic distance is about 8.9%, above the subspecies-level (Table 4.14). Geologic history has been a major factor in understanding evolution of flying squirrels. The geotectonic and paleoclimatic records reveal a series of episodic landscape transformations throughout the past millions of years coincident with changes in taxa and ecological diversity. Based on the rate at the third codon positions of cytochrome b gene (Table 4.18), the Pakistan population of *P. petaurista* diverged from the Yunnan population about 1.6 million years ago (Table 4.19). The distinct phylogeographic discontinuity between the eastern and the western lineages of *P. petaurista* suggests a major environmental impediment to gene flow. The possibility that the coincident pattern between these two populations were caused by a shared historical dispersal event is supported by a diverse array of vertebrate taxa that exhibit a similar genetic discontinuity in the trans-Himalayas (Woods, personal communication). The populations of *P. petaurista* delineated by large phylogenetic gaps are obviously associated with the biogeographic barrier of the great Himalayan chain to gene flow.

The climatic changes could lead to an expansion of the Palearctic fauna at the expense of the Oriental fauna, though some generalized species or population of Oriental forms would undoubtedly be able to adapt to the new conditions. The results of the

present study show that the genetic differences among the populations in SW China, SE Asia, and Pakistan are not closely associated with the geographic distances of sampling localities. This implies that *P. petaurista* rapidly extended into SW China, Pakistan, and SE Asia in a short time during the southward expansion of temperate forests during the glacial stage of Pleistocene. The estimations of the third codon positions of cytochrome b sequences indicate that all populations of *P. petaurista* diverged from each other during Pleistocene and Holocene. When all populations split from their common ancestor in Pleistocene, one branch migrated to the western side what is now in Pakistan where the climate in northern part and indeed the whole Indus plain, was evidently warmer and more humid in early Pleistocene times (Roberts, 1997). One moved to the eastern side what is now in SW China, and the third branch migrated to SE Asia. During the subsequent glaciation in the late Pleistocene and Holocene that was intervened with shorter periods of warmer moister climatic change, all populations gradually adapted to their present habitats. The similar cranial structures among the populations in SE Asia are apparently the adaptations of similar living conditions.

P. petaurista is a polymorphic species and is extensively distributed in various geographical locations. Like many wide-ranging taxa, it is divided into separate species or subspecies by zones of hybridization (Bull, 1991). Because hybrid zones involve closely-related taxa at various stages of speciation, they represent natural settings for study of speciation, gene flow, adaptation, and reinforcement of isolating mechanisms (Baker et al., 1989; Harrison, 1990, 1993; Bendict, 1999). Therefore, the further study of *P. petaurista* should focus on the populations between SW China and SE Asia, and

between the eastern and the western trans-Himalayas, such as the populations in Burma, India, Thailand, and Laos.

4.4.2 Taxonomic Status of *P. philippensis, P. yunanensis,* and *P. hainana*

P. hainana (Hainan giant flying squirrel) in Hainan, *P. yunanensis* (Yunnan flying squirrel) in Yunnan, and *P. grandis* (Taiwan giant flying squirrel) in Taiwan were referenced as valid species by Allen (1940) and Ellerman (1940), but Hoffmann et al. (1992) and Nowak (1999) regarded them as the subspecies or synonyms of *P. philippensis* (gray-backed giant flying squirrel). By checking the collections in Beijing and Yunnan, China, Zhang et al. (1997) and Wang (2002) elevate them as distinct species recently. Taiwan is an island situated on the contnental slope of mainland China and separated by the 150 km strait. It is known to have been connected by a landbridge to mainland China several times during the Pleistocene. In the last warm glacial period (10, 000 to 56, 000 years ago), sea levels decreased by 80 to 150m (Lin, 1966). This indicates that *P. grandis* migrated to Taiwan from south China during Pleistocene. The findings from both molecular and morphological data in this study support *P. p. grandis* to be a distinct species (Figure 4.2 and Figure 4.4), which are consistent with Oshida's et al. (2000a) result and suggest *P. grandis* to be a valid species.

The molecular and morphometric analyses on the remaining populations show some controversial results. The multivariate analyses indicate that *P. hainana* apparently differs from the rest populations in cranial morphology and *P. philippensis* in Yunnan is morphologically similar to *P. yunanensis* (Figure 4.2 to Figure 4.5). Whereas the molecular data suggest that *P. hainana* is genetically close to *P. philippensis* with short genetic distance (~ 4 - 5%). *P. yunanensis* is evidently distinguishable from *P.*

hainana and *P. philippensis* with genetic differences about 10.9% and 8.6%, respectively (Table 4.10).

Yunnan flying squirrel *P. yunanensis* is a very handsome maroon flying squirrel with white speckling over the back (Figure 4.24). The type specimen is a skin without a corresponding skull. It was collected from Momein (=Tengyueh), southwestern Yunnan, China, and is represented in the Indian Museum, Calcutta with a catalog number 9486. *P. yunanensis* resembles *P. alborufus* in size, but is morphologically similar to the population of *P. philippensis* in Yunnan in the general deep bay coloring (Figure 4.24). Both skulls are large but apparently show no special peculiarities. The distribution of *P. yunanensis* is from the extreme southwestern Yunnan probably into Burma and Indochina, extensively sympatric with *P. philippensis* in SW China (Zhang et al., 1997). Although at present it is not clear to their feeding habits, their morphological similarity in skull is clearly due to the adaptations to similar habitats. The similar morphological characters and the distinct genetic characters between them suggest that conservative systematic traditions or morphological stasis may be involved. According to the estimated divergence time, *P. yunanensis* diverged from the stock of *P. philippensis* about 4.8 million years ago, the early Pliocene. There are known to have been three major periods of successive prolonged glaciation when sea levels sank and huge ice caps developed over all the great mountain massifs during Pliocene, which dramatically affected the climate, biogeographic structures, and flauristic environments along the great Himalayan mountain chain. As a result, the eastern and the western extremes of Himalayas became the optimal shelter of refugees. A quite unique mammalian assemblage including flying squirrels migrates and survives today in the

western side of Yunnan of China. Although the range of *P. yunanensis* is overlapped with *P. philippensis* in Yunnan, there is no indication that the two interbreed. The significant differentiation in genetic characters suggest that *P. yunanensis* is a distinct species, notwithstanding the general similarity in size and body coloration with *P. philippensis*.

The type specimen of *P. hainana* is an adult female skin with the corresponding skull, which was collected from Namfong, the island of Hainan, China, on February 19, 1923, by Clifford H. Pope (Allen, 1940). It is now represented in the collection of American Museum of Natural history. *P. hainana* is a tropical species that reaches the northern limit of its range in extreme southern China. Allen (1940) predicted that no doubt Hainan giant flying squirrel (*P. hainana*) would be found to show relationship to some forms of the Indo-Chinese mainland. The present study demonstrates that *P. hainana* is phylogenetically related to *P. philippensis* in Yunnan. *P. philippensis* is distributed in mountainous coniferous forests at different elevations in W Yunnan; whereas *P. hainana* is confined to tropical forest on Hainan. Either the feeding habits or the living habitats are significantly different. The morphological difference between them is probably associated with their geographical variations and living conditions. But the close genetic distance between *P. hainana* and *P. philippensis* 5 – 7%, is not consistent with their geographic distances of sampling localities. The conflicting placement on the molecular and morphological trees is difficult to reconcile because the phylogenetic hypothesis suggested by the molecular tree requires convergent evolution in morphology and osteology to reflect similarities in these characters among the clades (Austin, 1996). The estimation based on the third codon positions of cytochrome b gene

shows that the divergence of *P. hainana* from *P. philippensis* is very recent. The reasonable explanation is that the population of *P. hainana* rapidly extended to its present distribution in a short time during the southward expansion of temperate forests in southern China during the last glacial stage of Pleistocene because the history of the island of Hainan is about 1 million years. The estimate of the times separating sequences in subspecies within different groups is ranging from 0.94 to 1.52 million years based on a rate estimate of six *Sciurus aberti* subspecies (Wettstein et al., 1995). Accepting this hypothesis, *P. hainana* is a subspecies or a synonym of *P. philippensis*.

4.4.3 Phylogenetic Relationship between *P. xanthotis* and *P. leucogenys*

Petaurista xanthotis is an endemic species in China. It is a large yellowish-gray species with an orange spot behind the ear. Muzzle, forehead, and cheeks shorter-furred than the neck and body, minutely mixed black and white, giving a gray appearance. It has been accepted as a valid species of *Petaurista* (Corbet and Hill, 1992; Nowak, 1999; Zhang et al., 1997; Wang, 2002). The type specimen of *P. xanthotis* is a mounted skin in the "galerie publique" of the Museum of Natural History at Paris, sent by Pere Armand David from Tibet, near Muping, Sichuan, China (Ellerman, 1940). *P.* (*Pteromys*) *xanthotis* was originally named by Milne-Edwards in the belief that it was, if not a distinct species, at least a variety of *Pteromys melanopterus* (Ellerman, 1940). Buechner (1892) verified that they were not the same animal, but undoubtedly represented *P. xanthotis*.

In China, *P. xanthotis* inhabits from Tsing Hai and Kansu, southeastward to Hubei and southward to Sichuna and Yunnan, China, at different elevations, ranging from 2,000 meters in Gansu to 3,300 meters in Yunnan. The major habitat in NW China is temperate forest. Due to its widely distribution, *P. xanthotis* shows morphological

variations. The population in Yunnan measures slightly less than those from Gansu and Tsing Hai, but the difference is not great and does not warrant their separation as a distinct form. Because of the semi-hypsodont molariform teeth, *P. xanthotis* had been considered as the closest relative of *Eupetaurus* (McKenna, 1962). But the molecular data suggest that the similarity of cheekteeth between *Eupetaurus* and *P. xanthotis* might be the convergent adaptation to the similar food selection (See Chapter 3 for detail).

In the historical classification, *P. xanthotis* was included in the Japanese *P. leucogenys* (Ellerman and Morrison-Scott, 1951). The molecular data in present study reveal a relative close phylogenetic relation between them. But their dental structures are very distinctive, and the cheekteeth of *P. xanthotis* is more complex than *P. leucogenys* and other *Petaurista* forms. *P. leucogenys* inhibits temperate forests in Japan (Nowak, 1999), which is similar to the habitat of *P. xanthotis* in NW China. According to pelage characteristics, *P. leucogenys* was classified into three subspecies, *P. l. leucogenys, P. l. nikkonis,* and *P. l. oreas* (Imaizumi, 1960). Although *P. xanthotis* is relatively close to *P. leucogenys* (Figure 4.8 and Figure 4.9), they are indeed genetically different with genetic distances about 9.2 to 14.1% (Table 4.11), suggesting two full species. The islands of Japan are separated from Korean penisula by the Korean Strait, which has a water depth of about 100 m (Kim et al., 1991). During the Pleistocene glacial periods, a landbridge was present between Korea and Japan, and the southern end of the Ryukyu Archipelago was connected with Taiwan in the early Pleistocene (Hikida and Ota, 1997). Based on available fossil records, Oshida et al. (2000c) thought that *P. leucogenys* migrated from southern China to Japan through the land bridge that was formed around the area of the present East China Sea in the early

middle Pleistocene. If these biogeographic hypotheses are correct, with the estimated divergence time (Table 4.19), *P. xanthotis* diverged from *P. leucogenys* and other *Petaurista* forms about 10 to 12 million yeas ago, the middle Miocene, much before the migration of *P. leucogenys* to Japan. The present distributions of *P. xanthotis* and *P. leucogenys* are apparently due to the geographic events of the Pliocene-Pleistocene period. During glacial stages in Pleistocene, they moved to southward shifting of temperate forests in both China and Japan.

4.4.4 Systematics of Chinese *Petaurista*

The eastern trans-Himalayan giant flying squirrels at species-level include *P. petaurista, P. philippensis, P. yunanensis, P. alborufus, P. elegans,* and *P. xanthotis.* With *Pteromys volans* as the outgroup, all *Petaurista* forms are genetically related to each other.

The red and white flying squirrel, *P. alborufus,* is distributed in somewhat more northern from Sichuan and Hupei to the mountains of the Likiang Range (Zhang et al., 1997; Wang, 2002) (Figure 4.1). The skull is of nearly maximum size for the genus and is heavily formed with the stout, triangular postorbital processes. The teeth are relatively simple in structure. This species includes three races in mainland China: the typical form of the mountains in southern Muping of Yunnan, with red feet; a more eastern race of Hupei with black feet and little whitish below; and a third form in Yunnan with the pale dorsal area and black feet (Thomas, 1923). *P. alborufus* is confined to forests of the central and eastern parts of Sichuan. Although *P. yunanensis* shares similar external structure and pelage coloration with *P. alborufus,* the two appear to be distinct species with allopatric distributions. The morphometric analysis on the cranial structure reveals that *P. alborufus* is significantly different from the rest

Petaurista groups (Figure 4.16 and Figure 4.18). The molecular data are not fully coincident with the morphological inference, however (Figure 4. 20 to Figure 4.22). *P. alborufus* in mainland is genetically related to *P. petaurista, P. philippensis*, and *P. yunanensis*. Whereas the population of *P. alborufus* in Taiwan is significantly different from other *Petaurista* groups, showing an early divergence (Figure 4.21 and Figure 4.22). Oshida's et al. (2000a) study suggests that the population of *P. alborufus* in Taiwan could be a distinct species because of its early deviation from *P. petaurista* and *P. philippensis*. With the geohistorical and biogeographical evidences (Lin, 1966), the population of *P. alborufus* might evolved independently from other *P. petaurista* species in the late Miocene and migrated to Taiwan island adapting itself to the alpine habitat. If this assumption is reasonable, *P. alborufus* in mainland might diverge from the lineage of *P. petaurista* and *P. philipensis* very recently, during the late Pleistocene or Holocene, and adapt to different living habitat.

Petaurista elegans is distributed in Sichuan and Yunnan, China (Hoffmann et al., 1992). Two forms used to be included in this species, *P. e. clarikei* with ochraceous rufous feet, and *P. e. punctatus* (or *marica*) with blackish head. Both forms were also treated as distinct species by some authorities (Allen, 1940; Ellerman, 1940; Zhang et al., 1997; Wang, 2002). The morphological data of this study shows that *P. elegans* is more similar to *P. xanthotis* rather than other giant flying squirrels (Figure 4.16 to Figure 4.19), which is consistent with their geographical distributions (Figure 4.1). In Yunnan and eastern Sichuan, the range of *P. xanthotis* is overlapped with that of *P. elegans*, showing sympatric distribution. But, *P. xanthotis* is not genetically related to

P. elegans for their highly different genetic characteristics. Both of them diverged from the ancestral stock of *Petaurista* in the middle Miocene, 11 to 14 million years ago.

Paleontological records can usually provide accurate morphological and genetic change information on the amazing diversity of life that existed in the past (Smith, 1998). The available fossil record suggests that there were multiple lineages of flying squirrels during Miocene (James, 1963; Mein, 1970). Several molecular studies have supported the general hypothesis of latest Pleistocene southward depression, followed by postglacial northward expansion of ranges in Palearctic and Nearctic taxa (Cooper et al., 1995). The further support is from the fossil evidence of similar distinctive dentition that was found in an Oligocene example (Bruijn and Uney, 1989). The fossil species, *Pteromys lopingensis*, which was discovered in Loping, Jiangxi, China and described by Young (1947), is considered from Pleistocene and referable to *Petaurista* or *Trogopterus*, proving that *Petaurisa* stock had distributed in China before Pleistocene.

Integrating the fossil records and the present distributions of these *Petaurista* forms, their biogeographic history can be interpreted with the following hypothesis. The radiation of *Petaurista* occurred in the Eurasian continent (Oshida et al., 2000a). After diverged from the ancestral *Petaurista* in the middle Miocene, both *P. xanthotis* and *P. elegans*, maybe the group of *P. alborufus* in Taiwan, and the ancestral stock of *Petaurisa* were restricted to the southern parts of the Eurasian continent. During the subsequent three major periods of successive prolonged glaciation, *P. xanthotis* had adapted to the cold environment and high elevation, and survived with special feeding habit. With the retreating of glacier and the further uprising of the Himalayas, *P. xanthotis* was expanded northward, where it inhabited temperate forests with mountainous coniferous

habitat; the Taiwanese *P. alborufus* migrated toward east; and the ancestor of P. *petaurista* and *P. elegans* remained in the south. During small upheavals or movements of tectonic plates that created the Himalayan foothill in the late Pliocene or Pleistocene, the other *Petaurista* forms, such as *P. yunanensis, P. petaurista,* and the mainland form of *P. alborufus*, explosively diverged. Within the successive periods of glaciation in the late Pleistocene, the intervening shorter periods of warmer, moister climatic changes forced them to dispersal to the present geographical ranges. These multiple dispersal events may have acted to increase haplotypic diversity within these eastern *Petaurista* groups.

On the other hand, phylogenetic analyses using both molecular and morphological information raise some controversial issues particular to the comparison of these data (Patterson, 1988, 1999). In some forms, the genetic results are not concordant with their geographic distances of sampling localities. For example, the population of *P. petaurista* in Pakistan is close to *P. yunanensis* rather than the population in Yunnan, which is relatively close to *P. philippensis* (Figure 4.20 and Figure 4.21). The similar conflict between morphological data and molecular data is also found in other mammals, such as Hugot's (1998) research on neotropical monkeys, in which the results based on morphological or molecular data were generally conflicting and the phylogeny of the group is debated. Riddle (1996) thinks that populations not clearly delimited by large phylogenetic gaps are genetically connected through ongoing or recent dispersal, and populations delineated by large phylogenetic gaps are usually associated with stable biogeographic barriers to gene flow. It is inferred that the Chinese *P. petaurista, P. philippensis, P. yunanensis*, and *P. alborufus* rapidly

extended its present distributions in a short time during the last glacial stage of Pleistocene.

4.5 Summary

In this chapter, the phylogenetic relationships among *Petaurista* groups in Pakistan and SW China were investigated. The following results are concluded based on the morphometric and molecular data:

1. The population of *P. petaurista* in Pakistan is significantly different from the population in W Yunnan of China in morphology and genetics. It is probably a new species, at least a new subspecies.

2. *P. yunanensis* is genetically different from the population of *P. philippensis* in Yunnan and *P. hainana*, and might be a valid species. *P. hainana* might be a subspecies or synonym of *P. philippensis* for their closed genetic relationship. The morphological similarity between *P. yunanensis* and *P. philippensis* is an adaptation to similar habitat.

3. *P. xanthotis* is a valid Chinese endemic species and morphological similar to *P. elegans* but genetically different. *P. xanthotis* shows a close phylogenetic relationship with *P. leucogenys* which is distributed in Japan and China.

4. Both *P. xanthotis* and *P. elegans* diverged from the ancestral stock of *Petaurista* in the middle Miocene. The remaining populations of *Petaurista* rapidly extended its present distribution in a short time during the last glacial stage of Pleistocene.

5. The controversial results from mophometric and molecular analyses in some forms can be interpreted with the Riddle's (1996) biogeographic theory. Populations not clearly delimited by large phylogenetic gaps are genetically connected through ongoing or recent dispersal and populations delineated by large phylogenetic gaps are usually associated with stable biogeographic barriers to gene flow.

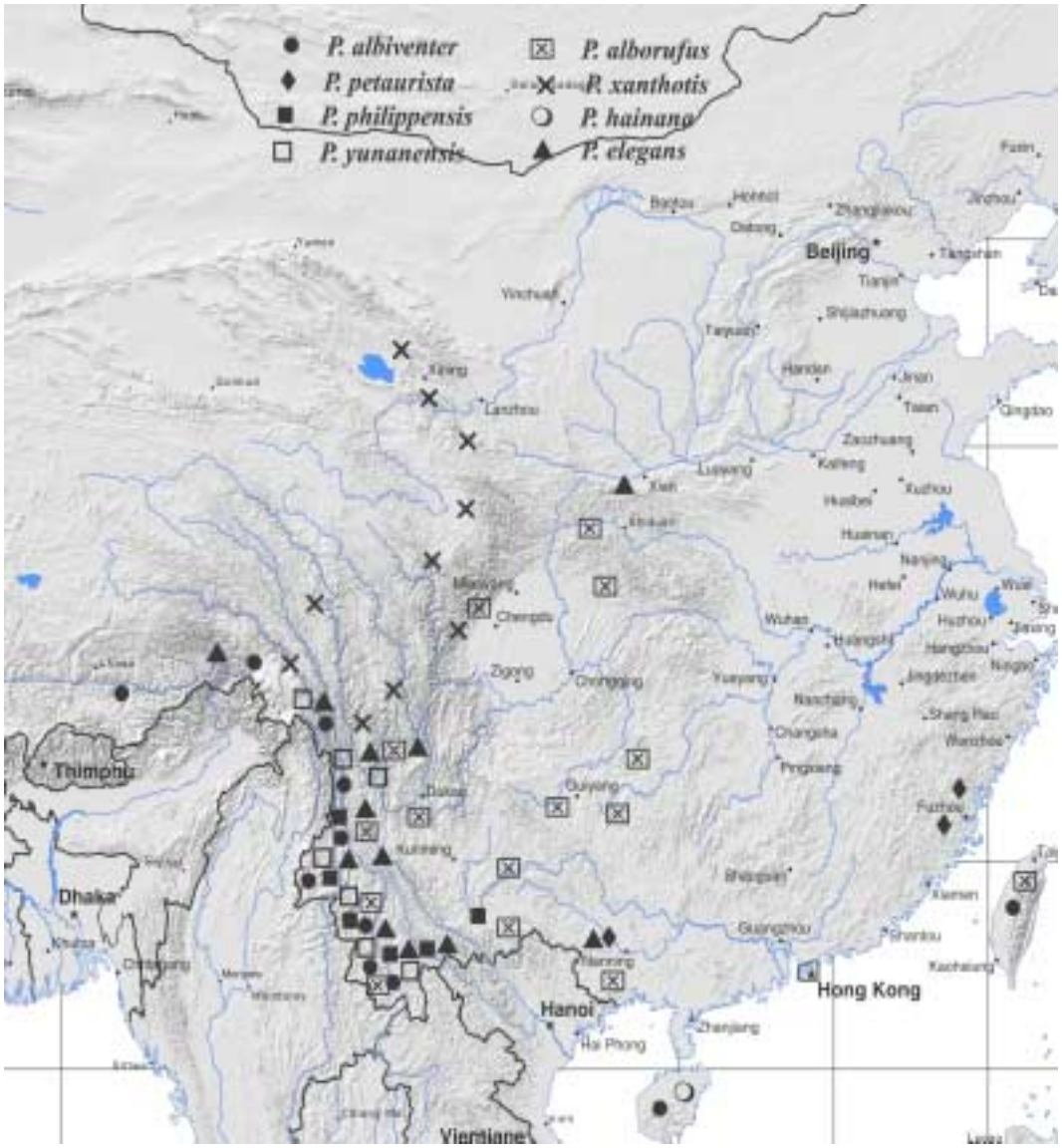

Figure 4.1 Chinese giant flying squirrels (*Petaurista*)

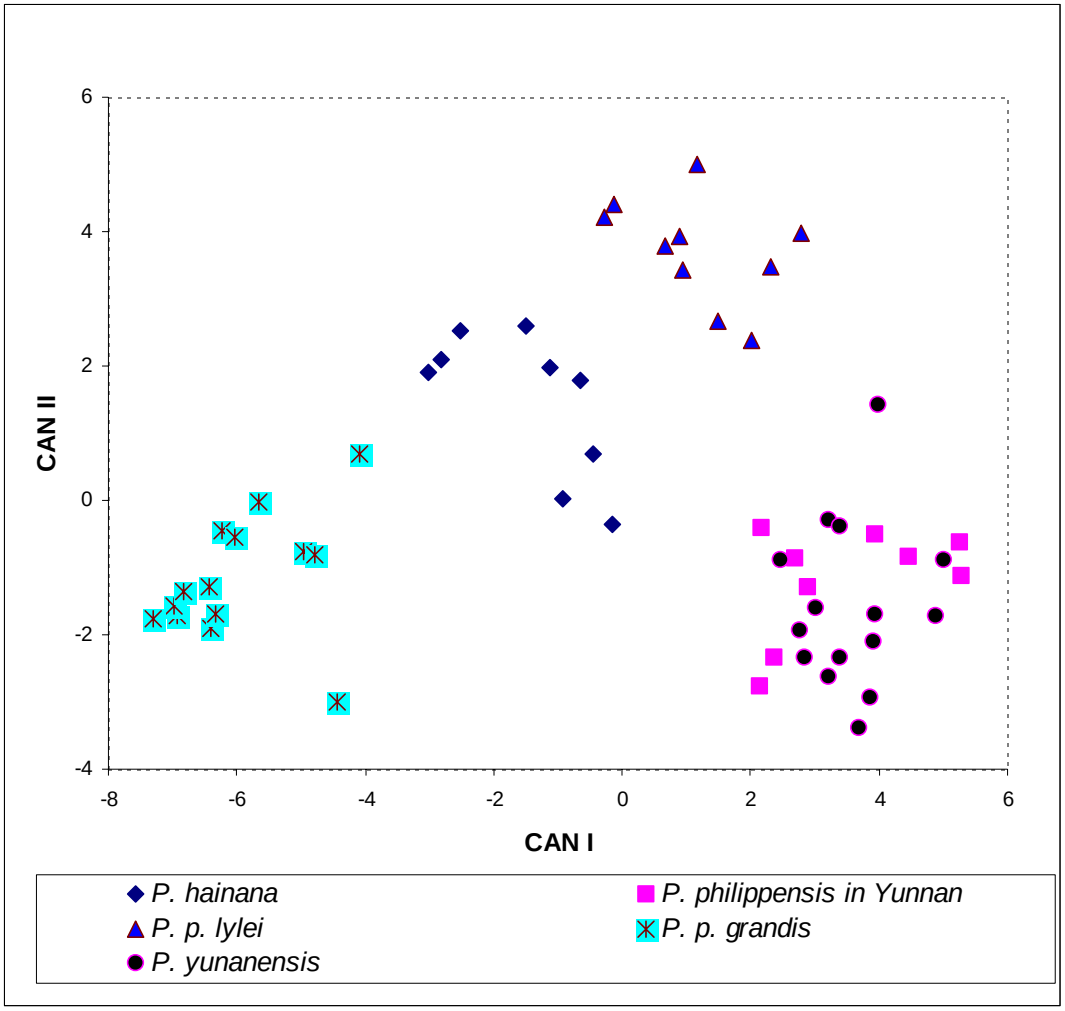

Figure 4.2 Plot of five *P. philippensis* forms onto discriminant function 1 (CAN I) and function 2 (CAN II)

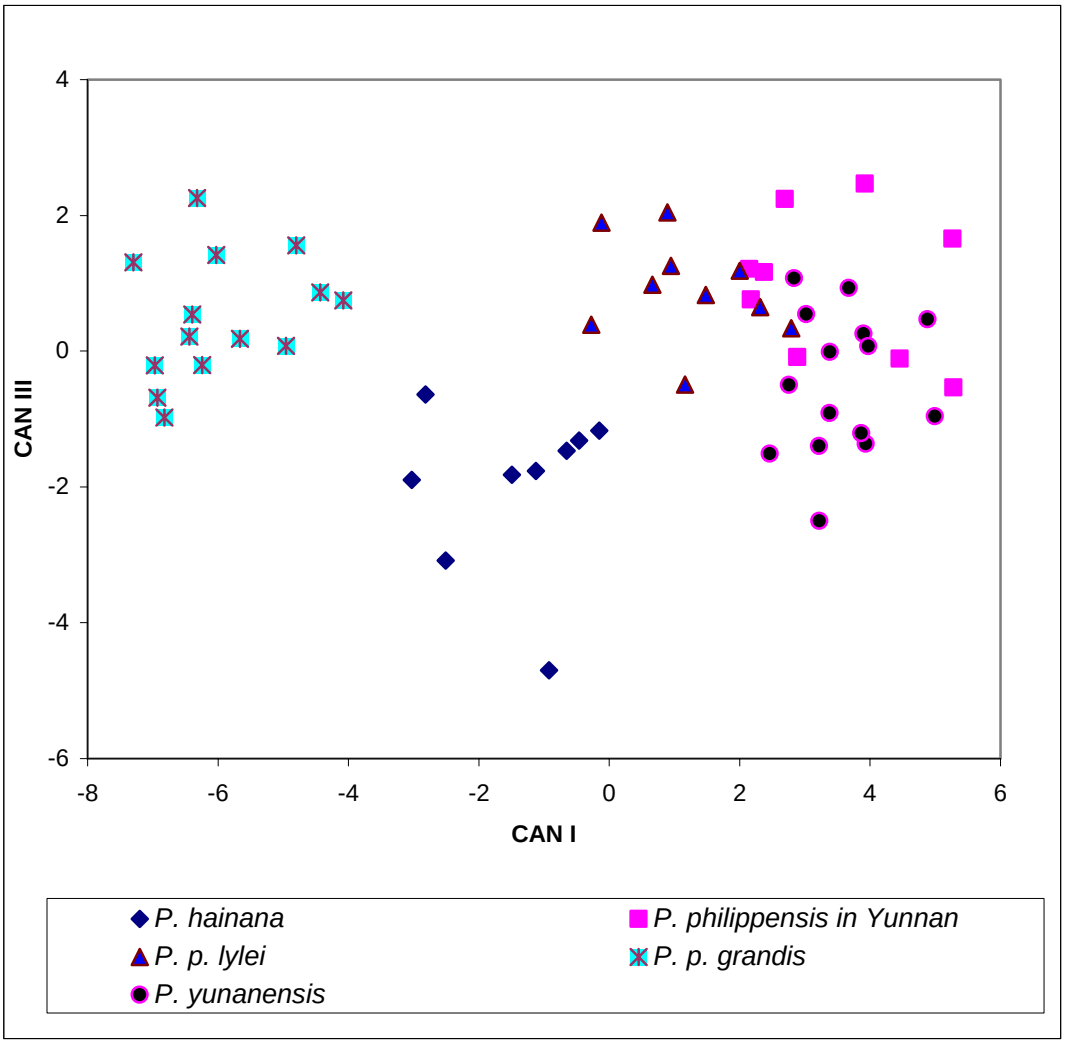

Figure 4.3 Plot of five *P. philippensis* forms onto discriminant function 1 (CAN I) and function 3 (CAN III)

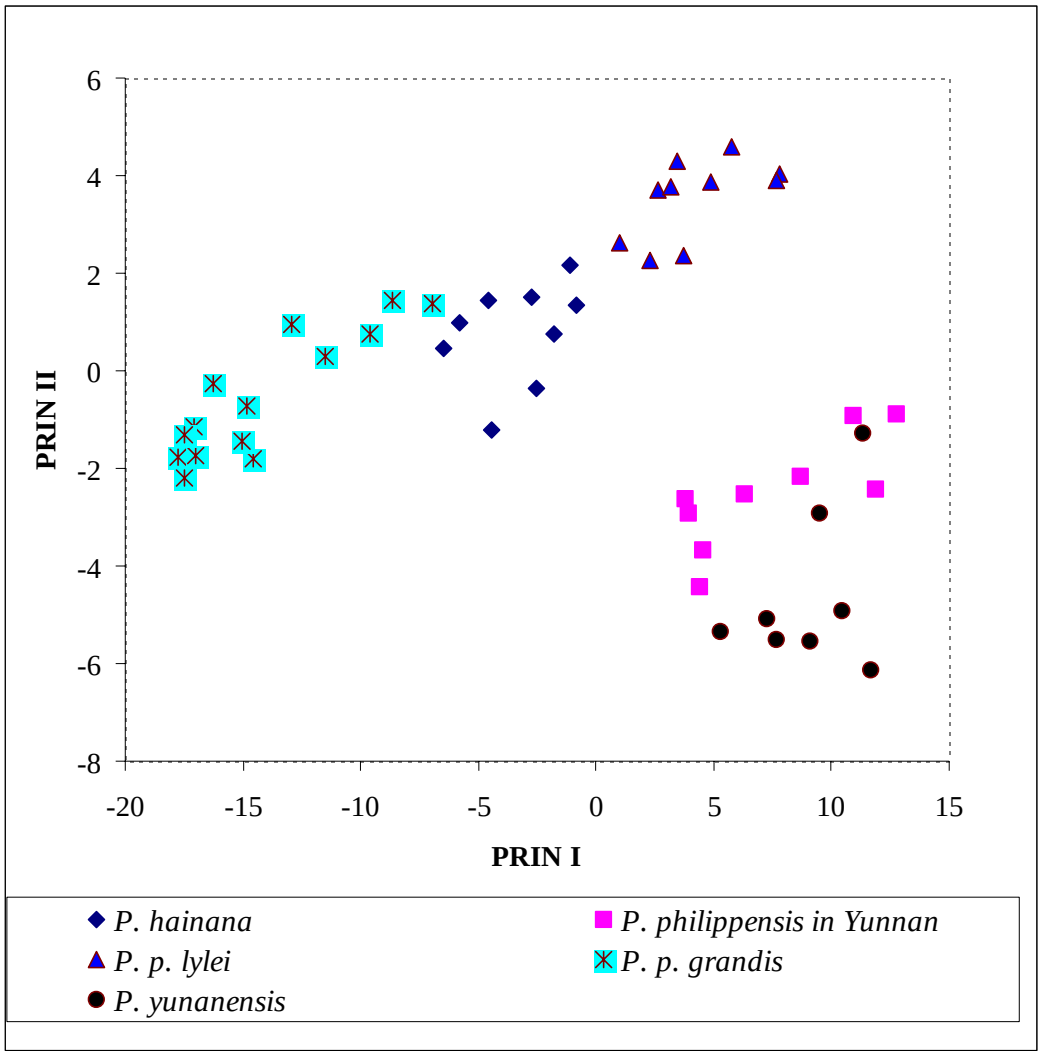

Figure 4.4 Principal components analysis of five *P. philippensis* forms onto factor 1 (PRIN I) and factor 2 (PRIN II)

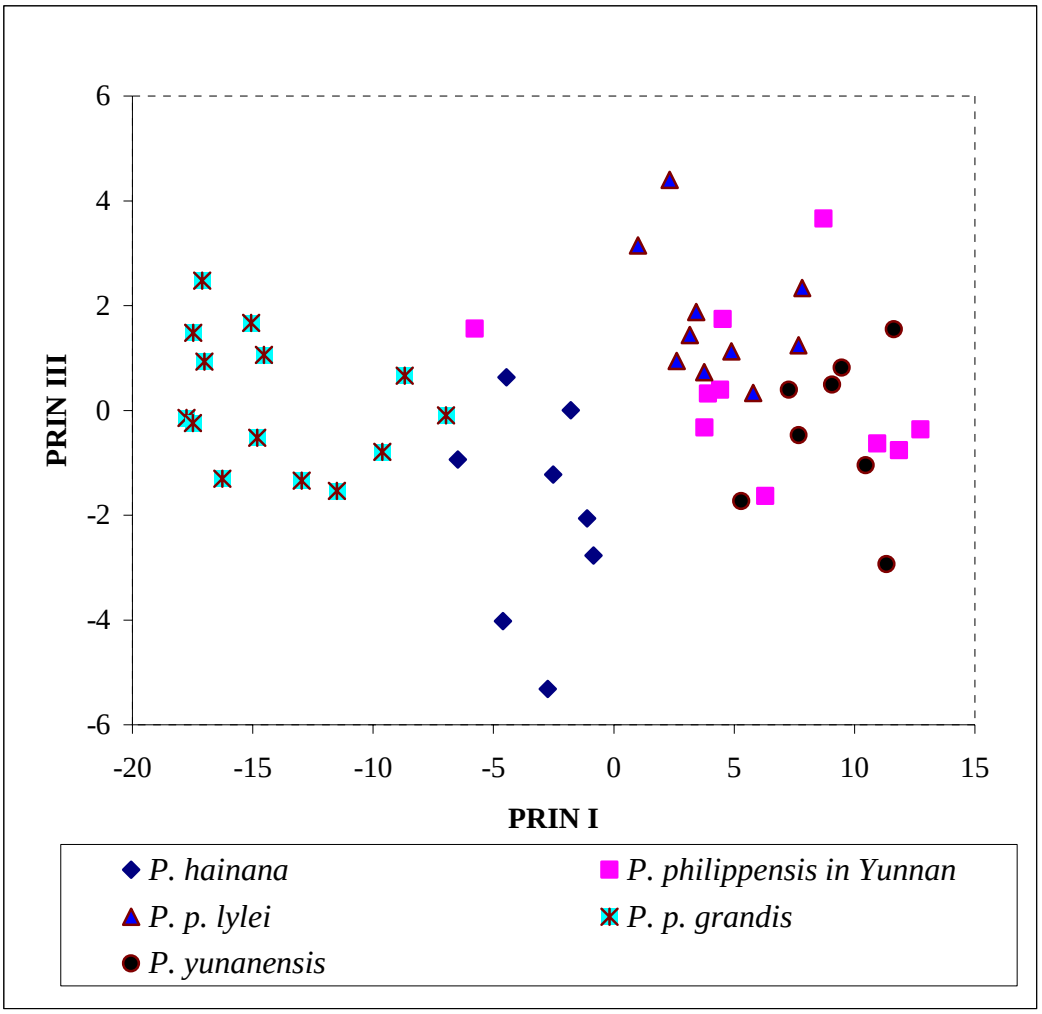

Figure 4.5 Principal components analysis of five *P. philippensis* forms onto factor 1 (PRIN I) and factor 3 (PRIN III)

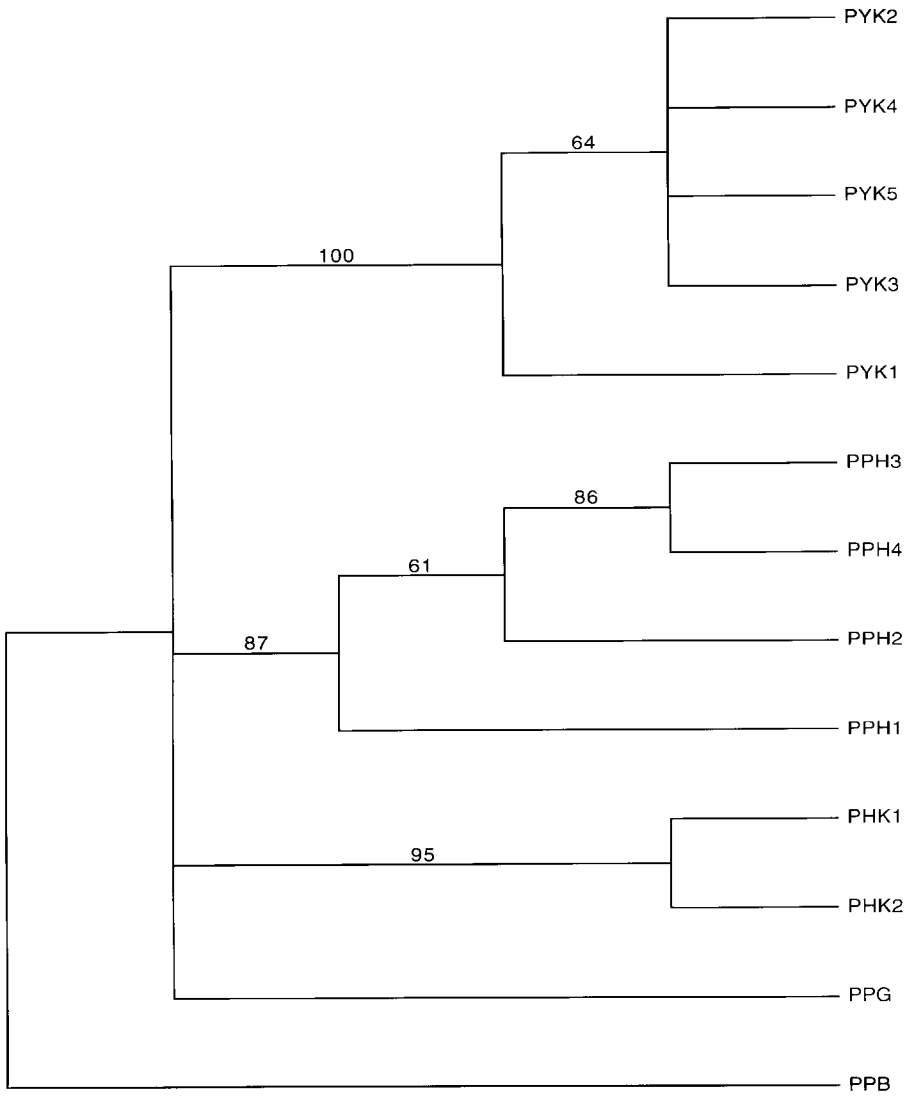

Figure 4.6 Phylogenetic relationships of *P. philippensis* forms based on the cytochrome b gene using maximum parsimony method (MP). Numbers above branches indicate the bootstrap values (%). Sample abbreviations are defined in Table 4.6 and Table 4.7.

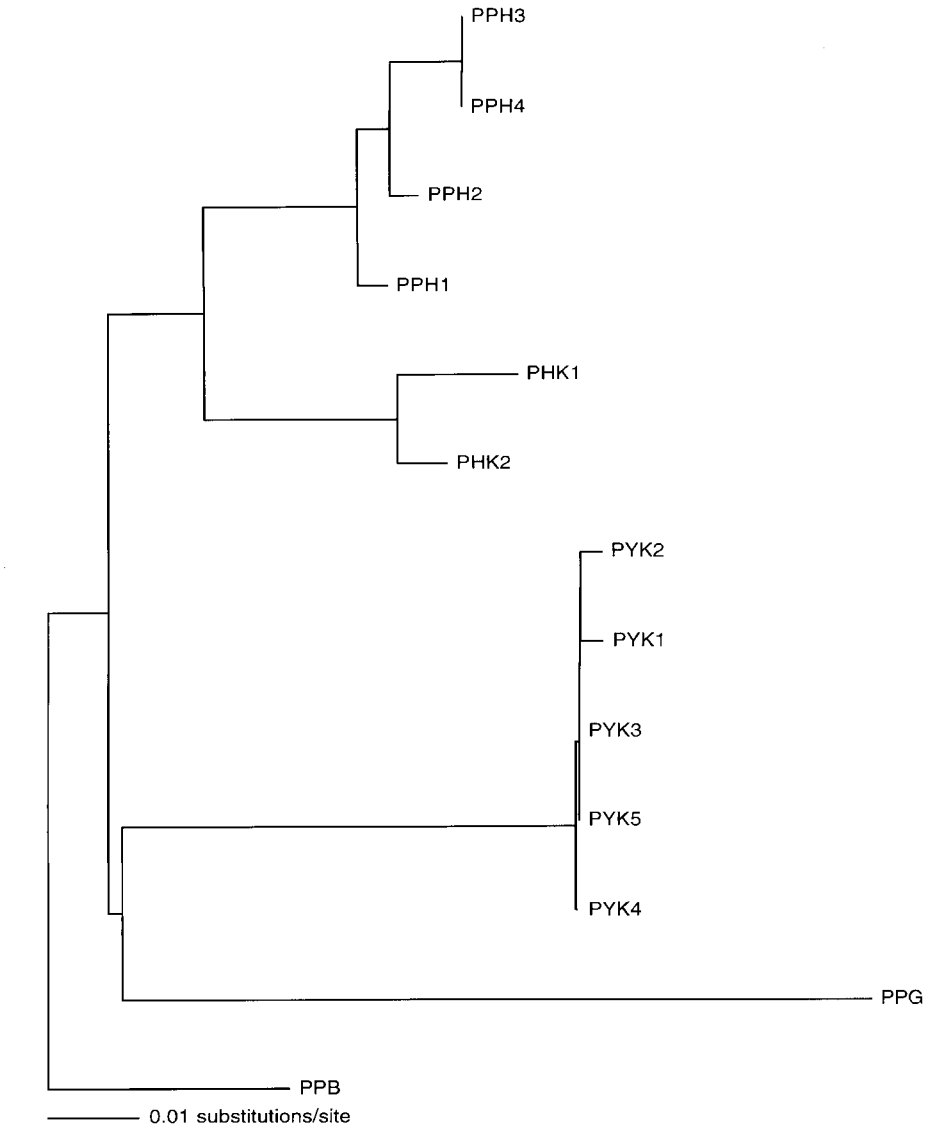

Figure 4.7 Phylogenetic relationships of *P. philippensis* forms based on the cytochrome b gene using neighbor-joining method (NJ). Scales in the tree represent branch length in terms of nucleotide substitutions per site. See sample abbreviations in Table 4.6 and Table 4.7.

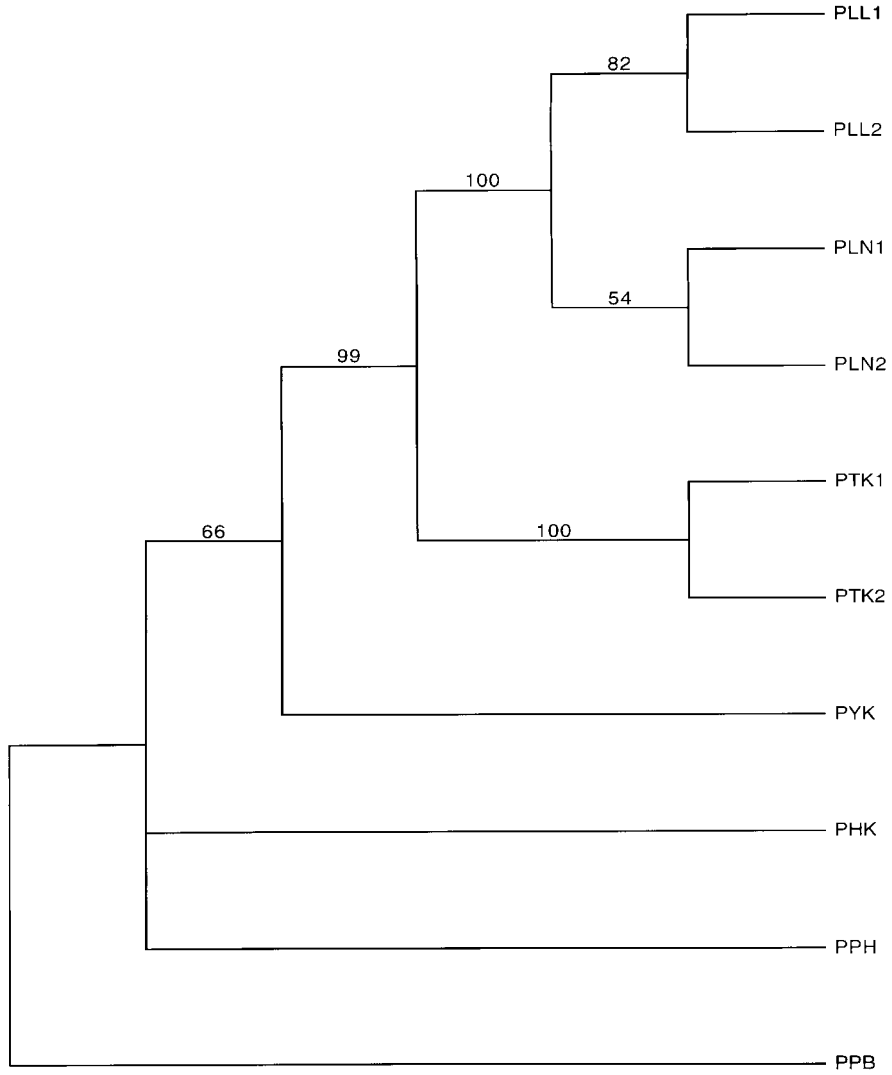

Figure 4.8 Phylogenetic tree of *P. xanthotis* and other giant flying squirrels constructed using maximum parsimony method (MP). Numbers above branches indicate the bootstrap values (%). Sample abbreviations are defined in Table 4.6 and Table 4.7.

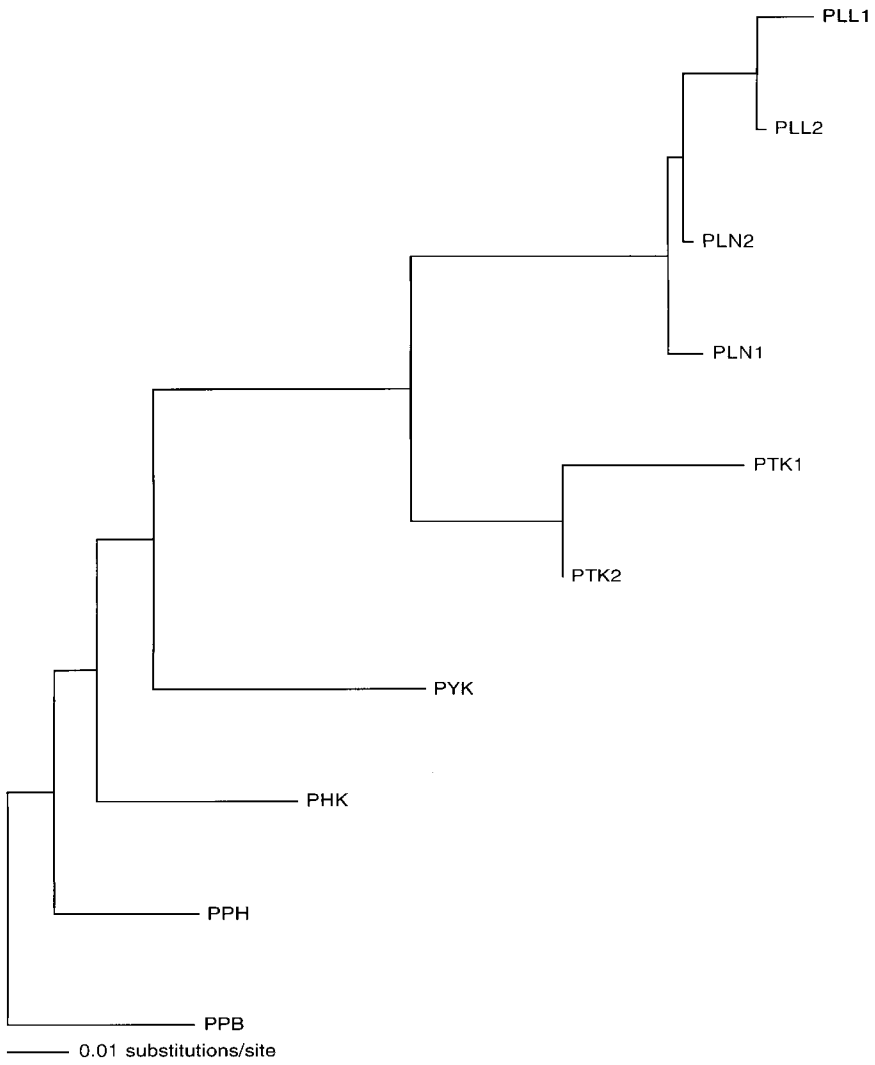

Figure 4.9 Phylogenetic tree of *P. xanthotis* and other giant flying squirrels constructed using neighbor -joining method (NJ). Scales in the tree represent branch length in terms of nucleotide substitutions per site. See sample codes in Table 4.6 and Table 4.7.

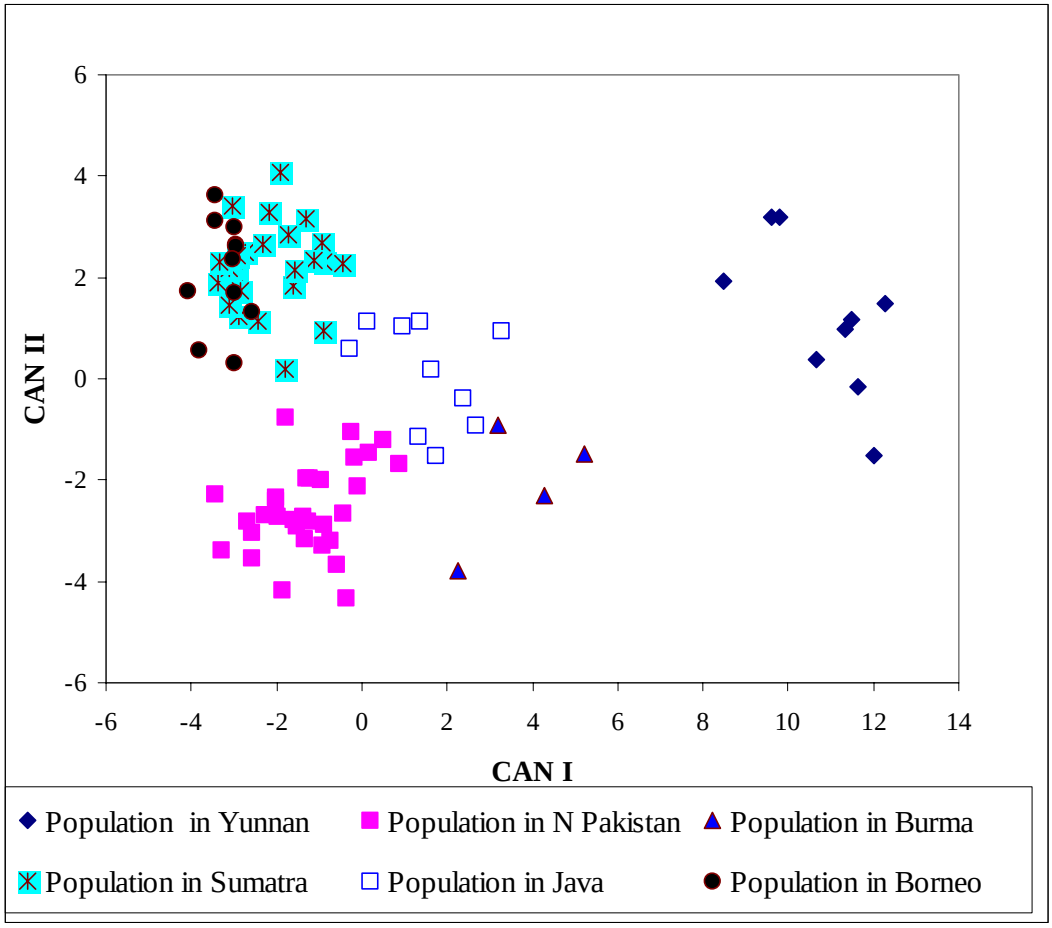

Figure 4.10 Plot of *P. petaurista* populations of discriminant function analysis onto the first and the second discrimiant function (CAN I to CAN II)

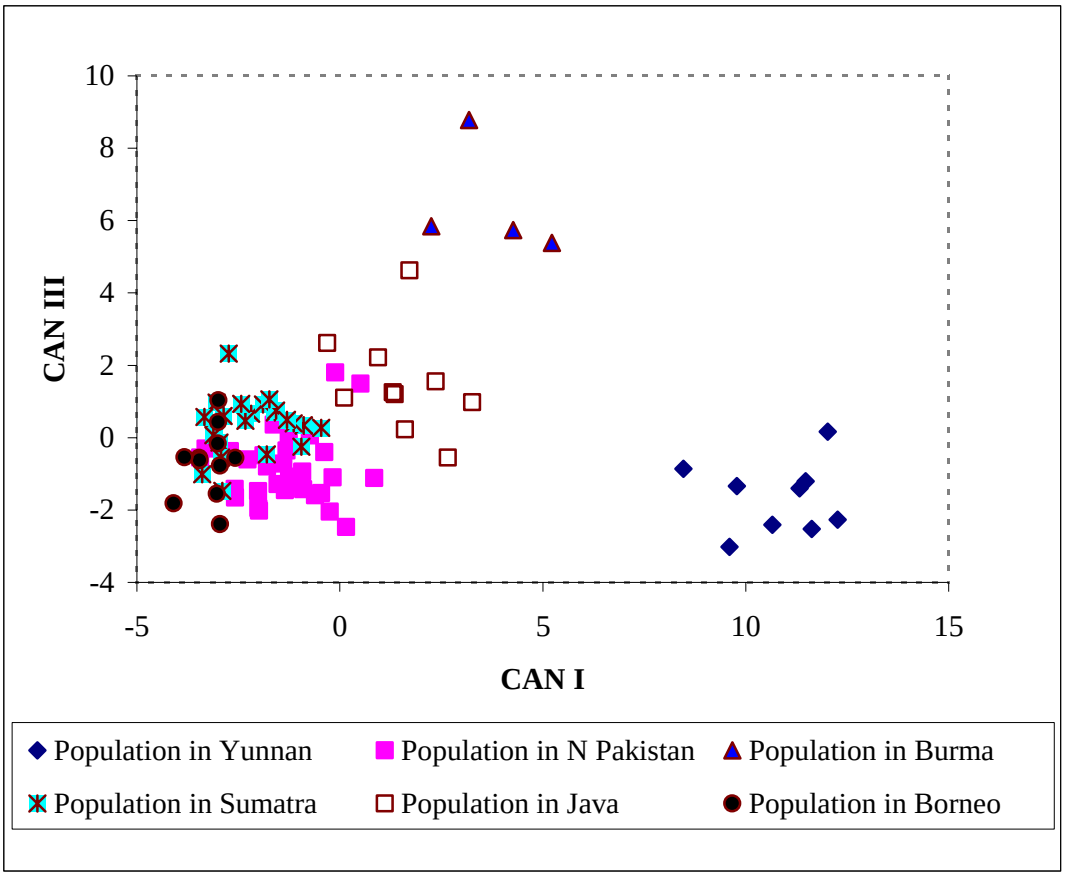

Figure 4.11 Plot of *P. petaurista* populations of discriminant function analysis onto the first and the third discrimiant function (CAN I to CAN III)

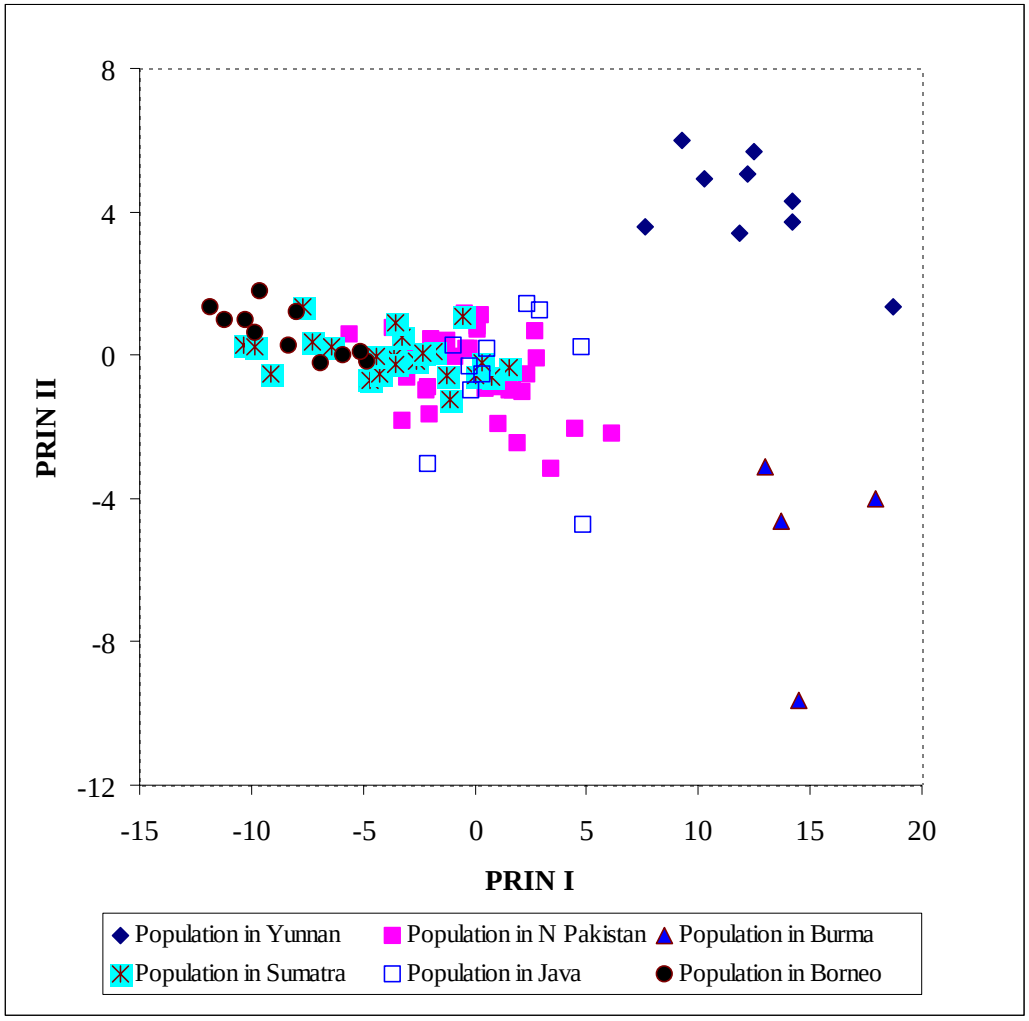

Figure 4.12 Principal components analysis of *P. petaurista* populations onto factor 1 and factor 2 (PRIN I to PRIN II)

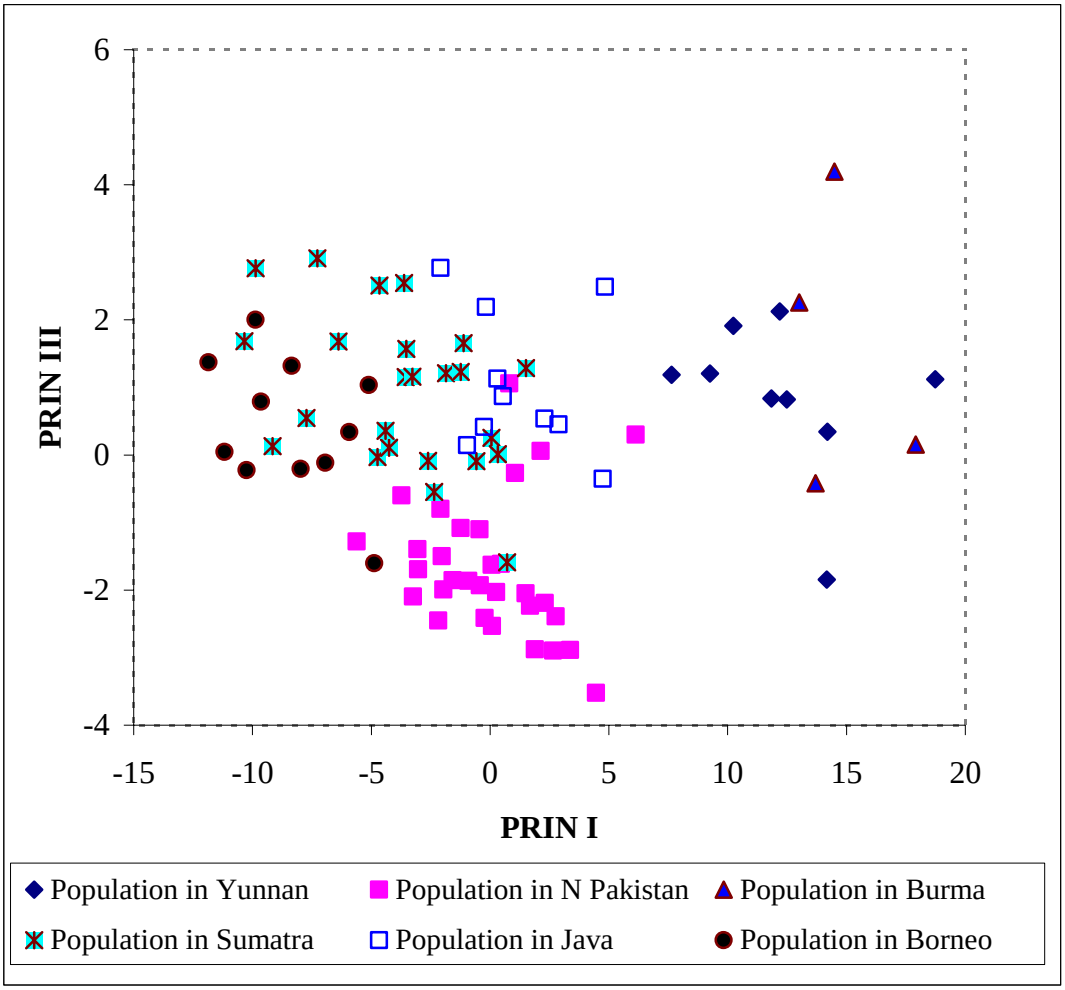

Figure 4.13 Principal components analysis of *P. petaurista* populations onto factor 1 and factor 3 (PRIN I to PRIN III)

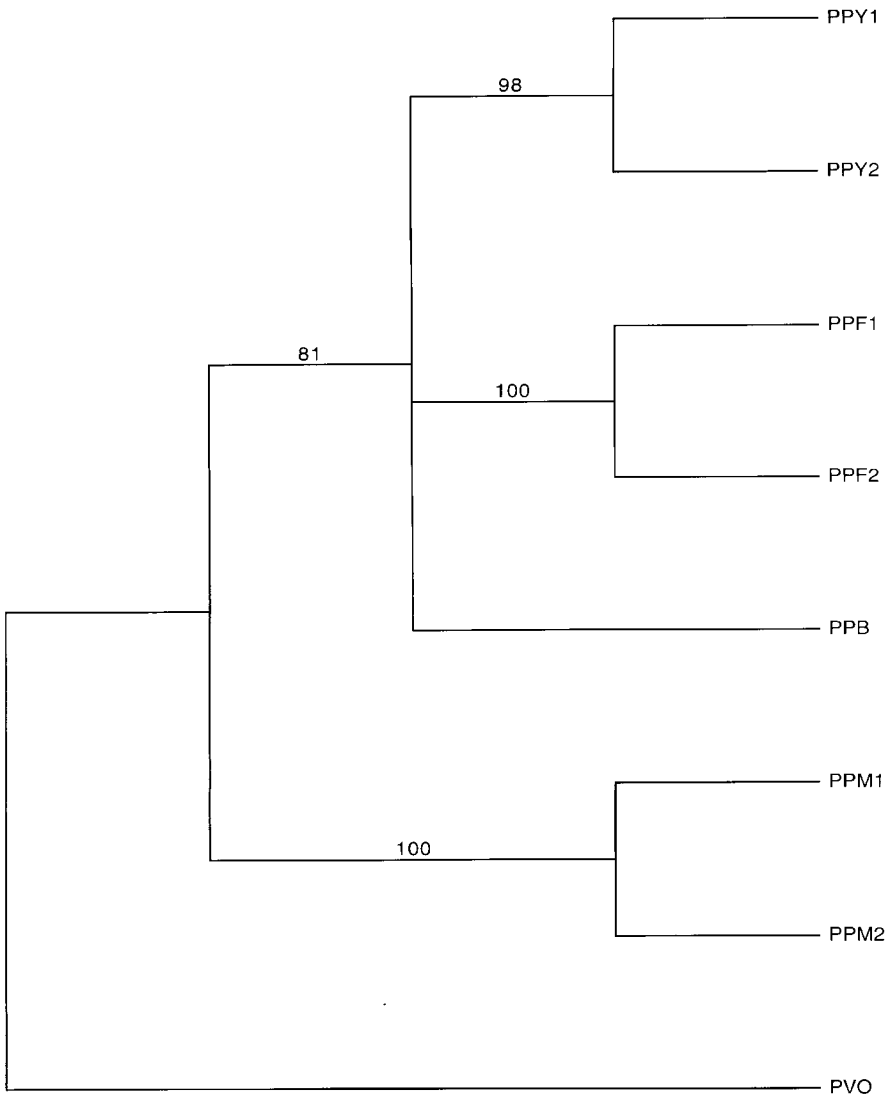

Figure 4.14 Phylogenetic relationships within the populations of *P. petaurista* reconstructed by maximum parsimony (MP) method with *Pteromys volans* as the outgroup. Numbers above branches are the bootstrap values (%). The abbreviations of taxa are defined in Table 4.6 and Table 4.7.

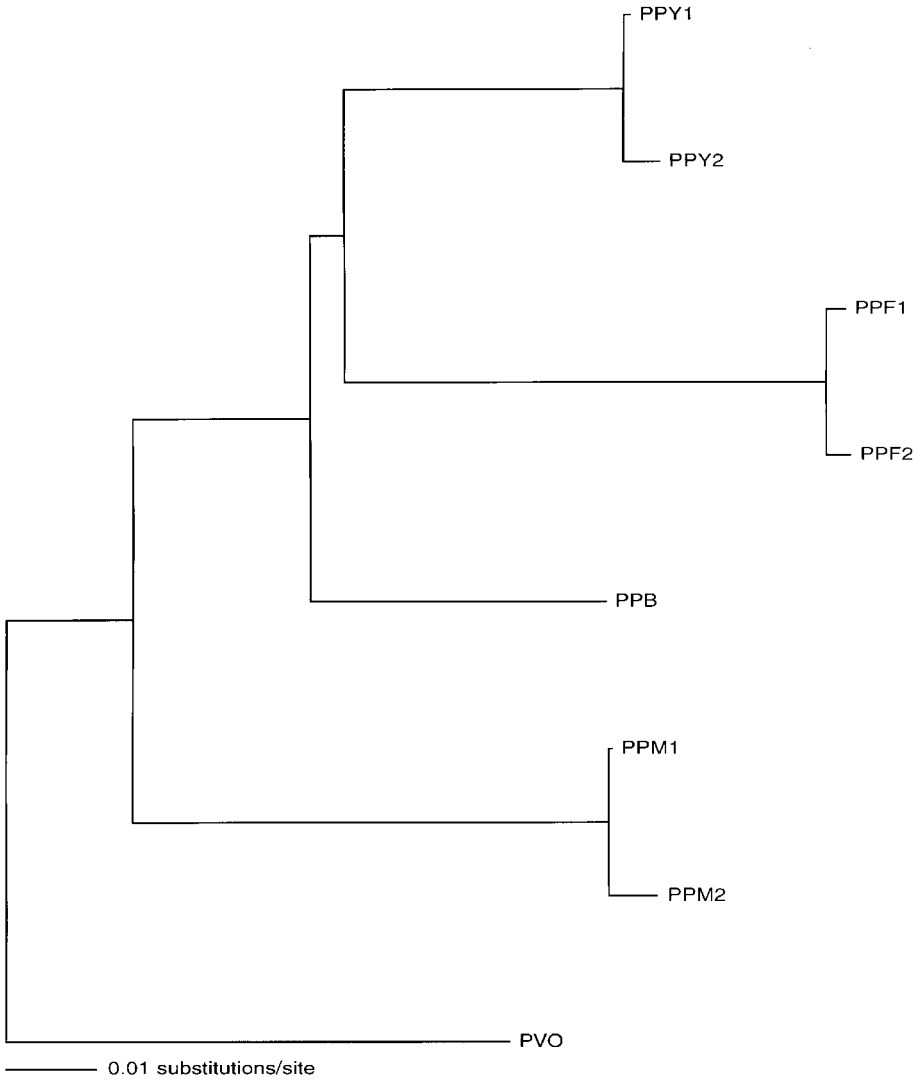

Figure 4.15 Phylogenetic relationships within the populations of *P. petaurista* reconstructed by neighbor-joining (NJ) method with *Pteromys volans* as the outgroup. Scales in the tree represent branch length in terms of nucleotide substitutions per site. The abbreviations of taxa are defined in Table 4.6 and Table 4.7.

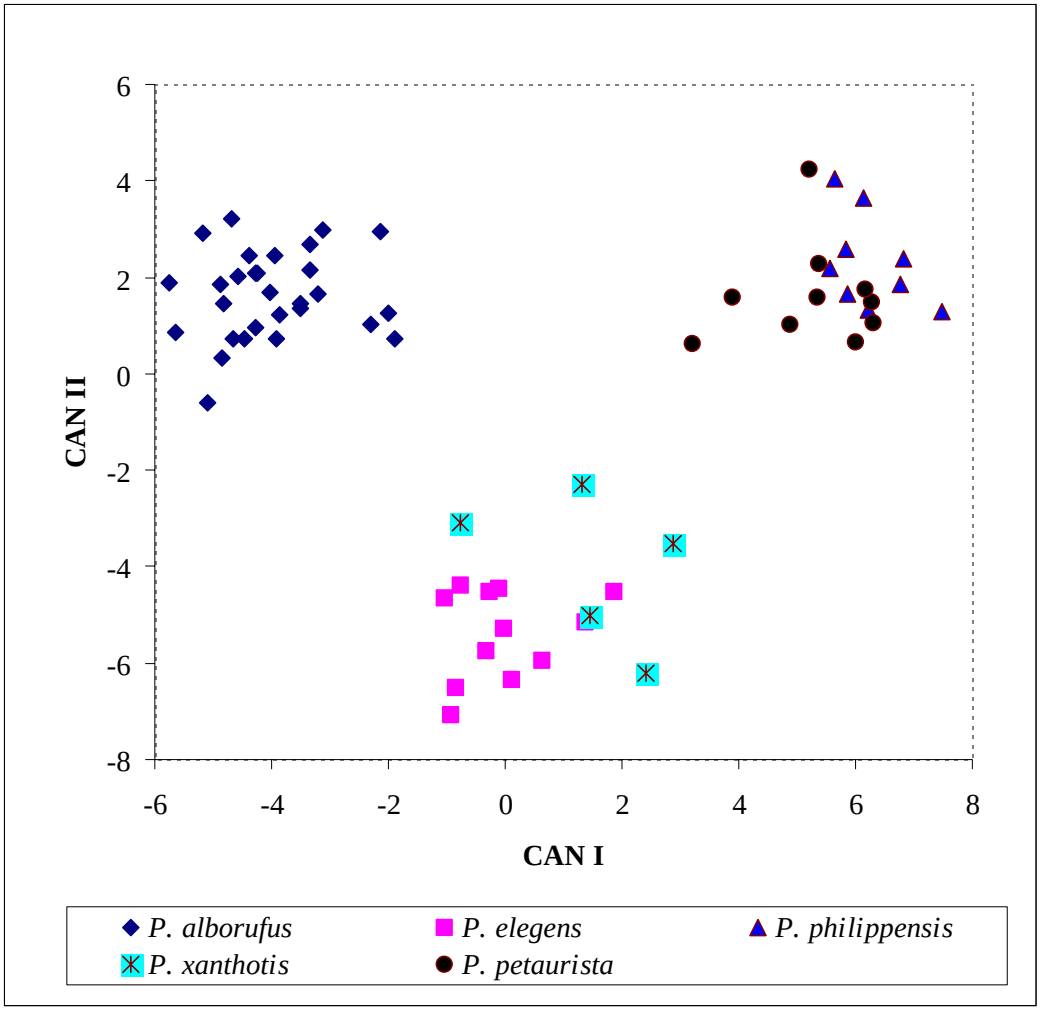

Figure 4.16 Discriminant function analysis of Chinese *Petaurista* on discriminant function 1 (CAN I) and function 2 (CAN II)

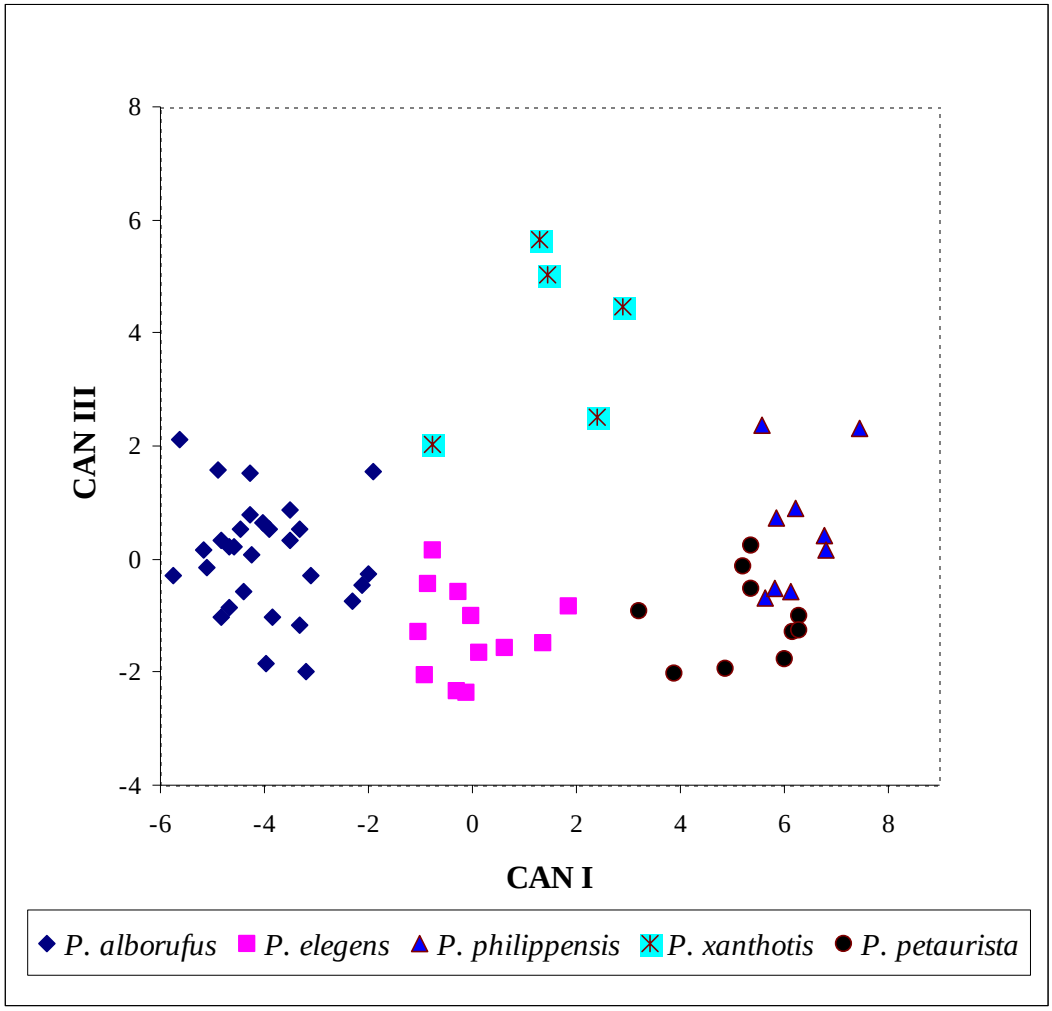

Figure 4.17 Discriminant function analysis of Chinese *Petaurista* on discriminant function 1 (CAN I) to function 3 (CAN III)

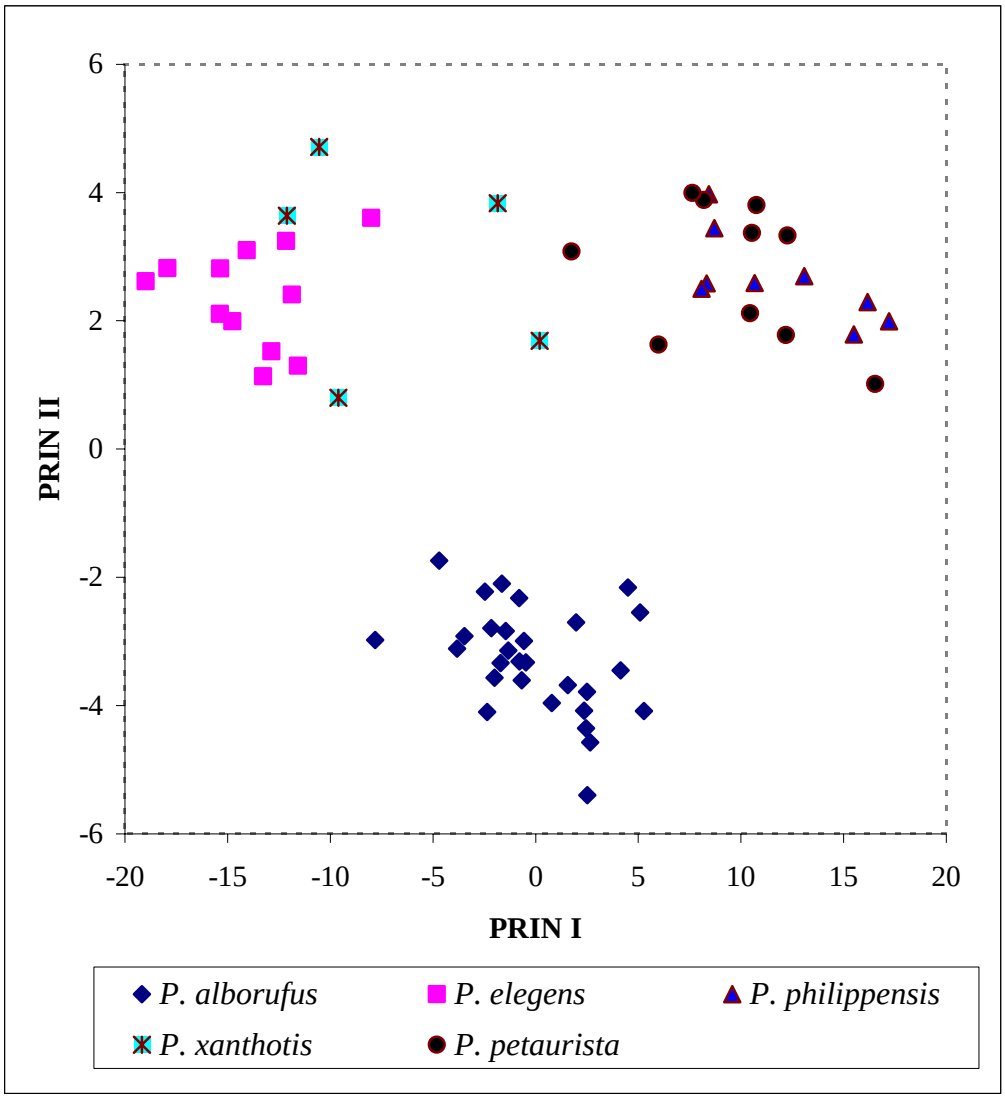

Figure 4.18 Principal components analysis of Chinese *Petaurista* on factor 1 (PRIN I) and factor 2 (PRIN II)

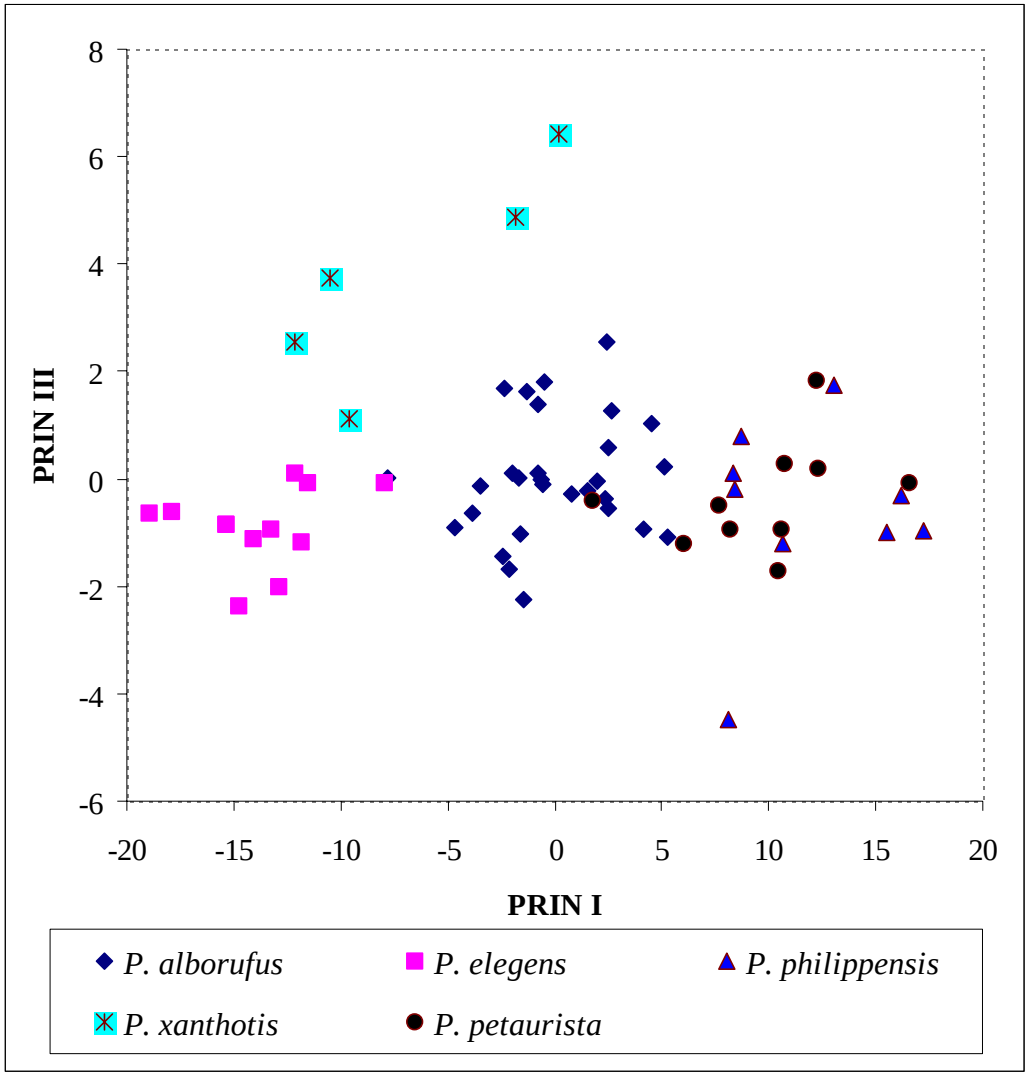

Figure 4.19 Principal components analysis of Chinese *Petaurista* on factor 1 (PRIN I) and factor 3 (PRIN III)

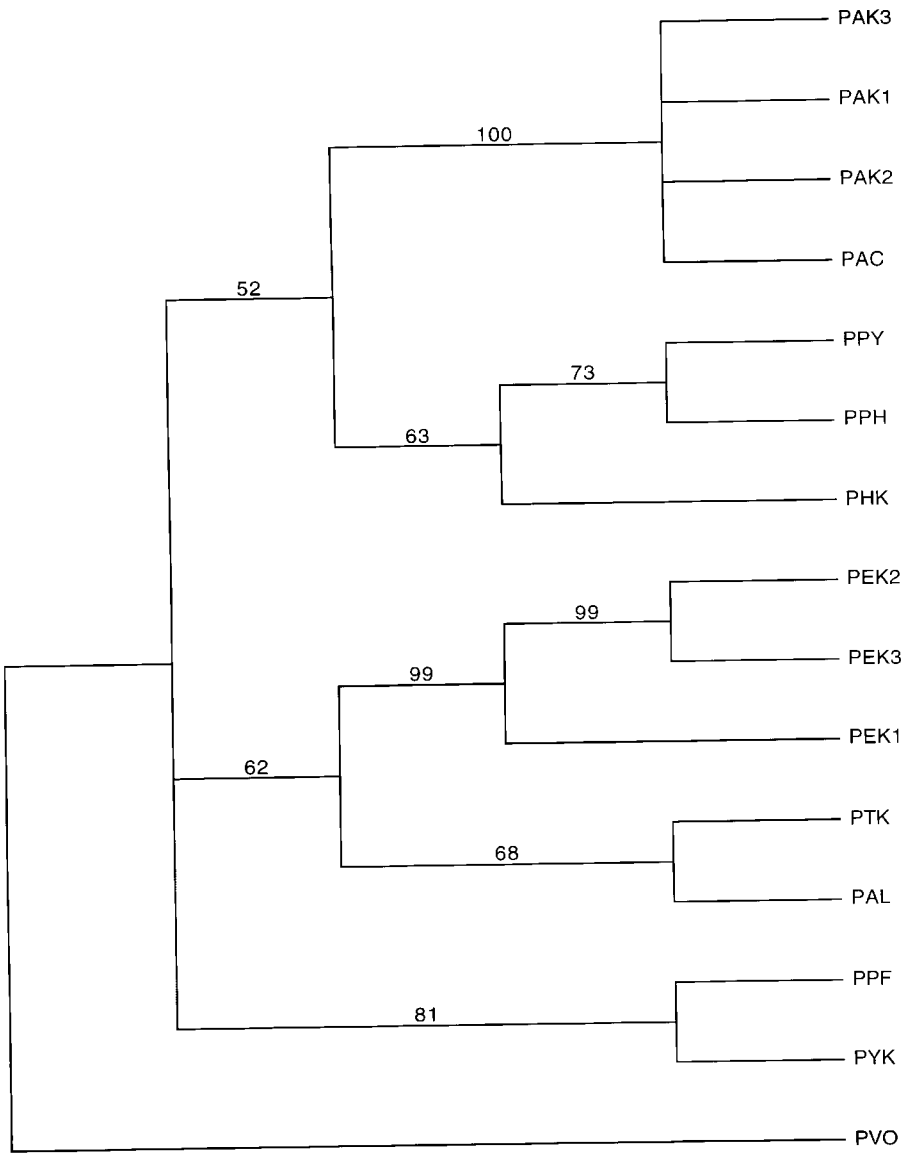

Figure 4.20 Phylogenetic topology of *Petaurista* based on the maximum parsimony method. The number of bootstrap value (%) is given above each branch. The abbreviations of taxa are defined in Table 4.6 and Table 4.7.

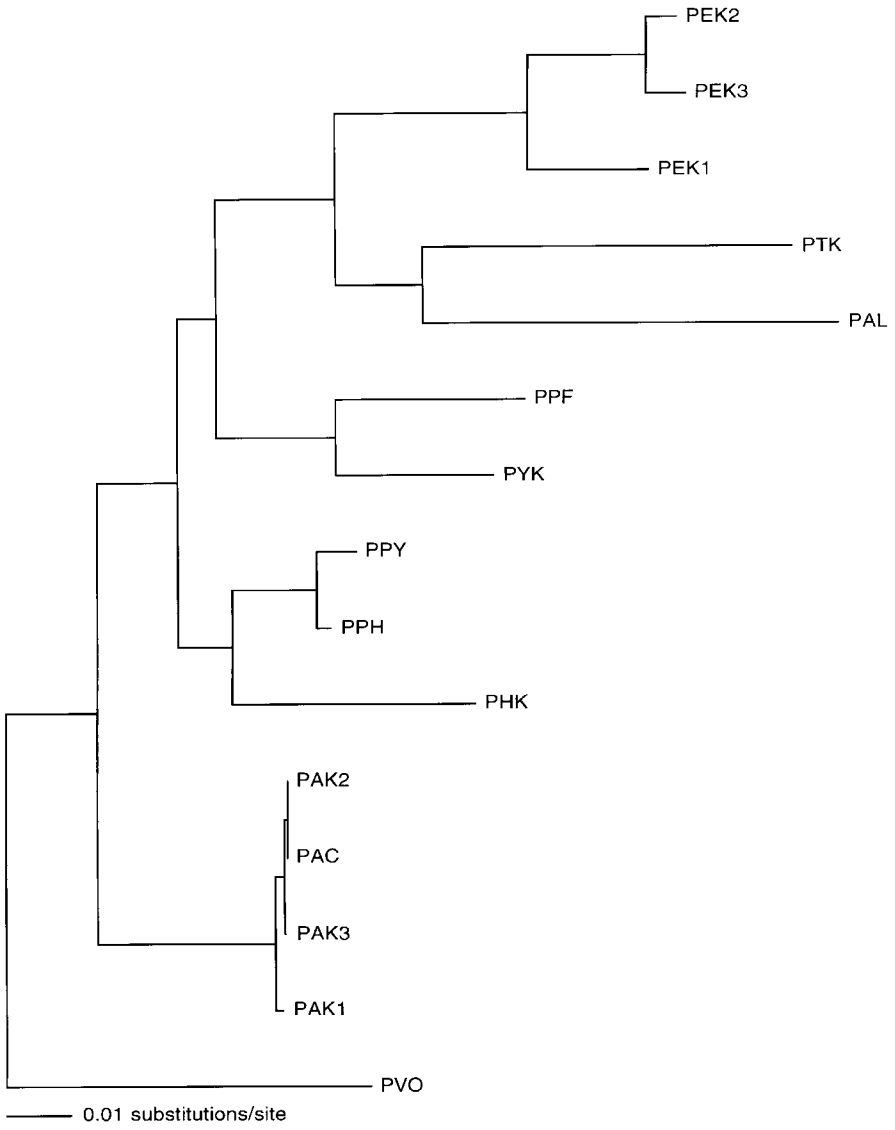

Figure 4.21 Phylogenetic topology of *Petaurista* based on the neighbor-joining method. Scales in the tree represent branch length in terms of nucleotide substitutions per site. The abbreviations of taxa are defined in Table 4.6 and Table 4.7.

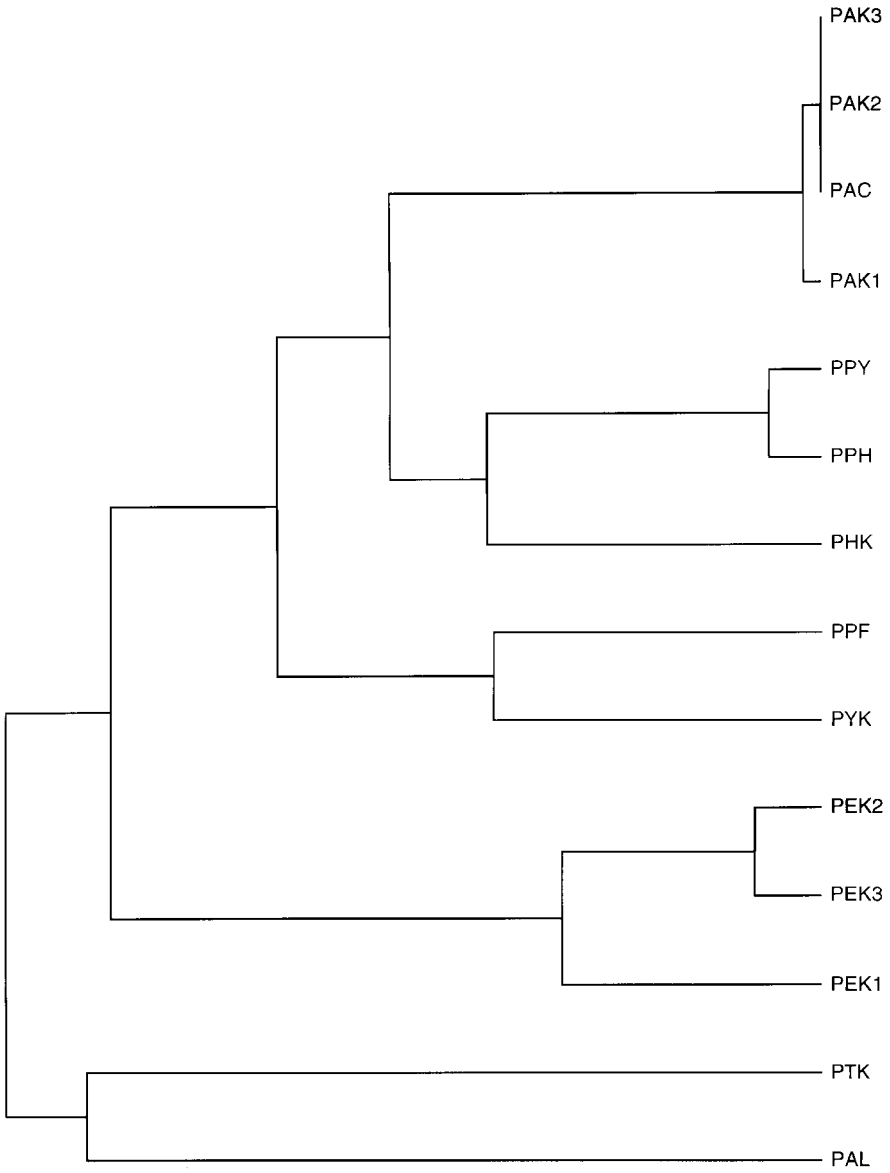

Figure 4.22 Phylogenetic topology of *Petaurista* based on the UPGMA method. The abbreviations of taxa are defined in Table 4.6 and Table 4.7.

Figure 4.23 *P. petaurista* in Pakistan and W Yunnan, China

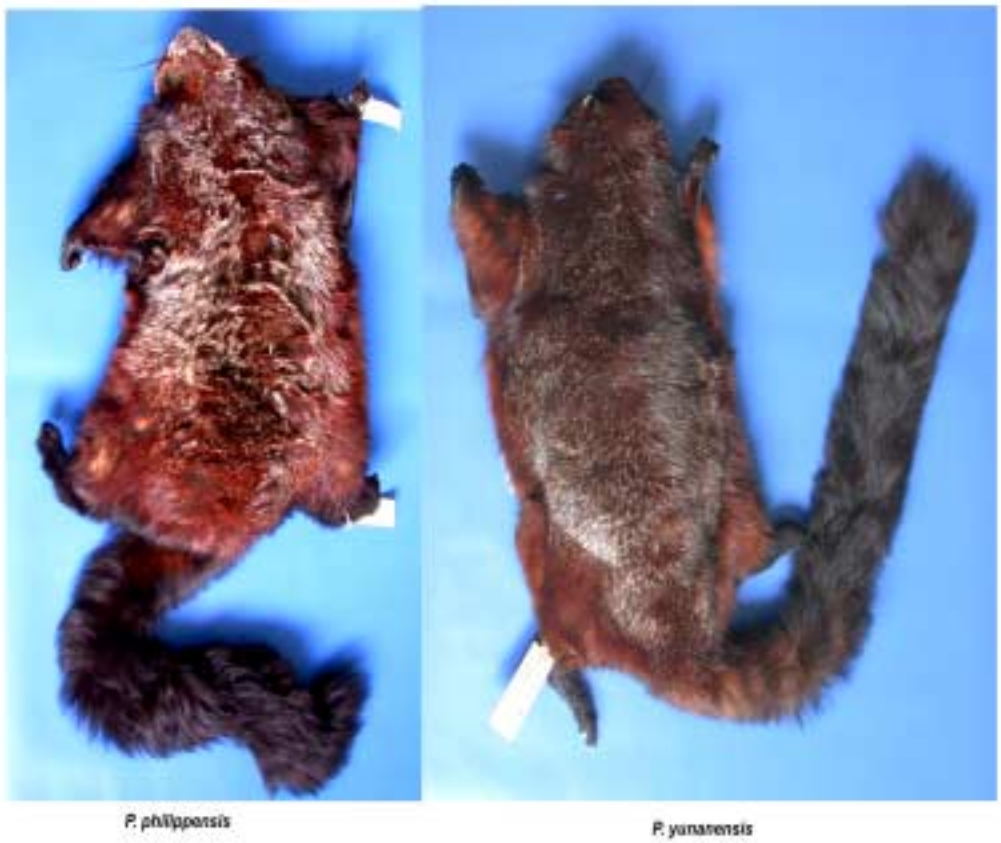

Figure 4.24 *P. philippensis* and *P. yunanensis* in Yunnan, China

CHAPTER 5
PHYLOGENY OF *EOGLAUCOMYS* AND *HYLOPETES* IN THE EASTERN AND THE WESTERN TRANS-HIMALAYAS AS INFERRED FROM MOLECULAR AND MORPHOMETRIC STUDY

5.1 Introduction

The generic status of *Hyloptetes* (arrow-tailed flying squirrel) and *Eoglaucomys* (small Kashmir flying squirrel) has been controversial for a long time. *Eoglaucomys* was maintained as a subgenus of *Glaucomys* (Thomas, 1908) until Howell (1915) first described it as a distinct genus. The morphological structures of the skull and baculum suggest that *Eoglaucomys* in the western extreme of the Himalayas could be a different genus from *Hylopetes* in the eastern Himalayas and SE Asia (Pocock, 1923; Johnson-Murray, 1977; Chakraborty, 1981; Roberts, 1997; Baillie and Groombridge, 1996). The main differences between *Hylopetes* and *Eoglaucomys* are related to the dental enamel, the cuspid of the third upper molar, and other dental structures of maxillary teeth. By carefully examining the baculum, footpads, musculature, crania including teeth, and postcranial structures (i.e., ankle and wrist joints) of the specimens from Afghanistan and Pakistan, Thorington et al. (1996) concluded that *Eoglaucomys* and *Hylopetes* are distinct clades. In the sixth edition of Walker's mammals of the world, Nowak (1999) accepted Thorington's recognition and treated *Eoglaucomys* as a valid genus. However, *Eoglaucomys* is still widely referenced as subgenus or synonym of *Hylopetes* because the morphological differences between them did not seem to be of more than subgeneric importance (Ellerman, 1947; Ellerman and Morrison-Scott, 1966; McLaughlin, 1967,

1984; Honacki et al., 1982; Corbet and Hill, 1991, 1992; Nowak, 1991; Wilson and Reeder, 1992).

Eoglaucomys is a monotypic genus with a single species *E. fimbriatus*, the small Kashmir flying squirrel. According to the difference in size between the crown areas of the first molar and the fourth premolar, Chakraborty (1981) elevated the population distributed in northeastern Afghanistan and northern Pakistan as another possible valid species, *E. baberi*. Corbet and Hill (1992) also accepted *Hylopetes* (*Eoglaucomys*) *baberi* as a full species. But Thorington et al. (1996) argued that *E. fimbriatus* and *E. baberi* are conspecific. By rechecking the type specimen and other specimens in the British Natural History Museum, Roberts (1997) found no evidence of consistent size differentiation between *fimbriatus* and *baberi* supports Chakraborty's (1981) result, and he thought that there is no basis for retaining *baberi* as a distinct species, even as a recognizable subspecies.

Hylopetes is a polymorphic genus and consists of 8 (Nowak, 1991) or 10 (Corbet and Hill, 1992) species (Table 5.1), occurring from the eastern Himalayas to Greater Sunda Island (SE Asia). Except for recognition of *Eoglaucomys* as a separate genus, the arrangements of *Hylopetes* proposed by Corbet and Hill (1992) were commonly accepted (Wilson and Reeder, 1992; Nowak, 1999). Three species, *H. alboniger*, *H. phayrei*, and *H. electilis*, are recognized in China, but the recognition of discrete species of *H. electilis* that is distributed in the island of Hainan, China, is not justified unanimously. *H. electilis* is treated as a subspecies of *H. phayrei* in recent references (Nowak,1999; Wang, 2002). Zhang et al. (1997) even combined *H. electilis* and the population of *H. phayrei* in China together as a valid species, *Petinomys electilis*, under the genus *Petinomys*. In Yunnan,

H. alboniger is mainly distributed in western mountainous areas (Figure 5.1) and is sympatric with *Petaurista*.

Table 5.1 Species of *Hylopetes* and *Eoglaucomys*

Nowak, 1991	Corbet and Hill, 1992	Locality
H. alboniger	*H. alboniger*	Nepal to Indochina
H. phayrei	*H. phayrei*	Burma, Thailand, Laos
H. nigripes	*H. nigripes*	Palawan Island, Philippines
H. lepidus	*H. lepidus*	Malay Peninsular, Sumatra, Borneo
H. spadiceus	*H. spadiceus*	S Burma, Thailand, Indochina, Sumatra, Java, Borneo
H. mindanensis		Mindanao
H. fimbriatus	*H. fimbriatus* *H. baberi*	Afghanistan, Pakistan, N India
H. electilis		Hainan, China
	H. sipora	W Sumatra
	H. bartelsi	W Java
	H. winstoni	N Sumatra

In the absence of experimental breeding data, the classification of geographically isolated populations as separate species or subspecies must rely on relative differences in morphology, behavior, and biochemical and genetic data (Garner and Ryder, 1996). The variability of mitochondrial DNA sequence is one of the many factors that should be considered in the classification of populations of flying squirrels. To date, most studies of *Eoglaucomys* and *Hylopetes* are mainly focused on the morphological, external, and ecological comparisons (McLaughlin, 1984; Honacki et al., 1982; Thorington et al., 1996; Roberts, 1997). The available data from either morphological or molecular study is fragmental and is not sufficient to construct the phylogenetic relationships. To test the

different hypotheses or revisions about the taxonomy and phylogeny of *Eoglaucomys* and *Hylopetes*, the morphological study with multivariate analysis and biochemical study with DNA sequencing analysis were conducted in this study. Finding the answers to the following questions would be important for reconstructing the phylogenetic relationships of the trans-Himalayan flying squirrels.

1. Is *Eoglaucomys* a valid genus or a subgenus of *Hylopetes*?
2. What is the phylogenetic relationship between *Eoglaucomys* in the western trans-Himalayas and the population of *Hylopetes* in the eastern trans-Himalayas?
3. Is *H. electilis* a full species or a subspecies of *H. phayrei*?
4. What's the phylogenetic relationship between *E. fimbriatus* and the populations of *Hylopetes*?

5.2 Materials and Methods

5.2.1 Materials

In the morphometric study, a total of 117 specimens of *Eoglaucomys* and *Hylopetes* were examined (Table 5.2). All specimens were represented in museums and institutes, including American Museum of Natural History, New York (AMNH), Florida Museum of Natural History, University of Florida, Gainesville (FLMNH), National Museum of Natural History, Smithsonian Institution, Washington DC (USNM), Chinese Institute of Zoology, Beijing (China, BIZ), and Kunming Institute of Zoology, Kunming (China, KIZ). Fourteen measurements of skull were used in multivariate analyses, which were defined in Table 4.5 (see Chapter 4).

Table 5.2 Species and localities of *Hylopetes* and *Eoglaucomys* examined in morphometric analysis

Species	Specimen	Sex	Locality	Museum
H. alboniger	8	4 F, 4 M	Yunnan, China	KIZ
H. electilis	24	12 F, 12 M	Hainan, China	KIZ, AMNH
H. phayrei	19	9 F, 10 M	Burma, Java	AMNH, USMNH
H. lepidus	10	5 F, 5 M	W Malaysia	USMNH
H. spadiceus	9	4 F, 5 M	W Malaysia	USMNH
H. nigripes	13	7 F, 6 M	Palawan, Philippines	USMNH, AMNH
Eoglaucomys fimbriatus	34	20 F, 14 M	W Pakistan, Kashmir	USMNH
Total	107	F = Female M = Male		

In the molecular study, the partial mitochondrial cytochrome b sequences were used for reconstructing the phylogenetic trees. Except for the sequence data of Phayre's flying squirrel (*H. phayrei*) and white-bellied flying squirrel (*Petinomys setosus*), which were retrieved from GenBank of NCBI, the sequences of *Hylopetes* and *Eoglaucomys* were determined from museum specimens, either hairs or skins. The detailed information of samples used was given in Table 5.3.

Table 5.3 Samples of *Eoglaucomys* and *Hylopetes* examined in molecular study

Species	Code	Museum ID	Locality
H. alboniger	HAK1	KIZ: 5654	Menla, Yunnan, China
	HAK2	KIZ: 74545	Luchun, Yunnan, China
	HAK3	KIZ: 74546	Luchun, Yunnan, China
	HAK4	KIZ: 55660	Menhai, Yunnan, China
H. electilis	HEK	KIZ: 29273	Hainan, China
E. fimbriatus	EFP1	USMNH: 353237	W Pakistan
	EFP2	USMNH: 353244	W Pakistan
	EFP3	FLMNH: 26513	Pakistan
H. nigripes	HNP	BIZ: 47792	Philippines
H. lepidus	HLP1	USMNH: 488619	W Malaya
	HLP2	USMNH: 488632	Malaya
H. spadiceus	HSP1	USMNH: 481112	W Malaya
	HSP2	USMNH: 48495	W Malaya
H. phayrei	HPT	AB030259	Thailand
Petinomys setosus	PSE	AB030260	Indochina peninsula
G. volans	GV	AF063066	Tennessee, US

5.2.2 Morphometric Analysis

Morphometric study of *Eoglaucomys* and *Hylopetes* was based on 7 species and 14 skull measurements. According to the localities, all specimens of *Hylopetes* were divided into two groups: the Chinese *Hylopetes* containing the populations of *Hylopetes* in the eastern trans-Himalayas, and the southeastern *Hylopetes* consisting of the populations in SE Asia. I first compared the morphological structures between

Eoglaucomys and the Chinese *Hylopetes*; then pooled both *Hylopetes* groups and *Eoglaucomys* together to assess the overall morphological relationships. Multivariate analyses including principal components analysis (PCA) and discriminant function analysis were applied to make these comparisons. The technical information of morphometric methods was described in the section 4.2 of Chapter 4.

5.2.3 Biochemical Study

The cytochrome b sequences of *Eoglaucomys* and *Hylopetes* in different localities determined from museum specimens were analyzed to rebuild the phylogenetic relationships. Two primer pairs, L14725 and H15149, and L14841 and H15149 (see Chapter 3 for detail), were synthesized at ICBR (Interdisciplinary center for biotechnology research, University of Florida, US) and KIZ (Kunming Institute of Zoology, China) for PCR amplification and PCR product sequencing. The techniques and protocols for DNA isolation, PCR amplification, PCR products purification, and DNA sequencing analysis were the same as those described in the section 3.2 of Chapter 3. The sequence data of some samples used for phylogenetic study were quoted from the GenBank of NCBI. The white-bellied flying squirrel (*Petinomys setosus*) from Indochina Peninsula was used as an outgroup for constructing the phylogenetic trees. Its sequence data was retrieved from GenBank of NCBI that is provided by Oshida et al. (2000a).

To avoid any confusion, only the specimens or samples that were collected in Pakistan and Kashmir in where *Eoglaucomys fimbriauts* is distributed were used in both mophometric and molecular studies. *G. volans* is used as the outgroup to reconstruct the phylogenetic trees.

5.3 Results

5.3.1 Comparison between *Eoglaucomys* and the Chinese *Hylopetes*

5.3.1.1 Morphological data

The multivariate analyses for comparative study of the Chinese *Hylopetes* and *Eoglaucomys* were discriminant function analysis and principal components analysis (PCA). The taxa included *H. alboniger*, *H. electilis*, *H. phayrei*, and *Eoglaucomys fimbriatus*. The results of discriminant function analysis were showed in Table 5.4. Along the first discriminant function (CAN I, 78% of variance), four species were discriminated as three groups. *Eoglaucomys fimbriatus* and *H. alboniger* were separated as two distinct groups, and *H. electilis* was clustered with *H. phayrei* as one group (Figure 5.2). All variables had equal contributions to this axis. The CAN II was strongly associated with the morphological variable BCASEL. The same grouping pattern was plotted in discriminant function analysis onto function 1 (CAN I) and function 3 (CAN III) (Figure 5.3). The major contributions to CAN III were from TBL and PORCL with a negative score.

Table 5.5 presented the results of principal components analysis of the Chinese *Hylopetes* and *Eoglaucomys*. The first principal component factor (PRIN I), the size factor, that accounted for 98% of the total original variance defined all specimens as three groups: *E. fimbriatus* and *H. alboniger* formed two distinct groups; *H. electilis* and *H. phayrei* combined as the third group for their extensively overlapping (Figure 5.4). The major contributions were from the variable CRANL and BCASEL. BCASEL also had the highest score on the second principal component factor (PRIN II). On the plot of the principal component factor 1 and factor 3 (PRIN III), *E. fimbriatus* was isolated from

Chinese *Hylopetes* and formed an independent group (Figure 5.5). There was a considerable overlap between *H. electilis* and *H. phayrei*. Along the third principal component factor (PRIN III), the main morphological variable contributing to this association was PGA.

Table 5.4 Discriminant function analysis between the Chinese *Hylopetes* and *Eoglaucomys*

CAN	Eigenvalue	Proportion	Cumulative
I	46.93	0.78	0.78
II	12.53	0.21	0.99
III	0.87		1.00
Canonical score			
Variable	CAN I	CAN II	CAN III
CRANL	0.99	0.08	0.01
BCASEL	0.97	0.22	0.03
CRANW	0.99	0.01	0.00
BPORW	0.99	0.00	0.05
PORCL	0.76	-0.03	-0.35
PGA	0.96	0.08	0.05
NAL	0.98	0.12	0.00
TBL	0.92	0.33	0.14
DSL	0.97	0.14	0.03
MTRL	0.98	0.00	0.06
MTRW	0.97	-0.06	0.03
LMDL	0.98	0.05	0.09
LMDH	0.98	0.06	-0.01
LMTL	0.99	-0.08	0.11

Table 5.5 Principal components analysis between Chinese *Hylopetes* and *Eoglaucomys*

PRIN	Eigenvalue	Proportion	Cumulative
I	366.71	0.98	0.98
II	2.56	0.01	0.99
III	1.58	0.00	0.99
Eigenvector score			
Variables	PRIN I	PRIN II	PRIN II
CRANL	0.55	0.01	-0.11
BCASEL	0.44	-0.70	-0.11
CRANW	0.18	0.20	-0.16
BPORW	0.29	0.42	-0.02
PORCL	0.05	0.04	-0.14
PGA	0.23	0.00	0.94
NAL	0.23	-0.10	-0.07
TBL	0.09	-0.28	0.07
DSL	0.13	-0.10	-0.05
MTRL	0.10	0.09	0.06
MTRW	0.11	0.18	0.01
LMDL	0.42	0.33	-0.09
LMDH	0.22	0.08	-0.11
LMTL	0.10	0.16	0.02

5.3.1.2 Molecular data

The molecular analyses were conducted on the basis of the partial sequences (400 bp) of cytochrome b gene. Nine samples from 4 species were used for studying the phylogenetic relationships of the Chinese *Hylopetes* and *Eoglaucomys*. Table 5.6 showed

the genetic differences and the substitutions of transversions and transitions based on the pairwise comparison between samples. The genetic differences between *Eoglaucomys* and *H. alboniger*, and between *Eoglaucomys* and *H. electilis* and *H. phayrei* were significant, varying from 12.8% to 17.1%. *H. electilis* was genetically distinct from *H. phayrei* and *H. alboniger* with 11.7% and 12% variations in sequence, respectively.

Table 5.6 Pairwise comparison of cytochrome b nucleotide sequences (400 bp) between Chinese *Hylopetes* and *Eoglaucomys*

	HAK1	HAK2	HAK3	HAK4	EFP1	EFP2	EFP3	HPT	HEK
HAK1		1.0	0.8	1.2	13.5	14.6	14.7	10.1	13.1
HAK2	2/1		0.1	1.7	14.6	15.6	15.6	10.8	12.2
HAK3	3/0	2/1		0.5	13.3	14.3	14.5	10.6	12.8
HAK4	5/0	5/1	2/0		12.8	13.8	14	10.7	12.2
EFP1	33/21	32/25	30/21			1.1	2.3	16.2	16.1
EFP2	35/23	33/26	34/23	32/23	3/1		1.5	17.1	16.8
EFP3	38/21	39/26	37/21	35/21	7/2	5/1		16.5	15.7
HPT	39/1	35/2	41/1	41/1	43/21	44/23	44/21		11.8
HEK	43/8	32/5	43/8	40/8	40/8	42/21	43/18	35/5	

Note: Data below the diagonal are the numbers of substitutions of transitions vs. transversions. Data above the diagonal represent the genetic differences between samples

The neighbor-joining analyses generated similar tree topology (Figure 5.6). Three distinct clades were formed in both trees: *H. alboniger*, *Eoglaucomys*, and the combination of *H. electilis* and *H. phayrei*.

5.3.2 Phylogenetic Relationships of *Hylopetes*

To assess the overall morphological relationships, *E. fimbriatus* and three other *Hylopetes*, including *H. nigripes*, *H. lepidus*, and *H. spadiceus* from SE Asia, were

included in discriminant function analysis and principal components analysis. Figure 5.7 and Figure 5.8 were the scatter-plots of discriminant function analysis on function 1 to function 2, and to function 3. In both plots, except for *H. electilis* and *H. phayrei* that were combined as one group, the rest were distinguished as distinct groups, showing different morphological structures of skulls. The principal components analysis onto the first factor and the second factor plotted similar morphological patterns as those of discriminant function analysis with an exception of *H. spadiceus,* which shared the similar skull characters with *H. electilis* and *H. phayrei* (Figure 5.9). The plot between factor 1 and factor 3 clustered the populations of *Hylopetes* in China as a complex group (Figure 5.10), and the rest were separated as different groups.

The sequence data of *H. nigripes*, *H. lepidus*, and *H. spadiceus* from SE Asia were isolated from museum skins. Table 5.7 presented the genetic results of pairwise comparison on the partial sequences, 375 bp, of cytochrome b gene. *E. fimbriatus* apparently differed from all *Hylopetes* with a high sequence differentiation, 16.2% - 17.8%. The genetic variation between *H. phayrei* and *Petinomys setosus* was about 3%. Considering *Petinomys setosus* as an outgroup, the NJ tree and MP tree generated similar branching patterns. All *Hylopetes* were separated as three clades: *H. lepidus* and *H. spadiceus* as one group with a high bootstrap value (89%), *H. phayrei* as the second group, the rest species, including *Eoglaucomys fimbriatus*, *H. nigripes*, *H. alboniger*, and *H. electilis,* as the third group (Figure 11 and Figure 12).

Table 5.7 Pairwise comparison of *Hylopetes* and *Eoglaucomsy* based on the partial cytochrome b sequences (375 bp)

	HAK	EFP	HPT	HEK	HLP1	HLP2	HSP1	HSP2	HNP	PSE
HAK		13.3	10.3	12.3	13.0	13.1	13.9	15.3	11.8	12.6
EFP	30/20		16.3	16.2	17.8	17.3	17.8	17.4	17.1	18.5
HPT	37/1	40/20		11.4	11.5	11.9	9.3	11.7	15.1	3.0
HEK	40/5	39/20	33/3		12.0	12.0	10.7	12.0	10.3	12.0
HLP1	41/7	44/21	36/6	39/5		0	7.5	8.4	12.6	13.6
HLP2	42/7	44/21	38/6	39/5	0		7.5	8.8	12.7	13.8
HSP1	45/6	43/22	28/4	32/5	20/6	21/6		1.1	9.8	11.4
HSP2	50/6	48/18	36/4	37/4	25/5	27/5	1/2		11.2	13.7
HNP	37/6	46/17	48/4	30/4	41/5	41/5	31/2	36/2		15.8
PSE	41/6	44/25	7/5	32/5	39/11	40/10	29/9	38/8	49/9	

Note: Data below the diagonal are the numbers of substitutions of transitions vs. transversions. Data above the diagonal represent the genetic differences between samples

The divergence time between *Hylopetes* and *Eoglaucomys*, was calculated based on the transversional substitution rates at the third codon positions of cytochrome b sequences (375 bp) (Table 5.8). Table 5.9 gave the divergence time between species that were calculated from the percentage of the transversional substitutions at the third codon positions of the sequences. The divergence rate used here is about 0.5% per million years (Irwine et al., 1991). The results indicated that *Eoglaucomys* diverged from its ancestor stock about 24 to 33 million years ago. The divergence time between the Chinese *Hylopetes* the SE Asian populations was about 6.4 to 9 million years ago. *H. electilis* was genetically distinguishable from *H. alboniger* as early as 8 million years ago.

Table 5.8 Transversional substitution rates at the third codon positions of the partial sequences (375 bp) of cytochrome b gene between species

	HAK	EFP	HEK	HPT	HLP	HSP	HNP	PSE
HAK		13.6	4	0.8	3.2	4	4.8	2.4
EFP	17		12	14.4	15.2	12	12	16.8
HEK	5	15		4.8	3.2	3.2	3.2	6.4
HPT	1	18	6		4.8	4	3.2	1.6
HLP	4	19	4	6		3.2	4	6.4
HSP	5	15	4	5	4		0.8	6.4
HNP	6	15	4	4	5	1		5.6
PSE	3	21	8	2	8	8	7	

Note: Data below the diagonal are the numbers of transversions at the third codon positions. Data above the diagonal represent the transversional percentage difference between samples.

Table 5.9 Estimated divergence time between species using the rate of the transversional substitutions at the third codon positions of mammalian cytochrome b gene of ca. 0.5% $*10^6$ years

	HAK	EFP	HEK	HPT	HLP	HSP	HNP
EFP	27.2						
HEK	8	24					
HPT	1.6	28.8	9.6				
HLP	6.4	30.4	6.4	9.6			
HSP	8	24	6.4	8	6.4		
HNP	9.6	24	6.4	6.4	8	1.6	
PSE	4.8	33.6	12.8	3.2	12.8	12.8	11.2

5.4 Discussion

5.4.1 Taxonomic Status of *Eoglaucomys*

The small Kashmir flying squirrel *Eoglaucomys* is a monotypic genus and is found in northeastern Afghanistan, Kashmir, adjacent parts of northern Pakistan, and India (Niethammer, 1990; Roberts, 1997). It is designated as a near threatened species by IUCN (Baillie and Groombridge, 1996). The single species, *Eoglaucomys* (*Hylopetes*) *fimbriatus*, was originally named as *Sciuropterus baberi* by Blyth (1874). The type locality is in Northwest India, Punjab, Simla (Hassinger, 1973). Based on the drawing of the specimen from the mountain district of Nijrow, Afghanistan, by Burnes, Blyth (1847) described *Sciuropterus baberis* as a larger species than *S. fimbriatus*. Ellerman (1940) described *fimbriatus* as a polytypic species under the genus *Eoglaucomys* Howell, and *baberi* was treated as a subspecies of it. Ellerman (1963) elevated *baberi* as a different race from *fimbriatus* according to the size of skull and the distribution, and considered *baberi* as a more western subspecies being found in Afghanistan, Pakistan (Northwest Frontier Province and Kagan Valley), Kashmir, and *fimbriatus* as an eastern subspecies found in Punjab, Kashmir (Islamabad district, Gilgit) and Himachal Prades. By comparing the cranial and dental characters of skulls that were collected around the town of Islamabad (formerly Anatnag) in the main valley of Kashmir -- 33^{o} 42’ E, 75^{o} 09’N (Shikargarh, Daksum Chaprot, Gilgit, Ladakh) and Sardalla and Chitral (Ellerman, 1963; Blanford, 1891), Chakraborty (1981) elevated *baberi* as a distinct species from *fimbriatus.* But Roberts (1997) argued that unless it can be proved that there is reproductive isolation between these two identical and certainly sympatric populations, there is no basis for retaining *baberi*, even as a recognizable subspecies.

Eoglaucomys fimbriatus is larger than *Hylopetes* (Figure 5.13). Both the molecular and morphological data of the present study yield the consistent results, supporting *Eoglaucomys* as a distinct genus. Although the phylogenetic differentiation between *Eoglaucomys* and *H. alboniger* is about 13%, the genetic differences between *Eoglaucomys* and other *Hylopetes* groups in Thailand (*H. phayrei*) and SE Asia (*H. lepidus* and *H. spadiceus*) have reached as high as about 17% (Table 5.7). This reveals that *Eoglaucomys* is quite distinctive from all *Hylopetes* groups. In multivariate analyses, the cranial structures of *Eoglaucomys* are significantly distinguishable in both discriminant function analysis and principal components analysis (Figure 5.2 to Figure 5.5). The major differentiation is associated with the skull size. The different morphological characters could be viewed as reflections of various adaptations to various ecological niches, which typically occur as a result of competition between species. Competition usually varies among habitats, and habitat selection is a major contributor to coexistence. The coexistence of competing species by partitioning microhabitat is very common among rodent species (Price, 1978; Jorgensen and Demarais, 1999). This phenomenon exists among the trans-Himalayan flying squirrels as well (Roberts, 1997; Wang, 2002).

In Pakistan, the small Kashmir flying squirrel (*E. fimbriatus*) is sympatric with the Pakistan giant flying squirrel (*P. petaurista*) and woolly flying squirrel (*Eupetaurus cinereus*) (Hassinger, 1973). The competitions for habitat and utilization of the available food resources are unavoidable. The habitat of *Eupetaurus* is characterized as high, cold desert dominated by *Artemisia* and *Juniperus* above 2,000 m, with many valleys having scattered forests of *Pinus* at high altitudes (Zahler and Woods, 1997). *E. fimbriatus* is

confined mainly to the Himalayan moist temperate forest that has a mixture of deciduous and coniferous tree species. Compared to *P. petaurista*, *Eogalucomys* is more adaptable to less mesic conditions and to living in drier regions where the forest is predominantly coniferous or pine forest, such as deodar (*Cedrus deodara*), Holly Oak (*Quercus ilex*) (Roberts, 1997), at elevation ranging from 2,000m to the limits of the tree-line. It is also found in spruce forest (*Picea smithiana*), such as in Indus Kohistan regions where *Petaurista* does not occur. *E. fimbriatus* feeds briefly upon the leaves and cones of pine, walnut, and barks of some trees (Chakraborty, 1981), which are partially similar to the food items of *P. petaurista* (Nowak, 1999).

The particolored flying squirrel *H. alboniger* is a small size flying squirrel and distributed in Yunnan, Sichuan, and S China, at altitudes of 1,500 – 3,300m China. It is smaller than *Eoglaucomys fimbriatus* (Figure 5.13). According to local people, *H. alboniger* feeds on oak trees and pine trees to which they are probably attracted by the acorns, cones, young shoots, and buds of fir or pine. In this region, other flying squirrels with large size, such as *Petaurista* and *Trogopterus*, were also observed in the same habitat, even in the same tree. I had heard them actively feeding on such trees at night during my field trips in Luchun of Yunnan, China, in 2000, implying their coexistence but distinctive in feeding preference. Because *H. alboniger* is strictly nocturnal and spend the day curled up asleep in hollow trees or the hidden holes, practically nothing is known about its biological activities.

5.4.2 Phylogenetic Status of *H. electilis*

Phayre's flying squirrel (*H. phayrei*) (Wang, 2002) and Hainan flying squirrel (*H. electilis*) (Corbet and Hill, 1992) are other valid species of *Hylopetes* in China, occurring in Fujian, and the island of Hainan. Hainan flying squirrel is a rather small species and is

restricted to the island of Hainan, China (Figure 5.14). The type specimen used for species elevation is an adult female skin with the corresponding skull, which was collected by Clifford H. Pope from Namfong, Hainan, China, during the central Asiatic expeditions in April 1923 (Ellerman, 1940). It is now represented in the collection of American Museum of Natural History with catalog number No. 58177. The skull shows short rostrum and low, uninflated bullae. The skin is with pale russet back, grading into fuscous on the upper part of the membrane, naked ears, and a distichous tail.

The validity of the Hainan flying squirrel has been controversial for a long time; even to date it is still referenced as different taxonomic group (Corbet and Hill, 1992; Zhang et al., 1997; Nowak, 1999; Wang, 2002). *H. electilis* used to be maintained as a distinct species (Ellerman, 1940; Corbet and Hill, 1991; Nowak, 1991), but is demoted as a subspecies of *H. phayrei* in recent references (Corbet and Hill, 1992; Wilson and Reeder, 1992; Nowak, 1999; Wang, 2002), or a species of the genus *Petinomys* (Zhang et al., 1997). The data in this study show that *H. electilis* is morphologically similar to *H. phayrei*, demonstrating their similar living environments. Both Hainan and Fujian belong to the same Chinese zoological region. A moist, monsoon climate during the summer gives way to cool, dry air in the winter. The habitat is a tropical, subtropical, or evergreen forest, and is within reach of the southwest and south monsoon. The simplest interpretation of the similar morphological structures between *H. electilis* and *H. phayrei* might be the adaptations to the similar living conditions. The molecular data presented in this study contrast with the morphological result. The genetic difference either between *H. electilis* and *H. alboniger* (12.3%) or between *H. electilis* and the population of *H. phayrei* (11.4%) in Thailand contradict the present view of the classification, which

assumes that *H. electilis* is a subspecies of *H. phayrei*. In fact, the most easily recognized symptom of disagreement between molecular and morphological character information manifests itself in different topologies resulting from separate analysis. The findings in this study are concordant with the recent claims that morphological data have less utility in systematic studies than do molecular data (Baker et al., 1998). The phylogenetic differentiation between *H. electilis* and both *H. phayrei* and *H. alboniger* revealed in this study confirms the species validity of *H. electilis*, which might be related to genetic isolation. The island of Hainan is about one million years old and has two endemic mammal species, the Hainan moonrat and Hainan flying squirrel. The isolation and rejoining between the island and the mainland of China alternatively reduced and facilitated the gene flow of populations as fluctuations of sea level. This is an ideal situation for speciation and many endemic species have evolved. As the island populations regained genetic contact with mainland, some could still interbreed with the mainland forms and were thus still the same genetic species, such as the Hainan giant flying squirrel (*P. hainana*) (See Chapter 4 for detail), but some had diverged enough for reproductive barriers to be erected and were thus new species, like *H. electili*s here.

One of the most serious gaps in the knowledge of Hainan flying squirrel is in the area of ecology, the study of other animals and plants in relation to each other and to its environment. For *H. electilis* and another flying squirrel *Petaurista* in the island of Hainan, virtually nothing is known, not even preferred habitat, so any field observation is likely to prove valuable. Much remains to be done in future.

5.4.3 Phylogenetics and Biogeography of *Eoglaucomys* and *Hylopetes*

Molecular sequences in extant taxa can be used to infer speciation and biogeographic historical distributions produced by range shifts in shallow time (Riddle,

1996), and thus provide a basis for constructing bridges between historical biogeographic, paleoecological, and ecological biogeographic perspectives. Genetic drift, adaptation to varying conditions, and genetic isolation can be expected to quickly evolve new species. *H. nigripes*, *H. lepidus*, and *H. spadiceus* are distributed in SE Asia, which are externally similar to *H. electilis* and *H. phayrei* (Figure 5.15 and Figure 5.16). The estimated divergence time inferred from the substitution rate at the third codon positions of cytochrome b gene suggests that *Eoglaucomys* had an early divergence from other groups, which is about 24 to 33 million years ago, the middle Oligocene. The split time between the populations of *Hylopetes* was in the late Miocene and the early Pliocene, about 4 to 6 million years ago (Table 5.8). This is consistent with the Oshida's et al. (2000a) conclusion. The patterns of morphological and molecular characters and species associations in this study correspond with variations of local geological formations in Pakistan, China, and SE Asia (Figure 5.6 to Figure 5.11).

The radical geological and climatological changes over relatively short periods of time have had remarkable effects on the evolution of mammals in southeastern Asia. South China is located at the crossroads of southeastern Asia and has been a bypass for animal dispersal from mainland Asia southward into the Indo-Malayan region. Of particular importance for the southeastern fauna were the changes in sea level which accompanied the isolation and rejoining of populations during the last few million years. In southeastern Asia, mammals are overwhelmingly affiliated with the Oriental fauna, with relatively few species of Palearctic affinities. Many species have migrated south from Burma and southern China along the forested mountains of the Thai-Burma border into Malaya, Sumatra, Java, and Borneo, such as gibbon (Lekagul and McNeely, 1988).

Similarly, many species have expanded northward and higher in elevation with warmer conditions. The morphological similarities and the genetic differences within *Hylopetes* groups concluded from the present study also prove this hypothesis. The genetic similarity between *H. alboniger* and SE Asiatic groups reveals their close phylogenetic relationship. For example, the early divergence of *Eoglaucomys* is closely associated with its unique morphological structures and high genetic difference. The similar morphological and genetic characters in the populations of *H. lepidus* and *H. spadiceus* are associated with their similar living conditions and recent divergence. The divergence between *H. phayrei* and *Petinomys* had occurred in southeastern or south Asia approximately 2 to 3 million years ago (Oshida et al., 2000a), suggesting a late and rapid divergence.

Based on the paleontological data, the Miocene fossil remains of *Petinomys* found in Europe imply a radiation of flying squirrels in Europe during the Oligocene-Miocene period. Accepting this hypothesis, there is a close biogeographical relationship between *Eoglaucomys* and *Hylopetes*. During the Oligocene-Miocene radiation in Europe, *Eoglaucomys* and *Hylopetes* diverged from their common ancestor stock. With the tectonic movements in the Miocene, these two stocks, *Eoglaucomys* and the ancestor of recent *Hylopetes*, migrated southward independently. *Eoglaucomys* migrated to the western extreme of the great Himalayan chain before the main uplift of the northwestern Tibetan Plateau that began ca. 4.5 million years ago (Zheng et al., 2000). About 6 million years ago, the late Miocene, the SE Asiatic and the Chinese *Hylopetes* diverged from their ancestor. During the Pliocene-Pleistocene period, the southeastern Asiatic

branch invaded to Southeast or South Asia along the forested mountains of the Thai-Burma border.

However, the ranges of these flying squirrels are still poorly known, with nearly every new collection revealing new distribution limits. The taxonomy and the phylogentic relationships among *Hyloeptes* are still unsettled, especially at the subspecies level. Further research will undoubtedly lead to changes in details.

5.5 Conclusion

This chapter focuses mainly on the phylogenetic relationships between *Eoglaucomys* and *Hylopetes* based on the morphometric and molecular analyses. The following results are concluded.

1. *Eoglaucomys* differs morphologically and genetically from *Hylopetes* in China and SE Asia. It is reasonable to recognize *Eoglaucomys* as a valid genus.

2. *H. electilis* is a possible valid species of *Hylopetes* based on its genetic characters, although it shares similar morphological characters with *H. phayrei* in skull features.

3. *Eoglaucomys* diverged from other *Hylopetes* populations as early as 24 million years ago, the middle to late Oligocene. All *Hylopetes* groups including the SE Asiatic and Chinese populations diverged from the ancestor stock of *Hylopetes* in the early Pliocene, after the European radiation of flying squirrels in Oligocene-Miocene. The migration of *Hylopetes* to the present geographical distribution is due to the tectonic movements of the Himalayas during the Pliocene-Pleistocene period.

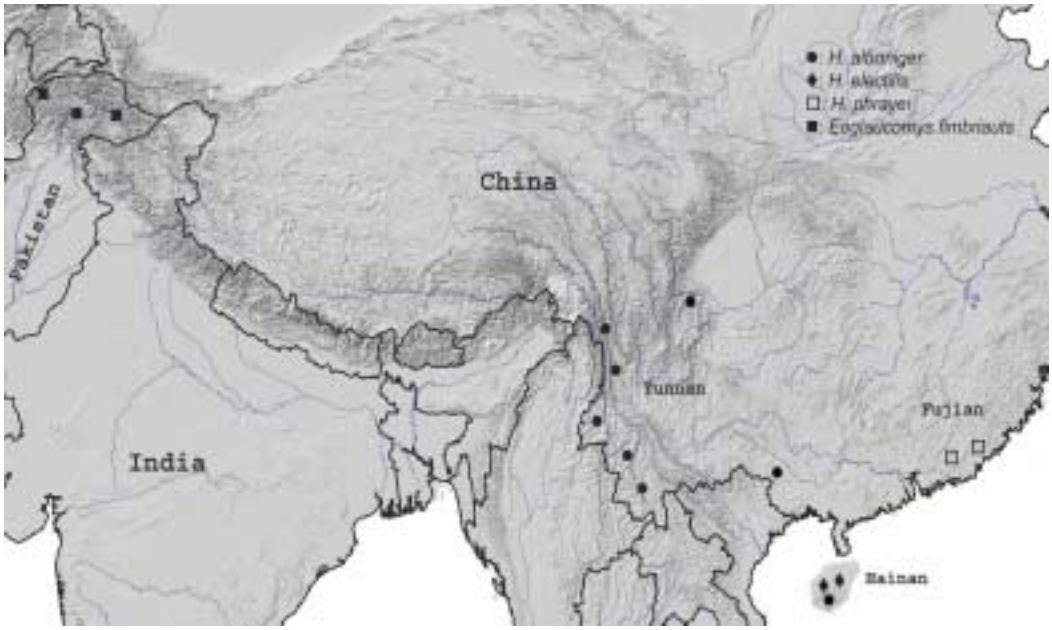

Figure 5.1 Distribution of Chinese *Hylopetes* and *Eoglaucomys*

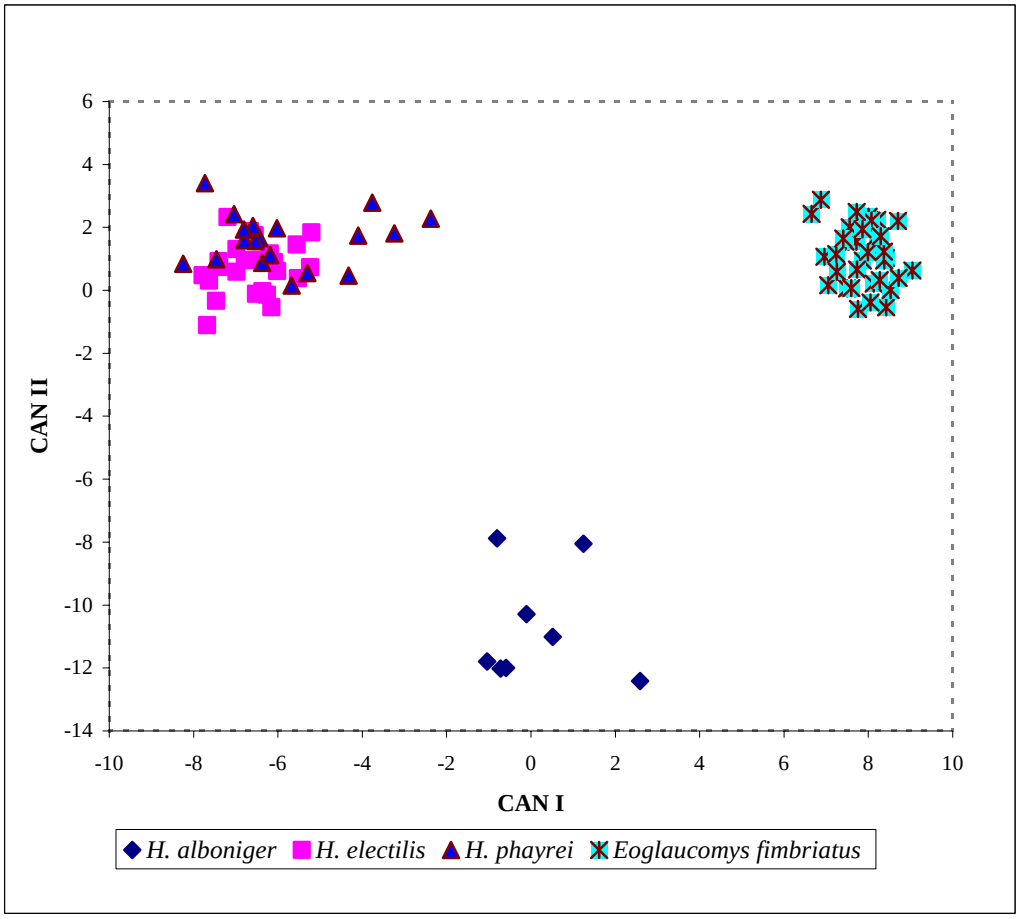

Figure 5.2 Discriminant function analysis between Chinese *Hylopetes* and *Eoglaucomys* onto function 1 and function 2

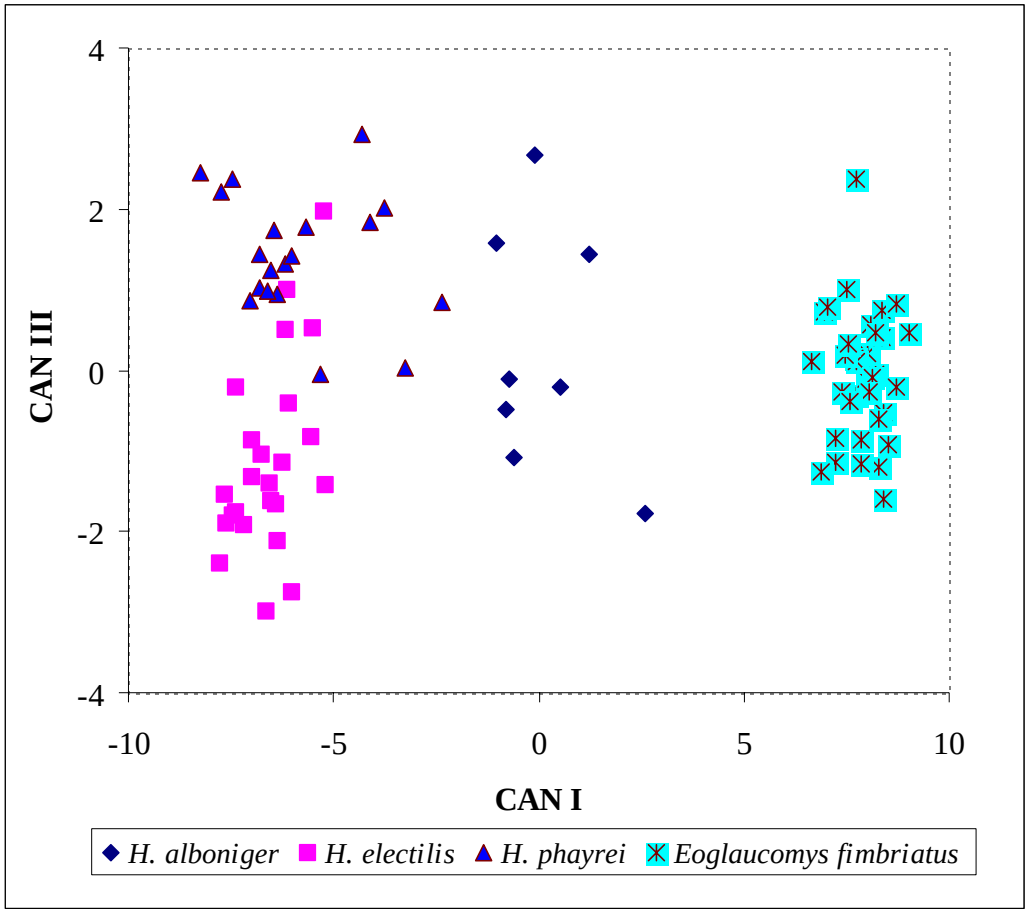

Figure 5.3 Discriminant function analysis between Chinese *Hylopetes* and *Eoglaucomys* onto function 1 and function 3

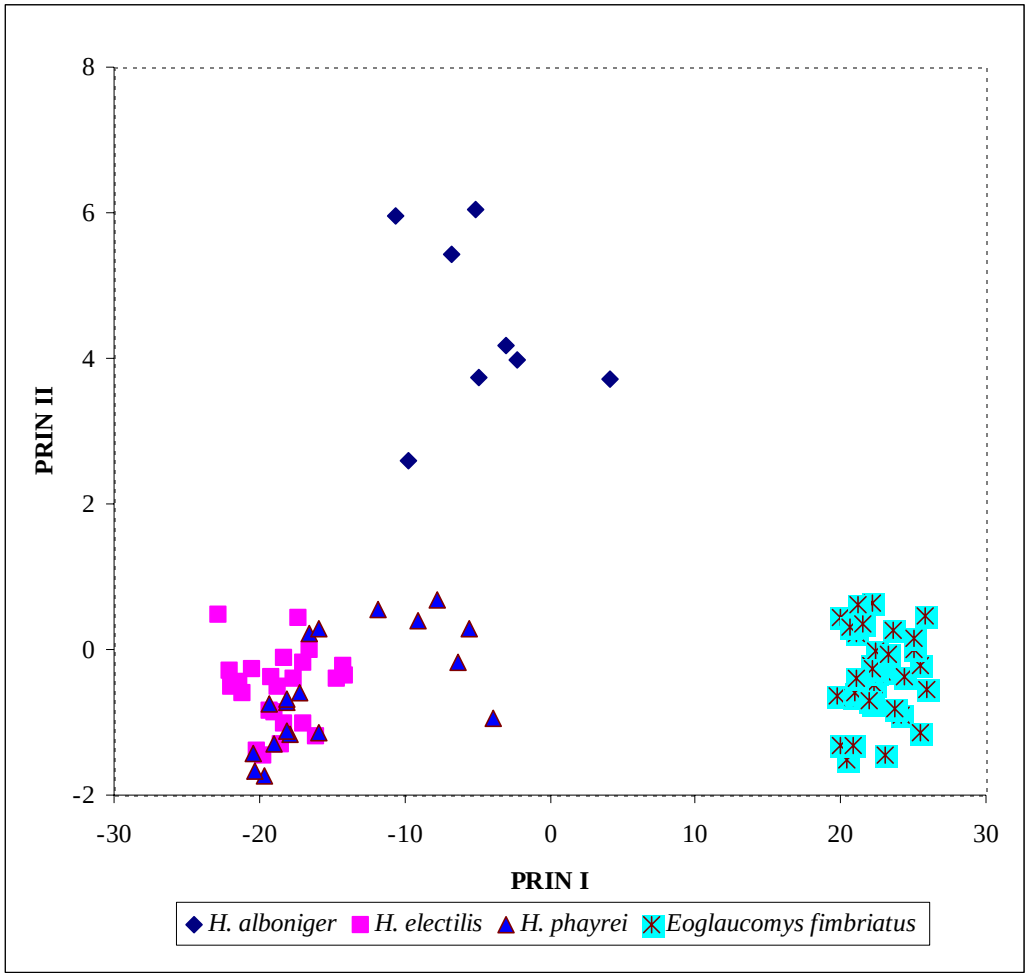

Figure 5.4 Principal components analysis of Chinese *Hylopetes* and *Eoglaucomys* onto factor 1 and factor 2

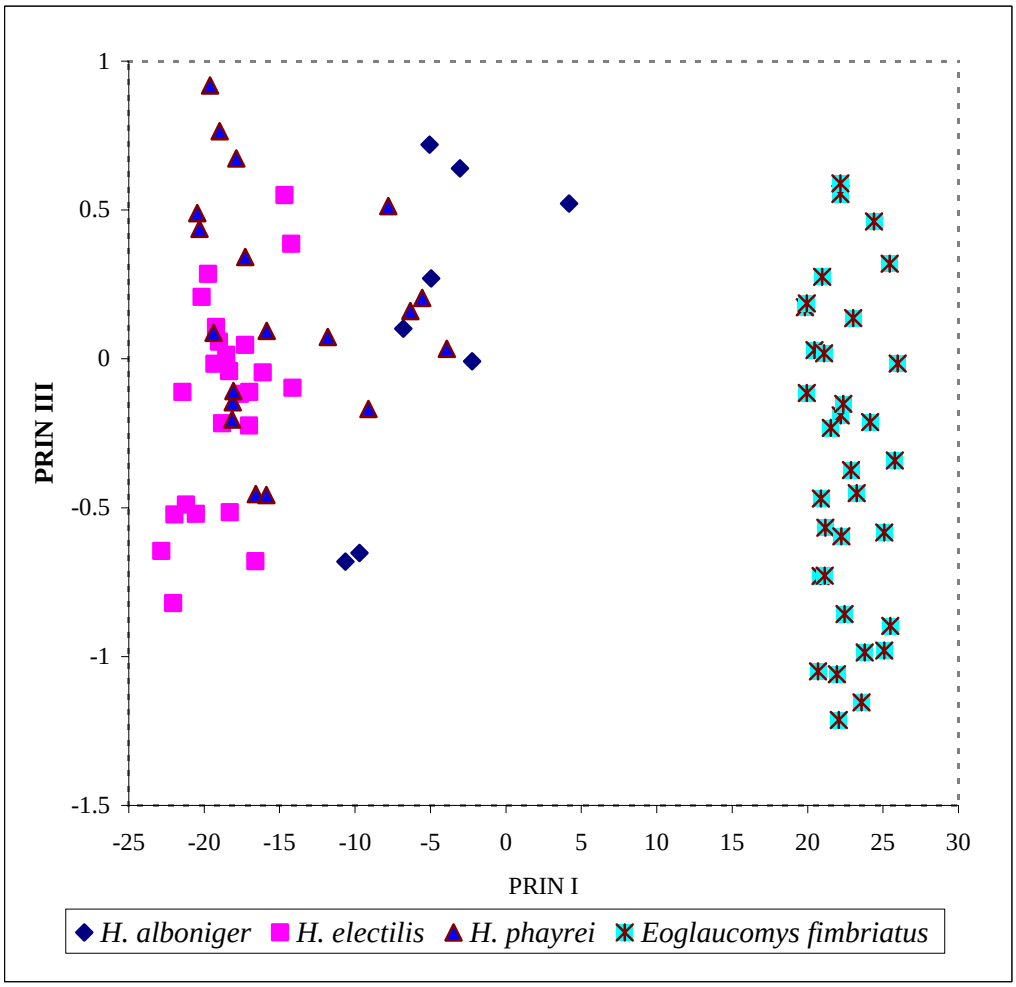

Figure 5.5 Principal components analysis of Chinese *Hylopetes* and *Eoglaucomys* onto factor 1 and factor 3

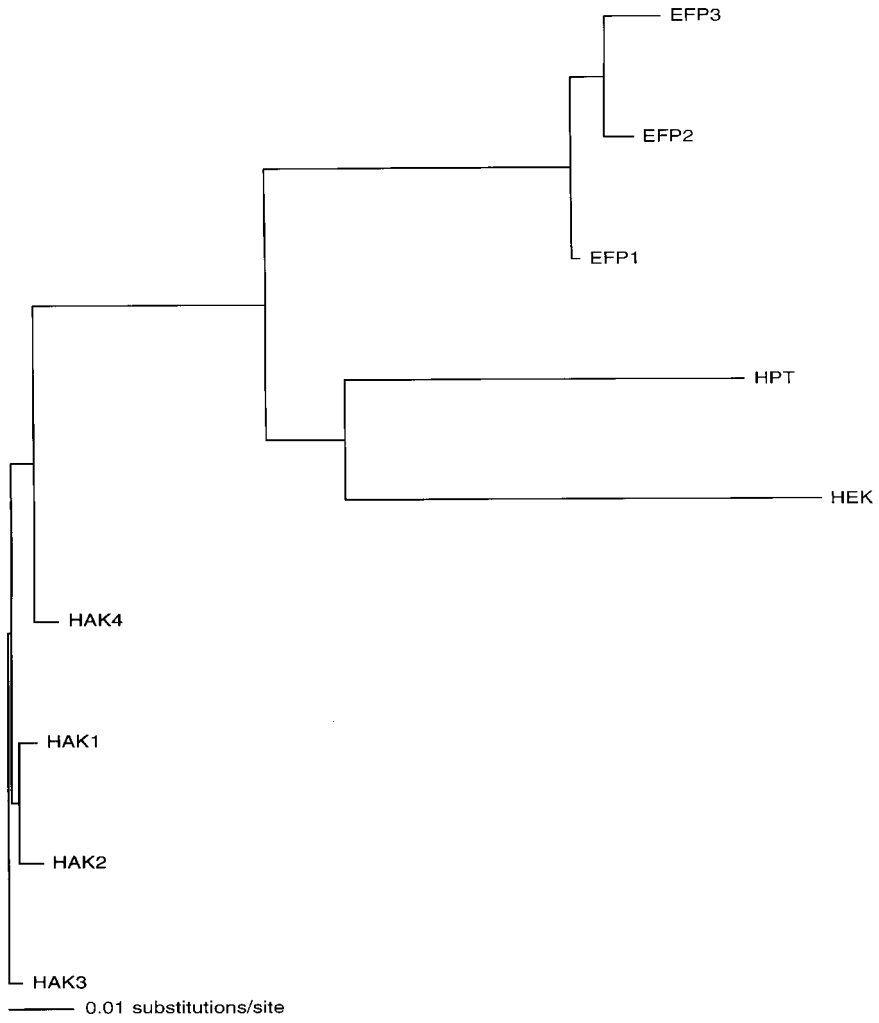

Figure 5.6 Phylogenetic topology of *Eoglaucomys* and Chinese *Hylopetes* constructed using the neighbor-joining method based on the partial sequences (400 bp) of cytochrome b gene. Scales in the tree represent branch length in terms of nucleotide substitutions per site. Sample abbreviations are coded in Table 5.3.

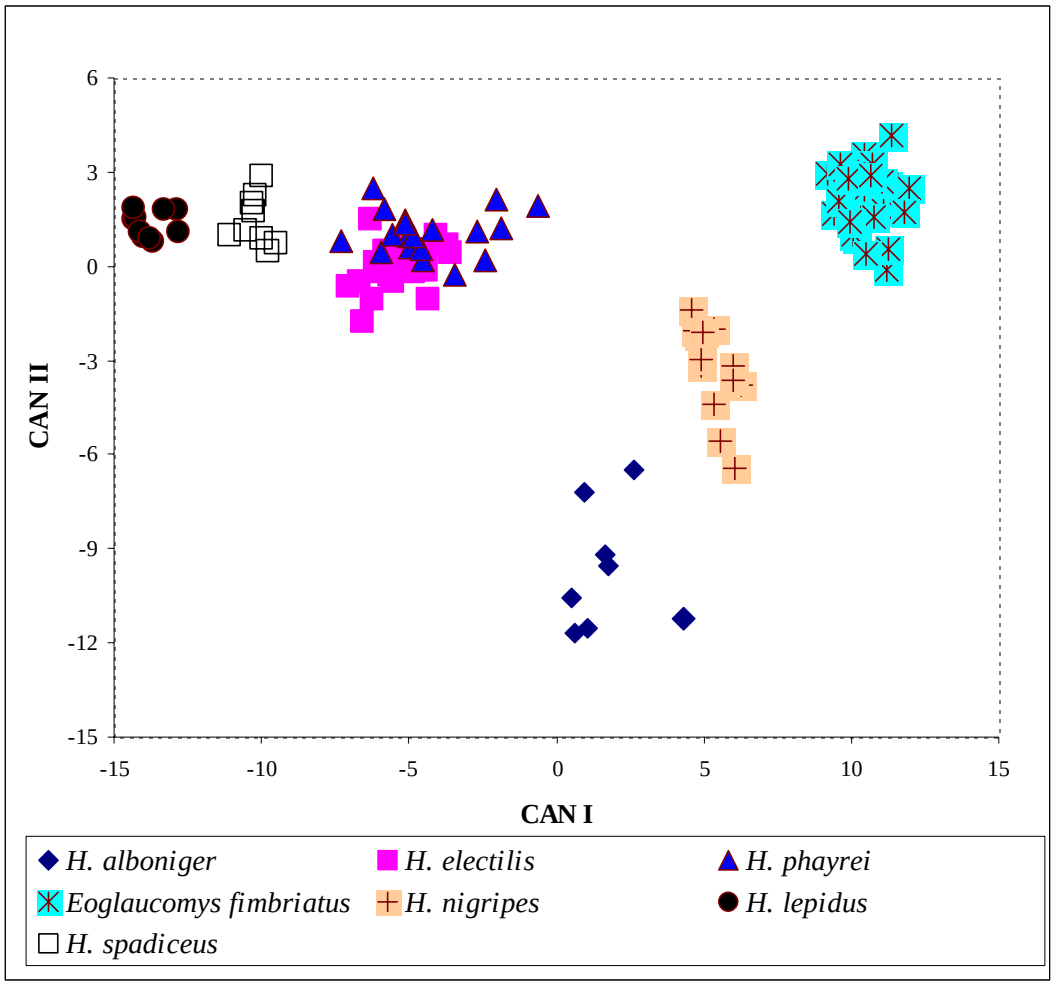

Figure 5.7 Scatter-plot of *Hylopetes* and *Eoglaucomys* along the first two discriminant functions

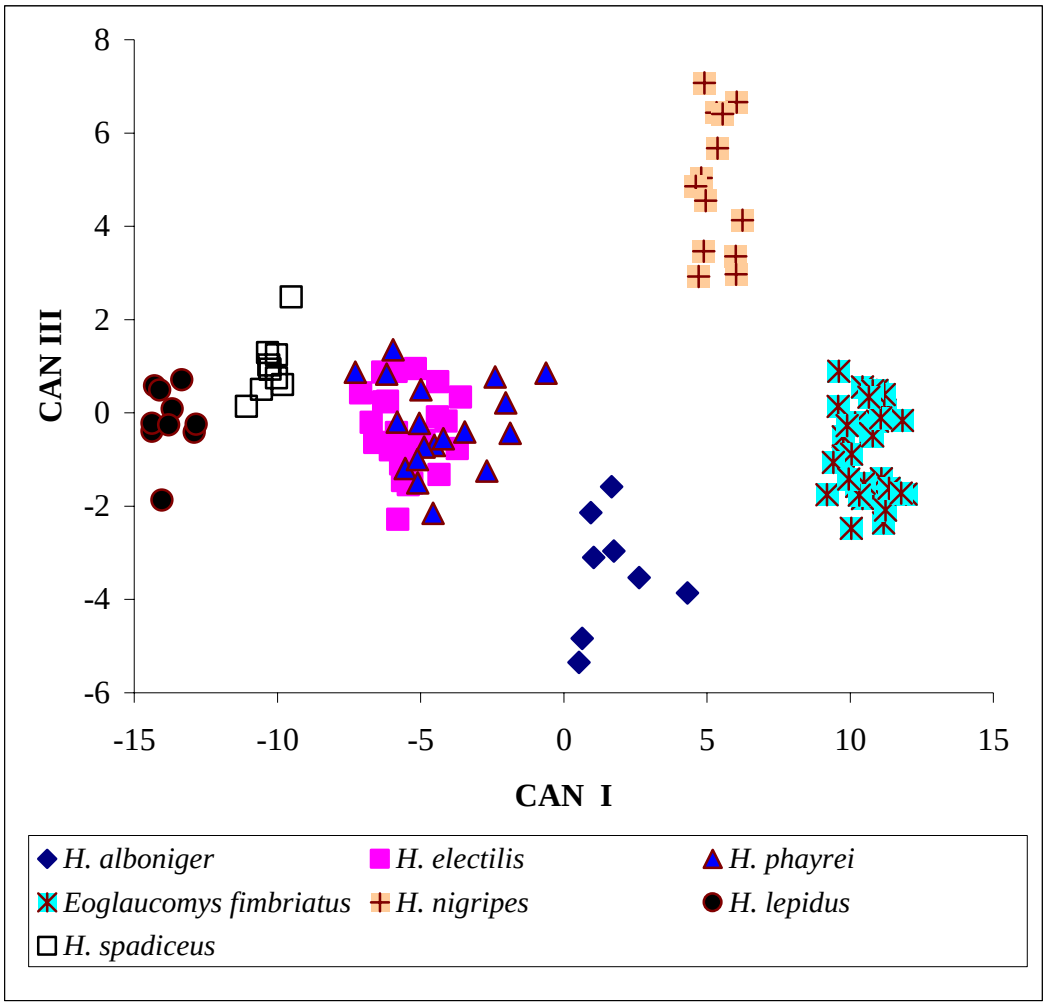

Figure 5.8 Scatter-plot of *Hylopetes* and *Eoglaucomys* onto the first and the third discriminant function

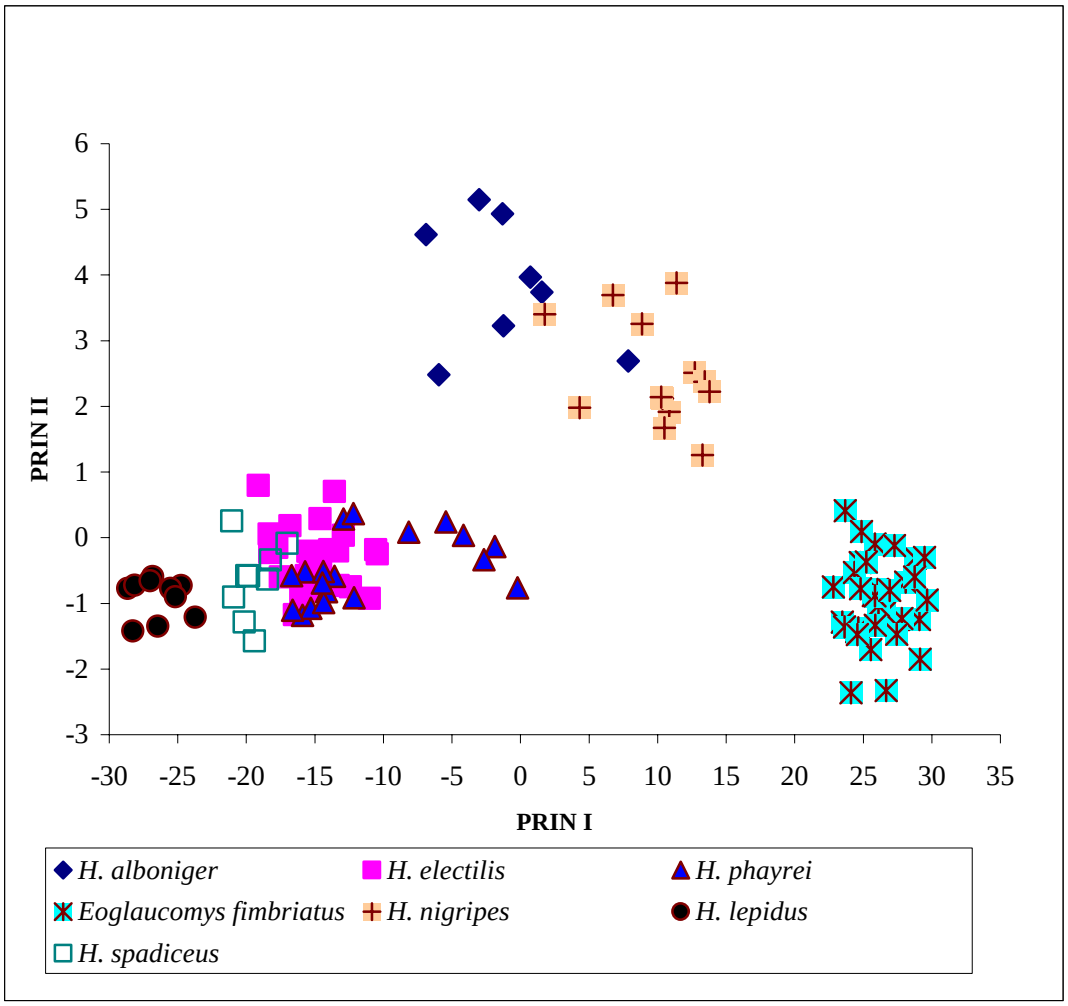

Figure 5.9 Plot of principal components analysis of *Hylopetes* and *Eoglaucomys* onto factor 1 and factor 2

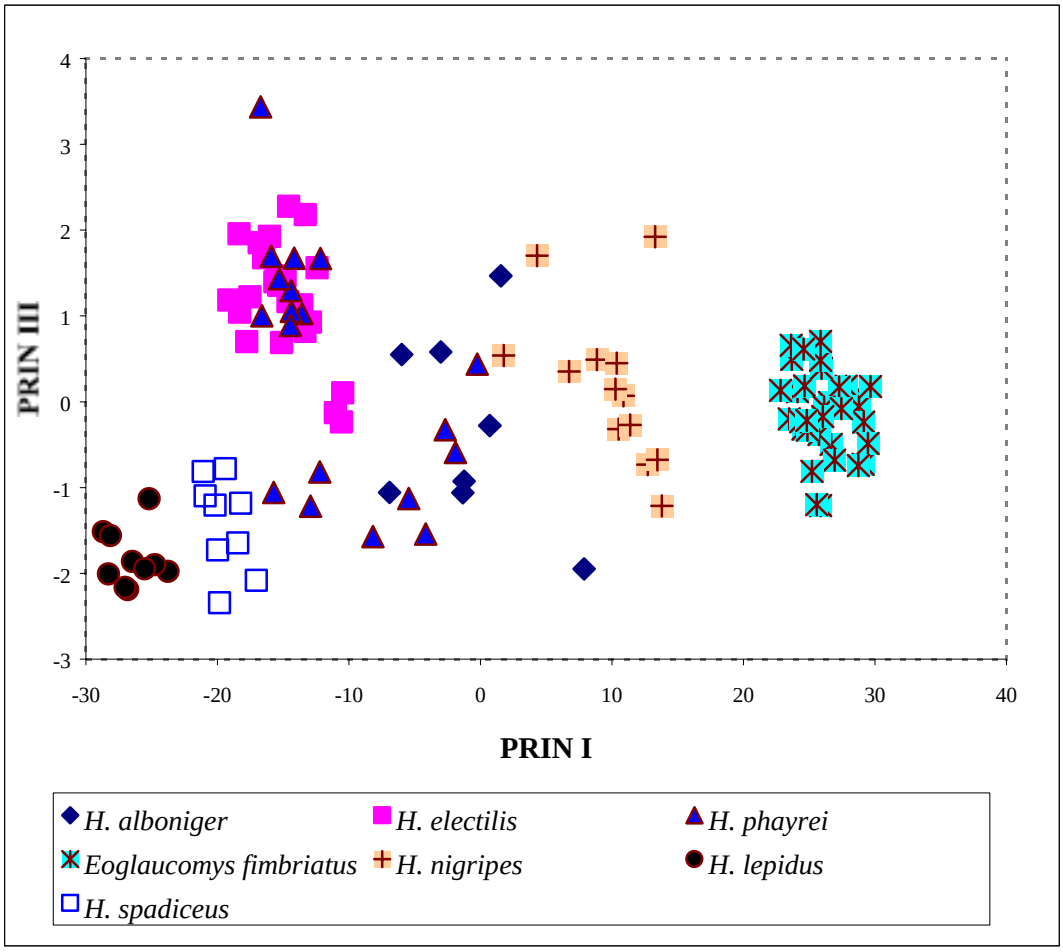

Figure 5.10 Plot of principal components analysis of *Hylopetes* and *Eoglaucomys* onto factor 1 and factor 3

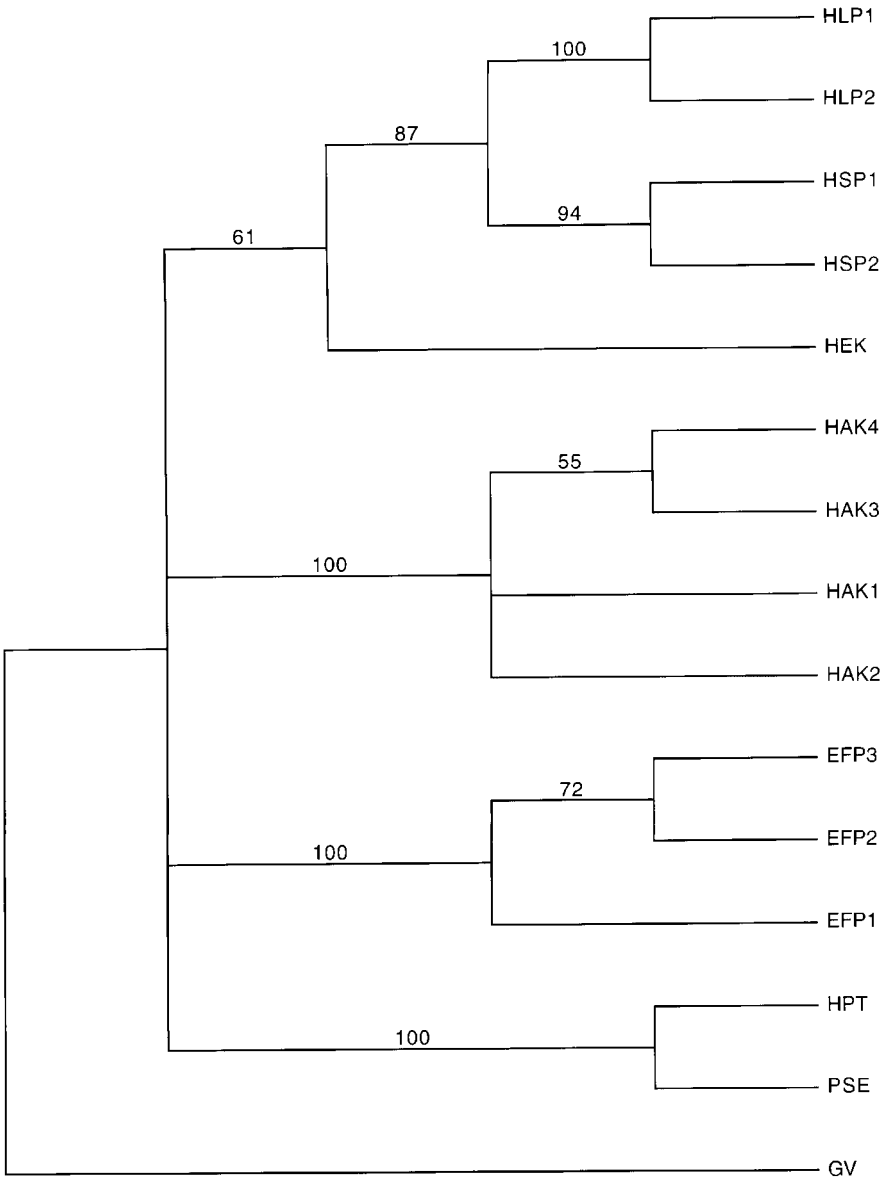

Figure 5.11 Phylogenetic tree of *Hylopetes* and *Eoglaucomys* generated by MP method on partial sequences (375 bp) of cytochrome b gene. Numbers above branches are the bootstrap values (%). Sample abbreviations are coded in Table 5.3.

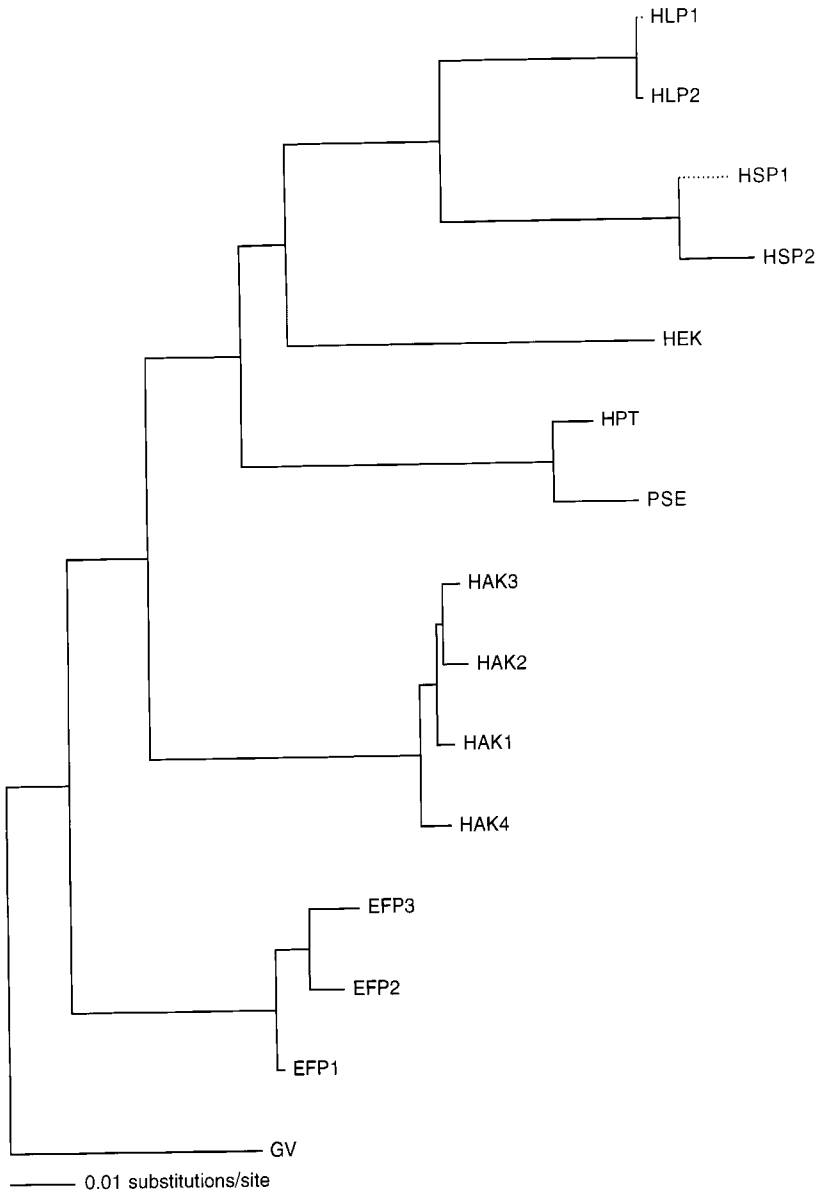

Figure 5.12 Phylogenetic tree of *Hylopetes* and *Eoglaucomys* generated by NJ method on partial sequences (375 bp) of cytochrome b gene. Scales in the tree represent branch length in terms of nucleotide substitutions per site. See sample abbreviations in Table 5.3.

Figure 5.13 *Eoglaucomys fimbriatus* and *Hylopetes alboniger*

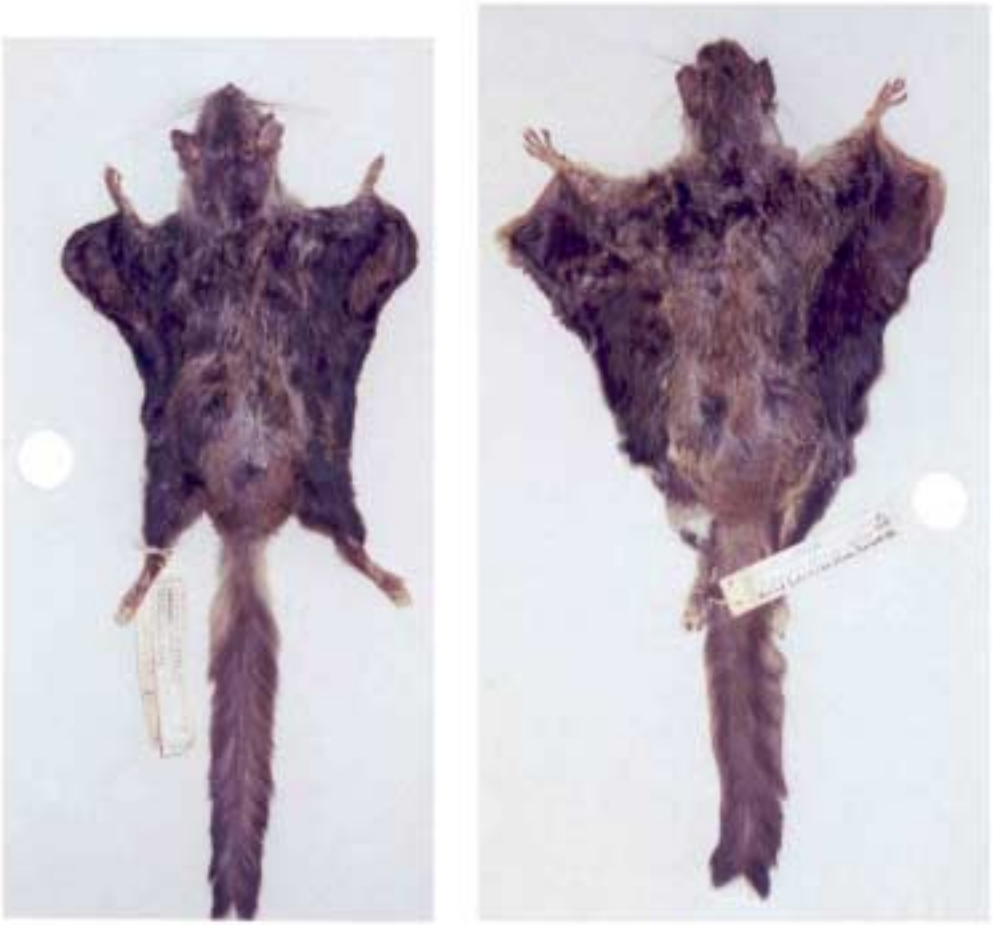

Figure 5.14 *Hylopetes electilis* and *Hylopetes phayrei*

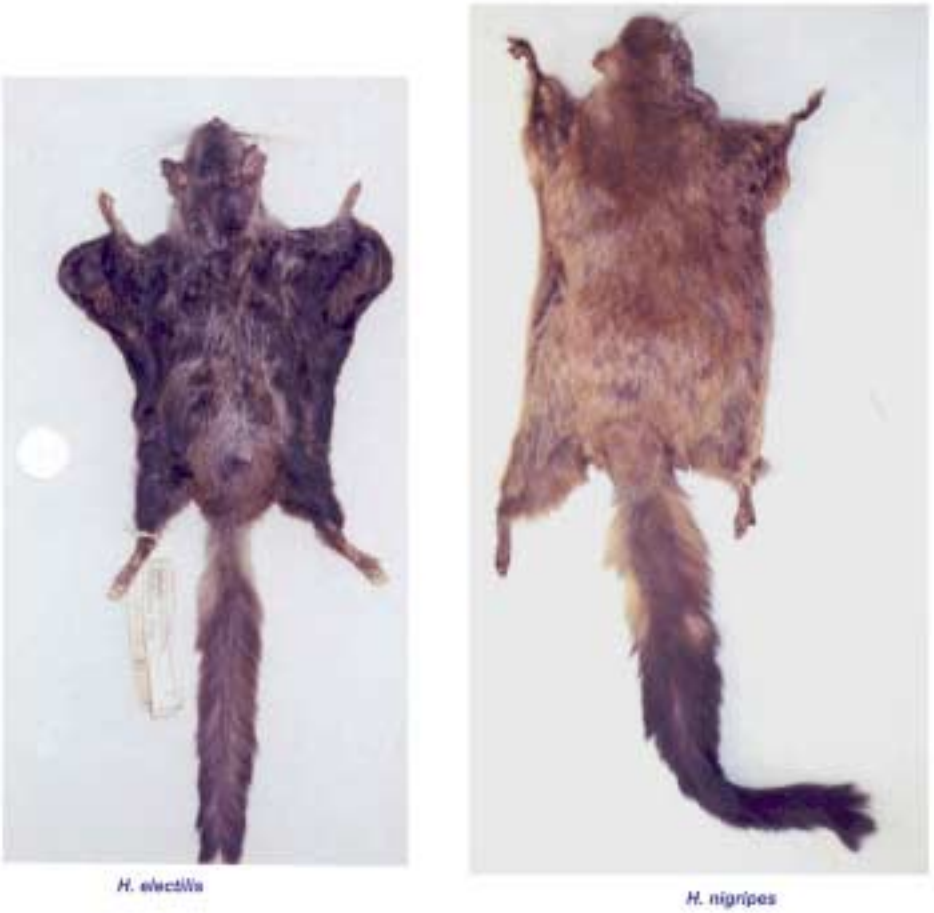

Figure 5.15 *Hylopetes electilis* and *Hylopetes nigripes*

Figure 5.16 *Hylopetes lepidus* and *Hylopetes spadiceus*

CHAPTER 6
PYLOGENY OF FLYING SQUIRRELS IN THE TRANS-HIMALAYAS AND OTHER PARTS OF CHINA

6.1 Introduction

Flying squirrels are better known by sight to people than most non-volant arboreal and terrestrial squirrels. It has a furred gliding membrane along the sides of body from the arms to the legs, even to the neck and tail in some genera. The membrane consists of sheets of muscles that can be tensed or relaxed. Varying the tension of membranes and the slant of the tail controls the direction of glide (Gupta, 1966). All flying squirrels are similar in postcranial anatomy and have evolved adaptations to the same locomotor problems (Thorington et al., 1997). Gliding has evolved among recent mammals at least six different times (Walker, 1975), but the complex wrist anatomy of flying squirrels provides evidence that gliding evolved only once among sciurids and that flying squirrels are a monophyletic group (Thorington, 1984; Thorington et al., 1998). This is contrast to Black's (1963) diphyletic and Mein's (1970) polyphyletic hypotheses.

Flying squirrels are nocturnal and are found in both the Old and New World. Fourteen or fifteen forms containing 38 - 52 species have been given generic rank in recent years. Based on the dental characteristics of the extant and extinct forms, Mein (1970) assembled all flying squirrels into three distinct groups (Table 1.2). However, the phylogeny does not support Mein's three-group hypothesis; instead, the extant flying squirrels are divided into four groups (Table 6.1). Figure 6.1 is the most recent

phylogenetic reconstruction of flying squirrels, tree squirrels, and fossil squirrels based on 44 dental characteristics (Pappas et al., 2002).

Table 6.1 Classification of the extant flying squirrels determined from 44 dental characters

Group	Genus
I	*Glaucomys, Eoglaucomys, Petaurillus, Iomys, Pteromys, Aeretes, Petaurista, Eupetaurus*
II	*Trogopterus, Pteromyscus, Belomys*
III	*Petinomys, Hylopetes*
IV	*Aeromys*

Table 6.2 Chinese flying squirrels other than *Petaurista*

Genera	Species	Distribution	Habitat
Trogopterus	*T. xanthipes*	NW and SW China, Hubei, Guangxi, Henan	Subtropical and warm-temperate forest
Belomys	*B. pearsonii*	Yunnan, Guizhou, Guangxi, Hainan, Guangdong, Taiwan	Tropical and subtropical forest
Aeretes	*A. melanopterus*	Heiberi, Sichuan, Gansu	Mountain forest
Pteromys	*P. volans*	NE and NW China, Hebei, Henan, Sichuan	Forest
Eupetaurus	*E. cinereus*	Yunnan	Subtropical evergreen broadleaf forest
Hylopetes	*H. alboniger*	Sichuan, Yunnan, Guizhou, Zhejiang, Xizang, Hainan	Mountain forest
Petinomys	*P. electilis*	Hainan, Fujian, Guangxi	Tropical and subtropical forest

There are seven or eight genera consisting of 14 or 15 recognized species of flying squirrels in China (Corbet and Hill, 1991, 1992; Wilson and Reeder, 1992; Nowak,

1999; Wang, 2002) (Figure 6.2). Table 6.2 shows the distributions and habitats of all Chinese flying squirrels except for *Petaurista*, which was discussed in Chapter 4.

Flying squirrels in mainland of China are mainly distributed in four geographical regions: southwestern China (Tibet, Yunnan, and Sichuan); southern China including Hainan island; northern China; and central China (see Table 1.6 for details). According to Pappas' et al. (2002) classification, the Chinese flying squirrels are categorized into three groups: *Pteromys*, *Aeretes*, *Petaurista*, *Eupetaurus* as one group, *Trogopterus* and *Belomys* as another group, and *Petinomys, Hylopetes* as the third group. The present taxonomic controversies at the specific level are mainly within the genera *Hylopetes* and *Petaurista*. The major information about Chinese flying squirrels is from the morphological comparisons and external descriptions, such as Allen's (1940) *The Mammals of China and Mongolia*, and Corbet and Hill's (1992) *The Mammals of the Indomalayan Region.* However, the specimens of flying squirrels, especially the skins and fluid, are not as common in museums or research institutes as the non-flying squirrels, and many of the forms are very little known. The latest taxonomical revisions are either a summary of the previous studies (Nowak, 1999; Zhang et al., 1997), or a pure morphological taxonomy that is based on the previous data and the observations of external structures (hairs, skins, pelage colors) (Wang, 2002). Many species remain nothing known and taxonomically controversial. Thus far, none of revisions in the literature is based on the morphologically quantitative comparisons or molecular analyses.

In this chapter, I focus on the comparative studies of the trans-Himalayan flying squirrels using both morphological and molecular techniques. The flying squirrels of the

eastern and the western trans-Himalayas, including *Eupetaurus*, *Petaurista*, *Eoglaucomys*, and *Hylopetes*, are compared (Figure 6.3), and all flying squirrels distributed in China are discussed. The purposes are to seek the answers for the following questions:

1. What are the phylogenetic relationships between the eastern and the western trans-Himalayan flying squirrels?
2. What are the phylogenetic relationships among Chinese flying squirrels?
3. Are the phlyogenetic relationships among Chinese flying squirrels obtained from morphological and molecular studies consistent with their geographical variations?

6.2 Materials and Methods

6.2.1 Specimens

In the morphometric study, 188 specimens representing 14 species were examined and 14 variables were measured (Table 6.3). A total of 25 samples including 18 species were used in the molecular study for reconstructing the phylogenetic relationships among the eastern and the western trans-Himalayan flying squirrels (Table 6.4). The tissues used in this study were collected from museums and academic institutes in China and US (See Chapter 3, 4, and 5 for detail). The sequence data of some species were retrieved from GenBank of NCBI that were provided by Oshida et al. (2000a, b, and c) and Arbogast (1999). The fresh tissues of flying squirrels and tree squirrels were either collected from the localities in where the species are distributed, or obtained from the mammal department of Kunming Institute of Zoology, the Chinese Academy of Sciences, Kunming, China. Two tree squirrels (*Callosciurus erythraeus*) in Kunming, China, were used as the outgroup taxon.

Table 6.3 Species and localities of flying squirrels used in morphometric analysis

Species	Specimen	Sex	Locality	Museum
Aertes melanopterus	3	2 F, 1 M	Hebei, China	AMNH
Belomys pearsoni	4	2 F, 2 M	Indochina	AMNH, USMNH
Trogopterus xanthipes	3	2 F, 1 M	Beijing, China	BIZ
Pteromys volans	11	7 F, 4 M	Jinin, China	BIZ
Petinomys setosus	5	3 F, 2 M	Mentawai Island	AMNH
Petaurista albiventer	9	5 F, 4M	Yunnan, China	KIZ
P. yunanensis	15	7 F, 6 M, 2 U	Yunnan, China	BIZ, KIZ
P. philippensis	9	3 F, 5 M, 1 U	Yunnan, China	KIZ
P. elegans	12	5 F, 5 M, 2 U	Yunnan, China	KIZ
P. alborufus	15	6 F, 8 M, 1 U	Hupei, China, Burma	AMNH
P. albiventer	32	13 M, 19 M	N Pakistan	USMNH
Eoglaucomys fimbriatus	34	20F, 14 M	W Pakistan	USMNH
Hylopetes alboniger	7	4 F, 3 M	Yunnan, China	KIZ, BIZ
H. phayrei	19	9 F, 10 M	Mandalaypopa, Burma	AMNH
Glaucomys volans	10	4 F, 6 M	Florida, US	UF
Total	188			

Note: F = Female, M = Male, U = Unknown sex

Table 6.4 Samples of flying squirrels used in molecular study

Species	Code	Museum ID	Locality
E. cinereus	ECK	KIZ: 73372	Yunnan, China
E. cinereus	ECF	UF: 26583	Gilgit, Pakistan
P. albiventer	PPF	USMNH: 353209	N Pakistan
P. philippensis	PPH	KIZ: Fresh tissue	Pianma,Yunnan, China
P. yunanensis	PYK	KIZ: Fresh tissue	Gongshan, Yunnan, China
P. hainana	PHK	KIZ: 22686	Hainan, China
P. xanthotis	PTK	QIZ: 85063	Gansu, China
P. alborufus	PAK	KIZ: 006679	Sichuan, China
P. elegans	PEK	KIZ: 84354	Mile, Yunnan, China
H. alboniger	HAK	KIZ: 74546	Luchun, Yunnan
Eo. fimbriatus	EFP	USMNH: 353237	W Pakistan
H. lepidus	HLP	USMNH: 488619	W Malaya
H. spadiceus	HSP2	USMNH: 48495	W Malaya
Tr. xanthepes	TRX1	KIZ: 73378	Deqing, Yunnan
Tr. xanthepes	TRX2	KIZ: Cell Bank	Yunnan
Pteromys. volans	PVO	KIZ: None	NorthEast, China
Belomys pearsonii	BPE	KIZ: 2970	Luchong, Yunnan
Callosciurus erythraeus	TSK1	KIZ: Fresh tissue	Kunniming
Callosciurus erythraeus	TSK2	KIZ: Fresh tissue	Kunniming
P. p. petaurista	PPB	AF063067	Borneo, E Malaysia
H. phyrei	HPT	AB030259	Thailand
Petinomys setosus	PSE	AB030260	Indochina peninsula
Pteromys volans	PVO	AB023910	Japan
Glaucomys volans	GV	AF063066	Tennessee, US

6.2.2 Methods of Phylogenetic Analyses

Most specimens used here were selected from the previous chapters. Multivariate analyses, discriminant function analysis and principal components analysis, were applied for the comparative studies between the eastern and the western trans-Himalayan flying squirrels, and among all Chinese flying squirrels. The detailed information of the morphometric techniques was described in Chapter 4. To reduce the sexual dimorphism, the equal male and female specimens of each species were selected in morphometrics.

Most samples in molecular study were the same as those used in Chapter 3, Chapter 4, and Chapter 5. In addition, the samples of *Trogopterus xanthepes* in Yunnan, *Pteromy volans* in NE China, *Belomys pearsoni* in Yunnan, and *Callosciurus erythraeus* in Kunming, Yunnan, were included. The techniques and protocols of DNA isolation, PCR amplification and purification, and DNA sequencing analysis were the same as those described in Section 3.2 of Chapter 3. The neighbor-joining method and maximum parsimony using a heuristic search algorithm with the 50% majority-rule consensus were used to construct the phylogenetic topology of flying squirrels. Two individuals of *Callosciurus erythraeus* were used as the out-group.

6.3 Results

6.3.1 Comparative Study of the Eastern and the Western Trans-Himalayan Flying Squirrels

To investigate the phylogenetic relationships between the flying squirrels in the eastern and the western trans-Himalayas, three genera consisting of 9 species were examined in multivariate analyses.

Table 6.5 Discriminant function analysis on the eastern and the western trans-Himalayan flying squirrels

Axis	Eigenvalue	Proportion	Cumulative
CAN I	275.49	0.72	0.72
CAN II	73.85	0.19	0.91
CAN III	22.62	0.06	0.97
Canonical score			
Variable	CAN I	CAN II	CAN III
CRANL	0.33	0.83	0.41
BCASEL	0.44	0.75	0.30
CRANW	0.40	0.85	0.32
BPORW	0.39	0.84	0.36
PORCL	0.47	0.79	0.28
PGA	0.36	0.80	0.43
NAL	0.26	0.78	0.42
TBL	0.42	0.70	0.23
DSL	0.14	0.74	0.60
MTRL	-0.48	0.82	-0.28
MTRW	0.09	0.90	0.39
LMDL	-0.77	0.55	0.30
LMDH	0.86	0.49	0.09
LMTL	0.92	0.37	0.07

The results of discriminant function analysis were presented in Table 6.5. The first two axes accounted almost for 91% of the original specimen variance. When all specimens were plotted onto the first two discriminant functions (CAN I and CAN II), five groups were identified (Figure 6.4). *Eoglaucomys*, *Hylopetes*, and *P. albiventer* in

Pakistan were distinguished as distinct groups; *P. alborufus* and *P. elegans* were combined as one group; and *P. albiventer* in Yunnan, *P. yunanensis, P. philippensis* were clustered as one group. The measurements of lower jaw were the key factors responsible for these separations along CAN I. The remaining variables contributing to the observed associations were strongly correlated with CAN II. Function 3 (CAN III), accounting for 6% of the total variance, described all trans-Himalayan flying squirrels as four groups (Figure 6.5). The clusters were similar to those in Figure 6.4, except for *Eoglaucomys*, which was merged into the group of *P. albiventer* in Yunnan, *P. yunanensis,* and *P. philippensis*. DSL was the major variable contributing to CAN III.

When all specimens used in discriminant function analysis were included in principal components analysis, a similar clustering pattern was generated. Table 6.6 presented the results of principal components analysis of trans-Himalayan flying squirrels. The first two principal component factors that accounted for 67% and 29% of the total variance, respectively, partitioned all species into four groups. These groups are concordant with the results of discriminant function analysis. Three genera, *Petaurista*, *Eoglaucomys* and *Hylopetes* are separated as distinct taxa (Figure 6.6). The populations in *Petaurista* were divided into two groups: one group containing *P. albiventer* in Pakistan, *P. alborufus*, and *P. elegans*, and another group comprising *P. albiventer, P. yunanensis*, and *P. philippensis*. CRANL and LMDL were the dominant variables on PRIN I and PRIN II, respectively. The clusters onto PRIN I and PRIN III showed that *P. albiventer* in Pakistan was morphologically similar to *P. albiventer* in Yunnan, *P. yunanensis,* and *P. philippensis*. The remaining species represented distinct taxa along PRIN III (Figure 6.7). MTRL was the morphological variable achieving this discrimination.

Table 6.6 Principal components analysis of trans-Himalayan flying squirrels on the first three factors

Axis	Eigenvalue	Proportion	Cumulative
PRIN I	559.30	0.67	0.67
PRIN II	241.53	0.29	0.97
PRIN III	10.04	0.01	0.98
Eigenvector score			
Variable	PCA I	PCA II	PCA III
CRANL	0.51	0.15	-0.25
BCASEL	0.38	0.02	-0.39
CRANW	0.27	0.05	0.25
BPORW	0.38	0.08	0.23
PORCL	0.13	0.00	0.20
PGA	0.21	0.05	-0.13
NAL	0.17	0.07	-0.21
TBL	0.07	0.00	-0.04
DSL	0.10	0.06	-0.19
MTRL	0.06	0.21	0.68
MTRW	0.12	0.09	0.08
LMDL	0.08	0.82	0.03
LMDH	0.41	-0.35	0.20
LMTL	0.29	-0.34	0.14

6.3.2 Phylogenetic Relationships among Flying Squirrels in China

Table 6.7 showed the percentage of genetic differences and the numbers of transversional and transitional substitutions based on pairwise comparison of the cytochrome b sequences (375 bp). When tree squirrel (*Callosciurus erythraeus*) was

used as the outgroup, except for *Eupetaurus*, all trans-Himalayan flying squirrels were branched into five similar groups in both MP tree and NJ tree. All populations of *Petaurista*, *Hylopetes*, and *Pteromys* were isolated as distinct clades (Figure 6.8 and Figure 6.9). *Glaucomys* and *Eoglaucomys* represented a clade. *Eupetaurus* was either separated as a distinct group in MP tree or combined with *Trogopterus*, and *Belomys* as a clade in NJ tree. The pairwise comparison of the partial cytochrome b sequences at the third codon positions between samples was given in Table 6.8.

Table 6.7 Pairwise comparison based on the partial cytochrome b sequences (375 bp) between species. See Table 6.4 for the sample abbreviations.

	ECF	ECK	PPF	PPH	PYK	HAK	HPT	EFP	TRX	PVO	BLP	PSE	GV	TSK
ECF		13.1	16.4	16.1	16.4	15.3	16.4	18	17.2	14	16.4	17.9	18.5	17.2
ECK	37/11		17.7	15.8	18.3	14.4	15.5	17.7	17.7	16.1	16.1	16.7	15.3	19
PPF	38/22	42/23		8.7	5.7	16.9	17.9	18.3	18.0	17.1	18.9	19.5	16.4	20.4
PPH	37/22	35/23	30/2		8.2	13.6	16.5	14.4	17.4	15.9	16.8	18.3	15.5	18.8
PYK	39/21	43/24	20/1	27/3		14.4	18.3	18.9	18.5	16.7	19.1	20.4	17.2	20.4
HAK	40/16	40/13	40/22	30/20	30/13		10.6	14	16.1	13.3	14.7	12.7	13.9	17.2
HPT	43/16	44/11	42/23	39/20	42/24	36/2		16.4	17.8	16.2	16.1	3	13.2	20
EFP	41/25	41/24	48/19	34/19	40/20	32/19	40/19		19.1	17.3	18.8	18.5	14.4	17
TRX	32/31	37/28	41/25	39/25	44/24	40/19	43/21	42/28		17	12.8	19.4	18.3	19.1
PVO	32/19	39/19	42/19	36/21	42/18	30/18	38/17	40/22	39/23		16.7	17.1	14.7	16.4
BLP	32/27	35/23	41/27	33/27	41/28	33/19	34/20	38/30	31/15	35/21		17.3	15.8	19.6
PSE	45/20	44/16	44/27	41/25	46/28	39/6	6/4	43/23	44/26	37/21	36/22		14.4	20.9
GV	49/19	42/14	37/23	36/21	39/24	40/11	37/11	31/22	47/20	32/20	37/20	37/15		17.2
TSK	40/23	42/28	48/27	42/27	49/26	38/25	64/24	39/22	46/24	37/22	47/24	40/23	39/24	

Note: Data below the diagonal are the numbers of transitions vs. transversions. Data above the diagonal represent the genetic differences between samples

Table 6.8 Pairwise comparison of the transversional substitutions at the third codon positions of the partial cytochrome b sequences (366 bp) between samples. Data below the diagonal are the numbers of transversions at the third codon positions. Data above the diagonal represent the transversional percentage difference between samples. Table 6.4 shows the information of samples.

	ECF	ECK	PPF	PPH	PYK	HAK	HPT	EFP	TRX	PVO	BLP	PSE	GV
ECF		4.9	13.1	14.7	13.1	10	10	16.4	17.2	10.6	13.9	11.4	11.4
ECK	6		13.9	14.7	13.9	8.2	8.2	16.4	18	11.4	13.1	10	10
PPF	16	17		1.6	0.8	15.5	14.7	13.9	18	12.3	17.2	16.4	16.4
PPH	18	18	2		2.4	13.9	13.9	13.1	18.8	14.7	16.4	16.4	15.5
PYK	16	17	1	3		17.2	16.4	13.9	18	12.3	18	18	18
HAK	12	10	19	17	21		0.8	13.9	13.9	12.3	13.1	2.4	10
HPT	12	10	18	17	20	1		14.7	15.5	12.3	13.9	1.6	9
EFP	20	20	17	16	17	17	18		20.5	13.9	19.6	15.5	17.2
TRX	21	22	22	23	22	17	19	25		15.5	10	17.2	15.5
PVO	13	14	15	18	15	15	15	17	19		15.5	14.7	13.9
BLP	17	16	21	20	22	16	17	24	12	19		14.7	12.3
PSE	14	12	20	20	22	3	2	19	21	18	18		10
GV	14	12	20	19	22	12	11	21	19	17	15	12	

The estimated divergence time between samples was calculated based on the percentage of the transversional substitutions at the third codon positions using the rate of divergence for the third codon positions of mammalian cytochrome b gene of ca. 0.5% $*10^6$ years and was presented in Table 6.9. With the inclusions of *Glaucomys* and *Eoglaucomys*, all Chinese flying squirrels, including 7 genera and 10 species, were analyzed using morphometric methods as well. The results were showed in Table 6.10. In discriminant function analysis, function 1 that accounted for 89% of total variance separated most populations into genus-based groups. The mixed group included *H. phayrei*, *Pteromys volans*, *G. volans*, and *B. pearsonii* (Figure 6.10). Principal

components analysis yielded the same results. The first two factors, accounting for 99% of the total variance, distinguished 12 populations as four major groups. Again, *Hylopetes*, *Pteromys volans, G. volans,* and *B. pearsonii* greatly overlapped with each other (Figure 6.11).

Table 6.9 Estimated divergence time between samples based on the rate of the transversional substitutions at the third codon of cytochrome b sequences proposed by Irwine et al. (1991). See Table 6.4 for sample abbreviations.

	ECF	ECK	PPF	PPH	PYK	HAK	HPT	EFP	TRX	PVO	BLP	PSE
ECK	9.8											
PPF	26.2	27.8										
PPH	29.4	29.4	3.2									
PYK	26.2	27.8	1.6	4.8								
HAK	20.0	16.4	31.0	27.8	34.4							
HPT	20.0	16.4	29.4	27.8	32.8	1.6						
EFP	32.8	32.8	27.8	26.2	27.8	27.8	29.4					
TRX	34.4	36.0	36.0	37.6	36.0	27.8	31.0	41.0				
PVO	21.2	22.8	24.6	29.4	24.6	24.6	24.6	27.8	31.0			
BLP	27.8	26.2	34.4	32.8	36.0	26.2	27.8	39.2	20.0	31.0		
PSE	22.8	20.0	32.8	32.8	36.0	4.8	3.2	31.0	34.4	29.4	29.4	
GV	22.8	20.0	32.8	31.0	36.0	20.0	18.0	34.4	31.0	27.8	24.6	20.0

Table 6.10 Multivariate analyses of Chinese flying squirrels

Axis	Eigenvalue	Proportion	Cumulative
Discriminant function analysis			
CAN I	173.38	0.89	0.89
CAN II	14.33	0.07	0.96
CAN III	2.46	0.01	0.97
Principal components analysis			
PRIN I	873.17	0.97	0.97
PRIN II	11.81	0.01	0.98
PRIN III	3.75	0.01	0.99

6.4 Discussion

6.4.1 Phylogeny of the Trans-Himalayan Flying Squirrels

The eastern trans-Himalayan flying squirrels include three genera, *Eupetaurus*, *Petaurista*, and *Hylopetes*, which are distributed mainly in western Yunnan, China. Three genera, each containing one species, *E. cinerus*, *P. albiventer*, and *Eoglaucomys fimbriatus*, occur in the western trans-Himalayas, mainly in N Pakistan. Usually, the populations of *Eupetaurus* and *Petaurista* in both regions are considered as the same species based on their morphological structures and external characters (Corbet and Hill, 1992; Nowak, 1999). *Eogalucomys*, the small Kashmir flying squirrel, which used to be considered as a species of arrow-tailed flying squirrels (*Hylopetes*), is merited as an independent genus (Thorington et al., 1996; Nowak, 1999). My results from both the morphometric and molecular analyses strongly support the latest classifications.

As discussed in Chapter 3, the populations of *Eupetaurus* in the eastern and the western trans-Himalayas are significantly different genetically. The genetic distance between these two clades is 11.0 – 13.3% (Table 3.4 and Table 6.7), implying two probable species. Unfortunately, since no skull was associated with the skins found in SW Yunnan of China, it is unreasonable to directly elevate these two populations as distinct species based only on the genetic information without evidence from morphological structures, especially from skull and teeth. The same situation exists between the populations of *P. albiventer* in Pakistan and W Yunnan, China, which are named as the same species or subspecies, *P. albiventer* or *P. p. albiventer* in recent references on the basis of their external and dental structures (Corbet and Hill, 1992; Wang, 2002). But the findings in both morphometric and molecular analyses in this study reveal that they are significantly different, at least at subspecies-level (See Chapter 4 for detail). When these two populations are compared with other trans-Himalayan flying squirrels, the morphometric results show that these two *Petaurista* populations are significantly different in cranial structures (Figure 6.4 and Figure 6.5). The principal components analysis indicates that their main differences are in skull size and the morphological structures of lower jaw. The population of *P. albiventer* in Pakistan shares more cranial characteristics with *P. elegans* and *P. alborufus* than with the population of *P. albiventer* in Yunnan, China. These two populations may occupy different habitats that result in different adaptations at different geographic locations during a long and complex geological, climatic, floristic and faunistic history.

The arrow-tailed flying squirrel (*Hylopetes*) in China and SE Asia used to be considered as the same genus with the small Kashmir flying squirrel (*Eoglaucomys*) in

the western trans-Himalayas (Corbet and Hill, 1992; Nowak, 1991; Roberts, 1997). They are elevated as two valid genera based on their different dental structures in recent references (Thorington et al., 1996; Nowak, 1999). The results in present study support their separation. The differences between these two groups are significant in both morphology (Figure 6.4 and Figure 6.5) and genetics (Table 6.7 and Table 6.9). They can be distinguished as two valid genera, namely *Hylopetes* in China and SE Asia, and *Eoglaucomys* in Pakistan and Kashmir.

With inclusion of the populations of *Eoglaucomys*, *Hylopetes*, and *Petaurista*, the differences among them are concordant with their geographical distributions. The present distributions of *Eupetaurus, Petaurista, Hylopetes,* and *Eoglaucomys* in these regions owe much to both major climatic changes in the late Pleistocene and the physical barriers to migration. The three flying squirrels in Pakistan occur sympatrically in some regions with the same living conditions, such as the Himalayan temperate forest with a mixture of deciduous and coniferous tree species, each occupying different microhabitat and having different food habits. Woolly flying squirrel (*Eupetaurus*) in Pakistan is confined to remote valleys in the extreme northern Himalayas. It lives in steppic mountainous conditions with isolated forest that consists of blue pine (*P. wallichiana*), edible seed or chilgoza pine (*P. gerardiana*), spruce (*Picea smithiana*), and *Juniperus macropoda* (Roberts, 1997). The unique tooth structures of *Eupetaurus* are closely associated with its special feeding habit. The developed molars suggest that *Eupetaurus* lives on a highly fibrous vegetable diet as its teeth are adapted to a high rate of abrasion and wear on their grinding surface (Woods and Howland, 1979). Giant flying squirrel in the western trans-Himalayas is distributed mainly in N Pakistan where the major habitat

is the Himalayan moist temperate forest. This flying squirrel feeds on the fir and pine cones, the nuts, even the young twigs and tree buds, or the acorns of the hill oak (*Quercus dilatata*). The food compositions are partially different in *Eupetaurus* and *Eoglaucomys* (Roberts, 1997). *Eoglaucomys* lives in dry temperate coniferous forests and is confined to the more sheltered lower slopes, or the Himalayan moist temperate forest. Compared to *Petaurista*, *Eoglaucomys* is apparently more adaptable to harsh conditions where the forest is predominantly coniferous, which has developed cell tissue with a high proportion of tough silicates. The morphological differences in skull size and the structures of lower jaw (Table 6.6) demonstrate that each of them has adapted to different living conditions.

The similar sympatric distribution of *E. cinereus, P. albiventer,* and *H. alboniger* also exists in SW China, where the two *Eupetaurus* skins were collected. Unfortunately almost nothing is known about the habitat, feeding habits, and geographical distribution of *Eupetaurus* in China except for two skins. *Hylopetes* is distinguishable from *Petaurista* by smaller body size and relatively shorter but broader tail, which has the hairs spreading laterally in a feather shape. The geographical distribution of *H. alboniger* and *P. petaurista* is overlapped extensively at different elevations (500m to 3,500m) from nouthwestern Yunnan to southern Yunnan, China. The habitat is mountainous conditions with coniferous forest. According to local people, *P. albiventer* frequently feeds on walnuts, acorns, and even corns in farm; whereas *H. alboniger* is always staying on the top of trees, such as Yunnan pine and spruce, and feeds on pine seeds, and young twigs, which I have watched in the field in Gongshan of Yunnan, China. But nothing else is

available about its feeding habits, geographic distribution, and food selection during different seasons.

Geological changes have strongly affected the evolution and distribution of the mammals by creating dispersal barriers and corridors, as well as by forming optimal habitats in both extremes of the trans-Himalayas. In Pakistan, there is a rich and varied mammalian fauna, affinitive to two of the major faunal regions, the Palaearctic region west of the Indus and the Oriental region east of the Indus (Roberts, 1997). The great Indus River and its drainage basin form a dominant physiographic feature over a large part of the country. Southwest China is comprised the hills of the eastern Himalayas and the Tibet plateau, particularly the Hengduan and Min mountain systems. Elevation varies from below 1,000m in the valley floors to glacial peaks of over 6,000m. The subtropical to sub-alpine mountains are cut into a number of subunits by major river gorges. The vegetation on mountain slopes has a relatively narrow vertical zonation and the vegetation on the plains has a broad horizontal zonation. In eastern Tibet and western Sichuan, among mountains within reach of the southwest monsoon, forests grow up to an elevation of 4,100-4,500m. A mixed coniferous and broad-leaved forest composed predominantly of spruce, fir, and oak with an understory of *Acer*, *Lindera*, *Litsea*, *Rhododendron*, and other trees predominate. Hoffmann (2001) thought that the mammalian fauna in this region belongs to either the Palaearctic region or the Oriental region. Below about 1,500m, tropical and subtropical communities typical of the Indomalayan region predominate, and above 2,500m, cool temperate to boreal communities typical of the Palaearctic region dominate the landscape. The region around 2,000m is a transition zone in which animals from both regions occur in varying

proportions depending on local environmental conditions. Accepting Hoffmann's hypothesis, flying squirrels in both regions are the elements of the Oriental region.

The genetic differences among these four genera are significant, too (Table 6.7). They diverged from their ancestor stock(s) about 20 to 32 million years ago (Table 6.9), the early Miocene. Migrations between the eastern and the western trans-Himalayan flying squirrels were after the secondary upheaval the great Himalayan mountain chain, which was about 13 million years ago. This dramatic tectonic movement led to an even more violent nature in Asia and Europe. Because of the changes of climate and environments that were influenced by geographical events, the populations in each genus were split into two branches. One branch migrated west into what is now Pakistan, Afghanistan, and NW India, another migrated eastward to what is now in SW China. During the smaller upheavals or movements of tectonic plates in the late Pliocene, flying squirrels in each region adapted to different habitats in the eastern and the western extremes of the Himalayan mountain chain. The same migrationspatterns also exist in some of the more primitive or earlier mammalian genera surviving today in Pakistan that came from the Oriental region, such as the grey goral, Himalayan langur, civet cats, and naked-tailed murid rodents (Roberts, 1997).

6.4.2 Systematics of Chinese Flying Squirrels

In structure, the morphology of tree squirrels is primitive and the morphology of flying squirrels is derived (Thorington and Heaney, 1981). The ability to glide depends upon the existence of membranes at the sides of the body, which are most fully developed in *Petaurista*. Based on the comparison of the cranial structures, all Chinese flying squirrels can be morphologically divided into four groups: *Petaurista, Aeretes, Trogopterus,* and the mixed group containing *Belomys, Petinomys, Hylopetes,* and

Pteromys (Figure 6.10 and Figure 6.11). This result is close to McKenna's (1962) and Mein's (1970) classification, although the classification of this study may not be very accurate because less than 5 specimens of *Belomys, Petinomys, Aeretes,* and *Trogopterus* were used in multivariate approaches. With the inclusion of *Eupetaurus*, the molecular data partitions all Chinese flying squirrels as five groups: *Petaurista, Pteromys, Eupetaurus, Hylopetes* and *Petinomys*, and the mixed group including *Trogopterus* and *Belomys*. The phylogentic reconstruction of Chinese flying squirrels (Figure 6.9) is in part identical to Oshida's et al. (2000a) study. The major differentiation is the divergence time between species. According to the estimated time calculated with the transversional rate at the third codon positions of cytochrome b gene, the generic divergences of Chinese flying squirrels occurred about 27 to 38 million years ago, the late Eocene to middle Oligocene.

Trogopterus originally contains two species, *Pteromys xanthipes* in China and *Sciuropterus pearsonii* in India for their similar external structures (Figure 6.13). Thomas (1888) erected *Sciuropterus pearsonii* as new genus *Belomys*. The chief distinction between these two genera lies in the structure of the teeth. *Trogopterus* is distributed in the forests of northeastern Hupei, Ichang, the area upper Min River, and Yunnan, China. Externally the flying squirrels of this genus show no striking peculiarities but appear to be characterized by the presence of a small tuft of long black hair at the inner and another at the outer base of each ear (Allen, 1940). The notable enlargement of the posterior upper premolar and the enamel pattern in checkteeth are somewhat complex and irregular. *Trogopterus* feeds on oak leaves, and the specifications of teeth are obviously associated with its feeding habit. To date, three

forms have been named, but it is probable that these are all races of a single wide-ranging species. *Belomys pearsonii* ranges from Nepal eastward into southern China and is represented in the island of Taiwan by a closely allied form. It is distinguished externally from *Pteromys* by the presence of a tuft of long delicate hair at the base of each ear (Figure 6.12). The results in this study support that *Trogopeterus* is better distinguished from *Belomys* by its actually and proportionately longer toothrow (Figure 6.10 and Figure 6.11). The molecular data suggest that *Trogopterus* was the first diverged flying squirrel. About 20 million years ago, the extant genera *Trogopterus* and *Belomys* were split from their common ancestor, inferring that the morphological differences in skull are the recent developments. But there are no available data from fossil records, ecological observations, and quantitative descriptions with which to compare the present morphological and molecular data.

Based on the morphology of cheekteeth (the key characteristic in the classification of flying squirrels), *Glaucomys*, *Hylopetes*, *Eoglaucomys* and *Petinomys* are closely related (Allen, 1940). They have short and broad heads, complex molars, and a tail that is bushy, cylindrical, and as long or longer than the head and body (Figure 6.13). The teeth of *Hylopetes* are like those of *Glaucomys* in essential structure, with transverse ridges of the upper molars complete, and partly joined internally to the outer slope of the internal wall, and lacking any notch extending part way across the tooth from the inner side. Molecular results based on the cytochrome b sequences support this conclusion, however the morphometric study based on the cranial characters reveals that *Eoglaucomys* is morphologically different from the other flying squirrels with small body

size (Figure 6.10 and Figure 6.11). This difference might be related to their different living conditions (see Chapter 5 for detail).

The skull of *Pteromys* closely resembles in general the contour of the genus *Hylopetes*. The species *Pteromys volans* in China is distributed mainly in SE China (Figure 6.3). Its developed wrist has a long rod that becomes more or less bony and serves to spread the anterior edge of the lateral parachute or membrane. *Pteromys volans* usually lives in a hollow tree and feeds on nuts and pine seeds; owls and the smaller cats frequently capture these squirrels at night (Nowak, 1999). Although Oshida's et al. (2000a) research indicates that a close phylogenetic relationship exists between *Pteromys* and *Petaurista*, the morphological and molecular data in this study present different results. Their cranial structures are significantly different in both the size and shape. Phylogenetic trees constructed with MP and NJ methods imply an early divergence of *Pteromys volans* from other flying squirrels (Figure 6.8).

Aeretes is a medium-sized species with a bushy flattened tail about the same length as the head and body (Figure 6.14). *A. melanopterus*, the species confined to northeastern China, has a very special skeleton, which shows a very long lumbar region, its eight vertebrae equaling in length the combined cervical and thoracic portions of the spine (Ellerman, 1940). The upper incisors are much broader than in any member of *Petaurista* or *Trogopterus*, and have a well-marked groove running vertically down the outer three-quarters of the width. Because of these characters in skull and skeleton, it seems worthy of generic distinction from smaller flying squirrels. Nothing is recorded of its habits, but the broad incisors and shortened rostrum may indicate a different feeding

habit from related species. My findings in morphometric analysis show that *Aeretes* apparently differs from the remaining flying squirrels, both large and small.

The most severe climatic cooling in the trans-Himalayan region was at about 33 Myr, slightly after the Eocene-Oligocene boundary, and was characterized by a drop in the mean annual temperature and by changes in vegetation from dense forests in the Eocene to more open country in the Oligocene (Meng and McKenna, 1998). In Oligocene faunas large species were few, medium-sized were rare or absent, and small mammals, such as rodents, became dominant. The first uplift of the great Himalayan mountain chain occurred at about 50 million years ago. This upheaval led to formation of a land bridge in the Bay of Bengal, connecting Gondwandland with southeastern Asia, particularly the Sino-Malaysian region. The paleontological records indicate that by the late Miocene the geography of the trans-Himalayas was similar to that of today (Wang, 1984). In middle Pleistocene, the Tibetan plateau was 3,000-3,500 m in elevation, and upheaved to 4,500-5,500 m during Holocene (Xu. 1981). The presence of the Himalayas caused the diversification of climate, and it became an important regulator of the Asian environment. Climatic and floristic fluctuations have led to fluctuations of species limits in both elevation and latitude through the effect on vegetation. The characteristically oriental faunal species invaded into the subcontinent and spread westward across the interface between Asia and Gondwanaland. The three or four major glacial periods in Asia, separated by warmer, interglacial periods in Pliocene-Pleistocene, played an important role for the present distributions of flying squirrels. This is corroborated in part by the evidence of a much greater diversity of species in the eastern Himalayas, compared with the northwest in what is now Pakistan.

The fossil records show that Sciurinae evolved from the Paramyinae, whereas the Petauristinae evolved from the Prosciurinae before the mid-Miocene (Mein, 1970). In Miocene, the European fauna evolved independently from the North American and Indo-Malayan forms (Arbogast, 1999). During the Pliocene flying squirrels were dominant in Europe, supplanting other squirrels. Considering the early fossil remains of *Petinomys* found in Europe (Black, 1972), all Chinese extant stocks had diverged from their ancestor(s) in the middle Oligocene. During the Oligocene-Miocene radiation of flying squirrels in Europe, each stock has migrated towards south. Their present geographical distributions are apparently associated with the recent tectonic movements during Pleistocene.

Several molecular studies have supported the general hypothesis of latest Pleistocene southward depression, followed by postglacial northward expansion of ranges in Palearctic and Nearctic taxa (Cooper et al., 1995). The levels of molecular divergence in several widespread neotropical bird and frog superspecies indicate that phylogenetic diversification preceded the latest Pleistocene by millions of years (Bush, 1994; Heyer and Maxson, 1982). If flying squirrels evolved from a tree squirrel during the Oligocene after the Chadeonian, 35 million years ago (Johnson-Murray, 1977), the molecular data in present study and in Arbogast's (1999) study suggest that the great difference between *Petaurista* and *Glaucomys* indicate that either flying squirrels are diphyletic or the divergence of lineages occurred very early (Thorington et al., 1998). The evolutionary history of flying squirrels, at least the New World flying squirrels that occupy the boreal and deciduous forest habitat of North America has been shaped profoundly by the Pleistocene glacial interglacial cycles (Arbogast, 1999).

To distinguish Chinese flying squirrels morphologically and externally, Table 6.11 is a genus-based key to identification of different genera. This key is based on the characters used for classification of flying squirrels by Ellerman (1940).

Table 6.11 Key to the genera of Chinese flying squirrels

Cheekteeth strongly hypsodont ……*Eupetaurus*
Cheekteeth not strongly hypsodont
Cheekteeth always in lower series and usually in the upper series

characterized by signs of extreme complication due to wrinkling;

the essential pattern of the cheekteeth usually more or less

masked

P4 conspicuously enlarged
Checkteeth semi-hypsodont; P4 extremely enlarged. ……*Trogopterus*
Cheekteeth brachyodont
P4 more moderately enlarged ……*Belomys*
P4 not specially enlarged; the basi-occipital narrowed……*Petaurista*
Cheekteeth with a more normal pattern, the wrinkling

though sometimes traceable never excessive, and never

masking the essential pattern

Bullae low and flattened, scarcely rising above general
level of the base of the skull ……..…….*Petinomys*
Ballae without special peculiarities
M3 with two clear ridges between the anterior and
posterior margins of tooth; M3 lower with four ridges
And three depressions, incisive foramina long…….*Pteromys*
M3 with only one ridge between anterior and posterior
margins of tooth; M3 lower never with four ridges
and three depressions; incisive foramina short.
Cheekteeth relative simpler, with small extra ridges
and depressions not or barely traceable ……*Eoglaucomys*
Cheekteeth relatively more complex, with small extra
ridges and depressions normally present …….*Hylopetes*

6.5 Summary

This chapter deals with the phylogeny of the trans-Himalayan flying squirrels and the systematics of Chinese flying squirrels. The results of morphometric and molecular

study provide some important insights into the complex evolutionary history of flying squirrels.

1. The genetic differences among *Eupetaurus, Eoglaucomys, Hylopetes*, and *Petaurista* are significant. They diverged from their ancestor stock(s) about 20 to 32 million years ago, the early Miocene.

2. The morphological and genetic differences among the populations of *Eupetaurus, Eoglaucomys, Hylopetes*, and *Petaurista* are concordant with their geographical variations along the trans-Himalayan mountain chain. The current distributions owe much to both major climatic changes in the late Pleistocene and the physical barriers to migration.

3. All Chinese flying squirrels can be genetically partitioned into five groups: *Petaurista, Pteromys, Eupetaurus, Hylopetes* and *Petinomys*, and the mixed group including *Trogopterus* and *Belomys*.

4. The estimated times from cytochrome b gene show that the generic divergences of all Chinese extant stocks of flying squirrels diverged from their ancestor(s) in the middle Oligocene. The present geographical distributions are apparently associated with the recent tectonic movements during Pleistocene.

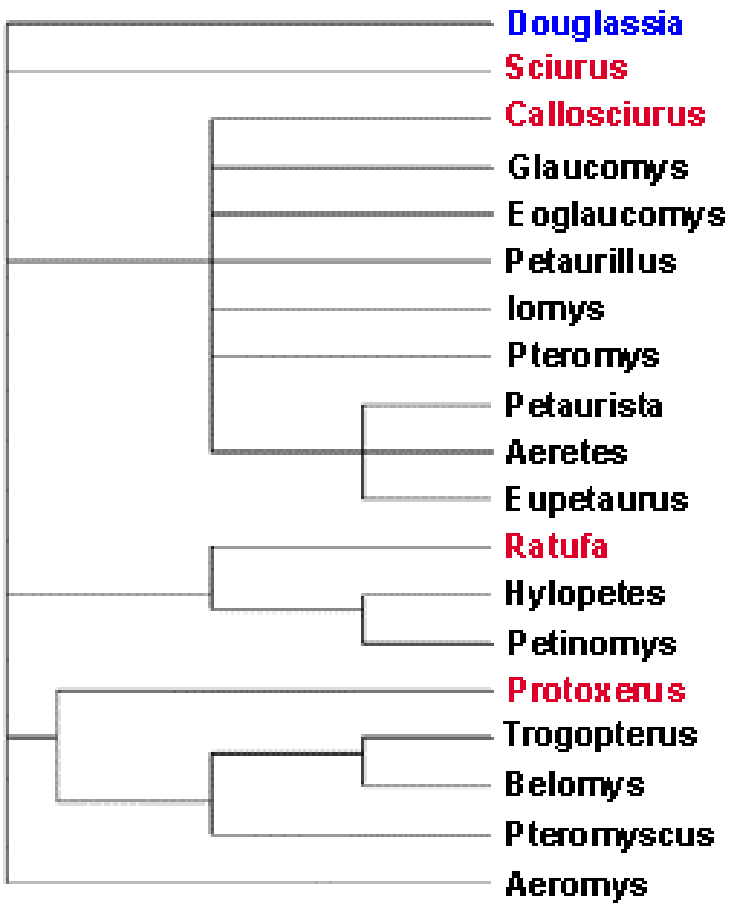

Figure 6. 1 Phylogenetic reconstruction of flying squirrels, tree squirrels, and fossil squirrels based on 44 dental characteristics

Figure 6.2 Chinese flying squirrels

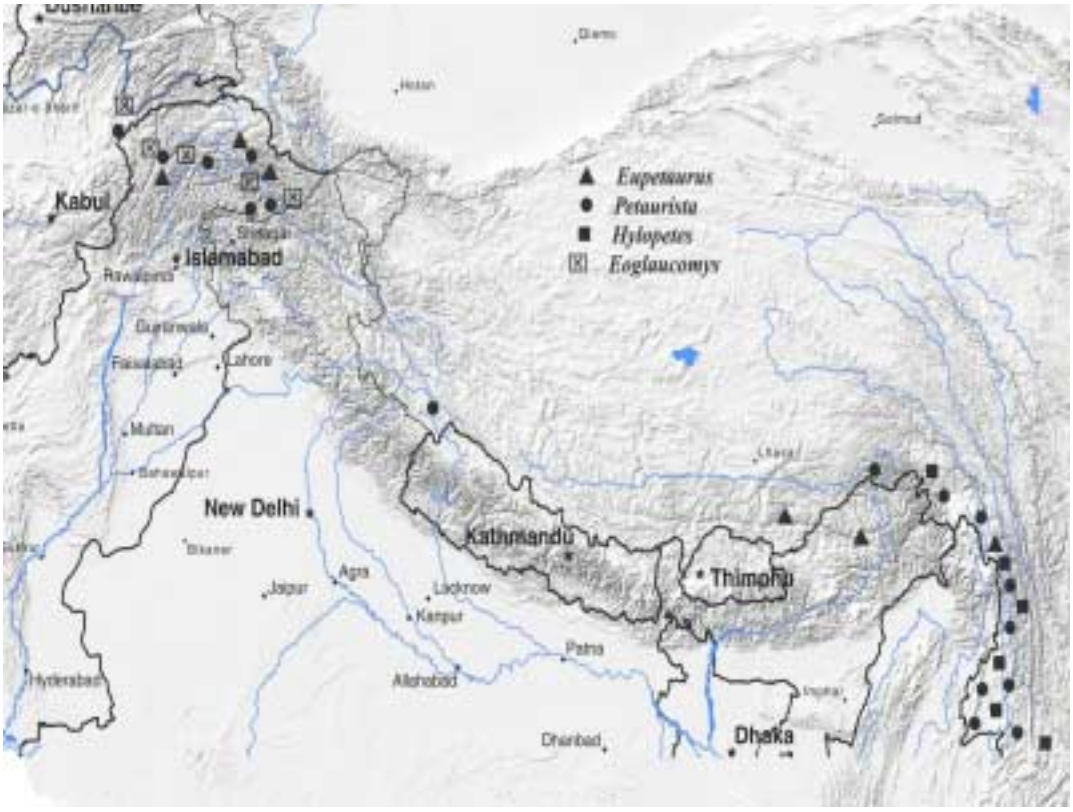

Figure 6.3 Trans-Himalayan flying squirrels

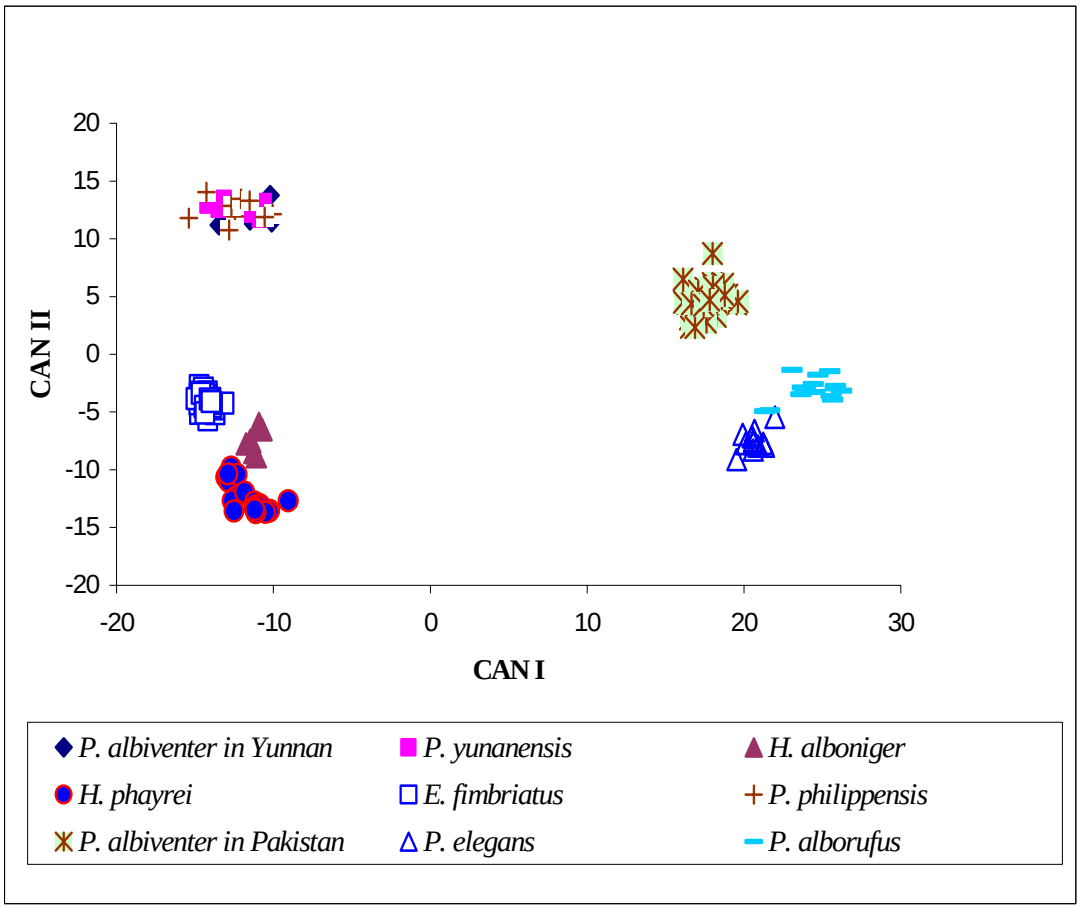

Figure 6.4 Scatter-plot of discriminant function analysis of the trans-Himalayan flying squirrels onto the first two functions (CAN I and CAN II)

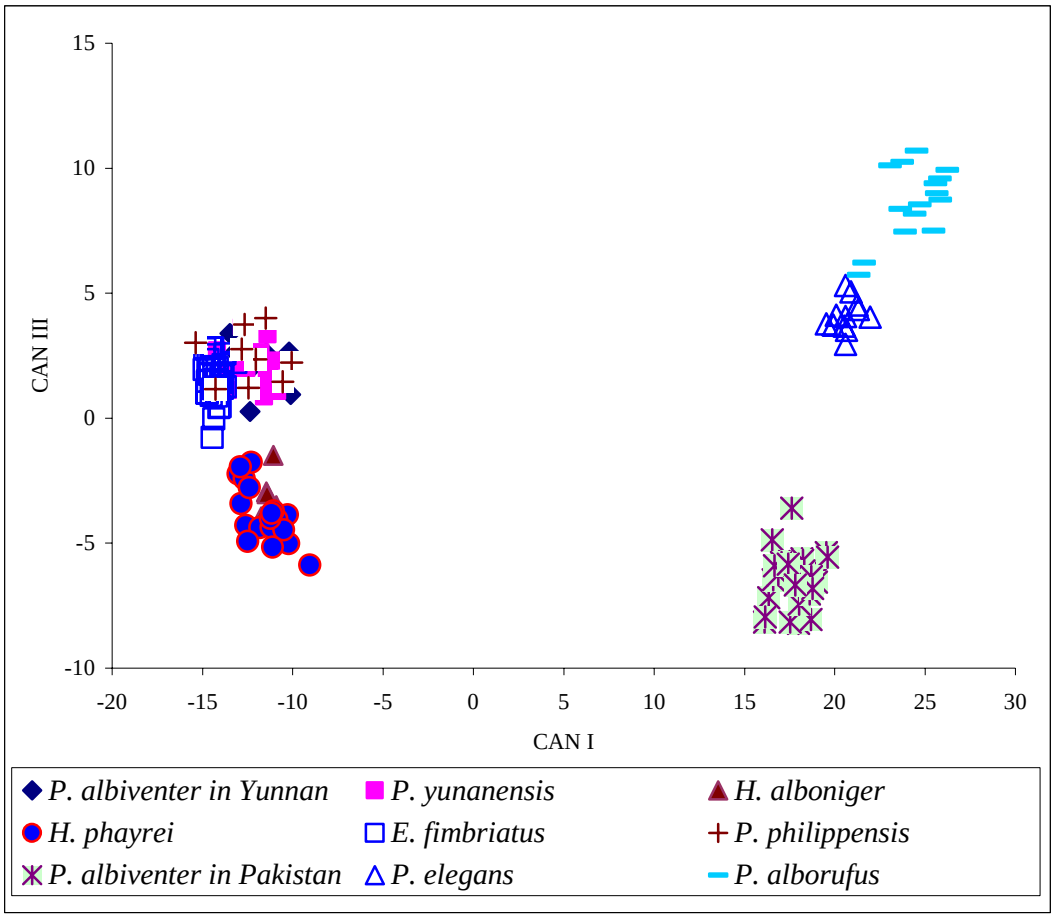

Figure 6.5 Scatter-plot of discriminant function analysis of the trans-Himalayan flying squirrels onto function 1 and function 3 (CAN I and CAN III)

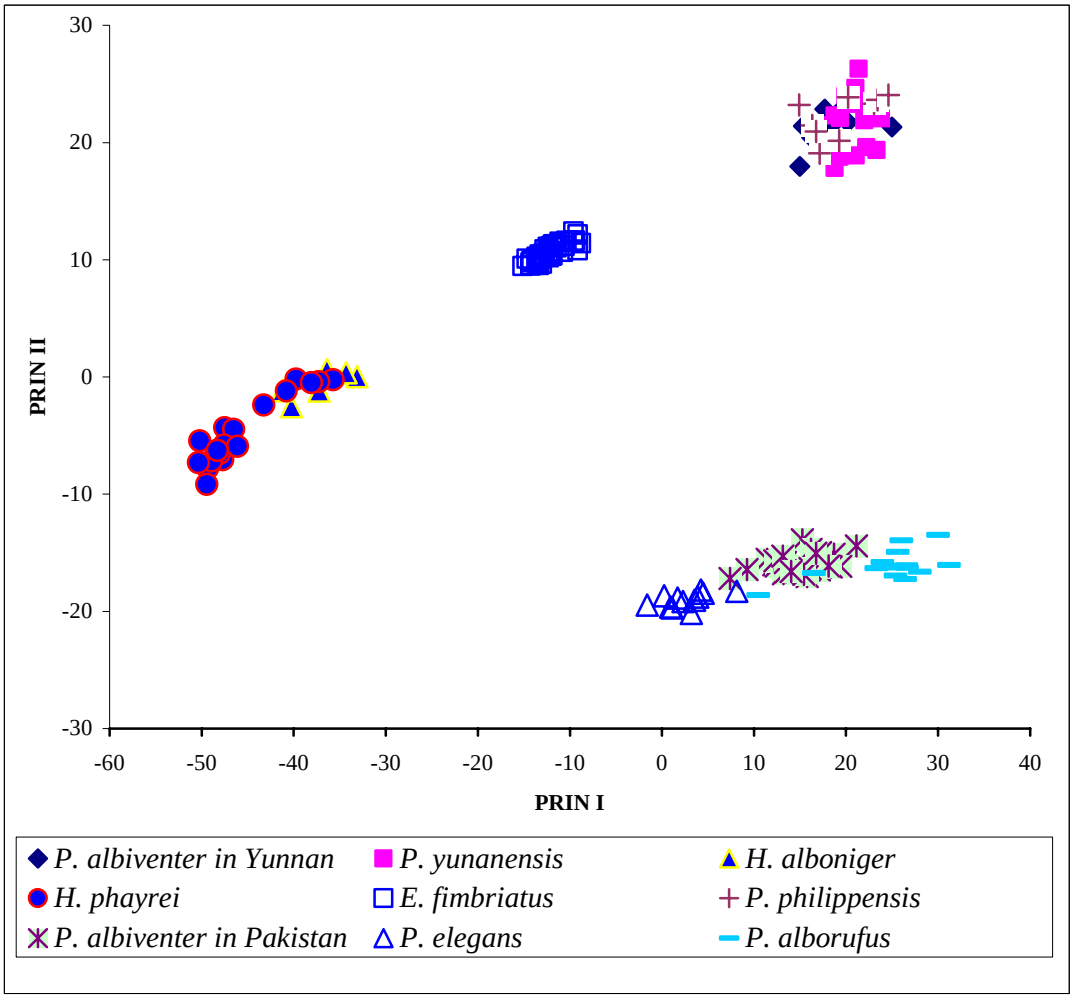

Figure 6.6 Principal components analysis of the trans-Himalayan flying squirrels onto the first two factors (PRIN I and PRIN II)

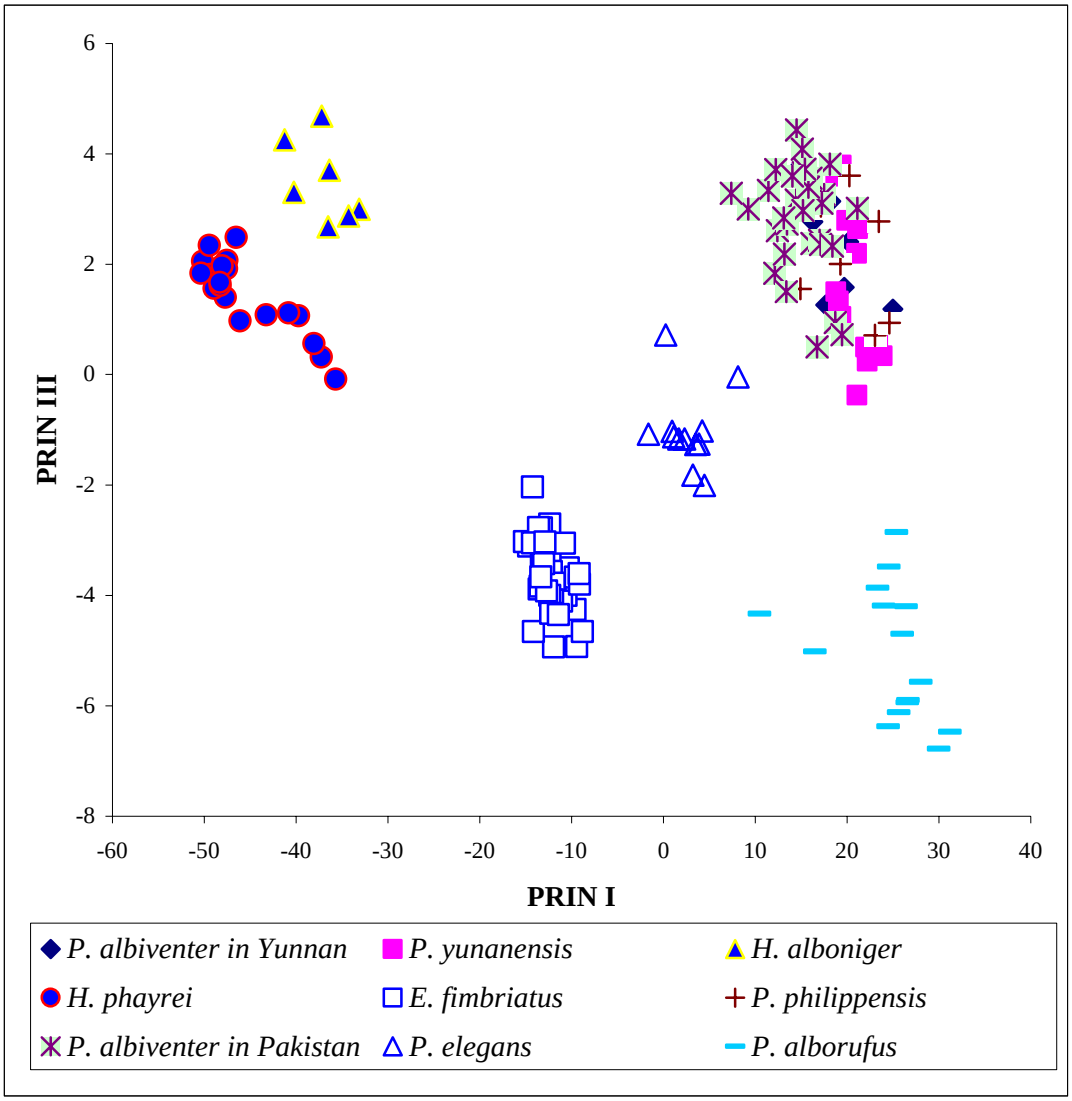

Figure 6.7 Principal components analysis of the trans-Himalayan flying squirrels onto the first and the third factors (PRIN I and PRIN III)

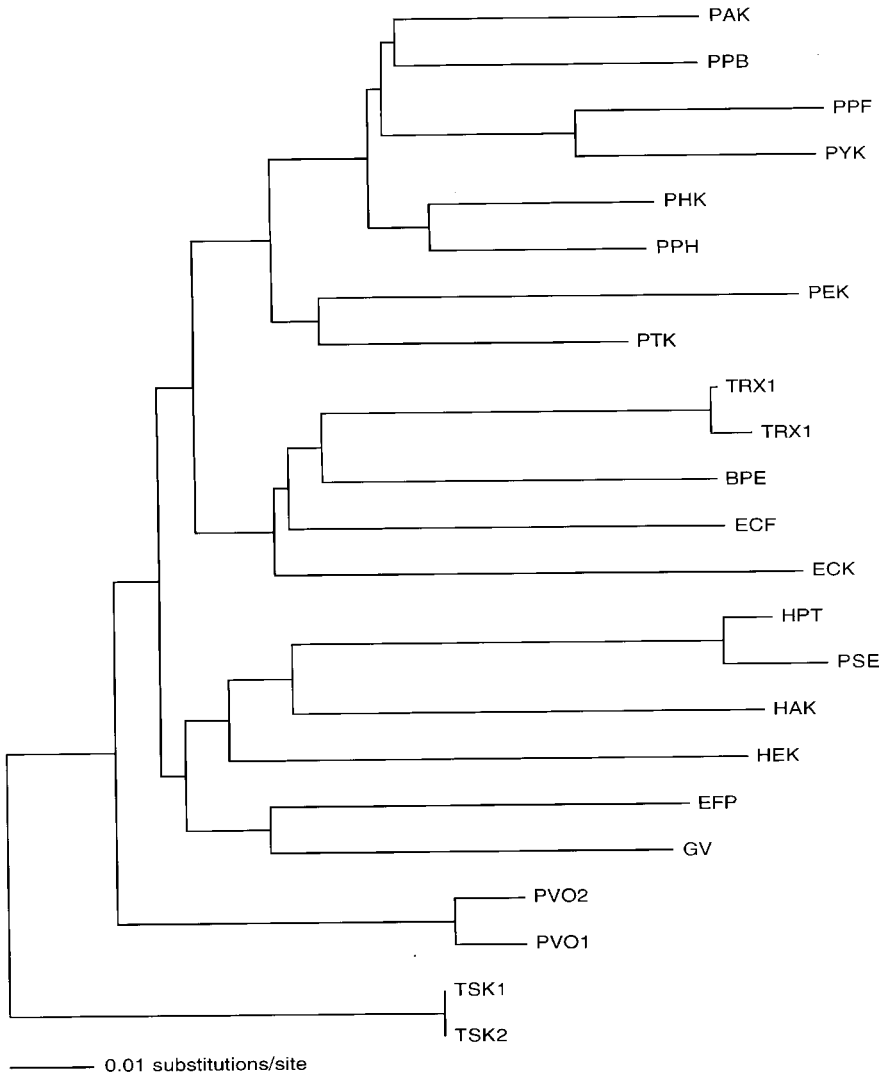

Figure 6.8 Phylogenetic relationships of all Chinese flying squirrels constructed using neighbor-joining (NJ) method. Scales in the tree represent branch length in terms of nucleotide substitutions per site. Sample abbreviations are defined in Table 6.4.

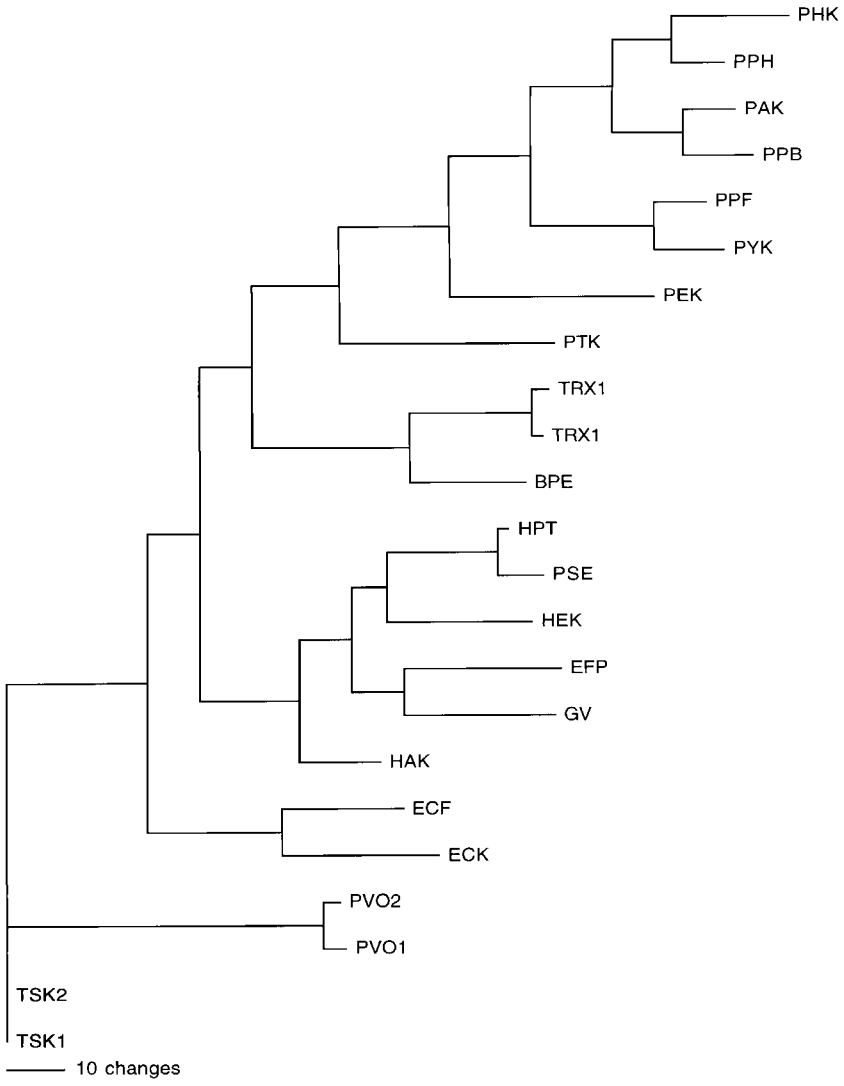

Figure 6.9 Phylogenetic relationships of all Chinese flying squirrels constructed via the maximum parsimony method using heuristic search algorithm. Scales in the tree represent branch length in terms of nucleotide substitutions per site. Sample abbreviations are defined in Table 6.4.

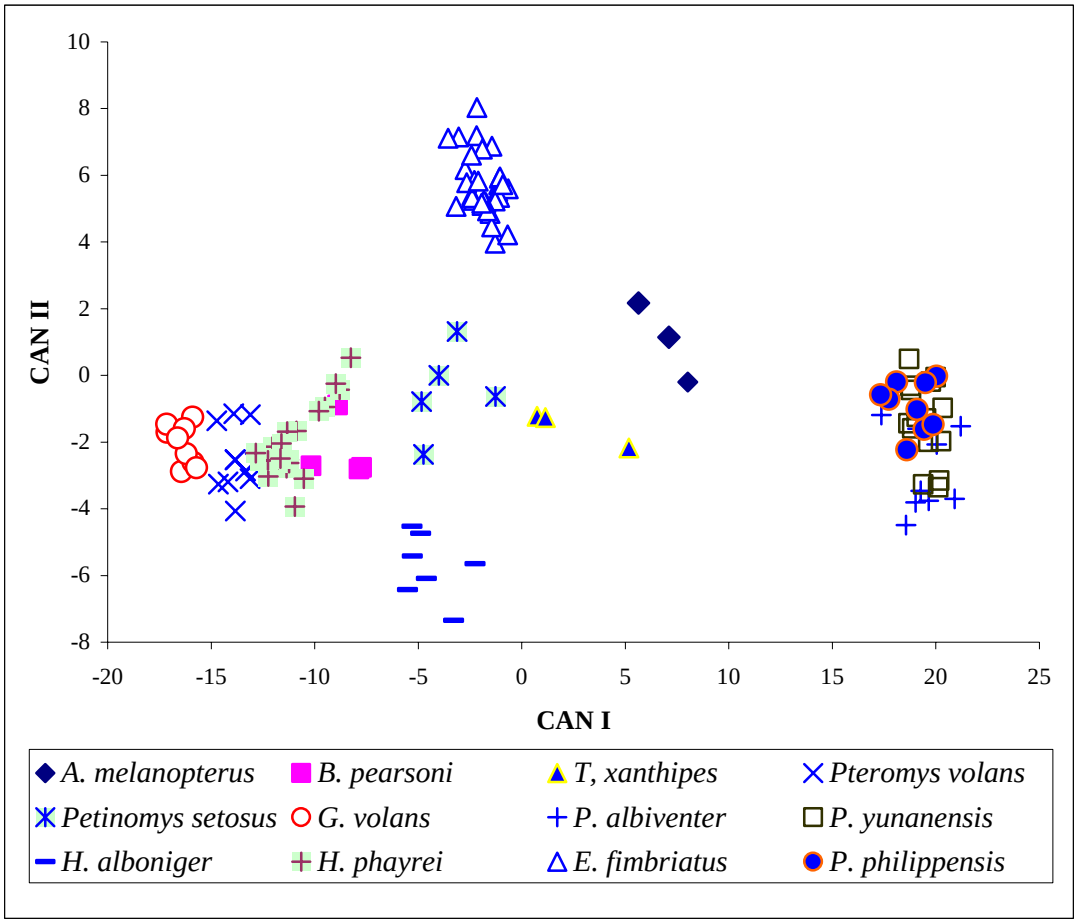

Figure 6.10 Plot of Chinese flying squirrels based on discriminant function analysis onto the function 1 and function 2 (CAN I and CAN II)

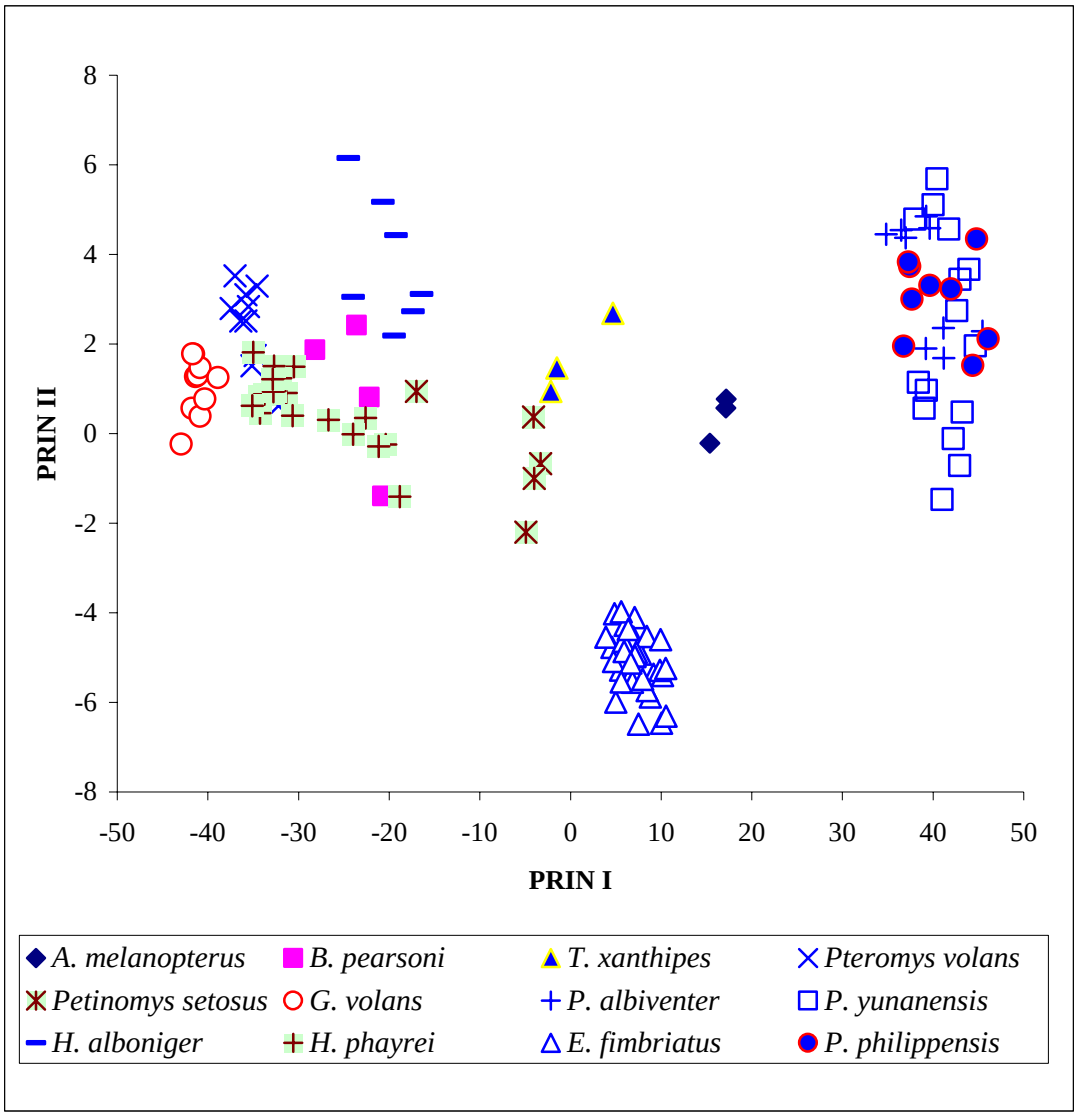

Figure 6.11 Plot of Chinese flying squirrels based on principal components analysis onto the factor 1 and factor 2 (PRIN I and PRIN II)

Figure 6.12 *Trogopterus xanthipes* and *Pteromys volans*

Figure 6.13 *Eoglaucomys*, *Hylopetes*, and *Glaucomys*

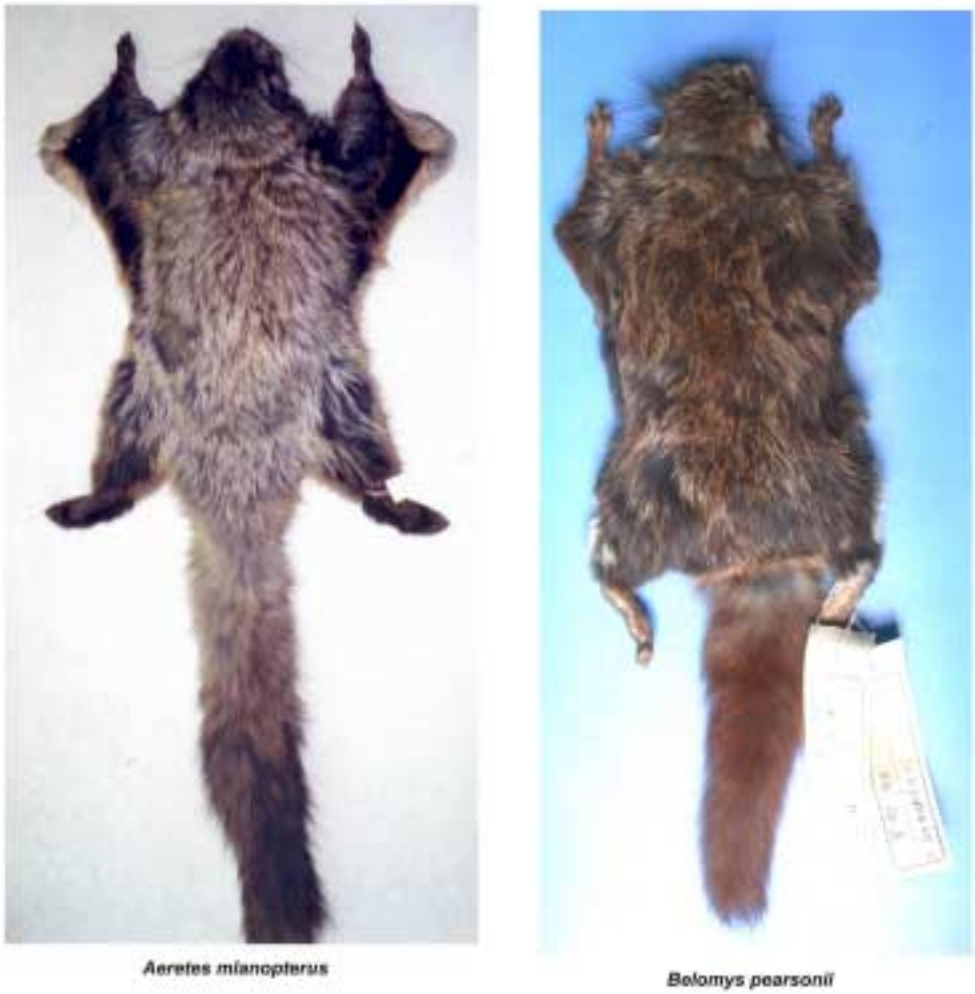

Figure 6.14 *Aeretes mlanopterus* and *Belomys personii*

CHAPTER 7
SUMMARY AND FUTURE WORK

7.1 Summary

The areas of the Himalayas where high mountain ranges meet the lowlands of Asia in a series of deep, narrow, and often xeric gorges are described as the "trans-Himalayas." The systematics, geographical distributions, and conservation status of many species in these rugged and remote regions are poorly understood. Flying squirrels are especially poorly understood because they occur in deep forest habitats and are nocturnal in habits. Despite the abundant taxonomic, ecological, and morphological information available for some flying squirrels, the phylogenetic relationships of many taxa remain uncertain. This taxonomic uncertainty is especially true for Chinese flying squirrels. Analyses of the partial sequences of mitochondrial cytochrome b gene and of morphological data were used to study the systematics and biogeography of flying squirrels in the eastern and the western trans-Himalayas. Detailed phylogenetic analyses have been carried out on forms or populations of *Eupetaurus*, *Petaurista*, and *Hylopetes* (*Eoglaucomys*) that are distributed in SW China and Pakistan. Taken together, the major contributions of this dissertation are as follows:

1. The two specimens that were collected in northwestern Yunnan, China, are members of the same genus *Eupetaurus*. There are significant differences in the population in the eastern and the western trans-Himalayas, indicating that two distinct species are present.

2. The possible divergence time of the two populations of *Eupetaurus* was at the end of Miocene, about ten million years ago. The glacial period and the uplift of the Himalayas and Qinghai-Tibet plateau during the Pliocene-Pleistocene period are

the major factors that secondarily affected on the present distribution of *Eupetaurus* in the trans-Himalayas.

3. The population of *P. petaurista* (*albiventer*) in Pakistan is significantly different from the population in W Yunnan of China in morphology and genetics. It is probably a new subspecies, even a new species.

4. *P. yunanensis* is genetically distinctive from *P. philippensis* and is a valid species. There is no basis for retaining *P. hainana* as a recognizable species; instead, it might be a subspecies or synonym of *P. philippensis*. *P. xanthotis* is a valid Chinese endemic species and shows a close phylogenetic relationship with *P. leucogenys* in Japan and China.

5. *H. electilis* is a possible valid species of *Hylopetes* based on its genetic characters, although it shares similar morphological characters with *H. phayrei* in skull.

6. *Eoglaucomys* in the western trans-Himalayas differs morphologically and genetically from *Hylopetes* in China and SE Asia. My data strongly support recognizing *Eoglaucomys* as a valid genus. *Eoglaucomys* diverged from other *Hylopetes* as early as 24 million years ago, the middle to late Oligocene. The migration of *Hylopetes* to the present geographical distribution is due to the tectonic movements of the Himalayas during the Pliocene-Pleistocene period.

7. All Chinese flying squirrels can be genetically partitioned into five groups: *Petaurista, Pteromys, Eupetaurus, Hylopetes* and *Petinomys,* and the mixed group including *Trogopterus* and *Belomys*. The morphological and genetic characters of *Eupetaurus, Eoglaucomys, Hylopetes,* and *Petaurista* are concordant with their geographical variations along the great Himalayan mountain chain. The present distributions owe much to both major climatic changes in the late Pleistocene and the physical barriers to migration.

7.2 Future Work

Despite the accomplishments of this dissertation, it is apparent that our understanding of the systematics of flying squirrels and the mechanisms responsible for their present geographical variations is far from complete and will continue to be an interesting area for future research. A few of further analyses I think would provide significant contributions to resolve the exact phylogenetic placement of Chinese flying squirrels.

In Chapter 3, I performed the comparative study between the populations of *Eupetaurus* in the eastern and the western trans-Himalayas, but the populations between the Himalayan extremes such as those in India and Sikkim are not included. It would be useful if the molecular data of the populations between Pakistan and China could be analyzed. Finding or collecting a skull or a skeleton of *Eupetaurus* in the eastern trans-Himalayas is also crucial for identifying the taxonomic status of *Eupetaurus* populations, and in describing the probably new *Eupetaurus* species in SW China.

South China is located at the crossroads of southeastern Asia and has been a bypass for animal dispersal from mainland Asia southward into the Indo-Malayan region. The further study of *Petaurista* and *Hylopetes* should focus on the populations between SW China and SE Asia, and between the eastern and the western trans-Himalayas, such as in north India, Myanmar, Thailand, Laos, and SE Asia. The inclusion of the population of *H. phayrei* in southeastern China will be significant for reconstructing the phylogenetic topology of Chinese *Hylopetes* by employing the similar analytical approaches.

Although I was able to construct the phylogenetic relationships of the Chinese flying squirrels at the generic-level, further analyses are necessary. Because specimens of flying squirrels, especially skins and fluid preserved specimens, are not as common in museums or academic institutes as specimens of non-flying squirrels, many of the forms are very little known. The collection of additional specimens of flying squirrels is crucial. The taxonomic affinities and phylogenetic relationships of taxa such as *Aeretes, Trogopterus, Belomys, H. electilis, H. phayrei,* and *P. petaurista* in various geographical locations could be resolved if specimens and tissues were available.

One of the most serious gaps in the knowledge of the Chinese flying squirrel is in the area of ecology, the study of other animals and plants in relation to each other and to its environment. The biology of the Chinese flying squirrels is still poorly known, with nearly every new collection or study revealing new distribution limits and providing very useful information. Further research will undoubtedly lead to changes in details.

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APPENDIX
GEOLOGICAL EPOCHS

Geological epoch		Time scale (million years)
Neogene	Holocene	0.1 - present
	Pleistocene	1.8 – 0.1
	Pliocene	5 – 1.8
	Miocene	23 - 5
Paleogene	Oligocene	34 - 23
	Eocene	55 - 34
	Paleocene	60 - 55
Cretaceous	>65	

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BIOGRAPHICAL SKETCH

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In 1984, the Northwest Normal University accepted him as an undergraduate student and he received his B.A. in 1988. From 1989 to 1992, as a graduate student, Fahong studied the evolution of primates at the Kunming Institute of Zoology (KIZ), the Chinese Academy of Sciences, with Professor Yanzhang Peng. In May 1992, he graduated with a M.Sc. in biology from KIZ. Then he continued his research in biology as an assistant researcher in KIZ.

In 1996, Fahong left KIZ to pursue his doctoral studies at the University of Florida, US. In the summer of 1999, when he became a Ph.D. candidate in zoology, he was also accepted as a graduate student at the Department of Computer and Information Science and Engineering (CISE). In 2000, he started his research with Dr. Stanley Su. In December 2001, he received his Master of Science from the Computer and Information Science and Engineering Department of the University of Florida. In 2002, under the guidance of Dr. Charles Woods, he was awarded the Ph.D. degree from the Department of Zoology, University of Florida.