1. INTRODUCTION

1.1. Natural hybridization and its evolutionary role

Over the last decade the study of natural hybridization has become one of the most dynamic areas in evolutionary biology and ecology. The renewed interest of the scientific community in hybridization appears to be in strong connection with big advances in genetic and molecular techniques.

The term of natural hybridization can be restricted to *crosses in nature between individuals from different species* or can be defined more broadly as *crosses in nature between individuals from two populations, or groups of populations, which are distinguishable on the basis of one or more heritable characters* (HARRISON 1993; ARNOLD 1997). Introgression or introgressive hybridization is defined as *the incorporation of genes of one species into the gene pool of another species by repeated backcrossing* (ANDERSON 1949). This results in individuals that approach a parental form (one species) but retain some genetic information of the other parent (a different species).

The evolutionary role of natural hybridization has been controversially debated for more than a century. Two contrasting viewpoints have emerged. According to the prevailing view, hybridization is considered to play an important creative role in speciation and adaptive evolution (BARTON 2001; RIESEBERG *et al.* 2003; ARNOLD *et al.* 2004). Among the evolutionary consequences of natural hybridization can be mentioned: formation of new species (see RIESEBERG 1997 for a review), increase of intraspecific genetic variation (ANDERSON 1948), origin and transfer of adaptations (see ARNOLD 2004 and references therein), invasion of one species into the range of other species (e.g. PETIT *et al.* 2004), demise of rare plant species through demographic swamping and genetic assimilation by an abundant related species (LEVIN *et al.* 1996). The opposing view accords little evolutionary importance to hybridization (except for allopolyploidy) at least in undisturbed natural populations (e.g. SCHEMSKE 2000).

Comparisons of occurrence of hybridization in plants and in animals have consistently shown that hybridization is more frequent in the former organismal group

(ARNOLD 1997). However, plant hybridization is less common than once thought. It appears to be confined to a small number of families and an even smaller number of genera, which may be viewed as potential 'hot spots' of contemporary hybridization (ELLSTRAND *et al.* 1996). One of such a genera is certainly the genus *Quercus* in which many species are known to hybridize (e.g. RUSHTON 1993).

1.2. The genus *Quercus*

1.2.1. Taxonomy and natural distribution

Oaks (*Quercus* spp.) are members of the family *Fagaceae*. The last checklist of the genus *Quercus* includes 531 species of trees and shrubs (GOVAERTS and FRODIN 1998). Several taxonomic treatments have been proposed for the genus *Quercus* based on morphological characters (e.g. CAMUS 1936-1954; SCHWARZ 1937; NIXON 1993). The most recent taxonomic scheme (NIXON 1993), the first to be based on explicit cladistic analysis, recognized fewer infrageneric groups than the previous ones. Within *Quercus*, two subgenera were recognized, *Cyclobalanopsis* and *Quercus*, the latter comprising three sections: *Lobatae* (red oaks), *Protobalanus* (intermediate oaks) and *Quercus* (white oaks). All European oak species are included in section *Quercus*, which corresponds to the white oaks in the broadest sense (sensu lato).

The oak species are distributed throughout much of the Northern Hemisphere (NIXON 1993). Oaks are conspicuous members of the temperate deciduous forests of Europe, North America, and Asia, as well as of the Mediterranean woodlands. They extend southward to the tropics where they occur at higher elevations (e.g. in Colombia) (NIXON 1993).

1.2.2. Reproductive biology

Quercus spp. are monoecious species, i.e. they produce male and female flowers on the same tree. As most of the temperate tree species, oaks are wind-pollinated. A recent study based on paternity analysis has indicated that pollen dispersal in oaks can be a combination of local pollen dispersal, i.e. prevalent matings with the neighboring trees, and long-distance transport (STREIFF *et al.* 1999). In general, oaks are thought to posses an incompatibility system (DUCOUSSO *et al.* 1993). Mating system studies (e.g. BACILIERI *et al.* 1993) and paternity analysis (STREIFF *et al.* 1999) revealed that oaks are nearly complete outcrossing species. Their annual regularity of flower production contrasts with their irregular acorn production, which may range from abundant crops (mast-years) in some years to poor or no crops in others (JOHNSON *et al.* 2002). Oak's fruits, the acorns, are dispersed by gravity, birds (e.g. jay – *Garrulus glandarius* L.) and rodents (DUCOUSSO *et al.* 1993).

1.2.3. Oak species in Romania

To date oaks cover approximately 1.13 million ha in Romania, i.e. 18.2% of the total forest area. They rank third among the forest tree species in the country, after beech (*Fagus sylvatica* L. – 30.7%) and Norway spruce [*Picea abies* (L.) Karst –22.9%] (STANESCU *et al.* 1997). The ranking was totally different in the past when oaks were the predominant species (56%) (GIURGIU *et al.* 2001). Oaks are a main source of timber and firewood, and oak forests have been cleared and fragmented to create land for agricultural and development purposes. The establishment of populations through plantation and artificial seed transfer has been relatively rare.

The genus *Quercus* is represented in Romania by 5-9 species depending on the taxonomic ranking (GEORGESCU and MORARIU 1948; SAVULESCU 1952; STANESCU *et al.* 1997). However, only five species are easily distinguishable and widely accepted among botanists: *Quercus robur* L. - pedunculate oak, *Q. petraea* (Matt.) Liebl. - sessile oak, *Q. pubescens* Willd. – downy or pubescent oak, *Q. frainetto* Ten. – Italian or Hungarian oak and *Q. cerris* L. - Turkey oak. The positions of these species according to different taxonomical schemes are illustrated in Tables 1-1, 1-2, and 1-3, respectively.

Table 1-1 Taxonomic classification of Romanian oaks (after CAMUS 1936-1954)

Genus	Subgenus	Section	Subsection	Species
Quercus	*Euquercus*	*Cerris*	*Eucerris*	*Q. cerris* L.
		Mesobalanus	*Macranthere*	*Q. frainetto* Ten.
		Lepidobalanus	*Sessiliflorae*	*Q. petraea* (Matt.) Liebl.
				Q. pubescens Willd.
			Pedunculate	*Q. robur* L.

Table 1-2 Taxonomic classification of Romanian oaks (after SCHWARZ 1937)

Genus	Subgenus	Section	Series	Species
Quercus	*Lepidobalanus* (*Quercus*)	*Roburoides*	*Sessiliflorae*	*Q. petraea* (Matt.) Liebl.
		Robur	*Pedunculatae*	*Q. robur* L.
		Dascia	*Confertae*	*Q. frainetto* Ten.
			Lanuginosae	*Q. pubescens* Willd.
	Cerris	*Eucerris*	*Cerrides*	*Q. cerris* L.

Table 1-3 Taxonomic classification of Romanian oaks (after NIXON 1993)

Genus	Subgenus	Section	Group	Species
Quercus	*Quercus*	*Quercus* s.l. (sensu lato)	*Quercus* s.s. (sensu stricto)	*Q. robur* L.
				Q. petraea (Matt.) Liebl.
				Q. pubescens Willd.
				Q. frainetto Ten.
			Cerris group	*Q. cerris* L.

The four other allied species that are mentioned to occur in Romania are as follows: *Q. pedunculiflora* K. Koch. which is usually treated as *Q. robur*, *Q. dalechampii* (Ten.) Soó and *Q. polycarpa* Schur. - Balcanic oaks – which are grouped with *Q. petraea*, and *Q. virgiliana* Ten. which is combined with *Q. pubescens*.

All five Romanian oak species (considered as sensu lato) are deciduous trees and have different ecological requirements. For instance, *Q. robur* (Fig. 1-1A) prefers nutrient-rich and wetter soils which can be subjected to flooding for a short period of time whereas *Q. petraea* (Fig. 1-1B) grows on more acidic and better drained soils (STANESCU *et al.* 1997). The three other species, *Q. pubescens* (Fig. 1-1C), *Q. frainetto* (Fig. 1-1D), and *Q. cerris* (Fig. 1-1E) are elements of the (sub-) Mediterranean flora which reach in Romania the northern edge of their natural distribution. *Q. pubescens* is usually found on sunny and dry slopes being better adapted to xeric conditions in comparison with the other species. *Q. frainetto* and *Q. cerris* are thermophile and xero-mesophile species, which tolerate different types of soil, including the compact ones (for example, heavy clay soils) (BUSSOTTI 1997; STANESCU *et al.* 1997; BARTHA 1998; BUSSOTTI 1998). In spite of substantial differences in ecological requirements the presence of two or more oak species in mixed stands is relatively common (STANCIU 1995; STANESCU *et al.* 1997).

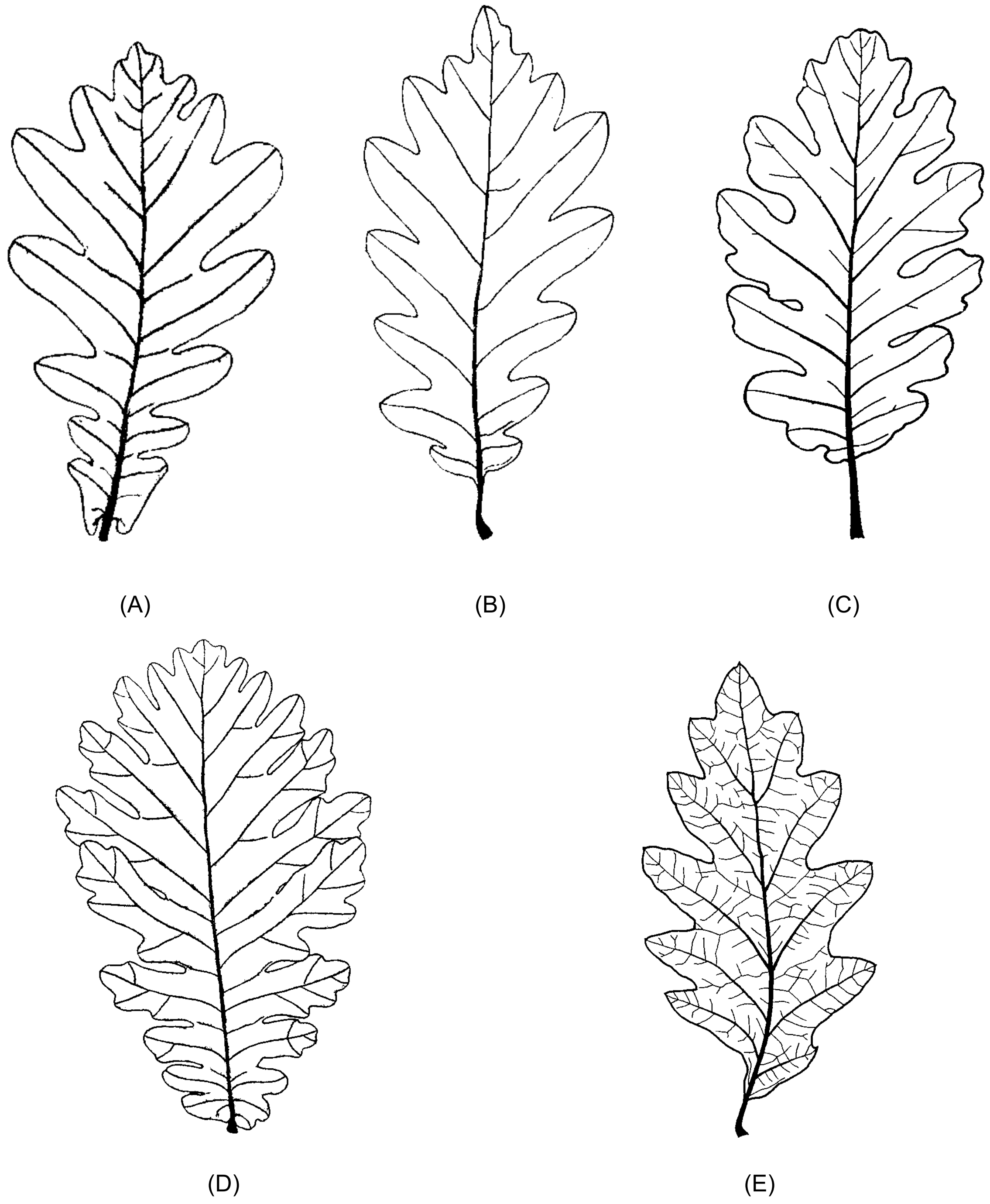

Fig. 1-1 Typical leaf shape for: (A) *Q. robur* (B) *Q. petraea* (C) *Q. pubescens* (D) *Q. frainetto* (E) *Q. cerris* - after SCHWARZ (1936)

1.2.4. Natural hybridization in oaks

Defining species boundaries in oaks has raised difficulties for decades. For this reason, SCHWARZ (1937) designated genus *Quercus* as „*crux botanicorum*". However, the '*taxonomically perplexing patterns of intraspecific morphological* *variation*' (MANOS *et al.* 1999) may be caused, in part, by hybridization between species (e.g. RUSHTON 1993; BACILIERI *et al.* 1996; HOWARD *et al.* 1997). The propensity of many oak species to hybridize has led the biological species concept (BSC - MAYR 1942), i.e. “species are groups of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups” to be challenged in *Quercus* (BURGER 1975). Moreover, the oaks served as model organisms in the development of species concepts that rely on ecological criteria (VAN VALEN 1976).

The occurrence of hybridization in oaks was commonly inferred based on morphological characters (RUSHTON 1993), which may be quite plastic and easily to misinterpret (RIESEBERG 1995). Studies based on cpDNA (chloroplast DNA) markers revealed that the most common variants are shared across closely related species (e.g. KREMER *et al.* 1991; WHITTEMORE and SCHAAL 1991). The finding of nearly identical geographical patterns of cpDNA variants for several sympatric species (e.g. DUMOLIN-LAPÈGUE *et al.* 1997; PETIT *et al.* 2002b) was interpreted as an evidence for hybridization and introgression between species during and/or after postglacial colonization. A mating system study (BACILIERI *et al.* 1996) and a paternity analysis (STREIFF *et al.* 1999) carried out in a mixed stand of *Q. robur* and *Q. petraea* showed that hybridization between the two species may be quite frequent. Accordingly, with only a few exceptions (the so-called *outlier* loci) several types of nuclear markers indicated a low genetic differentiation between *Q. robur* and *Q. petraea* (SCOTTI-SAINTAGNE *et al.* 2004 and references therein). Recently, shared ancestral polymorphism originating before the split of the two species was proposed as an explanation for the low levels of genetic differentiation detected between the two species (MUIR and SCHLÖTTERER 2005) which comes into contradiction with the prevailing view of important interspecific gene flow and selection on a subset of loci effectively maintaining species integrity (LEXER *et al.* 2006).

A few studies have used both morphological characters and random amplified polymorphic DNA (RAPD) markers to study hybrid zones between North American oaks (HOWARD *et al.* 1997; GONZALEZ-RODRIGUEZ *et al.* 2004; TOVAR-SANCHEZ and OYAMA 2004). Highly polymorphic microsatellite markers were also applied to study hybridization between two Californian oak species, *Q. lobata* and *Q. douglasii* (CRAFT *et al.* 2002) or between *Q. petraea* and *Q. pyrenaica* in Spain (VALBUENA-CARABANA *et al.* 2005).

Detailed studies of patterns of interspecific gene flow have focused on only a few European and North American oak species. In Europe, the two most widespread species, *Q. robur* and *Q. petraea*, have received much of the attention. However, little is known about the amount of gene flow between these two species and other oak species (e.g. *Q. frainetto*, *Q. pubescens*), in particular when they cohabit naturally in mixed forests.

1.3. Aim and objectives

The present work aims at characterizing hybridization in a mixed forest of five oak species (*Quercus* spp.) situated in west-central Romania. The main objectives of the study are:

- to assess the morphological variation based on pubescence and leaf characters in order to distinguish phenotypically pure species from intermediate individuals.
- to examine the genetic variation within and among oak species by using both chloroplast and nuclear genetic markers.
- to test the correspondence of morphological groups (phenotypically pure species and intermediate individuals) with those inferred from individual multilocus genotypes.
- to trace and quantify the level of contemporary gene flow and hybridization by using paternity analysis.

2. MATERIAL AND METHODS

2.1. Material

2.1.1. Study area

Trees were sampled at Bejan Forest (45°51’N, 22°53’E), an oak reserve situated at the foothill of the Carpathian mountains in west-central Romania (Fig. 2-1). At Bejan all five Romanian oak species cohabit naturally: *Q. robur*, *Q. petraea*, *Q. pubescens*, *Q. frainetto* - considered as sensu lato, and *Q. cerris* (e.g. STANCIU 1995; STANESCU *et al.* 1997).

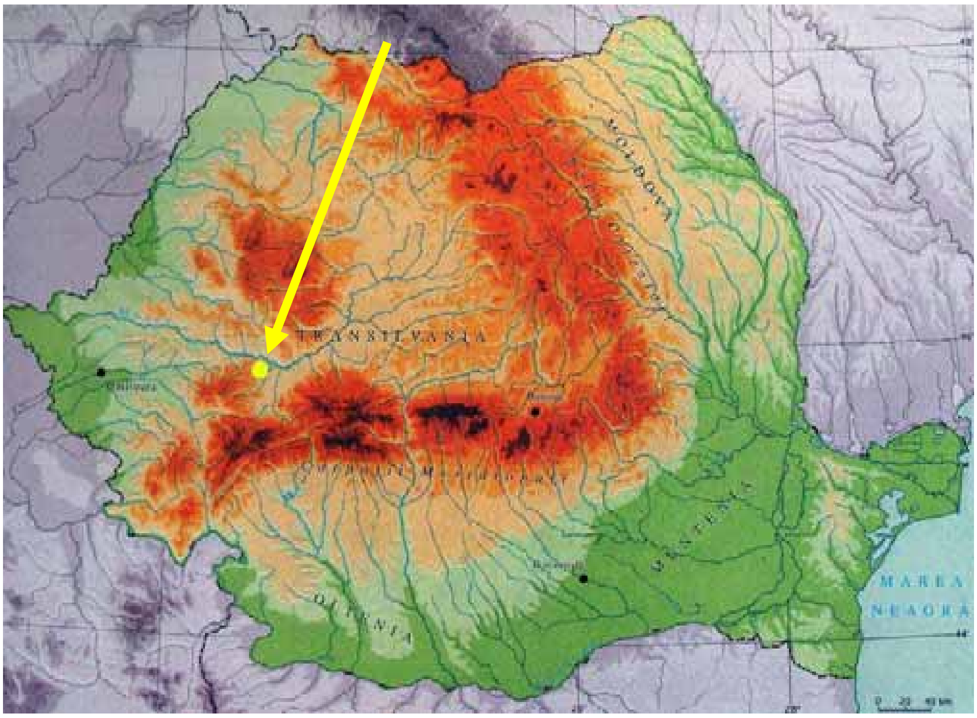

Fig. 2-1 Map of Romania. The study area in indicated by the arrow.

To preserve this unique area, Bejan Forest was declared a reserve in 1939 (STANCIU 1995). The study area is located on a south-east facing hillside at an elevation of 250-380m above see level and experiences a continental climate with Atlantic influences. Mean annual temperature and precipitation is 10.0°C and 578 mm, respectively. Different subtypes of brown forest soil were described throughout the reserve (STANCIU 1995). Silvicultural cuttings have been suppressed since the beginning of last century. According to the forest records oaks are the most abundant species making up to 90% of the forest composition. The oak trees are about 120-200 yr old and originate from natural regeneration.

2.1.2. Sampling

Adult trees. A study plot was established in the contact zone between the five oak species (Fig. III-1). In total 320 adult trees (269 white oaks – section *Quercus* s.s. Nixon and 51 individuals of *Q. cerris*) were sampled. Within an area of approximately 4.5 ha (the core-plot) the sampling of white oaks was exhaustive, with no *a priori* selection of trees. Since *Q. frainetto* was less abundant within the core plot, the sampling for this species was extended to the nearby area outside of the core plot (Fig. III-1). Trees were mapped using a LEDHA-GEO laser instrument.

Offspring sampling. In 2004 there was a lack of fructification for all species at Bejan. Only in autumn 2005 a total of 320 acorns could be collected from eight seed parents (four *Q. petraea*, two *Q. frainetto*, one *Q. robur*, and one *Q. pubescens*). The sampling design was severely affected by the nearly complete lack of fructification for *Q. robur* and *Q. pubescens* in 2005. All maternal trees are located in the central part of the study plot (Fig. IV-1). The acorns were collected on the ground near the trunk of each seed parent after shooting their crown with a gun.

2.2. Methods

2.2.1. Morphological assignment

Since *Q. cerris* could be unambiguously distinguished from the other white oak species in the field, it was not included in the morphological analysis. For the other species, three to five (on average 4.2) leaves and at least one current year shoot were sampled in the upper crown of each tree. Leaves and shoots were stored in a herbarium for further morphological measurements. To discriminate among species two methods were applied: the first one was previously used for the separation of *Q. robur*, *Q. petraea* and *Q. pubescens* in Central Europe on the basis of pubescence characters (AAS 1998); the second approach considers leaf morphological traits and was used by KREMER *et al.* (2002) for distinguishing *Q. robur* from *Q. petraea*. More details about the morphological methods are given in Chapter III.

2.2.2. Genetic analysis

DNA extraction

Total genomic DNA was extracted from buds using the Qiagen Dneasy96 Plant Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer.

cpDNA (chloroplast DNA) markers

As cytoplasmatic (chloroplast and mitochondrial) markers are uniparentally inherited, they are helpful in inferring the female or male origin. Analysis of chloroplast DNA