SYSTEMATIC AND ECOGEOGRAPHIC STUDIES ON CROP GENEPOOLS 12

IPGRI

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FOREWORD

Much has been said about the need to base biodiversity conservation strategies on solid scientific foundations, for example in international agreements such as the Convention on Biological Diversity and The State of the World's Plant Genetic Resources for Food and Agriculture. Of course, this requires data, often in large amounts. The task of collating and analyzing these data is a complex, expensive and time-consuming exercise, especially where wild species are concerned, requiring expertise in a number of disparate technical fields. This publication summarizes the results of an IPGRI project ('Studies on breeding systems: the case of a short-living perennial, alternatively outbreeder-inbreeder species—*Phaseolus lunatus* L.—and its consequences for germplasm conservation') which systematically gathered detailed ecogeographic, demographic, phenological and genetic data, and used these to develop and implement an effective, sustainable conservation strategy for a wild relative of a crop plant.

Wild and weedy relatives of cultigens are an extremely important source of genes for crop improvement, but these crucial genetic resources are coming under increasing pressure the whole world over, as wild habitats are altered, degraded and lost. Although wild Lima bean in the Central Valley of Costa Rica was the specific focus of the project, the approaches and tools used to analyze and apply the extensive and varied data that were amassed will be widely applicable. These are important achievements, and are a testament to the dedication of the project partners, the Escuela de Biología of the Universidad de Costa Rica (UCR) and the Unité de Phytotechnie Tropicale et d'Horticulture of the Faculté Universitaire des Sciences Agronomiques de Gembloux (FUSAGx), Belgium. They also illustrate the far-sightedness of the donor, Belgium's Direction Générale de la Coopération au Développement, which has supported the project over two phases lasting 8 years. IPGRI, and in particular its Regional Office for the Americas in Cali, Colombia, is proud to have initiated this project and to have been involved in its implementation as the coordinating institution. We look forward to, and will work towards, the wide dissemination of its results and their application to the conservation of biodiversity, and in particular wild relatives, in other parts of the world, so that their genetic diversity will be available to future generations for the improvement of the crops that our children and grandchildren will depend on for their food, health and income.

1. BACKGROUND TO THE PROJECT

The project 'Studies on breeding systems: the case of a short-living perennial, alternatively outbreeder-inbreeder species—*Phaseolus lunatus* L.—and its consequences for germplasm conservation' ran in two 4-year phases from 1992 to 2000, with funding from Belgium's Direction Générale de la Coopération au Développement. This was a collaboration between three partners: IPGRI's Regional Office for the Americas in Cali, Colombia, the Escuela de Biología of the Universidad de Costa Rica (UCR) and the Unité de Phytotechnie Tropicale et d'Horticulture of the Faculté Universitaire des Sciences Agronomiques de Gembloux (FUSAGx), Belgium. The field and laboratory work was carried out in large part by students at UCR and FUSAGx under the supervision of Dr Oscar Rocha and Prof Jean-Pierre Baudoin, respectively. IPGRI provided overall scientific coordination.

The objectives of the project were to study the influence of floral biology, breeding system, phenology and demography on the population dynamics and genetics of wild and weedy populations of Lima beans in the Central Valley of Costa Rica, and to integrate this information in a programme for *in situ* conservation of the species. These objectives are fully congruent with the policies of the Government of Costa Rica, which place strong emphasis on the development of strategies for the conservation and sustainable use of natural resources, including biodiversity.

The study was conducted in the Central Valley of Costa Rica because of the presence of numerous wild and weedy populations of *P. lunatus* (Figure 1), and because of their perceived risk of extinction as a result of increasing urbanization and changes in agricultural and land-use practices. These populations represent a very important genetic reservoir for the improvement of the various *Phaseolus* bean cultigens commonly found in many traditional smallholder cropping systems, not only in Latin America but also in other regions of the world (e.g. East Africa). *P. lunatus* is also a useful plant model due to its reproductive biology. Lima bean is a self-compatible annual or short-lived perennial species with a mixed mating system, i.e. predominantly self-pollinating, but with some degree of outcrossing.

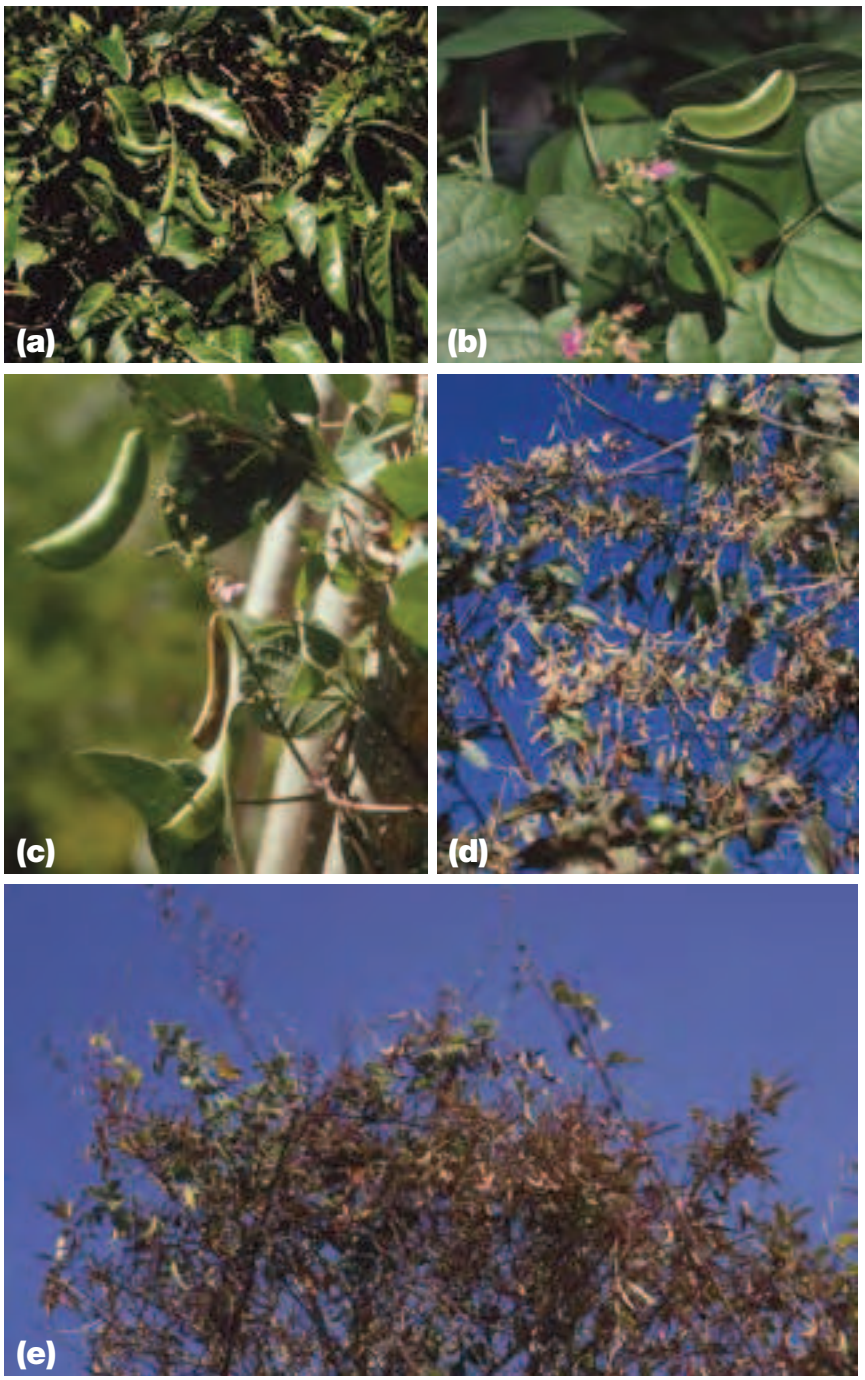


Figure 1. Wild Lima bean in the Central Valley of Costa Rica. (a) General view of Lima bean vines climbing over a coffee bush; (b) close-up of Lima bean inflorescence and leaves; (c) close-up of Lima bean inflorescence; (d) dry Lima bean pods; and (e) dry Lima bean pods in the canopy of a supporting tree.

2. THE MODEL SPECIES: *PHASEOLUS LUNATUS* L.

An understanding of the phylogenetic relationships among taxa within a crop genepool is of great importance both to germplasm curators and to plant breeders. Phylogenetic studies are useful in identifying the wild progenitors and other relatives of domesticated species (Page and Holmes 1998), and in clarifying the composition of their primary, secondary and tertiary genepools. This will help to define both sampling strategies for *ex situ* collections and management approaches for *in situ* conservation, as well as to prioritize material for utilization in breeding programmes. Gepts (1996) suggests that determining the closest relatives of cultivated beans, for example, has important implications for crop breeding because domestication has caused a 'genetic bottleneck' in all *Phaseolus* cultigens. Furthermore, breeding programmes that involve interspecific crosses are particularly important in *Phaseolus* (Hucl and Scoles 1985). This section provides background on *P. lunatus*, the model species studied by the project, by describing its taxonomic position within the genus. It also summarizes our knowledge of its ecogeography and of the large-scale patterns of genetic diversity within the species.

2.1 Taxonomy

The genus *Phaseolus* (subtribe Phaseolinae, tribe Phaseoleae, family Leguminosae) has a complex taxonomic and nomenclatural history, which is well illustrated by *P. lunatus*, the Lima bean (Maquet 1995). Recent phylogenetic investigations strongly support a monophyletic *Phaseolus*. In this modern circumscription, *Phaseolus* is strictly of New World origin, concentrated in tropical and warm temperate America. Whereas a definition of the genus is now agreed upon by most legume taxonomists, the exact number of species within the genus is still being debated (Debouck 1999). However, there are probably around 50 species in the genus, of which 5 are domesticated: common bean (*P. vulgaris* L.), Lima bean (*P. lunatus* L.), scarlet runner bean (*P. coccineus* L.), tepary bean (*P. acutifolius* A. Gray) and year bean (*P. polyanthus* Greenm.).

These five domesticated species belong to two distinct lineages. The *P. vulgaris* group includes *P. vulgaris*, *P. coccineus*, *P. polyanthus* and *P. acutifolius*. The fifth cultivated species, *P. lunatus,* is part of a separate, very well-defined clade which includes the South American and oceanic island diversification of *Phaseolus*, i.e. *P. augusti* Harms, *P. bolivianus* Piper, *P. lignosus* Britton, *P. mollis* Hook., *P. pachyrrhizoides* Harms, *P. rosei* Piper and *P. viridis* Piper (Caicedo *et al*. 1999; Delgado Salinas *et al*. 1999). A group containing *P. viridis* (from Oaxaca, Mexico) and *P. lignosus* (from Bermuda) is sister to the rest of this primarily South American group. The Andean *P. pachyrrhizoides*, *P. augusti* and *P. bolivianus* form a monophyletic group which is sister to a lineage containing both Mesoamerican and Andean accessions of *P. lunatus*, as well as one accession of *P. mollis*, a species endemic to the Galapagos Islands. *P. mollis* and a Peruvian accession of *P. lunatus* are resolved as a sister group, suggesting a mainland origin for the Galapagos species. *P. rosei* collected at the type locality (Chimborazo, Ecuador)

falls within the Andean group of wild Lima beans. *P. rosei* could thus be an Andean wild form of Lima bean (which would make its correct name *P. lunatus*) (Toro Chica *et al*. 1993; Caicedo *et al*. 1999).

The latest available taxonomic treatment of *P. lunatus* is by Baudet (1977). A botanical variety, *P. lunatus* var. *lunatus*, was created for all the cultivated genotypes and var. *silvester* for the wild form. The variety *lunatus* includes the three cultigroups defined by Mackie (1943). The primary genepool of *P. lunatus* comprises the wild populations and the landraces of Lima bean, which can be grouped into two main races: the Andean and the Mesoamerican. Each race is characterized by distinctive morphological characters (Debouck *et al*. 1987a; Maquet 1995), ecological adaptation (see below), seed storage proteins (Debouck 1989; Lioi 1994; Gutierrez Salgado *et al*. 1995; Maquet 1995; Lioi *et al*. 1999), allozymes (Maquet *et al*. 1997; Lioi *et al*. 1998) and molecular markers (Nienhuis *et al*. 1995; Fofana *et al*. 1997; Lioi *et al*. 1998; Caicedo *et al*. 1999). Escaped forms and weedy forms (natural hybrids between the wild form and a landrace) are observed throughout Latin America (see below). In addition, several collections made in northern Peru have shown either that more cultigroups of native varieties should be defined, or that the concept of a cultigroup is becoming obsolete because of the great diversity found in the field (Debouck *et al*. 1987b). Hawkes (1986) suggested avoiding Latin names altogether at the intraspecific level for cultivated species. The best taxonomic treatment may, in fact, be to use the binary combination (i.e. *P. lunatus* L.) with some additional indication of the biological status of the material (e.g. wild, weedy, hybrid, landrace, etc.) (Debouck 1991; Maquet 1995).

Studies of interspecific hybridization (reviewed by Debouck 1999) have been carried out to investigate the relationships between Lima bean and its wild allies from Mesoamerica in the context of the genepool concept of Harlan and de Wet (1971), as modified by Smartt (1990).

Currently, no natural interspecific hybrids involving *P. lunatus* have been reported. The secondary genepool of the Lima bean probably consists of the South American species (i.e. *P. augusti*, *P. bolivianus*, *P. pachyrrhizoides*). These species differ very little (Caicedo *et al*. 1999; Delgado Salinas *et al*. 1999) and may in fact constitute a single species with geographic variants.

The tertiary genepool of *P. lunatus* includes the following species from the USA and/or Mexico with varying levels of relationship and compatibility (Debouck 1999; Delgado Salinas *et al*. 1999; Delgado Salinas 2000): *P. jaliscanus* Piper, *P. juquilensis* Delgado, *P. maculatus* Scheele, *P. marechalii* Delgado, *P. polystachyus* Britt., Stern and Pogg, *P. ritensis* Jones, *P. salicifolius* Piper, *P. sonorensis* Standley and *P. xolocotzii* Delgado. Two variants of *P. polystachyus* are usually recognized: *P. sinuatus* Nutt. and *P. smilacifolius* Pollard.

All attempts to cross *P. vulgaris* with *P. lunatus* have so far failed to produce a viable and fertile hybrid (Al-Yasiri and Coyne 1966; Mok *et al*. 1978; Leonard *et al*. 1987; Kuboyama *et al*. 1991). The early success claimed by Honma and Heeckt (1959) with the use of heterozygous parents is problematic, as discussed by Hucl and Scoles (1985). The reciprocal cross *P. lunatus* × *P. vulgaris* is even more difficult to obtain (Rabakoarihanta *et al*. 1979). The difficulty in obtaining

fertile viable hybrids between common and Lima beans confirms their distinct taxonomic position in the genus. Some species could fall between these two extremes, but most have not been studied in sufficient detail so far.

2.2 Ecogeography

The wild form of *P. lunatus* is found only in the Americas, while the cultivated form is widely distributed all over the tropical regions of the world and in some temperate regions (e.g. Italy). Some short-cycle breeding lines are cultivated in southern Canada and escapes have been reported in the Democratic Republic of Congo. Despite this extensive distribution, we restrict our discussion to the New World, the origin and first centre of diversity of *P. lunatus*. With regard to the cultivated form, only the landraces are considered here.

The geographic distribution of wild and cultivated Lima beans extends from the extremely dry climate of the Peruvian coast, to the tropical humid climate of the Amazonian region in Ecuador and Peru, to the temperate climates of altitudes above 2500 m asl (metres above sea level). The small-seeded wild form of *P. lunatus* extends at low altitudes from Sinaloa, Mexico (23–26°N) to Salta, Argentina (25°S) and through the Caribbean islands (Maquet and Baudoin 1997). Wild populations are mainly distributed along the Pacific slopes of the mountains of Mexico and Guatemala, but are found exclusively on these slopes further south in Mesoamerica. Caducifoliate and semi-deciduous forests characterize the region at low altitudes (below 800 m asl), often referred to as *Tierra Caliente*, with an annual mean temperature (T_m) higher than 22°C. These forests are the transition between very humid and drier regions. In this zone, rainfall varies from 800 to 1500 mm per year, with a well-defined dry season of 3–5 months. At mid-altitudes (800–2000 m asl), the *Tierra Templada* is covered with evergreen forests, with T_m varying within the range 17–22°C. Temperature is a critical factor, and low temperatures (temperature of the coldest month <14°C) restrict the geographic distribution of wild *P. lunatus* populations both in altitude and in latitude.

Both small- and large-seeded wild populations are present in South America, but not in the same habitats. Small-seeded populations are distributed along the east slope of the Andean cordillera in central-northern Colombia, central-southern Peru and northern Argentina. The large-seeded wild form of *P. lunatus* extends in the Andean region from Cajamarca, Peru, to Imbabura, Ecuador, and possibly into Colombia (Toro Chica *et al*. 1993). In Ecuador and northern Peru, deciduous forests give way to steppes (*matorral*) due to low rainfall. This could suggest that large-seeded wild populations are best adapted to drier conditions. In addition, these populations are tolerant of lower temperatures ($T_m < 15$°C). No wild populations have been collected in the eastern part of South America. Although Piper (1926) reported the species in Brazil, he did not provide geographic data. Wild populations are also not found in Chile (probably due to extreme dryness in northern Chile) and in Bolivia, despite a similar climate in nearby Peru and Argentina.

Small-seeded landraces are grown in the semi-arid subtropical region of the southwestern USA, mainly in Arizona, home of the Hopi Amerindians

(Kuhnlein 1981). Core (1967) reported the use of Lima beans by Amerindians in the Appalachian Mountains when the first bush type appeared in the eastern USA at the end of the 19th century, showing adaptation to a subtropical climate. Landraces in Mesoamerica are also characterized by small seeds and are distributed from Mexico to southern Panama, an important part of the *milpa* subsistence agricultural system (SARH *et al*. 1984; Maquet and Baudoin 1997). A similar situation exists throughout the Caribbean, where Lima beans are present at low and mid-altitudes in the traditional homegardens known as '*conucos*' in Cuba (Esquivel and Hammer 1988).

Small-seeded landraces are also distributed throughout South America (Maquet and Baudoin 1997). In the eastern part, they are particularly common in northeastern Brazil. The frequency of large-seeded landraces increases in central and southern Brazil (Erickson 1982). Consequently, a hybrid zone has developed, characterized by landraces with intermediate seed sizes. Landraces are cultivated in the Andean region from Venezuela to Argentina, with small-seeded landraces found at lower altitudes (700 m asl on average) than large-seeded landraces (1880 m asl) (Maquet 1995). There is little information from Chile, but large-seeded landraces have been collected (M. Contreras, personal communication) in the dry north.

Lima bean landraces are thus adapted to ecological systems ranging from the dry Peruvian coast, to the tropical humid Amazonian region of Ecuador and Peru, to the temperate high altitudes above 2500 m asl. Weedy types resulting from gene flow between wild and cultivated forms have been observed where these grow sympatrically (Maquet 1995). For example, hybridization between the small-seeded wild form and the cultigen is known from Mexico, Guatemala, Costa Rica, the Bahamas, Cuba, Puerto Rico and Jamaica (Correll and Correll 1982; Liogier and Martorell 1982; Maquet 1991; Esquivel *et al*. 1993). The weedy type is also present in the Andean region and particularly in Ecuador and Peru (Debouck 1990). It is distributed from 1800 to 2000 m asl in Peru and even up to 2400 m asl in Ecuador.

2.3 Origin, domestication and genetic diversity

Central America, where approximately 40–50 *Phaseolus* species occur today, is considered the centre of origin of the genus (Sousa and Delgado Salinas 1993). *Phaseolus* species are numerous along the mountainous systems of the Sierra Madre Occidental and the Eje Volcánico Transversal of Mexico, which were formed during the Oligocene-Miocene and Late Tertiary or Pliocene, respectively. Therefore, current *Phaseolus* diversity may stem from the Oligocene or later (Delgado Salinas 1985).

With regard to *P. lunatus*, the existence of a secondary genepool in the Andes, with taxa such as *P. pachyrrhizoides*, would indicate a centre of speciation in this region more recent than that of Central America. This is supported by evidence from seed protein and isozyme studies presented by Maquet (1995), Maquet and Baudoin (1996) and Maquet *et al*. (1999). An Andean origin was also suggested by Fofana *et al*. (1999), Caicedo *et al*. (1999) and Sparvoli *et al*. (2001), using

random amplified polymorphic DNA (RAPD) markers, amplified fragment-length polymorphism (AFLP) fingerprinting techniques and lectins and lectin-related proteins, respectively. A wild Lima bean with small seeds has long been reported from several parts of Central America (Standley and Steyermark 1946). However, another wild type with slightly larger seeds was more recently discovered in northwestern Peru (Debouck *et al*. 1987a). Two separate domestication events have since been demonstrated, from two different wild forms, with different distribution ranges and distinct ecologies (Gutierrez Salgado *et al*. 1995; Maquet 1995; Fofana *et al*. 1997). Due to the restricted geographic distribution of the large-seeded wild type, it is assumed that one domestication event occurred within the southern Andes of Ecuador and the northwestern Andes of Peru. In contrast, the range of the small-seeded wild Lima bean is huge, and a precise location of the domestication of the small-seeded cultivated Lima beans is still to be determined.

Lima bean seems to be an ancient crop, although some dates have recently been revised downwards (Kaplan and Lynch 1999). It is more ancient in South America than in Central America. The earliest records are from the Central Andes of Peru and date to 3000 years BP (revised date), although a pod from the coast of Peru has been dated to 5600 years BP (Kaplan 1994). The evolution of the cultivated species under domestication has been reviewed recently (Debouck and Smartt 1995; Debouck 1999). As in most crops, larger pods and seeds are the most striking differences between the cultivated forms and their wild ancestors. Reductions in hard seededness, dormancy and antinutritional factors (i.e. antitrypsin factors, cyanide glycoside) have also occurred. Most landraces contain low levels of linamarin (Baudoin *et al*. 1991). White-seeded cultivars have been selected for by farmers. This mutation appears from time to time in wild populations, for example in Yucatan, Mexico (Debouck 1999). The loss of seed dispersal mechanisms is also a common feature of crop domestication. Major evolutionary changes have occurred in growth habit. The original viny habit with profuse branching, observed in the wild form, was selected against to obtain a quick harvest, leading to a bushy, determinate growth habit and annual landraces. In addition to these changes in plant habit, some selection has been undertaken for photoperiod insensitivity. As pointed out by Koinange *et al*. (1996), most of the evolutionary changes in beans are due to mutations of just a few genes; the increase in seed size is a noteworthy exception.

Domestication is generally associated with an overall reduction in genetic diversity, contrasting with an increased diversity in morphological traits (mostly those under direct, conscious selection by farmers). The reduction in genetic diversity during domestication due to a 'founder effect' has been best demonstrated using molecular markers, which are thought to be neutral to selection. Using seed-protein markers, Maquet *et al*. (1990), Gutierrez Salgado *et al*. (1995) and Maquet (1995) showed reduced genetic diversity among the small-seeded Mesoamerican landraces, and also among large-seeded Andean landraces, although not to the same extent and in different markers.

Allozyme studies complement the data obtained from seed proteins. The total gene diversity of the Lima bean at the species level (H_{es} = 0.26) is similar to

that of *P. acutifolius* (Schinkel and Gepts 1989) and *P. coccineus* (Escalante *et al*. 1994), but higher than that of wild *P. vulgaris* (H_{es} = 0.13) (Koenig and Gepts 1989). On the basis of allozyme data, the genetic diversity of the Lima bean is evenly distributed between the Mesoamerican and the Andean genepools (Maquet *et al*. 1997). This confirms the results obtained by Nienhuis *et al*. (1995) using RAPD markers. Allozyme data also showed that wild and cultivated forms of the Lima bean are characterized by similar levels of gene diversity and are not differentiated within each genepool. However, Fofana *et al*. (1997) showed, using RAPD markers, that the wild form and landraces of each genepool were indeed genetically differentiated. Such discrepancies between studies could result either from the different nature of the markers or from the samples used.

In the international collection of *P. lunatus* held by the Centro Internacional de Agricultura Tropical (CIAT) in Cali, Colombia, gene diversity is mainly distributed among rather than within accessions (G_{ST} = 0.755) (Maquet *et al*. 1997). Lima bean germplasm is characterized by a high inbreeding coefficient (f = 0.891). In spite of this, and a low rate of gene flow, the intrapopulation gene diversity estimated from the CIAT collection (H_S = 0.032) is significantly different from zero and higher than that of other selfing species, such as *P. acutifolius* (H_S = 0.0004) (Schinkel and Gepts 1989) and *P. vulgaris* (Koenig and Gepts 1989).

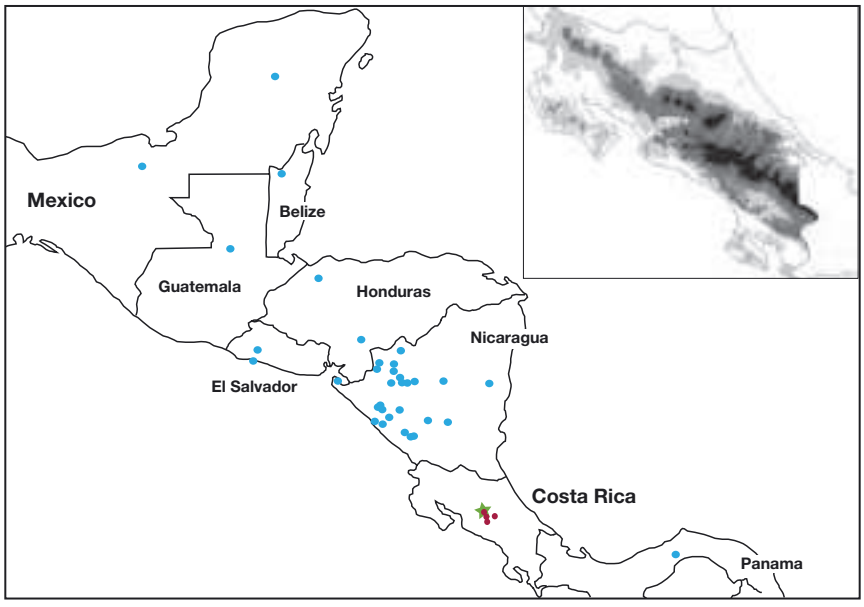
Because of the wide distribution of their wild ancestors and multiple domestication events, cultivated common bean and Lima bean probably have a wider genetic base than the other cultigens in the genus, i.e. *P. acutifolius*, *P. coccineus* and *P. polyanthus* (Debouck 1999). This can perhaps partly explain the tremendous ecological expansion of these two beans, in comparison with the limited success of the other three species.

In conclusion, *P. lunatus* shows high levels of genetic diversity, structured in Mesoamerican and Andean genepools, and also between the wild and cultivated forms. Recent germplasm explorations recorded eight wild *Phaseolus* species growing in the Central Valley of Costa Rica and its vicinity: *P. costaricensis* Freytag & Debouck, *P. leptostachyus* Bentham, *P. lunatus*, *P. oligospermus* Piper, *P. talamancensis* Debouck & Torres, *P. tuerckheimii* Donnell-Smith, *P. vulgaris* and *P. xanthotrichus* Piper (Debouck 1987; Freytag and Debouck 1996; Araya Villalobos *et al*. 2001; Torres González *et al*. 2001). Of these, wild Lima bean is perhaps the species with the lowest risk of extinction, even if many individual populations in the Central Valley are at risk. The study of *P. lunatus* thus gives us the opportunity to observe the mechanisms contributing to the maintenance of genetic diversity over many generations, and to obtain reliable data from a model system of relevance not just to the more critically threatened *Phaseolus* species, but also to other species with similar botanical characteristics.

3. THE STUDY AREA: THE CENTRAL VALLEY OF COSTA RICA

The Central Valley is an inter-montane valley located in the geographic centre of Costa Rica (Map 1). It is delimited by two major mountain ranges: the Cordillera Central to the north, and the Cordillera de Talamanca to the south (Flores 1991; Bergoeing 1998). Altogether it is estimated to enclose an area of approximately 1500 km², as defined by an altitudinal range between 800 and 1400 m asl. However, a broader definition of the Central Valley takes in a maximum area of 3246 km² (Bergoeing 1998). In this study, the area considered is within the altitude range 800–1800 m asl. The maximum length of the area is roughly 70 km, running from east to west, and the width is about 30 km, running from north to south.

The Central Valley is divided into two smaller valleys at the continental divide, by the Cerros de la Carpintera, an outlier of the Cordillera de Talamanca, and the lava flow of Ochomogo (Flores 1991; Castillo-Muñoz 1991; Bergoeing 1998). The western valley, or Valle de San José, is the larger, and includes about two-thirds of the total area. It is bounded by the Cerros de Escazú, La Candelaria and El Tablazo to the south, the Cerros de Aguacate to the west, and the Cordillera Central to the north. The eastern valley, or Valle de Cartago, is located between the Cordillera Central to the north and the Cordillera de Talamanca to the south. Both valleys have access to the coastal lowlands; the Valle de San José by



Map 1. Map of Central America showing location of herbarium specimens of wild *P. lunatus* conserved at the Missouri Botanical Garden. The locations in red are in the Central Valley of Costa Rica. The capital, San Jose, is shown by a star.

descending slowly along the basin of the Grande de Tárcoles River, and the Valle de Cartago along the basin of the Reventazón River. The eastern valley drains into the Caribbean Sea, while the western valley into the Pacific Ocean.

Most of this study was conducted in the upper valley and watershed of the Río Grande de Tárcoles (latitude range 9º54'–10º07'N, longitude range 83º50'–84º28'W). A portion of the Valle del Guarco and the small valley of Acosta were also included.

Geologically, the Central Valley of Costa Rica, together with the Cordillera Central, are part of the Nicaraguan graben (Bergoeing 1998). In general, the geological history of the valley begins with the deposition of sedimentary rocks before the Upper Eocene. This process ends in the Lower Miocene. These sediments later experienced folding, faulting and intrusion. In the Upper Tertiary, a period of volcanic activity originated the Aguacate formation, which also later experienced folding, faulting and erosion. A new period of volcanic activity began in the Quaternary, giving rise to the Cordillera Central.

The valley lies on top of slightly folded Tertiary marine sediments, over which are deposited Quaternary volcanic sediments (Flores 1991). The Central Valley volcanic field is made up of two sequences of rocks that outcrop in the western and eastern portions of the valley. The volcanic rocks of the western valley are made up of andesitic and basaltic lava flows, tuffs and ignimbrites of latitic and andesitic-basaltic composition, mudflows derived from the highlands to the north and northeast, and pyroclastics recently erupted by the volcanoes of the Cordillera Central (Castillo-Muñoz 1983). On the other hand, the volcanic rock sequences of the eastern valley are made up of lava flows, breccias, tuffs, mudflow deposits and recent pyroclastic material (Castillo-Muñoz 1983; Bergoeing 1998).

The population of Amerindians living in Costa Rica before the arrival of the Spaniards has been estimated at approximately 337 000 people (Pérez-Brignoli 1997). As in the rest of Central America, the Costa Rican Amerindian population declined with the coming of the Conquistadors, primarily because of a succession of severe epidemics, slavery and war (Seligson 1980). The central part of Costa Rica was primarily inhabited by one native group, the Huetares. Typically, they lived in small settlements, practising a little agriculture, hunting and fishing. Their main crops were maize, beans, cassava, squash and peppers (Meléndez 1978). However, most of the valley was covered with dense forests.

The conquest of Costa Rica started in 1561, with the arrival of Juan de Cavallón and the establishment of Garci-Muñoz in the Central Valley. Most Spaniards settled in the valley, where they started small family farms growing subsistence crops, such as maize and wheat (Seligson 1980; Boucher *et al*. 1983). It was accepted that the settlers could use as much land as necessary to subsist: land was viewed as essentially limitless in supply. However, because of the rapid decline in the Amerindian population, which resulted in insufficient labour, colonial haciendas did not take root in the Central Valley, proving to be economically unviable. The availability of land attracted the few Spanish migrants who eventually came to Costa Rica. The new settlements had an important impact on the vegetation cover, as the settlers cleared the forest to establish their agricultural fields and pastures.

The most striking change in land use in the Central Valley, however, began with the introduction of coffee to Costa Rica in 1808. Governor Tomás de Acosta attempted to strengthen the economy of the poverty-stricken colony, and considered that this crop could become a much needed commodity to trade with Europe. However, the cultivation of coffee evolved slowly, and little progress was made before 1920 (Seligson 1980; Boucher *et al*. 1983). In 1821 the town council of San José passed a decree providing free State land and free coffee seedlings to any individual who agreed to plant them. Later, after independence from Spain, the Chief of State, Juan Mora Fernández, exempted coffee cultivation from taxes. In addition, a decree was issued in 1931 to the effect that anyone who cultivated coffee on State lands would automatically become the owner of those lands if he worked them for 5 years (Seligson 1980).

As a result of these measures, sufficient quantities of coffee beans became available for export, but these actions also resulted in the establishment of most of the Costa Rican population in the Central Valley. The landscape changed dramatically as the forest was almost totally replaced with agricultural fields (Figure 2). Coffee and sugarcane, with smaller areas of other crops and pastures, now dominate the landscape, and most Costa Ricans still inhabit the Central Valley.

In general, the soils of the Central Valley are highly fertile. According to the preliminary soil map of Costa Rica (Pérez *et al*. 1978), there are 11 soil

Figure 2. General view of the Central Valley of Costa Rica.

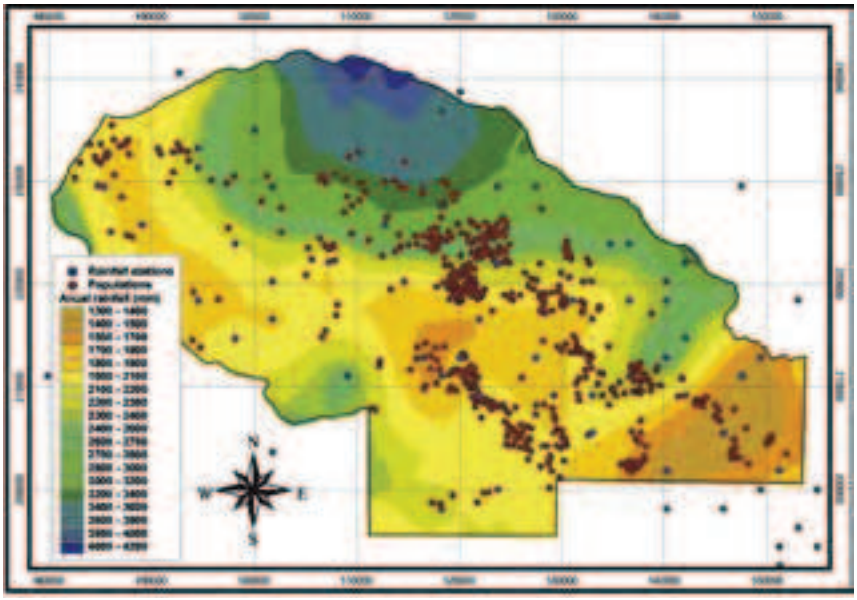
associations in the study area, belonging to 9 main soil types. Most soil types have been affected by volcanic activity in the Cordillera Central and typically have developed from volcanic ash deposits (i.e. Typic Dystrandept, Typic Humitropt, Andic Ustic Humitropt and Ustic Humitropt) or other volcanic tuffs. Most soils can be described as deep, rich in organic material, and well-drained. Some notable exceptions are the poorly drained Aeric Tropaquet in Cartago, and the thin Typic Dystropept in the Cerros de La Carpintera, Cerro Tablazo in Cartago and the counties of Aserrí, Santana and Mora in San José, and the towns of Cebadilla and Río Grande in Alajuela. The variation in soil types closely reflects the diversity in topography, climate and geological history that characterizes this area.

As far as climate is concerned, great variation in the microgeographic distribution of rainfall results from the orientation of the mountain ranges and the location of wind passes (Table 1, Map 2). Rainfall is seasonal, with a well-defined dry season during December–April. The dry season in the southwestern portion of the Central Valley, especially in those areas located west of the Cerros de Escazú (namely Acosta, Tabarcia de Mora and Puriscal), is not as severe as in the rest of the valley. This is because these areas do not experience the strong winds that affect the rest of the valley at this time of the year (Fournier *et al*. 1985). The rainy season also varies in intensity, and there is less rain in the middle of the year (typically in July) than during the months just before and after. This period of less rain is called *veranillo*, which means little summer (Coen 1983). Typically, March and September are the months with the lowest and highest precipitation, respectively (Protti *et al*. 1983). In general, annual rainfall is lower in the eastern valley than in the western valley.

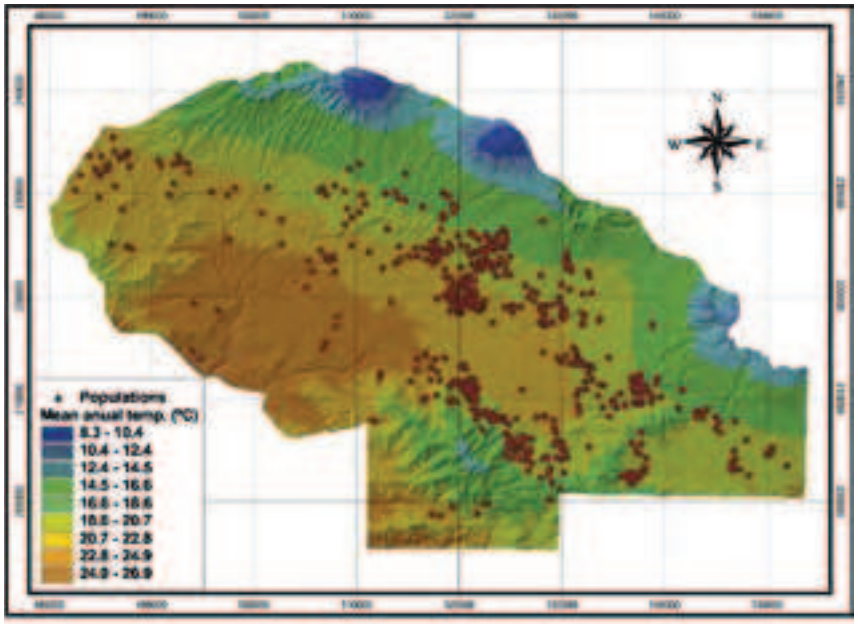
Because of its geographic location, temperature is not particularly variable in the Central Valley (Protti *et al*. 1983; Fournier *et al*. 1985). Mean annual temperature ranges from 15°C, at the top of the Cordillera Central and the

Table 1. Elevation, mean annual rainfall and location of 14 cities within the study area (from Fournier et al. 1985).

City	Elevation (m asl)	Mean annual rainfall (mm)	Valley
Desamparados	1162	1963	West
Ojo de Agua	850	1767	West
Acosta	1905	2552	West
Coronado	1382	2255	West
Atenas	696	1813	West
Curridabat	1100	2424	West
Grecia	525	2303	West
Naranjo	1042	2505	West
Alajuela (El Coco)	920	1875	West
Alajuela (San José)	840	2017	West
San José	1172	1977	West
Ochomogo	1499	1883	Divide
Cartago	1434	1201	East
Paraíso	1325	1733	East



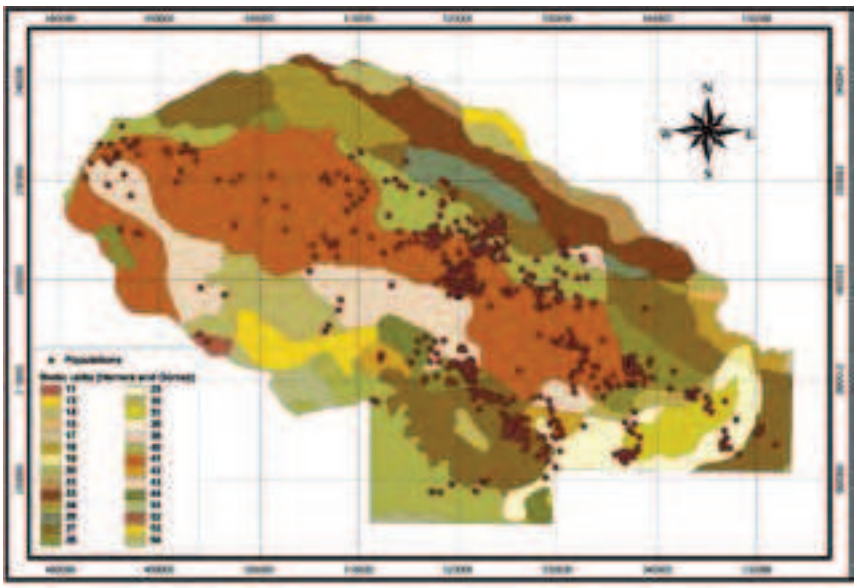
Map 2. Variation in mean annual rainfall in the study area. Red dots indicate populations of Lima beans; blue dots indicate location of weather stations.



Map 3. Variation in mean annual temperature in the study area. Red dots indicate locations of Lima bean populations.

Cordillera de Talamanca, to 25°C in the southeastern part of the study area (near the city of Atenas) (Map 3).

The Central Valley of Costa Rica is very diverse biologically. Two distinct approaches have been used to describe the interactions of its physical environment with biological diversity. These are the ecological map of Costa Rica, which is based on the Holdridge life zone system (Holdridge 1966), and the map of biotic units, which is based on the structure and floristic composition of the plant communities (Gómez 1986) (Map 4). Digitized coverages were obtained from 1:200 000 and 1:685 000 maps, respectively.



Map 4. Distribution of biotic units in the study area. Red dots indicate location of Lima bean populations.

4. WILD *P. LUNATUS* IN THE CENTRAL VALLEY OF COSTA RICA

Wild populations of Lima bean can be found throughout the Central Valley of Costa Rica (Standley 1937; Rocha *et al*. 1997, 2002). The populations are usually found in open and disturbed areas in association with grasses and scattered trees or bushy thickets; they also colonize the coffee plantations from perennial hedges (usually *Erythrina* and euphorbs) bordering the plots (Standley 1937; Debouck 1987). They are also found where coffee is grown under shade (traditional coffee plantations), as well as in the wasteland around these plantations. This habitat has a forest-like structure, with a diverse mixture of shade trees and also bananas. Typically, agricultural activities are less intense in this agroecosystem, and do not rely on the heavy use of herbicides for the elimination of weeds (Rocha *et al*. 1997). However, it has been demonstrated that because of changes in agricultural practices and in land use due to urban development, the populations of Lima bean in the Central Valley are fragmented and regularly undergo local extinction and recolonization (Rocha *et al*. 1997).

The project investigated the following aspects of the biology of wild Lima bean in the Central Valley of Costa Rica: (1) ecogeography and metapopulation dynamics, (2) population demography and phenology, and (3) genetic structure of populations, using morphological traits, seed storage proteins, allozymes, RAPD markers, AFLP fingerprinting techniques and microsatellites. A computer simulation was also developed based on the results of these studies.

4.1 Ecogeography

The geographic location of approximately 450 populations (defined as groups of Lima bean individuals separated at least 500 m from any other) was determined during 1992–1994 (Phase I of the project). Further surveys were carried out in subsequent years, which found new populations as well as recording the disappearance of old ones. In order to analyze the physical and ecological attributes of all locations where *P. lunatus* was found during the course of the project (565 populations in total), a detailed classification of each site was conducted using a geographic information system (GIS). This work was carried out in collaboration with Centro de Investigaciones en Desarrollo Sostenible (CIEDES) of the Universidad de Costa Rica. The results may be summarized as follows:

- **Altitude.** Analysis was conducted using a digital elevation model produced from the contour lines of the 1:50 000 topographic map of Costa Rica. Overall, *P. lunatus* was found in sites located between 800 and 1800 m asl, but most populations were found between 1100 and 1600 m asl.
- **Annual precipitation.** Spatial distribution of annual precipitation was obtained by interpolating (using a kriging model) the annual average rainfall values of 95 weather stations located in the study area and its surroundings. The data revealed that *P. lunatus* populations are more likely to be found in locations with annual precipitation between 1900 and 2400 mm.

- **Mean annual temperature.** A digital map of the distribution of the mean annual temperature for the Central Valley was produced using a multiple regression model based on altitude and annual precipitation. Populations of *P. lunatus* in the study area are more frequently found in areas with annual temperatures between 19 and 22°C.
- **Relative humidity.** Relative humidity was obtained using an interpolation (again using kriging) of values from 14 weather stations in the study area and its surroundings. Lima beans were found in large numbers in all relative humidity categories, showing no clear association with population abundance.
- **Soil type.** This analysis was conducted using a digitized 1:200 000 soil map of Costa Rica (Acón *et al*. 1991). Lima beans were found growing on 30 different soil types. However, 46% of the populations (260) were found on deep Inceptisol, well drained and permeable soils with low fertility.
- **Ecological and vegetation type.** Lima beans were found in seven different life zones, being most abundant in the humid premontane (38% of populations) and the very humid premontane forests (54%). Similarly, the species was observed in 15 different biotic units. However, 72% of the populations were found in only three of these, i.e. humid, temperate and subtropical areas with a marked dry season lasting 3–6 months (see Map 4).

Overall, the project's findings indicate that Lima beans are not randomly distributed in the study area, and that their location may in fact be influenced by physical environmental factors. This information can be used to make predictions about additional locations where Lima beans are likely to be found, both within and outside the Central Valley. The relationship between Lima bean distribution and the physical factors examined can also be used to explain the genetic structure of the species revealed by isozyme and other markers.

4.2 Metapopulation dynamics

During the numerous censuses and surveys that have been conducted in the study area during the course of the project, over 500 locations where Lima beans grow were identified. During the first census, conducted between November 1992 and April 1993, Lima beans were found at 450 locations. During the following year, all plants had disappeared from 206 of these locations (Figure 3). Similarly, Lima beans became extinct at 82 locations during the following year. However, populations were found at 33 new locations (Rocha *et al*. 1997). These findings clearly illustrate that wild populations of Lima beans undergo episodes of local extinction and recolonization in the study area.

Given the occurrence of local extinction and recolonization events, it was felt that a metapopulation approach would be the most suitable method to use to study the effects of factors such as population size, population distribution, and breeding systems on the genetic structure of the species. The term metapopulation is used to describe a situation where a species is distributed as an assemblage of ever-changing, interacting populations. Metapopulation models have the advantage of recognizing that local populations are dynamic

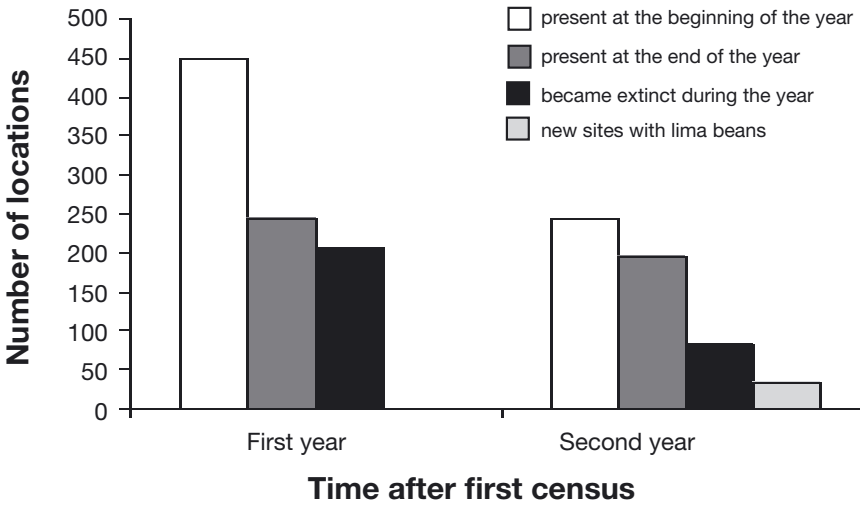


Figure 3. Lima bean metapopulation dynamics in the Central Valley, showing number of locations where Lima beans were found in the first and second years following the first census (November 1992 to April 1993).

and are subject to local extinction (Harrison and Quinn 1989; Olivieri *et al*. 1990; Hanski 1991). Moreover, Gliddon and Goudet (1994) concluded that the study of local extinction and recolonization under a metapopulation approach could generate useful predictions for particular problems in conservation biology.

A metapopulation approach has been used to examine the potential effects of local extinction and recolonization events on the genetic differentiation of local populations (Slatkin 1977; 1987; Wade and McCauley 1988; McCauley 1993; Gliddon and Goudet 1994). Slatkin (1987) highlighted two factors that determine the outcome of these processes: first, the sampling of individuals from the previous populations to form the propagules that recolonize the site (seed or seedling bank); and, second, the dispersal of propagules among populations. The direction and the magnitude of the effects of local extinction and recolonization on genetic structure depend on the roles that genetic drift, founder effect and gene flow (including pollen flow and migration) play in a given situation (Slatkin 1977, 1987; Wade and McCauley 1988; McCauley 1993).

In order to study the metapopulation dynamics of Lima beans in the Central Valley, six sampling transects were established along main roads in the study area (Map 5). In 1994, 103 populations were found along these transects. All populations were visited every 2 weeks from January 1995 to April 2000. During each visit, the phenological status of each population was recorded by counting the number of individual plants that had foliage, flower buds, flowers, immature fruits and/or mature fruits with seeds. In addition, any disturbance experienced by each population was recorded; these might include fire, weeding (manually or with herbicides) and habitat destruction due to urban development.

Map 5. Location of the study area showing the six transects defined to monitor metapopulation dynamics. The number of populations along each transect varied between 14 and 23 (Rocha *et al*. 2002).

This study confirmed that local extinction of Lima bean populations is a common event in the Central Valley. Two types of extinction may be recognized:

(1) *transient extinction*, when all plants at the location disappeared but recolonization occurred during the same year
(2) *effective extinction*, when all plants in the location disappeared and no recolonization had occurred by the end of the year.

Most locations where extinctions occurred were recolonized during the same year, indicating that the soil seed bank plays a major role in restoring populations. However, in 5 years of observations, there were 91 episodes of effective local extinction. The number of effective extinction episodes varied between years (Table 2, Figure 4). For example, during the period between October 1994 and April 1995, Lima beans disappeared from a total of 70 locations. In contrast, during the period between May 1995 and April 1996 and the period between May 1997 and April 1998, no episodes of local extinction were recorded. In addition, the number of plants growing in a location appears to be negatively associated with the risk of local extinction. In general, small groups of plants (< 10 plants) were more likely to experience local extinction. Furthermore, local extinction was not observed in locations with more than 50 Lima bean plants. Most of the

Table 2. Number of populations that experienced local extinction and number of extinct populations that were re-colonized.

Time period	Number of populations undergoing extinction	Number of population sites re-colonized
Oct 1994 – April 1995	70	51
May 1995 – April 1996	0	0
May 1996 – April 1997	15	0
May 1997 – April 1998	0	10
May 1998 – April 1999	2	1
May 99 – April 2000	4	0

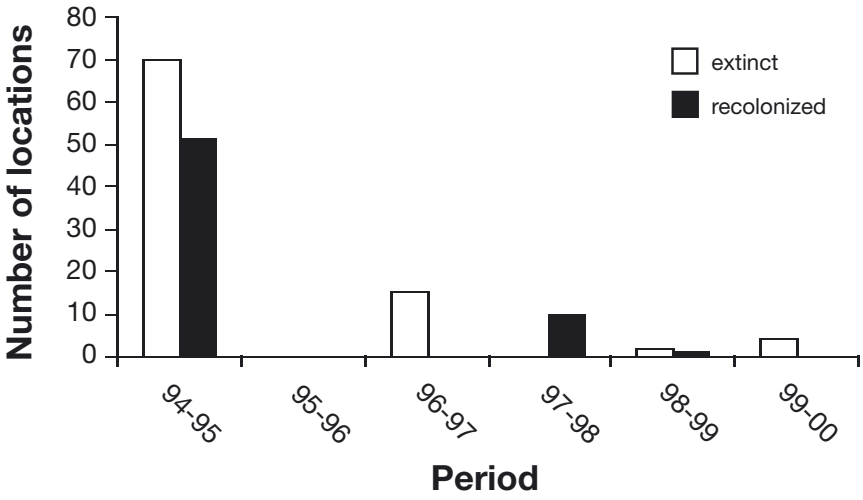


Figure 4. Number of local effective extinction events and number of recolonization events for each year during the study (1994–2000).

effective extinction events were observed in locations with fewer than ten plants (Figure 5). Moreover, once a group of plants had become extinct from a given location, the risk of experiencing another extinction after recolonization was also high. In other words, the risk of extinction was not evenly distributed among the locations that we monitored, indicating different intensities of disturbance in the study area.

Metapopulation dynamics was studied by determining the transition probabilities among five possible population stages, namely (1) populations that remained vegetative during the year, (2) populations that flowered during the year, (3) populations that produced seeds during the year, (4) populations that became extinct, and (5) populations that recolonized a site. Years were defined on the basis of the phenology of the species, and were thus considered to begin in May, at the beginning of the rainy season, which is accompanied by a flush of new leaves, and

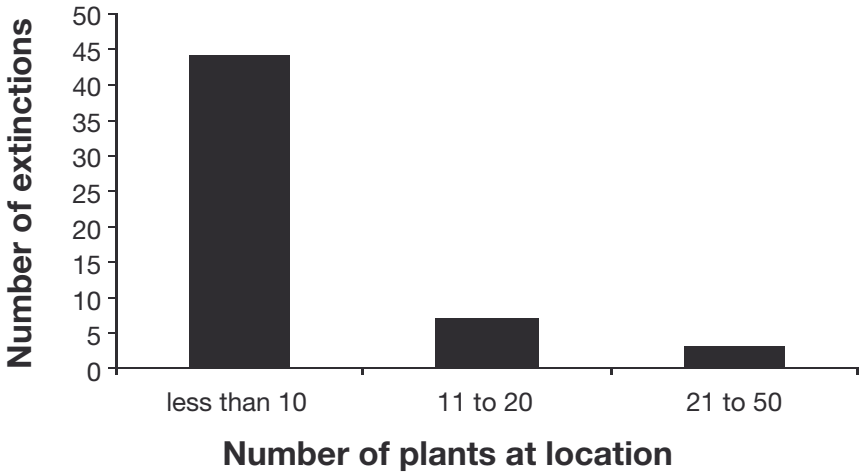


Figure 5. Number of effective extinction episodes according to the number of plants of Lima beans at each location. No effective extinction events were observed for locations with more than 50 plants.

to end at the end of the dry season, when plants are leafless and seeds have already been dispersed. Lefkovitch matrices (Lefkovitch 1965) were used to describe the dynamics of these populations.

Not all populations were equally likely to produce seeds. Nearly one-fifth of the populations (18.8%) in the study area failed to produce flowers every year. In addition, only 49.1% of the populations produced seeds each year. These findings suggest that populations differ in their ability to maintain a seed reservoir in the soil. Moreover, populations that produce seeds in a given year have a high probability of producing seeds in future years (probability of remaining in same group = 0.824), while those that become extinct also have a high probability of staying extinct (probability of remaining extinct = 0.787).

The unified life model (ULM) software program (Legendre and Clobert 1995) was used to determine demographic parameters for the metapopulation, in particular, metapopulation growth rate (λ, the rate at which the number of populations in the metapopulation increases or decreases). In spite of the frequent extinctions recorded in this study, the metapopulation growth rate calculated from the matrix of average transition probabilities is close to unity (λ = 0.990). However, the trajectory of the number of populations in the metapopulation varies according to the initial scenario, i.e. the distribution of populations among the four stages considered in this study. For example, if we consider only the 33 active populations recorded in 1995 as our initial metapopulation size, the number of populations will decline rapidly (Figure 6). However, if we consider that all recorded populations were vegetative at the beginning of the study (initial metapopulation size = 103), the number of populations will decline for a few years but will then become relatively stable (Figure 7). There is another possible

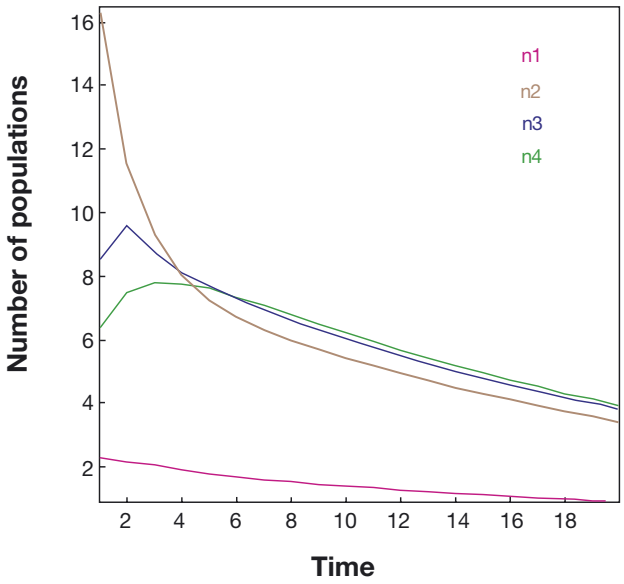


Figure 6. Simulation of metapopulation dynamics considering the 33 populations that were active in 1995 as the initial metapopulation (variation in the number of populations in each stage over time (years) is shown as follows: n1 = extinct populations, n2 = vegetative populations, n3 = populations that flowered and n4 = populations that produced seeds). Time is measured in years.

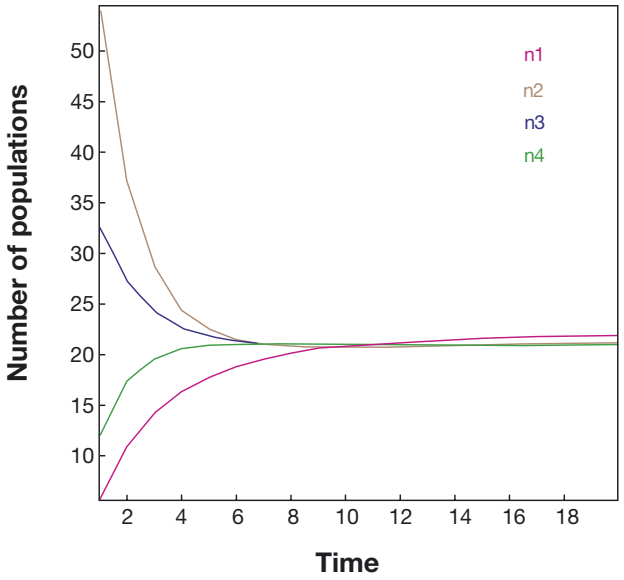


Figure 7. Simulation of metapopulation dynamics under the assumption that all 103 initial populations were vegetative (variation in the number of populations in each stage over time (years) is shown as follows: n1 = extinct populations, n2 = vegetative populations, n3 = populations that flowered and n4 = populations that produced seeds).

scenario, using the mean number of active populations in each category as the initial metapopulation size. In this scenario, the number of populations also shows a decline, but eventually the number of populations becomes stable (Figure 8). Despite the fact that only 24% of the locations where populations experienced extinction were colonized again, overall this analysis shows that wild populations of Lima beans in the Central Valley are not under an overall threat of extinction.

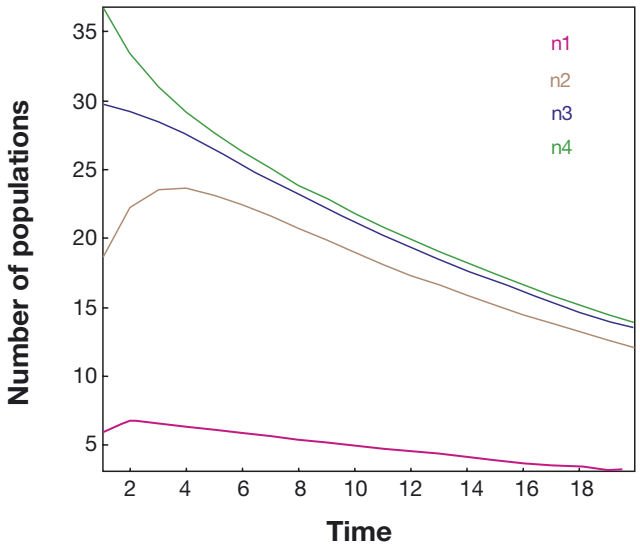


Figure 8. Simulation of metapopulation dynamics considering the mean number of populations in each category as the initial metapopulation (n1 = extinct populations, n2 = vegetative populations, n3 = populations that flowered, n4 = populations that produced seeds).

4.3 Demography

Very few data are available regarding the demographic behaviour of herbaceous plants in the tropics (Silva *et al*. 1991; Horvitz and Schemske 1994, 1995), and small legumes such as *Phaseolus*, despite their importance as wild relatives of crop species, have until now received almost no attention as far as this topic is concerned (Degreef *et al*. 1997). However, demography is widely considered to be a key to the formulation of *in situ* conservation strategies (e.g. Olmstead and Alvarez-Buylla 1995; Oostermeijer *et al*. 1996; Ratsirarson *et al*. 1996; Ehrlen and van Groenendael 1998). Matrix models have been used to determine which stages of the life cycle of a plant are the most vulnerable (Werner and Caswell 1977; Charron and Gagnon 1991; Damman and Cain 1998) and which life-stage transitions most strongly affect population growth (Caswell 1989), hence improving our understanding of how plant populations respond to changes in the environment, including the impact

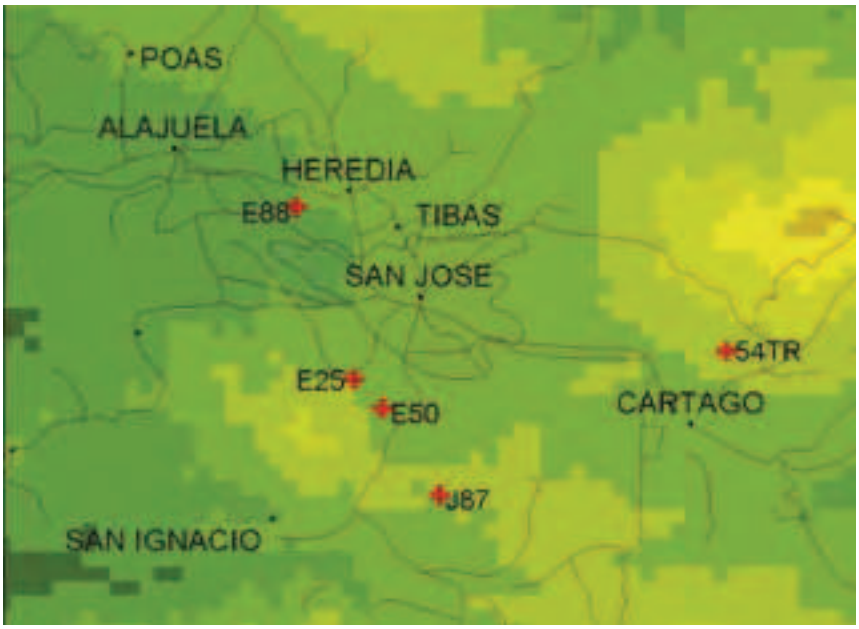
of human activities. The project examined the population dynamics of five wild Lima bean populations in the Central Valley of Costa Rica for 3 years in order to gather basic information for the in formulation of *in situ* conservation strategies.

4.3.1 Field observations

The populations studies demographically were located in very disturbed sites along trails (population 54TR), at the border of coffee plantations (J87) or both (E88), or in more natural sites as at the edge of secondary regeneration forests (E25, E50). Table 3 gives some information on the locality of each wild population (see also Map 6).

Table 3. Parameters of five wild Lima bean populations in the Central Valley. λ_1 is the dominant eigenvalue of the transition matrix.

Population	Latitude	Longitude	Ecology	λ_1
54TR	9°54' N	83°54' W	Trail	0.5340
J87	9°49' N	84°04' W	Border of coffee plantation	1.4654
E88	9°59' N	84°09' W	Trail and border	0.4725
E25	9°53' N	84°07' W	Edge of secondary forest	0.9280
E50	9°52' N	84°06' W	Edge of secondary forest	1.1769



Map 6. Localities of the five wild Lima bean populations monitored in the demography studies.

The following parameters were monitored in each population: (1) seed germination and longevity in the soil seed bank, (2) survival and growth of seedlings and adult plants, and (3) reproductive output of individual plants. Matrix population models were used to compare the response of the species to different environments and to identify the most critical life stages in each population.

The life cycle of the species was described on the basis of monthly field observations at each study site. In all, 49 quadrats (1 m^2 each) were sampled; all adult individuals present and all seedlings appearing during the course of the study were labelled and identified by a code number. Developmental stage (vegetative or lignified stem), stem diameter (at 2 cm height), fecundity of each individual (number of seeds produced per year) and mortality rate in each quadrat were documented. Germination tests were conducted with painted seeds placed in plastic plates in the vicinity of each quadrat (Degreef 1998).

4.3.2 Seed bank dynamics

Germination rate ranged from 70 to 86% after 1 year in the soil, and from 89 to 94% after 2 years, for the five populations. Seed-coat dormancy is likely to be the major factor responsible for this delay in germination. This dormancy is induced by drought stress, which may occur on the soil surface just after seed dispersal (Degreef 1998). Hypothesizing that the annual germination rate is similar from year to year, we estimated that 96–99% of seeds will have germinated at the end of the third year. The design of the model was simplified by assuming that all seeds germinated within 3 years of their dispersal.

4.3.3 Matrix demographic model

A life cycle graph was prepared for the species (Figure 9), in which each node is associated with a specific stage in the life of Lima bean individuals. Due to the presence of a soil seed bank, seed classes were identified according to age. Juvenile and adult plants were grouped according to both their developmental stage and stem diameter.

The six classes were defined as follows (see Degreef (1998) for more details):

- G_1 and G_2: seeds remaining in the soil seed bank 1 and 2 years after dispersal, respectively
- J: juvenile individuals; the upper limit of this class is the minimum diameter which a lignified plant must reach in order to be able to produce seeds
- L_1, L_2 and L_3: ligneous, potentially fertile individuals with increasing diameter.

The projection interval is 1 year, which means that an arrow joining two nodes expresses the probability of an individual moving from one class to the other (a), or contributing by its reproduction to an increase in the size of the other class (b) within 1 year. The probability of stasis in the same class is represented by a loop (c).

At the end of a 1-year interval, a seed produced by a L_1, L_2 or L_3 individual will have met with one of three possible fates:

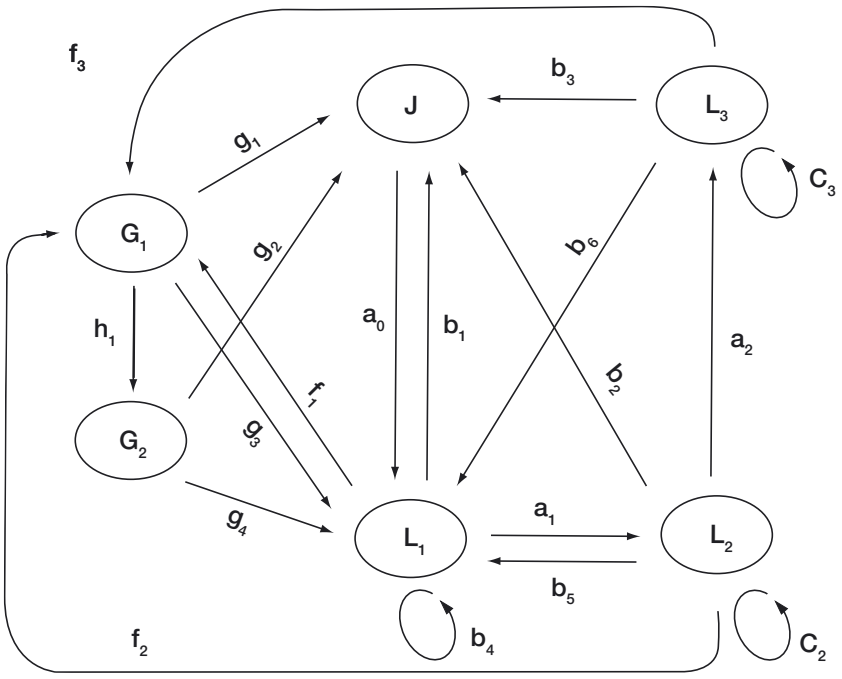


Figure 9. Life cycle graph of wild Lima bean (for explanation, see text).

(1) it germinates and increases the number of individuals in class J (b_1–b_3) or L_1 (b_4–b_6), illustrating the recruitment of individuals from seeds
(2) it germinates and then dies
(3) it fails to germinate and enters the soil bank as a class G_1 individual (f_i). The fate of seeds in the soil bank is either germination (g_i) or staying dormant (h_i).

The fact that no individual stays in class J after a time interval of 1 year is also apparent in the life cycle graph. In one population (E88), the growth of lignified individuals was so much reduced that they did not reach stage L_3.

The contribution of an average individual belonging to one class to the number of individuals in another class in one year is called its transition rate. All transition rates recorded in a specific population can be summarized in a matrix (Caswell 1986, 1989; van Groenendael *et al*. 1988). A generalized projection model was developed to describe the demography of Lima bean populations. Transition matrices for each Lima bean population are presented in Figure 10.

The dominant eigenvalue λ_1 of the transition matrix (Table 3) corresponds to the asymptotic growth rate of the population when it reaches its stable structure, and can be used as a measure of fitness for the population in its particular environment. The λ_1 values indicated a decrease in the number of individuals in populations 54TR, E88 and to a lesser degree E25, and an increase in the number of individuals in populations E50 and J87. Rocha *et al*. (1997) reported that wild

$$A_{54TR} = \begin{pmatrix} 0 & 0 & 0 & 0.187 & 1.802 & 2.270 \\ 0.486 & 0 & 0 & 0 & 0 & 0 \\ 0.014 & 0.028 & 0 & 0.032 & 0.305 & 0.384 \\ 0.030 & 0.059 & 0.027 & 0.067 & 0.642 & 0.809 \\ 0 & 0 & 0 & 0.120 & 0.176 & 0 \\ 0 & 0 & 0 & 0 & 0.059 & 0.333 \end{pmatrix}$$

$$A_{E25} = \begin{pmatrix} 0 & 0 & 0 & 0.226 & 1.459 & 138.750 \\ 0.448 & 0 & 0 & 0 & 0 & 0 \\ 0.111 & 0.201 & 0 & 0.261 & 1.688 & 160.549 \\ 0.003 & 0.006 & 0.009 & 0.008 & 0.050 & 4.793 \\ 0 & 0 & 0 & 0.218 & 0.149 & 0 \\ 0 & 0 & 0 & 0 & 0.075 & 0.750 \end{pmatrix}$$

$$A_{E50} = \begin{pmatrix} 0 & 0 & 0 & 1.026 & 45.514 & 238.442 \\ 0.140 & 0 & 0 & 0 & 0 & 0 \\ 0.132 & 0.154 & 0 & 0.678 & 30.076 & 157.566 \\ 0.004 & 0.005 & 0.009 & 0.022 & 0.977 & 5.116 \\ 0 & 0 & 0 & 0.118 & 0.143 & 0 \\ 0 & 0 & 0 & 0 & 0.429 & 0.800 \end{pmatrix}$$

$$A_{E88} = \begin{pmatrix} 0 & 0 & 0 & 0.224 & 4.956 \\ 0.196 & 0 & 0 & 0 & 0 \\ 0.023 & 0.029 & 0 & 0.014 & 0.299 \\ 0.088 & 0.109 & 0.077 & 0.051 & 1.122 \\ 0 & 0 & 0 & 0.030 & 0.250 \end{pmatrix}$$

$$A_{J87} = \begin{pmatrix} 0 & 0 & 0 & 0.399 & 27.533 & 21.401 \\ 0.218 & 0 & 0 & 0 & 0 & 0 \\ 0.063 & 0.081 & 0 & 0.103 & 7.101 & 5.520 \\ 0.027 & 0.034 & 0.031 & 0.043 & 2.981 & 2.317 \\ 0 & 0 & 0 & 0.334 & 0.200 & 0 \\ 0 & 0 & 0 & 0 & 0.600 & 0.364 \end{pmatrix}$$

Figure 10. Transition matrices for the five wild populations of Lima bean.

Lima bean populations in the Central Valley experience frequent extinctions and population fragmentation. These phenomena were recognized as resulting mainly from human perturbations, although some populations not subject to weeding and other disturbances also showed oscillations in the number of their individuals over the course of time. Therefore, the critical status of some populations, indicated by low values of their asymptotic growth rate, probably results from perturbations which occurred at a very early developmental stage and are still

Table 4. Elasticity values corresponding to some transitions of the life cycle graph (b_4 to b_6 = direct transition of seeds to the L_1 stage within one year; f_i = entry of seeds into the soil seed bank; h_1 = seeds remaining in the soil seed bank for a second year; a_1 = transition from L_1 to L_2 stage; c_3 = survival of L_3 individuals).

Pop.	Elasticities b_4 to b_6	Elasticity f_i	Elasticity h_1	Elasticity a_1	Elasticity c_3
54TR	0.2036	0.0921	0.0588	0.2451	0.1096
J87	0.2901	0.0608	0.0097	0.3518	0.0346
E88	0.1267	0.1642	0.0559	0.2219	0.2493
E25	0.0824	0.0211	0.0099	0.1285	0.3479
E50	0.1334	0.0320	0.0039	0.1914	0.5283

having an impact on their dynamics. The values of λ_1 give a useful indication of the current trend in population size for each population, assuming that conditions do not change in the future. Even if the situation changes, however, these data are of particular interest for the development of management practices. The status of each population, whether declining or recolonizing a site, has to be taken into account when designing a conservation plan and determining the urgency and the intensity of the management to implement.

The calculation of the elasticity of λ_1 to a change in a transition matrix element is a particularly efficient way of determining which phase of the life cycle of the individuals is the most critical for the survival of the population, to quantify the contribution of each vital rate to population growth, and to evaluate the effects of environmental perturbations on population dynamics (de Kroon *et al*. 1986; Moloney 1988; Caswell 1989; Carlsson and Callaghan 1991; Aberg 1992; Mesterton-Gibbons 1993; Svensson *et al*. 1993; Okland 1995; Valverde and Silvertown 1997; Menges and Dolan 1998). The larger the value of the elasticity e_{ij} for an element of the transition matrix, the greater that element's influence on the value of λ_1. In our situation, the analysis of the elasticity matrices shows the importance, for the population growth rate, of the direct transition of seeds to the L_1 stage, i.e. their germination and the lignification of the seedlings within 1 year (Table 4). This fate is only possible if seeds germinate soon after dispersal. Consequently, rapid germination can be considered as a key factor in the population dynamics of wild Lima beans in the Central Valley of Costa Rica.

Second, the elasticity matrices reveal the importance of the soil seed bank for the dynamics of populations. The growth rate of populations that are decreasing in size (54TR, E88, and to a lesser extent E25) is particularly influenced by the recruitment of seeds into the soil seed bank. In increasing populations E50 and J87, elasticities for this particular transition are low. Similar situations can be pointed out for the elasticities of the transition of G_1 seeds to G_2 (Table 4).

Third, the analysis also reveals the importance of the growth of ligneous individuals. This is clearly illustrated by the elasticities of the transition from L_1 to L_2 (Table 4). According to the meteorological data recorded in the vicinity of each population (Degreef 1998), the highest mortality of L_1 individuals occurred

during the driest months (November and December), indicating that air and soil moisture probably favour the survival of L_1 individuals and their transition to L_2.

Finally, the analysis shows the importance of the survival of L_3 individuals in populations E25 and E50, located in the most 'natural' sites (Table 4). In the case of these two populations, the growth rate appeared to be mainly dependent upon the survival of L_3 individuals.

As the elasticities of λ_1 with respect to recruitment of new individuals from seeds (e_F), growth (e_G) and stasis (e_L) sum to unity (de Kroon *et al*. 1986; Mesterton-Gibbons 1993), they may be used to compare the relative importance of selected regions of the projection matrix on the value of λ_1 (Aberg 1992; Silvertown *et al*. 1993, 1996; Oostermeijer *et al*. 1996). The relative positions of the five wild populations of Lima bean in the triangle of elasticities (Figure 11) confirms field observations on the ecology of the species.

The central zone of the triangle characterizes iteroparous herbaceous plants from open areas (Silvertown *et al*. 1996). Populations located in the most disturbed sites (54TR, E88 and J87) are characterized by high values of e_G and e_F. As shown by the position of these populations in the triangle, growth is very important for their dynamics. In perturbed sites, the transition of L_1 individuals to the L_2 stage and the recruitment of new individuals (J or L_1) from seeds germinating within 1 year played a major role in determining growth rate. In contrast, populations located in the most natural sites (E25 and E50) are characterized by the largest e_L

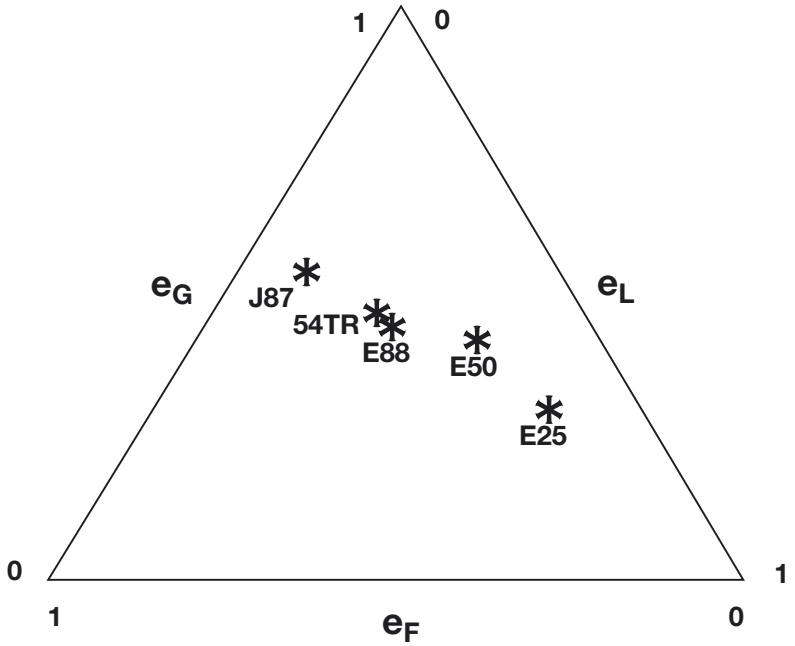


Figure 11. Triangular representation of combinations of elasticities of λ_1 with respect to recruitment of new individuals from seeds (e_F), growth (e_G) and stasis (e_L) for the five wild Lima bean populations.

values, indicating that the survival of individuals at the same stage (particularly L_3) is the most important factor for their dynamics.

It is generally recognized that the life-history component which most strongly affects population growth depends on the habitat where the population grows (Bierzychudek 1982; Silvertown *et al*. 1993, 1996; Damman and Cain 1998). These findings show that higher growth and fecundity elasticities are typical of open habitats, and higher survival elasticities are characteristic of closed habitats (Menges and Dolan 1998). In particular, for wild Lima bean, this pattern is best shown by the extent and the frequency of disturbances to which the populations are exposed. Higher growth and fecundity elasticities were obtained in populations experiencing perturbations and lower environmental stability. In contrast, populations in more stable habitats had higher survival elasticities. The significance of these results for conservation is discussed in Section 6.

4.3.4 Phenology

Phenology is the study of the seasonal timing of life cycle events. The timing of reproductive events within a plant population affects not only the persistence, abundance and diversity of that population, but also all the animals that depend on plant resources (Newstrom *et al*. 1994a). The study of phenological patterns is thus very important for understanding the ecology and evolution of species and communities.

Newstrom *et al*. (1994a) classified the long-term patterns of flowering of lowland wet tropical plants at La Selva Biological Station in Costa Rica. They found that the most common pattern is irregular sub-annual flowering, followed by regular annual flowering. In general, neotropical plant populations reproduce synchronously and at regular intervals (Newstrom *et al*. 1994a, b). However, most available studies show that there are pronounced variations between seasons and/or between years (van Schaik *et al*. 1993). Borchert (1983) proposed that, for tropical plants growing in seasonal climates, flowering is discontinuous, with flower initiation and anthesis being separated by a prolonged rest period and being controlled separately.

The general phenological pattern in different parts of the study area was investigated using the six sampling transects along main roads already mentioned (Map 5). In 1994, 103 populations were found along these transects. All populations were then visited every 2 weeks from January 1995. During each visit, the phenological status of each population was recorded by counting the number of individuals that had foliage and the number bearing flower buds, flowers, immature fruits and/or mature fruits with seeds.

It was found that, in general, there are usually at least a few populations with plants bearing foliage all year round (Table 5, Figure 12). However, there is a clear period of leaf flush at the beginning of the rainy season (May–July), which results in all populations bearing foliage by late June or early July. Most populations experience at least a few weeks without any leaves.

Flowering starts very early after leaf flush, with bud differentiation typically beginning in August and ending at the end of the dry season (March–April). Anthesis starts a few weeks after bud differentiation, but the likelihood of producing

Table 5. Timing of each phenological event for the wild populations of Lima beans in the Central Valley of Costa Rica.

Stage	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Vegetative	___	___	___	___	___	___	___	___	___	___	___	___
Flower buds		___	___	___				___	___	___	___	___
Flowers	___	___	___					___	___	___	___	___
Fruit (green)	___	___	___	___	___	___				___	___	___
Fruit (mature)	___	___	___	___	___	___				___	___	___
Seeds	___	___	___	___	___	___						

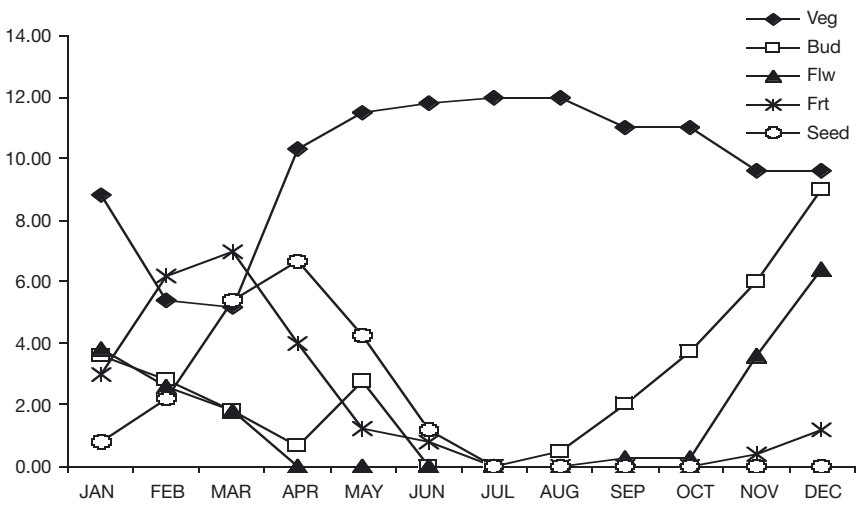


Figure 12. Mean monthly number of populations experiencing each phenological stage among the 17 wild populations of Lima beans monitored in the first transect.

fruits declines during the season, and flowers opening during the dry season usually fail to produce fruits and their seeds fail to mature. In general, the peak of flower production occurs between November and January, and seed production and dispersal is maximal between February and May. It is also clear that not all populations produce flowers or fruits in any given season.

A more detailed study was carried out on two populations selected from each of the sampling transects, monitoring the proportion of plants at each phenological phase every 2 weeks. The proportion of plants at each phenological stage at any one time varied significantly among populations, as did the peak of each phase. Thus, even adjacent populations did not necessarily overlap in their flowering time, and were therefore not likely to experience gene exchange. These analyses

also showed differences in the duration of each phenological phase. On average, flowering only lasted for a few weeks in most populations, but for location E104 flowering lasted from September through to December. Similarly, seed maturation and dispersal typically occured between January and July, but in location J59, it only lasted from February through to May. Microclimatic conditions, along with soil properties, are probably responsible for such differences, although there may also be a genetic component. Whatever their cause, these differences could play an important role in determining the genetic structure of Lima beans at the metapopulation level.

4.4 Genetic diversity

Genetic diversity within and among populations of wild Lima beans in the Central Valley of Costa Rica was quantified using a variety of morphological, protein and DNA markers.

4.4.1 Phaseolins

Storage proteins represent nearly 50% of the total proteins found in seeds of flowering plants. Upon germination, these proteins are the main source of nitrogen for the developing seedling (Gepts 1990). Osborn (1988) classified storage proteins according to their solubility. According to this classification system, albumins dissolve in water, globulins dissolve in saline solutions, prolamines dissolve in 90% alcohol, and glutelins dissolve in weak acid or base (Bewley and Black 1983). The relative abundance of the different storage proteins varies from one group of plants to another; for example, legumes are rich in globulins, while in cereals the most important storage proteins are the prolamins (Gepts 1990).

Globulins represent between 50 and 75% of the total proteins found in the seeds of common beans (*P. vulgaris*) (Müller 1983; Alli *et al*. 1993). Two types of protein are included in this group; phaseolins and lectins or phytohaemagglutinins (Staswick *et al*. 1986). Phaseolins are the dominant component, representing 35–50% of the total protein content, while lectins represent only 5–10% (Gepts *et al*. 1992). Phaseolins have been widely used to investigate the domestication and dispersal of cultivars of *P. vulgaris* (Gepts 1988, 1990), and to analyze the phylogenetic relationships among species in the genus *Phaseolus* (Pusztai and Watt 1970; Ma and Bliss 1978; Suzuki *et al*. 1983; Deshpande and Nielsen 1987; Imungi and Jackson 1989; Gepts *et al*. 1992; Alli *et al*. 1993).

Different species of the genus *Phaseolus* show different phaseolins electrophoretic patterns among (Sullivan and Freytag 1986; Maquet 1995; Sparvoli *et al*. 1996). In *P. lunatus*, phaseolins can be easily distinguished by SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) from those of *P. vulgaris*, *P. coccineus* and *P. acutifolius* because the different subunits exhibit lower molecular weights. The weights of the subunits observed in *P. lunatus* ranged from 21 to 38.5 kDa, contrasting with those observed for *P. vulgaris*, *P. coccineus* and *P. acutifolius,* which ranged from 45 to 50 kDa (Sparvoli *et al*. 1996).

In comparison with common beans, other species of the genus *Phaseolus* have received little attention with respect to the content or the molecular characteristics of their storage proteins. For example, Maquet (1995) considered that phaseolins were not present in *P. lunatus*. He examined the electrophoretic mobility of storage proteins from material of the Mesoamerican and the Andean genepools. When he compared the banding patterns that he observed with those of *P. vulgaris*, he noted the absence of bands in the range corresponding to phaseolins. However, Lioi *et al*. (1993; Lioi 1994) were able to identify and characterize phaseolins among the storage proteins of *P. lunatus*. This finding was very important because it provided another marker with which to study genetic differences among the species in the genus, and genetic variation between and within genepools (Lioi 1994).

Results are presented here on total levels of phaseolins (G1 globulin) among 37 wild populations of Lima beans in the Central Valley of Costa Rica. In addition, the variation in phaseolins among different geographic regions within the study area was studied. A protocol has been proposed for the isolation and purification of phaseolins when they are used as molecular markers (Vargas *et al*. 2000).

A random sample of 12–15 seeds was taken from a bulk sample of seeds from each population. These samples were subdivided into three groups of four or five seeds. These groups were used as replicates for protein extraction, isolation and quantification. The methodology described by Bradford (1976) was used to determine phaseolin.

The phaseolin content in seeds of wild Lima beans was generally low, with a mean of only 3.2±0.9 mg g^{-1} of cotyledon. In addition, the data revealed that phaseolin content varied significantly among populations, with the lowest concentration found in population TR28 (1.73±0.08 mg g^{-1}) and the highest in population KM59 (5.04±0.06 mg g^{-1}). It was found that 80% of the populations examined had protein contents higher than 2.0 mg g^{-1}.

In order to investigate variation in phaseolin content according to geographic area, the 37 populations were arranged according to their spatial distribution within the Central Valley into eight groups defined according to their proximity (Table 6). A nested model analysis of variance revealed that there was a significant effect of

Table 6. Ranges of mean annual temperature, annual precipitation and relative humidity for the eight geographical regions defined in the phaseolin study.

Geographical region	Temperature (°C)	Precipitation (mm)	Relative humidity (%)
I	17.5–20.0	1700–2000	80–85
II	22.5–25.0	1700–2000	75–80
III	20.0–22.5	2000–2500	80–85
IV	15.0–17.5	1700–2000	80–85
V	15.5–17.5	2000–2500	85–90
VI	20.0–22.5	2000–2500	80–85
VII	17.5–20.0	2000–2500	80–85
VIII	20.0–22.0	1500–1700	75–80

region on phaseolin content. Mean values found for the different regions ranged from 2.23 to 4.97 mg g^{-1}. Region VI, with five populations sampled, showed the widest range of variation (1.76–5.04 mg g^{-1}), while region VIII showed the narrowest range (3.60–3.63 mg g^{-1}) (Figure 13). Furthermore, the analysis also revealed that there is a significant effect of populations within regions. However, most of the variation (about 87%) was found among populations within the same region and only about 11% was found between regions.

Phaseolin variation was determined using one-dimensional SDS-PAGE (Vargas *et al*. 2001). This showed that G1 globulins are present in *Phaseolus lunatus*, and that their molecular weights ranged from 21.5 to 31 kDa. In addition, four main polypeptides or banding regions were revealed, differing in their molecular weight.

There was significant variation in the molecular weights within some of the G1 globulin polypeptides. For banding regions A and D, there was a single polypeptide in each. However, for banding regions B and C, there was more than one polypeptide. These polypeptides differ in their molecular weights and can easily be identified in the gels.

There were differences in the frequencies of the different polypeptides within banding regions B and C. Overall, there were four different banding patterns, namely $B^{11}C^{11}$, $B^{22}C^{22}$, $B^{12}C^{12}$ and $B^{33}C^{23}$. The frequencies of these banding patterns varied among populations. For example, in population E76, there were two seeds with banding pattern $B^{11}C^{11}$, one seed with banding pattern $B^{22}C^{22}$ and two seeds with banding pattern $B^{12}C^{12}$. In contrast, all seeds from population SR3 showed banding pattern $B^{11}C^{11}$, while in population E1 all seeds showed banding pattern $B^{22}C^{22}$. In general, banding patterns $B^{11}C^{11}$ and $B^{22}C^{22}$ were the

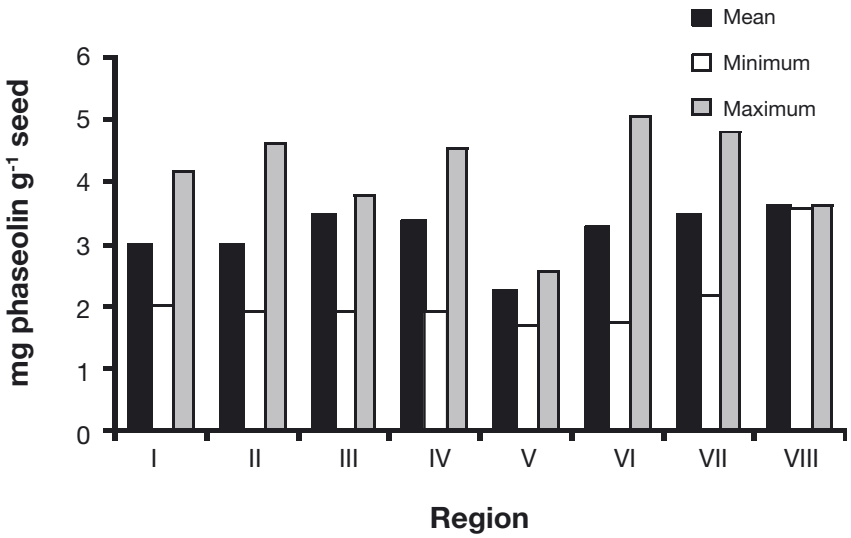


Figure 13. Mean, minimum and maximum values for phaseolin content (mg g^{-1} of seed) in each of the eight geographic areas studied.

most abundant and banding pattern $B^{33}C^{23}$ was only found in one population, namely E104. The banding patterns $B^{11}C^{11}$ and $B^{22}C^{22}$ were both present in more than 50% of the populations.

There was also variation in the frequency of the different banding patterns when the 37 populations were grouped into the eight geographic areas (Figure 14). Some geographic areas appeared to be dominated by one pattern, but all areas presented at least two banding patterns. For example, regions IV, V and VII were dominated by banding pattern $B^{11}C^{11}$, while regions I, II, III, VI and VIII were dominated by banding pattern $B^{22}C^{22}$.

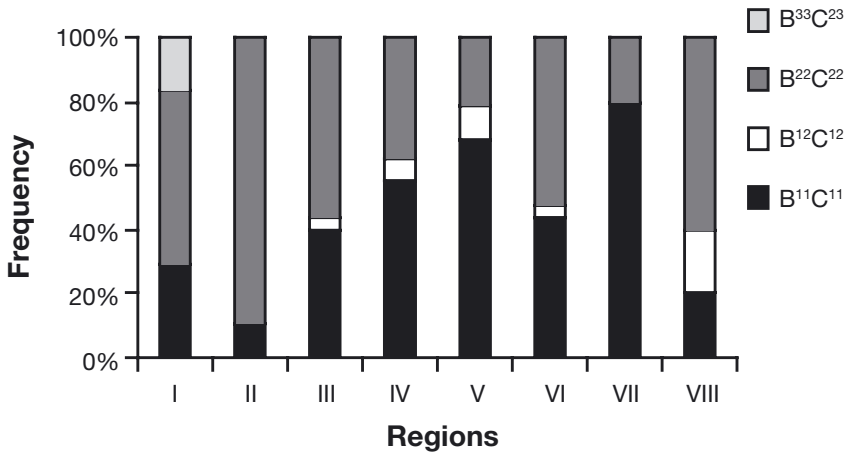


Figure 14. Frequency of the four banding patterns ($B^{33}C^{23}$, $B^{22}C^{22}$, $B^{12}C^{12}$ and $B^{11}C^{11}$) for each of the eight geographic regions.

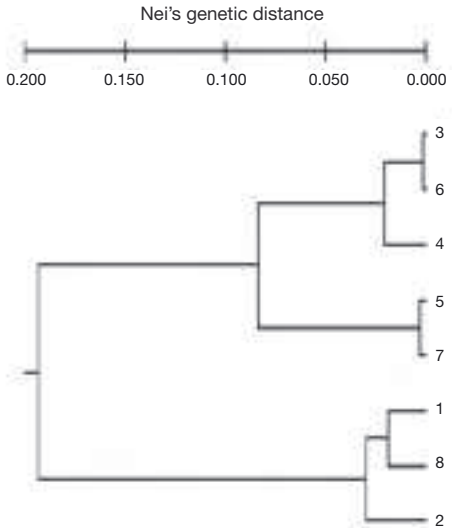


Figure 15. Dendrogram showing the relationship among the eight groups of populations based on their phaseolin.

An analysis of population structure based on the presence of the different bands revealed that nearly 80% of the variation was found among populations (θ = 0.823). Moreover, the variation among the eight groups of populations was smaller (θ = 0.109). The relationship among regions, based on their allelic frequencies, is shown in Figure 15. Two well-defined clusters were established on the basis of genetic distance (*sensu* Nei 1972), reflecting the relative abundance of the two most frequent banding patterns.

The results of the cluster analysis conducted with the eight groups of populations may be explained, at least in part, by climatic factors (Vargas *et al*. 2001). For example, the first main cluster includes regions III, IV, V, VI and VII, which are located in areas that, on average, experience higher annual precipitation than the other regions. Regions V and VII experience lower mean annual temperatures than the other regions in this cluster, and are very close to the main cluster (Herrera 1985). Moreover, regions III and VI, which have the lowest average temperatures and are both under the influence of wind paths into the Central Valley, also show similar phaseolin patterns.

4.4.2 Isozymes

Since 'isozymes' were first defined by Markert and Moller (1959), they have become widely used as genetic markers in plant diversity studies. Isozymes (or isoenzymes) are structurally different molecular forms of an enzyme system with, qualitatively, the same catalytic function. Isozymes which are encoded by different alleles of the same gene locus are referred to as 'allozymes' or 'alloenzymes'. Allozymes have proved to be useful as nuclear, species-specific markers in the quantification of heterozygosity, genetic diversity, genetic differentiation and other measures of intra- and interpopulation genetic variation. They possess many important advantages, but also have significant limitations: (1) the genes encoding enzymes represent a non-random (and small) sample of structural gene loci; (2) only nucleotide substitutions which change the electrophoretic mobility of the enzyme molecules are evident; (3) the possibility cannot be excluded that two bands with identical mobility in fact represent two different alleles; and (4) the question as to whether allozyme polymorphisms are adaptive or neutral is still being hotly debated (Müller-Starck 1998).

Knowledge of the mode of inheritance of isozyme markers is a prerequisite for their use in population genetic studies. Zoro Bi *et al*. (1999) described procedures for horizontal starch gel electrophoresis, including preparation of extracts, electrophoretic buffer systems and specific staining assays for enzyme activity. These authors also analyzed the genetic aspects of allozyme variation, including the quaternary structure of the resolved isozymes and the linkage relationships, allowing them to more accurately designate isozymes and allozymes. Suitable electrophoretic separation methods for 34 isozymes from 17 enzyme systems resolved in *P. lunatus* were also developed. Data from the migration of the staining zones indicated that three loci control EST, GPI, IDH and MDH, while two loci control ACO, ADH, DIA, G6PDH, GDH, PER, PGDH, PGM and SOD. A single locus controls END, β-GLU, LAP and SKDH. The quaternary structure was also inferred for 11 enzyme systems. DIA isozymes were identified as tetrameric;

ADH, fEST, GPI, MDH and PGDH as dimeric; and ACO, cEST, END, G6PDH, PGM and SKDH as monomeric.

These enzyme systems allowed Zoro Bi (1999) to investigate the main factors contributing to the maintenance of genetic variability in wild *P. lunatus* populations. An analysis of the combination of seed and plant numbers to be sampled per population using the theoretical model developed by Yonezawa and Ichihashi (1989) showed that 10–80 plants and one to two seeds per plant are necessary in order to quantify genetic diversity when considering polymorphism at the 5% level (Zoro Bi *et al*. 1998). At any level of polymorphism, one to four seeds and 200–300 plants per population are required in order to collect all alleles at six enzyme loci.

Zoro Bi (1999) sampled 29 wild populations from the Central Valley and quantified the genetic diversity at 22 loci. His estimates of the percentage of polymorphic loci (*P*), the mean number of alleles per locus (*A*), and the mean effective number of alleles (A_e) were 10.32%, 1.10 and 1.05, respectively. These values are similar to those recorded previously by Maquet *et al*. (1996) for 20 wild populations from the same region, but lower than those reported by Hamrick and Godt (1990) for all the 473 species they reviewed (P = 34.2, A = 1.53), with a wide range of life forms and breeding systems.

The genotypic composition of the populations analyzed showed a deviation from the expected Hardy-Weinberg proportions (F_{IT} = 0.878). This divergence from Hardy-Weinberg equilibrium (HWE) is due to genetic differentiation among populations (F_{ST} = 0.444) and non-random mating within populations (F_{IS} = 0.777). The estimates of the total heterozygosity (H_T), the intra-population gene diversity (H_S), and the inter-population gene diversity (D_{ST}) were 0.193, 0.082 and 0.111, respectively. About 52% of the total gene diversity was thus due to inter-population genetic variation (G_{ST} = 0.519), with 48% being intra-population genetic variability. This value is similar to that of autogamous species (G_{ST} = 0.510) and higher than that of annual species (G_{ST} = 0.357), as reviewed by Hamrick and Godt (1990). The D_{ST} and G_{ST} values obtained for the wild Lima bean populations may be due to limited pollen and seed dispersal (see Section 5.5 on gene flow). On the basis of ten populations showing at least three polymorphic loci, Zoro Bi (1999) obtained average simple locus (t_s) and multiloci (t_m) estimates of, respectively, 0.067 $\pm$ 0.030 and 0.072 $\pm$ 0.030. These results are very close to the values reported by Brown (1989) for predominantly inbreeding species.

Several factors could help explain departure from HWE:

- *The overlap of distinct generations* occurring at the same time at the same site, a very common occurrence for such a short-lived perennial species.
- *Founder effect* (see Sections 5.2 and 5.3), whereby only a few plants survive along hedges in some populations, with recolonization of the site relying therefore only on these plants and on the soil seed bank. This could explain why small populations (which are common for *P. lunatus* in the Central Valley of Costa Rica), in which no subpopulations are present, also diverge from HWE (Motro and Thomson 1982).

- *The presence of subpopulations* in a single natural population. In this situation, individuals could have been sampled from subpopulations among which no natural crosses occurred (see Section 5.5). Such populations might also have different allelic frequencies.

Zoro Bi *et al*. (1997) analyzed the spatial patterns of allozyme variants within three wild populations of *P. lunatus* in the Central Valley. Results showed spatial genotypic subdivision in two of these populations. The observed spatial structure was attributed to demographic phenomena rather than to the existence of two distinct neighbourhoods that diverged by genetic drift. The two structured populations were probably founded by a coming together of two subpopulations presenting different allele frequencies. This situation, typical of other wild populations in the Central Valley, is probably the result of the frequent occurrence of colonization and extinction events in areas prone to human disturbance (see Section 5.2).

Maquet *et al*. (1996) and Zoro Bi (1999) observed that some alleles were distributed uniformly in the Central Valley, while others appeared to have a narrow distribution in climatically distinct regions. Therefore, spatial distribution of genes was analyzed by Masumbuko (1999) using 117 populations from the Central Valley connected by a Gabriel network (see Sokal and Oden 1987). In this network, two sites A and B are neighbours if no other site (C) is on or included in the circle having a diameter AB. Two significant correlograms were obtained, showing a spatial structure at short (3–18 km) and long distances (47–71 km) for the locus *ADH-2*, and at medium (25–47 km) and long distances (60–71 km) for the locus *Pgm-2* (Maquet *et al*. 2001). These correlograms are characteristic of a 'patch' structure due to alternately positive and negative Moran *I* values. The observed spatial structure seems to be the result of a restricted gene flow (see Section 5.5), although confined selection pressure cannot be ruled out.

In conclusion, isozyme studies of *P. lunatus* in the Central Valley indicate that genetic variability occurs at both the intra- and inter-population levels, and that the narrow distribution of some alleles is likely to have been influenced by ecological factors. In any sampling strategy for *ex situ* or *in situ* conservation, therefore, it would be essential to identify the most representative populations for each ecological zone within the Central Valley. As subdivisions may also occur within some populations, germplasm collectors should take care to sample in different areas within a population, and *in situ* management of these populations should attempt to maximize internal gene flow.

4.4.3 DNA markers

Recent developments in molecular genetics have made important contributions to conservation biology (Smith and Wayne 1996; Karp *et al*. 1998). For example, the development of molecular markers has significantly improved our ability to address complex problems in the management of plant genetic resources, in particular the characterization of plant genetic diversity. The development of the polymerase chain reaction (PCR) technique has provided a new way to study the plant genome (Fritsch and Riesberg 1996; Mace *et al*. 1996). RAPD (random

amplified polymorphic DNA) and AFLP (amplified fragment-length polymorphism) markers provide an arbitrary sample of the genome, and can generate information on an essentially unlimited numbers of loci for use in genetic analysis (Vos *et al*. 1995; Karp *et al*. 1998). These markers have already proved to be useful in a wide variety of theoretical and applied studies, including the construction of genetic maps, the identification of genetic markers linked to specific phenotypic traits, clone and variety determination, and population genetics.

Other important PCR-based markers are microsatellites or simple sequence repeat (SSR) (Goldstein and Schlötterer 1999). These markers consist of tandemly repeated units of up to six base pairs (6 bp) (Karp *et al*. 1998; Hancock 1999). Such sequences are widely spread throughout the genome of most organisms, especially those with a large genome (Hancock 1999). Microsatellite markers have been used to investigate such questions relevant to conservation biology as hybridization, reproductive success, the fitness consequences of inbreeding, mutation rates, and breeding systems (Beaumont and Bruford 1999; Pemberton *et al*. 1999; Rossetto *et al*. 1999).

RAPD, AFLP and microsatellite markers were used in this study to investigate the genetic structure of wild populations of Lima beans in the Central Valley of Costa Rica, in particular to document how genetic variation is distributed among populations and to determine the rate of migration among populations. Microsatellites were also used to examine the impact of local extinction and recolonization on genetic diversity of target populations. The AFLP and microsatellite results are summarized below.

AFLP analysis. DNA was obtained from five seeds from each of 31 populations. AFLP analysis was carried out following the method of Vos *et al*. (1995), according to the protocol proposed by Applied Biosystems (1996).

AFLP analysis revealed substantial variation in wild Lima bean populations in the Central Valley of Costa Rica. A total of 121 DNA fragments were analyzed using four arbitrary primer combinations (Table 7). Overall, 60 of these fragments were variable, yielding a high proportion of polymorphic loci (P = 0.496). The level of polymorphism varied among populations. Overall, 93 haplotypes were found in 202 individuals examined, and the number of haplotypes found in each population also varied. Some populations presented only one haplotype, while others presented as many as four haplotypes.

Table 7. Primer combinations used to produce AFLPs during the selective amplification of *P. lunatus* DNA, and the number of variable and non-variable bands generated.

Restriction enzyme	**Primer combination**				
***Eco*RI**	**ACC**	**ACT**	**ACA**	**AGC**	**Overall**
***Mse*I**	**CAT**	**CTT**	**CTG**	**CAC**	
Number of bands					
Variable	9	7	30	14	60
Non-variable	14	28	12	7	61
Total	23	35	42	21	121

The level of differentiation among populations was high. The mean estimate of θ across all fragments was 0.7643 (with a 95% confidence interval of 0.7223–0.8043). An analysis of molecular variance (AMOVA) was carried out on the matrix of inter-individual genetic distances generated from pairwise comparisons of all individuals within and between populations. This indicated that there was substantial variation among the populations examined, as 60% of the total variation was found between populations (Table 8). However, the analysis also revealed a level of population differentiation, as indicated by a Wright's F_{ST} index value of 0.399. The genetic relationships among populations are illustrated in Figure 16.

Table 8. Analysis of molecular variance (AMOVA) based on AFLP phenotypes consisting of 121 band states. The level of significance is based on 1000 iteration steps.

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation
Among regions	7	66.579	–0.09282	–2.22
Among populations within regions	23	259.488	1.76577	42.18
Within populations	123	309.200	2.51382	60.04
Total	153	635.266	4.18677	

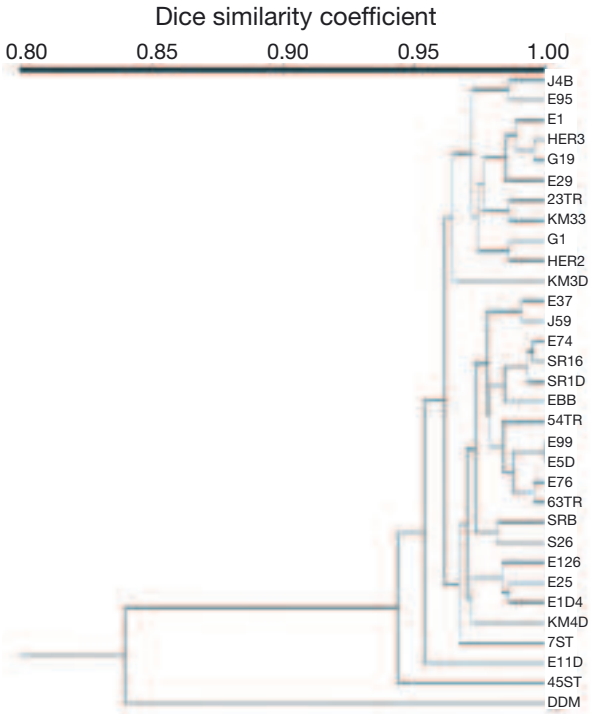


Figure 16. UPGMA cluster analysis of Dice similarity coefficient calculated of 121 AFLP markers from 31 wild populations and 1 cultivated accession of Lima bean from the Central Valley of Costa Rica.

Microsatellite analysis. To develop microsatellite markers for wild Lima bean, 73 primers isolated from *P. vulgaris* L. and provided by the Unit of Biotechnology at CIAT (Cali, Colombia) were tested on *P. lunatus* from the Central Valley. Out of the primer pairs isolated from *P. vulgaris*, 57 (78%) amplified in *P. lunatus*, with good polymorphism obtained from the following 11 primer pairs: AG1, BM98, BM114, BM141, BM149, BM156, BM159, BM160, BM161, GATS54 and GATS91. Among these, 9 primers were applied to amplify 359 individuals belonging to nine populations distributed near Heredia, Costa Rica. Sequencing gels were analyzed and population genetics parameters determined. Tables 9 and 10 give the results obtained for the population genetics parameters and Nei's genotypic diversity indices, respectively (Ouédraogo 2003).

Table 9. Intrapopulation polymorphism indices estimated in nine wild populations of *P. lunatus*.

Population	P^*	$A^\dagger$	$A_e{}^\ddagger$	$H_O{}^\S$	$H_E{}^\P$
E85	60	1.700 (0.675)	1.285 (0.460)	0.011 (0.024)	0.156 (0.209)
E088	80	2.100 (0.738)	1.372 (0.414)	0.015 (0.024)	0.213 (0.215)
E085	60	1.700 (0.675)	1.285 (0.460)	0.011 (0.024)	0.156 (0.209)
E093	20	1.200 (0.422)	1.165 (0.348)	0.014 (0.045)	0.090 (0.190)
E091	20	1.200 (0.422)	1.143 (0.317)	0.014 (0.045)	0.082 (0.175)
E068	30	1.300 (0.483)	1.151 (0.314)	0.000 (0.000)	0.091 (0.167)
E078	50	1.600 (0.699)	1.178 (0.385)	0.005 (0.016)	0.101 (0.174)
E076	70	2.200 (1.033)	1.350 (0.343)	0.016 (0.028)	0.216 (0.191)
E062	50	1.500 (0.527)	1.208 (0.354)	0.006 (0.018)	0.122 (0.189)
E094	60	2.000 (1.054)	1.392 (0.458)	0.031 (0.045)	0.215 (0.230)
Standard deviation	21.47	0.384	0.099	0.009	0.058

*Percentage of polymorphic loci; †allelic richness; ‡effective number of alleles; §observed heterozygosity; ¶genetic diversity. Numbers in parentheses are standard deviations.

Table 10. Nei's genotypic diversity indices estimated in nine wild populations of *P. lunatus.*

Microsatellite locus	$H_T{}^*$	$H_S{}^\dagger$	$D_{ST}{}^\ddagger$	$G_{ST}{}^\S$
AG1	0.398	0.286	0.112	0.281
BM98	0.599	0.328	0.271	0.452
BM141	0.386	0.296	0.090	0.233
BM142	0.323	0.226	0.097	0.301
BM149	0.022	0.021	0.001	0.040
BM156	0.124	0.063	0.061	0.493
BM160	0.015	0.015	–0.000	–0.013
BM161	0.082	0.069	0.013	0.158
GATS54	0.202	0.185	0.017	0.084
GATS91	0.047	0.044	0.004	0.084
Mean	0.220	0.153	0.067	0.303

*Total genetic diversity; †intrapopulation gene diversity; ‡interpopulation gene diversity; §genetic differentiation coefficient.

Overall, the proportion of polymorphic loci (*P*) ranged from 20 to 80%, depending on population, with an average value of 49%. Genetic variation may also be measured by the number of alleles per locus (*A*), and the effective number of alleles (A_e). These values indicated that one or few alleles are often over-represented at each locus. Observed heterozygosity (H_O) was low and different from the expected heterozygosity value (H_E) under Hardy–Weinberg equilibrium (HWE). Deviation of observed genotypic frequencies from the expected proportions under Hardy-Weinberg equilibrium was significant.

Microsatellite markers showed a larger total genetic diversity (H_T) within the wild Lima bean populations and also a larger intra-population genetic diversity (H_S) than isozyme markers and AFLPs. With microsatellites, the contribution of intra-population genetic diversity (H_S) to total diversity (H_T) was higher than the contribution of inter-population genetic diversity (D_{ST}). The coefficient of genetic differentiation, representing the contribution of intra-population genetic diversity to total genetic diversity is also typical of a predominantly selfing species, as indicated by the high values of coefficient of consanguinity (F_{IT} = 0.945) and coefficient of intra-population consanguinity (F_{IS} = 0.916).

The impact of anthropogenic extinction of local populations on genetic diversity was investigated using microsatellite markers to compare the level of genetic diversity before an extinction event and after the subsequent recolonization. Overall, the impact of local extinction on genetic diversity was variable, depending on the length of time before the recolonization, and the reproductive history of the population before the extinction event. The likelihood of restoration of genetic diversity of a given population depended on the health of the soil seed bank.

4.4.4 Morphological characters

Despite the advances in molecular marker technology, morphological characterization continues to be the foundation of genetic diversity research at any taxonomic level (Chandran and Pandya 2000). It is still an important tool for the management of crop germplasm collections (Ariyo 1993; Polignano *et al*. 1993; Annicchiarico and Pecetti 1994), having been used to identify duplicates, to discriminate among material from different geographic areas, to establish core collections, to investigate relationships between landraces and their wild relatives, and to prioritize material for use in breeding programmes.

Seed morphology has been one of the key traits in the study of the origin and diversity of Lima bean (Debouck *et al*. 1989; Baudoin 1991; Maquet 1995). It has been shown that the morphotype ‘Big Lima’, which typically exhibits large seeds, belongs to the Andean genepool, while the morphotypes ‘Potato’ and ‘Sieva’, with small and intermediate seed sizes, belong to the Mesoamerican genepool. However, very little is known about the levels of morphological variation in Lima beans within a given morphotype, and particularly about the levels of morphological diversity among populations present within a relatively small geographic area.

In order to study morphological diversity among wild populations of Lima beans in the Central Valley of Costa Rica, 37 populations were selected in the study area to cover the different parts of the Central Valley. All populations

were visited between March and May 1997, and at least 100 mature fruits were collected from each population prior to dehiscence. Fruits were taken to the laboratory at the University of Costa Rica, where they were measured and the seeds from each fruit counted. The length and the width of 30 fruits and the length, width and thickness of one seed from each of the fruits were measured with an electronic caliper. In addition, three replicates of 100 seeds from each population were weighed.

The mean size of the fruit was less than 1 cm in width and about 6 cm in length (Table 11). The seeds were about 3 mm thick, 6 mm wide and 7 mm long. The weight of 100 seeds was 21.7 g. However, the results also indicated that there were significant differences among populations for all descriptors of fruit and seed size (Table 12). Moreover, when the populations were grouped according to geographic areas, significant differences were revealed between these groups (Figure 17).

The levels of morphological variation of various descriptors of the size of the flower were also investigated. Flowers were collected from 12 populations. Length and width of each wing petal, the length and width of the corolla, the length and

Table 11. Mean and standard error for the descriptors of fruit and seed size.

Character	Mean ± S.E.
Fruit width (cm)	0.97 ± 0.13
Fruit length (cm)	4.15 ± 0.42
Seed thickness (mm)	2.97 ± 0.36
Seed width (mm)	6.00 ± 0.66
Seed length (mm)	7.07 ± 0.69
Weight of 100 seeds (g)	7.6 ± 1.3

Table 12. *F*-values and significance levels for 10 descriptors of size of fruit and seed for all wild populations of Lima beans considered in this study.

Character	Geographical area	Populations within geographical areas
Fruit width (W)	51.16†	41.77†
Fruit length (L)	36.27†	31.48*
Seed thickness (ST)	31.46†	12.47†
Seed width (SW)	18.47†	14.44†
Seed length (SL)	32.03†	19.00†
Weight of 100 seeds	21.68*	8.87†
L/W (fruit)	10.04 †	26.87†
LS/SW (seed)	2.80*	5.54†
LS/ST (seed)	14.08†	8.21†
SW/ST (seed)	13.00†	9.91†

Significance level: * = 0.0001 > *P* >0.001; † = *P* > 0.0001.

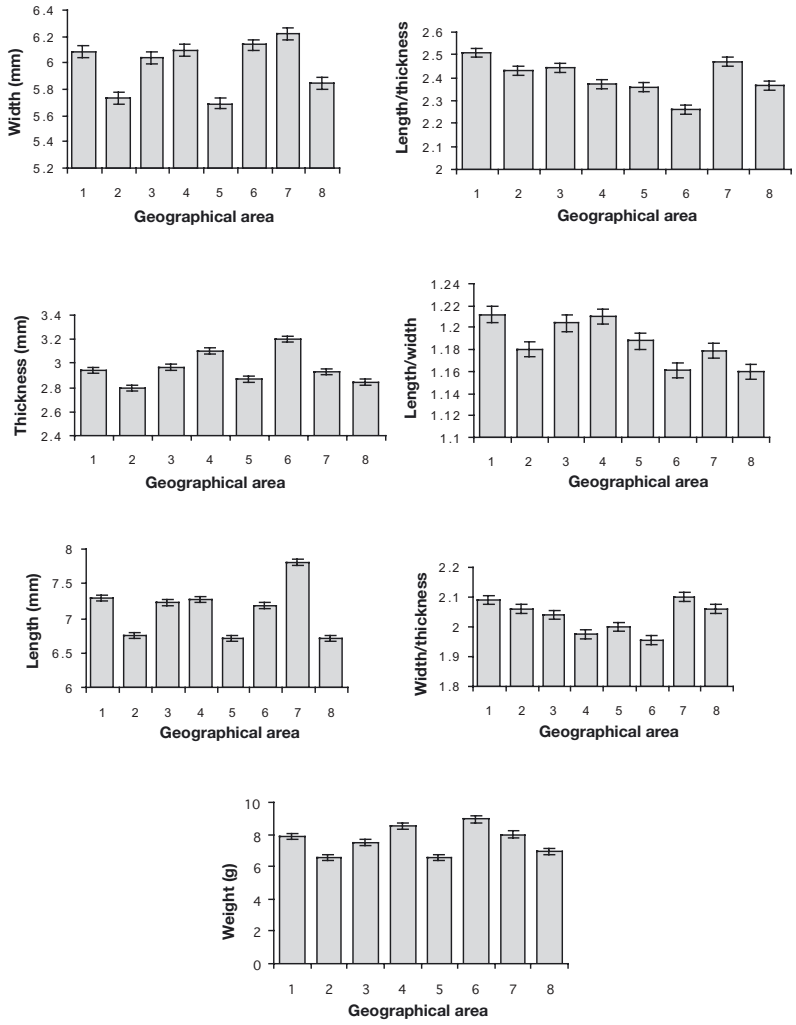


Figure 17. Variations in seven descriptors of seed size and shape: thickness (mm), length to width ratio, length (mm), width/thickness ratio and weight (g), among the eight geographic areas considered in this study.

width of the stamen, and the length of the ovary were measured on 10 flowers from each of these populations. There were significant differences among the populations sampled for all traits examined. In addition, multivariate analysis using principal components analysis (PCA) showed that most of the variation can be explained by the first two components. This analysis also showed that there is a clear differentiation in the size of the flower between some of the populations (Figure 18).

These findings show that there is morphological variation in the Central Valley for the traits examined, and that a significant fraction of this variation is found among

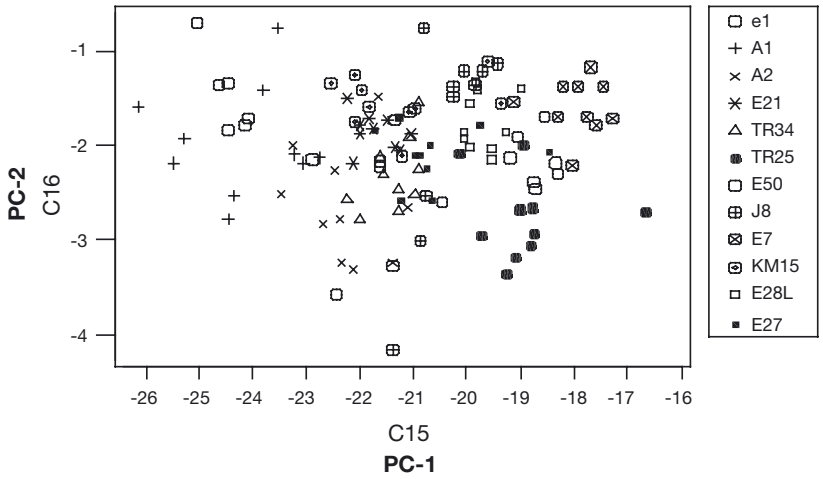


Figure 18. Principal components analysis (PCA) of the variation in 10 descriptors of flower size: see text for details.

populations. However, it was not possible to correlate the geographic distribution of this variation with that of major climatic factors such as annual precipitation, mean annual temperature and mean annual relative humidity. The levels of morphological variation described here probably indicate genetic differentiation between populations due to processes such as genetic drift, genetic isolation, and changes in reproductive behaviour involving high levels of biparental inbreeding and selfing, resulting from habitat fragmentation and population disruption.

4.4.5 Summary of genetic diversity studies

A major component of this project was to evaluate the genetic diversity represented by the wild Lima bean populations found in the Central Valley of Costa Rica. This diversity was studied at both the intra- and inter-population level, and the results served as a guide to the understanding and maintenance the genetic structure of the species in the area. Once biochemical (protein, isozyme) and molecular (microsatellite) markers had been developed, the genetic diversity investigations included the following: determination of an appropriate seed sampling strategy within populations, assessment of the genetic diversity between and within populations, analysis of factors responsible for the genetic organization of the wild Lima bean genepools, and study of the microgeographic patterns of genetic diversity.

Electrophoretic profiles of phaseolins showed four main polypeptides, with molecular weights ranging from 21.5 to 31 kDa. Two of these polypeptides were polymorphic, and their combination resulted in four distinct genotypes. Most of the variation (80%) was found among populations. Nevertheless, two genotypes were found in most of the studied populations. There was also a lack of heterozygotes among the individuals examined, which strongly suggests a

gametic disequilibrium for the encoding regions involved in the synthesis of polypeptides. Despite the relatively small size of the area considered in this study and the small sample size taken from each population (five seeds from a bulked sample), there was significant phaseolin variation among the wild populations studied, all belonging to the Mesoamerican genepool.

Assessment of genetic variability with enzymatic systems was made from 330 populations, with 1–60 individuals per population and 22 selected loci. At the intra-population level, results showed a low percentage of polymorphic loci (10%), few alleles per locus, and <1% heterozygotes. Wright's consanguinity coefficient showed deviation of populations from Hardy–Weinberg equilibrium. At the interpopulation level, Nei's genotypic diversity indices and *F*-statistics showed that 52% of the total heterozygosity came from genetic diversity among populations (G_{ST}), while 48% came from genetic diversity within populations. This was a relatively high value.

Microsatellites showed similar results for F_{IT}, F_{IS} and F_{ST}, but higher values for H_O, H_S, H_T and D_{ST}, compared with isozyme markers. Because microsatellites are more polymorphic and are codominant markers, a better estimation of genetic diversity would be expected, particularly if those markers are isolated directly from the plants (rather than from seeds).

Despite small population sizes, frequent bottlenecks, a low allogamy rate, common alleles at several loci, a restricted gene flow within populations and very few heterozygous individuals, significant polymorphism within populations was observed. Significant intra-population diversity could be due partly to the existence of gene flow over long distances. A significant correlation was observed between population size and genetic variability. The loss of genetic diversity in small-sized populations could be attributed to inbreeding and bottleneck effects in some populations.

Using phaseolins, populations could be arranged in groups according to their proximity and phenology. This could be explained, at least in part, by climatic factors, such as dry season. A study of microgeographic patterns was also carried out using isozyme markers on 95 populations well distributed throughout the valley. Results showed a very heterogeneous allelic distribution through all the polymorphic loci. Alleles were present in either very few or numerous populations. Geographic distribution of the alleles was very irregular, with some alleles being only found in specific parts of the Central Valley. This non-random spatial distribution of alleles might result from limited gene flow between populations, and/or very localized selection pressure caused by biotic or abiotic stresses.

Investigations were carried out to design an optimum seed sampling methodology for populations, which would be needed to preserve the integrity of each population an to understand the genetic organization within the population, and thus for the eventual implementation of *in situ* conservation strategies. The application of Yonezawa and Ichihashi's (1989) model showed that the best strategy for capturing all alleles consisted of sampling a large number of plants per population (80), and 1 to 4 seeds per plant.

Genetic variation among populations and its geographic pattern will be affected by several factors, including the dynamics of the populations as well

as environmental conditions in the Central Valley. For example, wild populations of Lima beans might undergo repeated bottlenecks, as weeding and other agricultural practices only allow a few plants to survive and reproduce. These processes lead to significant reductions in effective population size, and to high levels of inbreeding, favouring the decrease of heterozygotes in the populations. The recurrent reduction in population size will also favour genetic differentiation among populations. The discontinuity of the habitats where wild Lima beans are most likely to be found in the Valley also promotes genetic differentiation between populations. Such fragmentation is mainly the result of replacement of traditional coffee plantations by modern, intensive plantations and accelerated urban development. Differences in abiotic (climate and soil) and biotic factors will also have affected levels and patterns of genetic variation in the Central Valley.

4.4.6 The impact of local extinction on genetic structure

Monthly monitoring of 106 populations of Lima beans during the period from 1994 to 2000 revealed the occurrence of multiple extinction and recolonization events (see Figure 4). The data showed that 39 populations experienced local extinction. Only 16 of these locations were recolonized (41%) in later years. This figure does not include those locations where the population was totally eliminated but where seedlings of Lima beans were found again before 1 year (transient extinction).

The data also indicated that many populations that remained active failed to produce seeds for several annual cycles. Only 28 (42%) populations produced seeds every year. Moreover, on average, 17% of the populations never produced flowers. A sizable fraction of the populations clearly do not contribute to genetic flow among populations. However, many populations, including those that produced seeds every year, often experienced anthropogenic disturbances which diminished their size (Barrantes 2003).

In order to determine the effect of local extinction and recolonization on the genetic diversity of populations in a given location, the genetic structure of 13 populations was studied using microsatellite markers. The populations from each location were classified into three groups according to the occurrence of extinction episodes during the previous 5 years: (1) locations where local populations experienced extinction and were recolonized; (2) populations that did not experience local extinction, but were cut to simulate one extinction episode; and (3) control populations that did not experience local extinction. The selected populations were distributed throughout the Central Valley, and their geographic locations are shown in Table 13. For each location, genetic diversity from the samples taken from the populations present at two different times was compared. A sample of 25 individuals was randomly chosen from the population present at each collecting date.

Four locations were included in the first group (recolonizations). The populations present in these sites suffered at least one period of local extinction and recolonization. In addition, these locations were selected because there were seeds gathered prior to the extinction episode in the project's seed

Table 13. Location of the populations included in this study. Populations are grouped according to criteria described in the text.

Location	Community	Latitude North	Longitude West	Elevation (m asl)
Recolonized				
E17	San Antonio, Escazú	09°53' 376	84°07' 708	1382
E29	Quebrada Honda, Mora	09°53' 194	84°13' 446	988
E38	Salitrillos, Aserrí	09°51' 063	84°05' 435	1423
Her2	Centro, Barba	10°01' 754	84°07' 568	1332
Experimental				
E104	Jericó, Desamparados	09°50' 140	84°04' 436	1625
KM15	Vuelta de Jorco, Aserrí	09°48' 533	84°06' 921	1638
KM51	Centro, Naranjo	10°06' 492	84°23' 797	1138
KM53	Alto Murillo, Naranjo	10°05' 471	84°24' 190	1250
E25	San Antonio del Llano, Alajuelita	09°53' 293	84°07' 302	1473
Control				
E88	Barreal, Heredia	09°58' 875	84°08' 560	1067
J11	San Antonio del Llano, Alajuelita	09°53' 486	84°07' 040	1441
J58	Piedades, San Ramón	10°05' 244	84°07' 769	1088
E25	San Antonio del Llano, Alajuelita	09°53' 293	84°07' 302	1473

collection. This group included sites Her2, E17, E29 and E38. Location E17 was recolonized 2 years after its extinction, and the rest of the locations only remained extinct for 1 year.

The second group (manipulated locations) included five locations with conditions similar to that of the control group, i.e. no history of local extinction. A sample of 25 individuals was collected from each of these populations, and then all Lima bean plants present were cut between May 2000 and April 2001, before the time of fruit maturation and seed dispersal. All sites were recolonized the following rainy season. In order to compare the genetic diversity of the populations present before and after the experimental manipulation, leaf tissue was collected from 25 individuals from each newly established population. Two of these populations (E104 and E25) were exposed to a partial disturbance, letting a small patch of isolated individuals complete their reproduction period and disperse seeds. These transient extinction events are frequently observed in Lima bean populations throughout the Central Valley.

Populations in the last group had not experienced local extinction since November 1994 (control populations). In addition, these populations were successful in producing seeds every year. In this case, genetic diversity was compared between a sample of seeds collected in the same location in 1995, and a sample of the individuals present in May 2001. Three microsatellite marker genes were used to determine genetic diversity. Primers for these loci were developed by Yu *et al*. (2000) for common bean (*P. vulgaris*).

The results indicated that genetic differentiation and genetic distance between populations sampled in each location were affected by the occurrence

Table 14. Indicators of effective number of alleles, levels of genetic differentiation, and genetic distance for populations sampled in the same location for each of the three treatments. Numbers in parentheses indicate standard errors.

Treatment	Location	Population	Effective number of alleles	Genetic differentiation (F_{ST})	Nei's genetic distance
Recolonization					
	Her2	1995	1.45 (0.25)	0.2620	0.1548
		2001	1.12 (0.09)		
	E17	1997	1.18 (0.18)	0.6371	0.4639
		2001	1.08 (0.08)		
	E38	1994	1.16 (0.16)	0.1678	0.0815
		2000	1.37 (0.29)		
	E29	1994	1.34 (0.29)	0.0407	0.0142
		2000	1.16 (0.16)		
Control					
	J11	1995	1.09 (0.09)	0.0605	0.0186
		2001	1.36 (0.21)		
	E88	1995	1.58 (0.33)	0.0860	0.0790
		2001	1.48 (0.27)		
	J58	1995	1.32 (0.17)	0.0263	0.0133
		2001	1.48 (0.29)		
	E25	1995	1.42 (0.24)	0.0578	0.0493
		2000	1.15 (0.07)		
Experimental					
	KM51	2000	1.40 (0.26)	0.0099	0.0063
		2001	1.44 (0.29)		
	KM 15	2000	1.03 (0.03)	0.0213	0.0005
		2001	1.00 (0.00)		
	KM 53	2000	1.14 (0.07)	0.0069	0.0013
		2001	1.09 (0.08)		
	E104	2000	1.01 (0.01)	0.0258	0.0033
		2001	1.08 (0.08)		
	E25	2000	1.15 (0.07)	0.0304	0.0037
			1.03 (0.03)		

of extinction events (Table 14). In locations where Lima bean populations experienced long-term extinction and subsequent recolonization, the level of genetic differentiation (F_{ST}) ranged between 0.0407 and 0.6371 (mean F_{ST} = 0.2769). In contrast, in control locations, genetic differentiation between the population sampled in 1995 and that of 2000 or 2001 ranged between 0.0263 and 0.0860 (mean F_{ST} = 0.0576). Similar findings were observed for genetic distance between populations, where for recolonized locations genetic distances were, on average, larger than those of control populations (Nei's genetic distance, 0.1786 and 0.0400, respectively).

The impact of transient extinction on genetic diversity is illustrated by the experimental populations (Table 15). These successfully produced seeds every year, except for the year when they were cut. In these locations, a new population of Lima beans was easily re-established after the intervention. Analyses at these locations showed the lowest level of genetic differentiation (mean F_{ST} = 0.0189), and also the lowest genetic distance (mean Nei's genetic distance = 0.0030) between the plants present before and after the transient extinction. These findings indicate that populations of Lima beans may experience short anthropogenic extinction but still preserve their genetic diversity.

Variation in the effective number of alleles provides additional information (Table 14). For example, three of the four locations in the recolonization group showed a reduction in the effective number of alleles. In contrast, one location (E38) showed an increase in the effective number of alleles. This finding suggests that local extinctions might play an important role in the dynamics of Lima beans, as they allow the recolonization of the site by seeds stored in the soil bank. If there is a healthy seed bank, the new population may provide a better sample of the genetic diversity once present at that location, counteracting the negative effects of genetic drift. However, variation in the effective number of alleles was also observed in two locations in the control group, suggesting temporal variation in the genetic diversity within populations or sampling problems due to genetic structure within locations.

The potential impact of local extinction and recolonization on the genetic structure of Lima beans thus depends on the duration of the period of extinction, the status of the seed bank, and the likelihood of gene flow from another population. In general, locations where the existing populations experience multiple, long-term extinction episodes and fail to produce seeds every year are more likely to experience genetic change and loss of genetic diversity than locations where the populations never experience long-term extinction and produce seeds every year.

Table 15. Analysis of the linear regression between geographical distance and the mean rate of migration.

	R^2 *	*F*-ratio	*P* †	*b* ‡
All populations	0.009	50.469	0.000	–0.155
Locus *Adh-2* Short distance	0.018	3.274	0.072	–0.229
Locus *Adh-2* Long distance	0.003	0.473	0.493	–0.070
Locus *Pgm-2* Medium distance	0.000	0.114	0.736	–0.025
Locus *Pgm-2* Long distance	0.000	0.028	0.867	–0.018

* Determination coefficient; † probability at the 0.05 level; ‡ slope.

4.5 Gene flow

Lima bean is a self-compatible annual or short-lived perennial species with a mixed mating system. The cross-pollination mechanism, mediated by insects, has been described by Webster *et al*. (1979). Field and laboratory investigations have revealed a considerable amount of information on wild *P. lunatus* in the Central Valley of Costa Rica. Most relevant in relation to gene flow are the high frequency of small population sizes (66% of populations with fewer than 30 individuals), the low allogamy rate ($t \leq 10\%$), the presence of major alleles at several loci, the low frequency of heterozygous individuals, and the high intra-population polymorphism (indicated by significant G_{ST} values for both isozyme and microsatellite markers).

The origin of this significant intra-population diversity is in part related to the importance of short- and long-distance gene flow. Estimation of gene flow was therefore an important objective of the project's investigations. According to Slatkin (1981, 1985a), gene flow encompasses several mechanisms of gene exchange among populations, including movement of gametes, zygotes, individuals or groups of individuals from one place to another, and extinction and recolonization of entire populations. Indeed, pollen or seed flow, or both, have been used as indices of gene flow in plants. This movement of individuals and genes in space affects many important ecological and evolutionary properties of populations (Hanski and Gilpin 1997). In particular, the rate of movement of genes from one population to another helps to determine the possibility of local adaptation and of adaptive evolution in complex landscapes.

Gene flow can be estimated at the within- and between-population levels, using direct (field measures) and indirect methods, as described below.

4.5.1 Direct methods of gene flow estimation

According to Wright (1943, 1946), Crawford (1984) and Gliddon *et al*. (1987), estimation of gene flow is based upon the number of individuals in a local random breeding unit, i.e. a 'neighbourhood' defined more precisely as the genetic neighbourhood area (NA) and the effective neighbourhood size (Nb). These two parameters are determined from two equations:

$$NA = 4.\pi\,(1/2.t.\sigma_p^2 + \sigma_s^2 + \sigma_v^2) \qquad (1)$$

$$Nb = NA.d\,(1 + t)/2 \qquad (2)$$

where σ_p^2, σ_s^2 and σ_v^2 are dispersal variances, respectively, for pollen, seed and flowers (or vegetative growth), t is the outcrossing rate and d is adult plant density.

Gene flow was measured in three selected Lima bean populations of the Central Valley. The methods used to estimate pollen, flower and seed dispersals have been described by Janart (1996), Hardy *et al*. (1997) and Baudoin *et al*. (1998). Pollen grains and seeds were labelled *in vivo* using stains and fluorescent dye. Actual pollinators were distinguished from simple visitors by observing each population at different dates during its flowering period and estimating the Lima bean pollen load on an insect's body.

Lima beans in the Central Valley bloom during the dry season, from about mid-November to mid-February. The mean pollen:ovule ratio is about 863 (Hardy *et al*. 1997). According to Cruden (1977), this ratio is typical of species with a mating system qualified as facultative allogamy. Flower visitors in the target area include Thysanoptera, Coleoptera, Lepidoptera and Hymenoptera, but the major pollinator is the honey bee, *Apis mellifera* L. (Hardy *et al*. 1997). In all studied populations, most pollen transfers occurred across distances of less than 1 m, confirming that common bees disperse pollen mostly over short distances. The frequency of pollen transfers dropped rapidly above 1 m, although transfer could reach a maximum value of 5.5 m. The corresponding dispersal variance for pollen was σ_p^2= 1.7 m². This value is probably somewhat underestimated because it does not take into account pollen transfers over distances of >6.5 m. Measures of flower dispersal showed great variability in the vegetative growth of wild Lima bean individuals. Distances separating each inflorescence from its respective plant base ranged between 0.37 and 6.5 m, according to the presence of supporting vegetation, allowing the plant to climb. In the populations tested, the mean flower dispersal variance was σ_v^2 = 2.7 m². Seeds of wild Lima beans are too heavy to be carried by the wind. The most important contribution to seed dispersal occurs when dehiscent pods open and propel their seeds onto the ground. In the populations studied, the maximum distance of seed dispersal was 5.5 m from the pod. The mean seed dispersal variance was σ_s^2 = 1.68 m².

In the Central Valley, Maquet *et al*. (1996), using isozymes, and Hardy *et al*. (1997) calculated a mean outcrossing rate and a mean adult plant density of, respectively, t = 0.1 and d = 0.235 plants/m². From equations (1) and (2), the neighbourhood area (NA) and the effective neighbourhood size (Nb) were, respectively, 56 m² and 7.23 individuals. According to Wright (1943, 1946), the low value for Nb (fewer than 20 individuals) corresponds to a high probability of random local genetic differentiation within the population. As the populations in the region could spread over areas ranging from about 100 m² to more than 1000 m², they could contain several to many neighbourhood areas. Therefore, allelic distribution in a single population is expected to be highly structured and there is a need to collect germplasm systematically at many sites within the population in order to sample the whole genetic diversity present.

4.5.2 Indirect methods of gene flow estimation

A common approach to quantifying gene flow has been to transform measures of population structure into indirect estimates of the average number of migrants exchanged per generation, most commonly by using an island model (Wright 1951). One can also apply an isolation-by-distance model, in which the rate of gene flow is expected to decline monotonically with increasing geographic distance between continuous populations. This model incorporates spatial information and thus permits tests of hypotheses about relationships between the effective migration rate and spatial patterns of inter-population connectivity. Both methods were used in the Central Valley.

Enzyme markers were analyzed to estimate the gene flow among 139 wild populations in the Central Valley (Ouédraogo 2003). Isolation by distance and spatial autocorrelation analyses were carried out on 117 populations for which the geographic coordinates were available, in order to build a distance matrix. For microsatellite analysis, nine populations from Heredia were scored for 10 pairs of microsatellite primers isolated as explained above.

Gene flow was evaluated as follows:

- ***Wright's island model*** (Wright 1951). The mean rate of migration (Nm) was calculated by analyzing 10 enzymatic loci and 10 microsatellite loci using the formula of Crow and Aoki (1984).
- ***Slatkin's private alleles model*** (Slatkin 1985b) and the corrected estimate of Nm by Slatkin and Barton's method (Slatkin and Barton 1989). Private alleles are alleles found in one population only. The estimation of gene flow was made using the module '*Dist*' included in the *GENEPOP* software (Raymond and Rousset 1995).

The spatial pattern of distribution of genes was analyzed using 5 polymorphic loci in all 117 selected populations. The Moran index of spatial autocorrelation (*I*) was estimated on the basis of the allelic frequencies for each polymorphic locus and each distance class.

From the autocorrelation and the geographic distances matrix, four groups of populations were selected:

(1) 20 populations at short distances, for the locus *Adh-2*
(2) 20 populations at long distances, for the locus *Adh-2*
(3) 31 populations at medium distances, for the locus *Pgm-2*
(4) 13 populations at long distances, for the locus *Pgm-2*.

The mean rate of migration was regressed against geographic distance for these populations, to test the hypothesis of isolation-by-distance (Slatkin 1993).

With microsatellite data, an average of the genetic differentiation coefficients was calculated according to classes of distance between pairs of wild populations (Hardy and Vekemans 2002). An analysis of variance of the regression between genetic differentiation coefficient (F_{ST}) and geographic distance was also carried out.

4.5.3 Results of gene flow studies

Using isozymes, Wright's method produced a G_{ST} value of 0.575, corresponding to a number of migrants per generation (Nm) of 0.18, suggesting restricted gene flow among subpopulations (Nm < 1). By the method of Slatkin using rare alleles, Nm was estimated at 0.074 for n = 10, 0.058 for n = 25, and 0.047 for n = 50. The mean number of individuals per population was 18 and the mean frequency of the private alleles was 0.4. The corrected rate of migration was thus 0.08. The sample size ranged from 4 to 334 seeds per population, whereas Slatkin

(1985b) suggested sampling an identical number of seeds per population. To test the effect of this factor, 42 populations were selected in which a maximum of 10 seeds were sampled. The mean number of individuals per population was 9 and the mean frequency of the private alleles was 0.16, which corresponded to a corrected rate of migration of 0.51. Even this higher number of migrants per generation was still <1.

Using microsatellites, the fixation index (F_{ST}) was 0.346, and the average inbreeding coefficient within populations was high (F_{IS} = 0.916). The number of migrants per population and per generation from Wright's method was 0.47. By the method of Slatkin using private alleles, Nm was estimated at 0.099 for n = 10, 0.075 for n = 25, and 0.060 for n = 50. The mean number of individuals per population was 33 and the mean frequency of the private alleles was 0.35. The corrected number of migrants per population and per generation was 0.06.

4.5.4 Spatial structure of genes and isolation by distance

Two loci showed significant autocorrelation. *Adh-2* exhibited a spatial structure at short (3–18 km) and long distances (47–71 km), and *Pgm-2* at medium (25–47 km) and long distances (60–71 km) (Figure 19). Restricted gene flow among populations could imply isolation by distance, but analysis indicated that geographic distance explains less than 2% of the variability in gene flow (Figure 20, Table 15).

Nonetheless, for distances ranging between 0 and 997 m, a linear relation between genetic coefficient differentiation and geographic distance was obtained based on microsatellite data (Figure 21). Geographic distance explained 18% of the divergence among populations. A linear relation between gene flow and geographic distance was also observed for this range of distances. Beyond 1000 m, however, no relation between genetic coefficient differentiation or gene flow and geographic distance was found.

Assuming no selection on populations and equilibrium between genetic drift and gene flow, the genetic differentiation coefficient between populations is

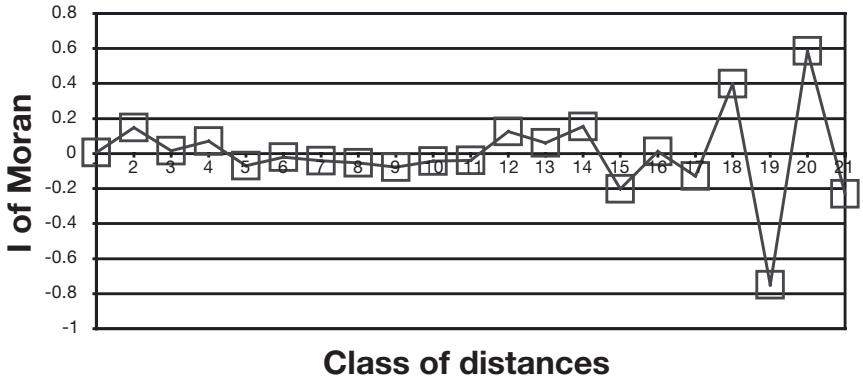


Figure 19. Spatial autocorrelation among 117 wild populations of *P. lunatus*.

inversely related to gene flow between populations (Nm = (1 – F_{ST})/(4F_{ST}); Slatkin and Barton 1989). The genetic differentiation coefficient decreased from 0.64 to 0.43, while Nm increased from 0.14 to 0.33 when comparing distance classes 227–997 m and 0–226 m. Such genetic differentiation coefficients characterize populations with very significant divergence and weak to moderate gene flow (Wright 1978).

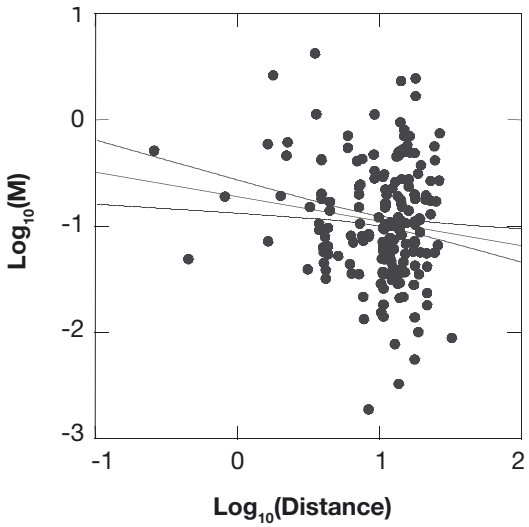


Figure 20. Relation between geographic distance (km) and the rate of migration (M) at short distances among 20 wild populations of *P. lunatus* possessing the allele *Adh-2*[61].

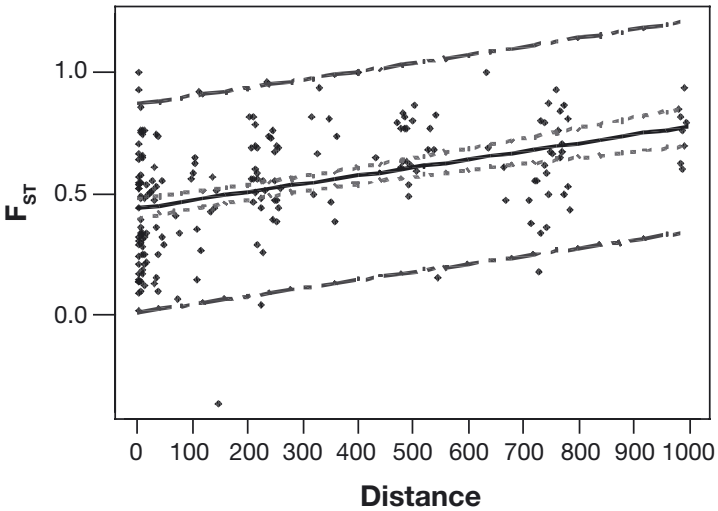


Figure 21. Linear relation between geographic distance (m) and genetic coefficient differentiation (F_{ST}).

4.5.5 Conclusions from gene flow studies

The distribution of alleles in space is, broadly speaking, influenced by seed- and pollen- mediated gene flow, random genetic drift, and different forms of natural selection, mutation and genetic recombination. Quantifying migration, and especially long-distance migration, has always been one of the more difficult tasks in plant biology. Estimating gene flow directly in the field or indirectly with genetic markers essentially involves assessing the distribution of alleles or the structure of populations in space. Direct gene flow estimation in the field showed that neighbourhood areas were limited within Lima bean populations, at least in the populations selected for study. At this level, genetic drift could therefore be important.

The island model and isolation-by-distance models were employed to measure the cumulative effects of gene flow indirectly. Such indirect methods are useful for understanding the evolution of genetic structure in plant species with a history of metapopulation dynamics. However, strictly speaking, Wright's method is appropriate for situations where equilibrium between genetic drift and gene flow has been reached in a large number of populations which are constant in size and never become extinct (Whitlock and McCauley 1999). Slatkin and Barton (1989) compared indirect methods for estimating average level of gene flow and showed that F_{ST} and rare allele methods yielded comparable estimates over a wide variety of conditions; two maximum-likelihood methods tended to yield biased estimates when a relatively small number of locations were sampled (10 demes), but more accurate estimates when a larger number were sampled (40 demes). F_{ST} is likely to be more useful under realistic conditions. In metapopulations with frequent extinctions and (re)colonizations, the relationship between genetic differentiation and gene flow is not straightforward, and the rate of migration is underestimated. Nm is also underestimated with Slatkin's method when the seed number per population is heterogeneous.

In summary, low to moderate levels of gene flow (0.06–0.47) were documented in wild Lima bean populations in the Central Valley of Costa Rica, although heterogeneity in the number of individuals per population could cause underestimation (Slatkin 1985b). Both enzyme and microsatellite markers showed that very great divergence (Wright 1978) occurs among populations. This is probably due to restricted gene flow, with genetic drift therefore playing a major role in the genetic structure of Lima bean populations in the study area.

Gene flow was not related to geographic distances at larger scales. Several factors could explain this fact. One could be that only a small number of loci (7–10) were analyzed. However, Slatkin (1993) demonstrated that ten loci could be sufficient. Other possibilities are heterogeneity among the gene flow values when all populations are considered and, in the isolation-by-distance model, the assumption that populations must be in equilibrium between migration and genetic drift.

4.6 Computer simulation of population genetic structure

Software was developed by the project to simulate the genetic behaviour of wild Lima bean populations, taking into account information on the local population

density around each plant, the spatial distribution of individuals in the population and the pattern of pollen dispersal. All the available data on mating system, gene flow, demography and genetic variation were integrated in the program to build the model. The simulation program used a 'cellular automaton' approach (Gardner 1970; Dresden and Wrong 1975). The behaviour of each individual (plant or seed) in a population was simulated separately and had its own characteristics (Judson 1993). The main difficulty in developing such simulations is the need for detailed knowledge of gene flow patterns between the various group levels. Each individual is located on an orthogonal two-dimensional grid and its behaviour is simulated separately. Crosses between plants are simulated on the basis of several factors, including allogamy rate, spatial distribution of gene flow, and population size, shape and density. For each generation, allelic and genotypic frequencies, fixation index, heterozygosity and gene diversity parameters are calculated. Genotypes are chosen randomly to constitute the next generation, without any form of selection on fitness.

The simulation model adopted relied on the balance between mechanisms responsible for inbreeding (favoured in Lima bean by the proximity of anthers and stigma within the keel at the time of pollen shedding) and outcrossing (due to pollinator behaviour). The simulation gave an allogamy rate of 5–10%, which was in good agreement with the rates calculated directly from isozyme studies. Simulated data after several mating generations revealed stable spatial genetic structuring (clusters) within populations. This also coincided with the isozyme data. The model takes into account the demography of wild Lima beans, integrating age structure and life cycle data, as suggested by Degreef (1998). The coefficients used by the simulation program are the transition probabilities from stage L_1 to stage L_2 and from stage L_2 to stage L_3 (the three successive stages of ligneous individuals).

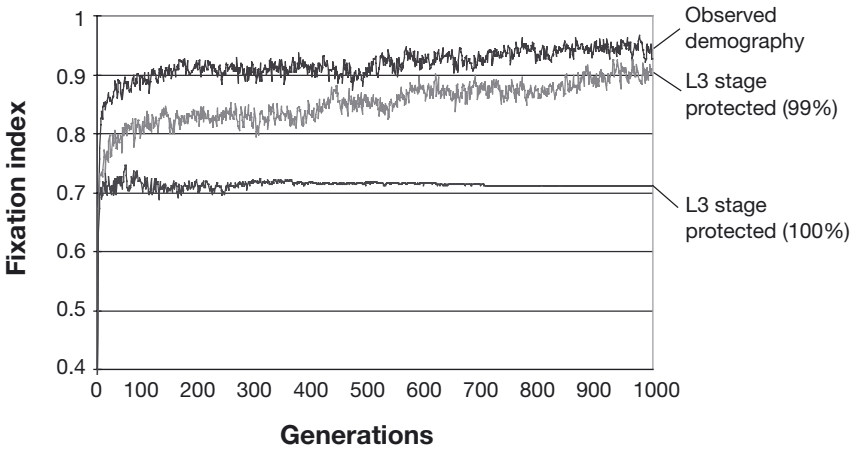


Figure 22. Evolution of the fixation index according to three different *in situ* conservation strategies (population E95). L_3 is the largest stem diameter class among the three adult life classes.

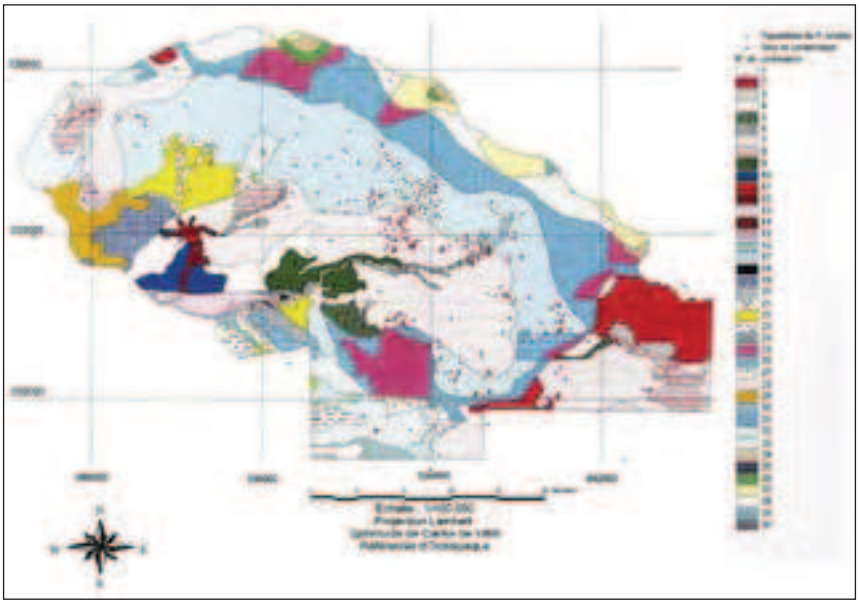
The program also integrates survival rates supplied by the user for each of these three stages. As an example of the impact of population dynamics on genetic structure, Figure 22 shows the evolution of the fixation index *F* according to three different *in situ* conservation strategies. Population E95 (with the *Adh-2* locus) was selected for this simulation. At the onset of the simulation, it was assumed that all plants in the initial population were at the L_2 stage. Such a simplification has very little effect on the results because population genetic parameters, such as fixation index, are apparently quite stable in relation to age structure. The first curve in the graph indicates the evolution of the population based upon the mean observed coefficient values of the life cycle. The second curve results from a very high protection of the L_3 stage (survival rate set to 0.99). A third curve is obtained when the L_3 stage is fully protected (survival rate = 1). Results show that it is possible to influence the population genetic structure (the relative importance of heterozygotes) depending on the stage protected. In particular, the L_3 stage appears to have the most striking effect on genetics. A very slight difference between the protection intensity on this stage can have very important consequences. Therefore, even if some differences in fitness exist between genotypes, population management could still have significant consequences for genetic conservation.

5. *IN SITU* CONSERVATION OF WILD *P. LUNATUS* IN THE CENTRAL VALLEY

The reason for investigating and documenting the ecogeography, demography and genetic diversity of wild *P. lunatus* in the Central Valley as thoroughly as has been described in the preceding pages was to make possible the development of a scientifically sound strategy for its conservation in the area and beyond. The diversity studies led to the formulation of a sampling strategy for *ex situ* conservation (Zoro Bi *et al*. 1998, 1999; see Section 6.2 for details). In this section, we look in depth at the complementary approach of *in situ* conservation. Two options (not mutually exclusive) were investigated. One was to identify and protect specific existing populations. The other was to establish new, synthetic populations in protected areas.

5.1 *In situ* conservation areas

In a preliminary step, life-zone and soil-type maps were used to identify all the possible environmental combinations in which Lima beans occur. This work was related to the study of spatial distribution in *P. lunatus* genetic variability over the whole Central Valley (Maquet *et al*. 1996; Zoro Bi 1999; Vargas *et al*. 2000). A first map combining 12 life zones and 6 soil types was prepared in 1997 (Map 7). Of the 72 possible combinations, 40 were observed in the Central Valley, but their area and the number of populations present in each one varied widely. Two combinations



Map 7. Distribution of Lima bean populations in the Central Valley. The map combines life zones and soil types.

were found to be particularly important: combination 7 (bh-P [bosque humedo premontano] life zone on Inceptisol) with an area of 449 km² and 135 populations, and combination 32 (bmh-P [bosque muy humedo premontano] life zone on Inceptisol) with an area of 474 km² and 171 populations. These two combinations include more than 75% of the total number of populations of wild Lima beans we recorded in the Central Valley.

The map combining life zones and soil types was used in conjunction with other maps (communication, water courses, etc.) in order to verify the accessibility of the sites and to identify landmarks. The 40 combinations identified were visited with the aim of locating sites potentially useful as conservation areas. The choice was guided mainly by the need to retain only those sites likely to maintain their integrity in the long run. Considering the two main agents of genetic erosion in the central Valley, i.e. urban expansion and intensifying agricultural practices, and the small size of many Lima bean populations, sites prone to human pressure (such as coffee plantations, small-scale farms, hedges near roads) were discarded. Special attention was paid to locations representative of the diverse ecological regions of the Valley and characterized by a micro-environment well-suited to wild Lima beans. Some isolated sites, remote from cultivated lands or human settlements, mainly located along water streams and steep slopes, and legally protected from cutting and weeding, were identified as potential conservation areas. The secondary, stratified, scattered vegetation growing at these sites is very suitable for Lima beans, with light easily penetrating to the leaves of the plants, and several woody species capable of serving as shade or support for the climbing vines. During the field survey, some ecologically favourable sites were rejected because of various drawbacks, for instance a low frequency of woody plants, very small area, lack of access, or close proximity to the Irazu Volcano National Park.

In order to preserve the widest array possible of the genetic diversity of wild Lima beans available in the Central Valley of Costa Rica, several micro-conservation sites should be established in each ecological zone, but particularly in the zones where the highest proportion of the populations are present (i.e. life zone/soil type combinations 7 and 32). This approach takes into account the results obtained by Zoro Bi (1999), who analyzed the microgeographic pattern of the genetic variability of 95 populations and found a structuring in the spatial distribution of allelic frequencies, with some populations being characterized by the presence of localized and private alleles, and therefore deserving priority in preservation.

In the end, the survey of the Central Valley identified 30 conservation sites, representing 24 combinations of life zone and soil type and distributed at elevations of from 340 to 1980 m asl. These sites had an average area of 7500 m², ranging from 725 to 570 000 m². The micro-environment was very often characterized by a diverse overstorey, producing a layered vertical structure but allowing light penetration (Figure 23). The conservation sites were classified into two major categories according to their vegetation structure and fitness for Lima bean population development:

- *Category 1* (10 sites), the most appropriate for *in situ* conservation, had a very scattered vegetation, mainly made up of trees and bushes, with very few herbaceous species, located on slopes of more than 30°, and with the presence of abundant litter covering the soil.
- *Category 2* (16 sites), had woody but relatively open vegetation, with abundant herbaceous strata and vines.

Figure 23. Typical wild *P. lunatus* conservation site in the Central Valley of Costa Rica.

Some large populations, covering an area of 1000 m² or more and containing at least 100 individuals, are present in the Central Valley, in environments considered appropriate for *in situ* preservation, for example those located at the margins of secondary forest or near a river or stream. These large populations should be maintained in their original sites and will only require regular monitoring (perhaps every year). Other populations, of smaller size and at risk of anthropogenic disturbance, could be moved to conservation sites in the previously defined 24 environmental combinations. This process should follow specific guidelines, taking into account the population itself and the surrounding micro-environment (see Section 6.2).

Specific recommendations for *in situ* conservation can be derived from the results of the demographic studies described above. The management strategies proposed for wild Lima bean populations must be adapted to the disturbance level they experience. In perturbed sites, such as in the vicinity of coffee plantations or along trails, conservation must favour the growth of young lignified individuals, which closely depends upon the humidity of the environment. The suggested management is to spread a mulch on the soil surface at the end of the rainy season or to install a vegetation cover, which will favour both high air and soil moisture. In addition, the recruitment of new individuals from seeds can be promoted by weeding the soil surface just after the dispersal of the seeds at the end of the dry season. In contrast, in the more natural, undisturbed sites, mainly located at edges of secondary forests, it is important to encourage the survival of large lignified individuals. Selective clearing should be carried out at these sites to maintain potential reserves of lignified adult plants.

5.2 Establishment of synthetic populations of wild Lima beans in the Central Valley of Costa Rica

A synthetic population is defined here as a group of individuals derived from seeds collected from the original sites of wild Lima bean populations, then sown in a protected site selected (and subsequently managed) to allow optimal plant development and gene flow within and among populations. The protected sites should be located in the same life zones as the original wild Lima bean populations. To test this approach to *in situ* conservation, a number of synthetic populations were established, starting in June 1998, in protected micro-conservation sites covering four life zones of the Central Valley (Table 16).

At each of these sites, synthetic populations were sown using seeds collected from nearby populations found in natural areas. The micro-conservation plots containing the synthetic populations were designed to fulfil several requirements, with regard to gene flow, plant dispersal, population size and fragmentation, extinction and recolonization processes. Such requirements are discussed, on a broad level, by Gilpin (1987), Given (1993), Maxted *et al*. (1997), Shafer (1990, 1997), Tiebout and Anderson (1997) and Yonezawa (2000). The project's investigations in the Central Valley, particularly on gene dispersal (Hardy *et al* 1997), population dynamics (Rocha *et al*. 1997; Dubois 1998), demography (Degreef 1998) and population genetics (Zoro Bi 1999) were also crucial in complementing these general principles. On the basis of all this previous work, two types of micro-conservation reserve (Figure 24) were designed and established, taking into account the patchy distribution of wild Lima beans in the Central Valley, the generally small population size, the twining vegetative growth, and the possibility of gene exchange between nearby populations through pollen and seed dispersal.

Table 16. Synthetic populations established in protected micro-sites of the Central Valley of Costa Rica.

	Alfredo Baulio	**Agricultural Research Station Fabio Baudrit**	**Lankester Botanical Garden**
Coordinates	09°54′N–83°57′W	10°00′N–84°16′W	09°51′N–83°53′W
Type	Circular	Circular and linear	Circular and linear
Climate	Humid type D	Sub-humid humid	Humid type C
Holdridge life zone	bh-MB	bh-P	bh-P
Populations sown	A1-KM30-23TR-54TR	E123-E126-J67-KM67	E110-E111-E114-G19
	Ciudad Colon	**Tabarcia**	**Chirracal**
Coordinates	09°55′N–84°1′W	09°51′N–84°15′W	09°55′N–84°10′W
Type	Circular and linear	Linear	Circular and linear
Climate	Sub-humid humid	Humid type D	Humid type C
Holdridge life zone	bh-T	bm-P	bh-P
Populations sown	E28-E29-E37-E115	KM12-KM14-KM15-E125	E1-E25-E127-KM1

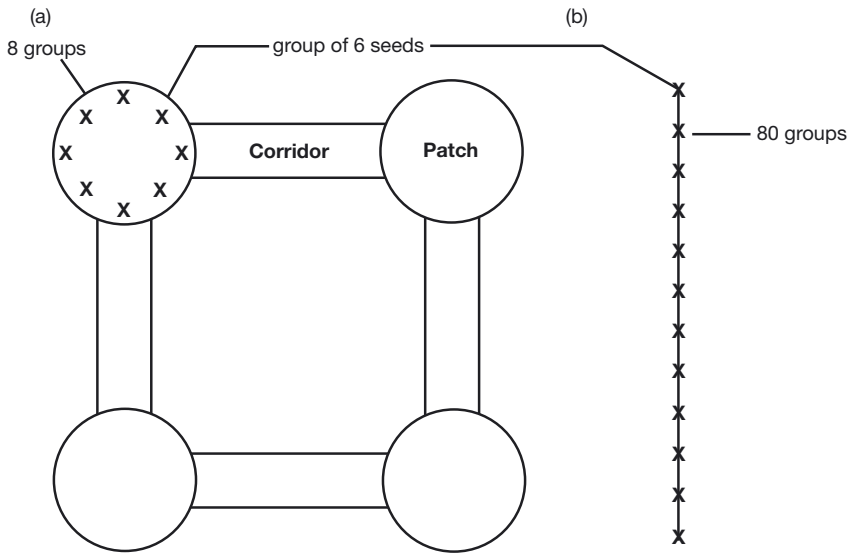


Figure 24. Schematic representation of (a) a circular and (b) a linear conservation site for synthetic populations.

The ***circular conservation design*** consisted of four circular cleared patches, 10 m in diameter each, linked by corridors 2–3 m wide and 8 m long. In each patch, eight groups of six seeds were sown either in mixture (seeds from four different nearby populations) or on their own (seeds from the same population). Groups of seeds were planted at the base of an existing tree or shrub, to provide support for the vines. Each of the four nearby populations was duplicated in each circular patch. The corridors, where natural vegetation was also cleared, were meant to facilitate gene flow between patches through pollen movement or vegetative development (wild Lima beans being characterized by very long twining branches, bearing racemes at leaf nodes).

The ***linear conservation design*** consisted of 80 groups of 6 seeds (20 from each of 4 nearby populations) sown every metre along a hedge or thicket. Groups of seeds from different populations were sown alternately. In the two conservation models, plant growth was favoured through various management practices (see below). A map of each site was made to locate precisely each group of six seeds within the plot.

The area of each patch was determined on the basis of several parameters: total area available in the life zone/soil type combination, number of interesting populations in the target combination, population size. According to the neighbourhood area calculation (Hardy *et al*. 1997), the minimum area of the patches should vary between 56 and 150 m^2. The number of seeds to be introduced in each patch was determined with a view to reaching a mean density of 0.35 adult plants/m^2, a situation frequently observed in the valley (Degreef 1998). The seeds to be sown in each patch represented the original genetic variability of the population in its original site. In order to achieve this objective,

Zoro Bi *et al*. (1998) and Zoro Bi (1999) have made some recommendations regarding how to sample seeds:

- for *small populations* (10–20 individuals), an equal number of seeds should be sampled from each plant;
- for *populations with 50 or more individuals*, one or two seeds should be sampled from as many plants as possible; the choice of plants should consider intra-population genetic diversity, requiring stratified sampling;
- for *very large populations* (more than 100 individuals), sampling should be made along transects; a larger number of seeds should be sown to cover the original genetic variability, requiring larger patches to maintain the average plant density.

Following the results of the demographic studies, the vegetation in each patch was slightly disturbed during the development stage of introduced populations in order to obtain optimal germination and rapid growth of the plantlets, and to avoid uncontrolled dispersal.

To assess the effectiveness of this methodology, the demography of the synthetic populations was studied and compared with that of four natural populations located in the same environments. Field observations and measurements similar to those described in Section 5.3 were taken (Degreef 1998). In particular, the demographic parameters of germination percentage, death rate, lignification rate and mean fecundity were evaluated in each synthetic population 1 year after their establishment.

The results of this comparative demographic study between synthetic and natural populations are presented in detail by Meurrens *et al*. (2001). In summary, the data obtained after 1 year of management in micro-conservation plots showed a positive impact for at least three important demographic parameters: germination percentage of seeds sown, lignification, and death rates. On the other hand, there was apparently no positive influence on fecundity, as represented by the mean number of seeds per plant. Preliminary data also showed a better performance of the circular model compared with the linear model, which could be partly attributed to the buffer zone established around circular patches, protecting them from micro-climatic variations.

The increase in germination percentage in synthetic populations can be explained by the management method used to break seed dormancy, i.e. successive weeding after sowing. This practice moves the seeds to the soil surface, where they are exposed to high temperature, a factor favouring the breaking of seed coat dormancy (Degreef *et al*. 2002). When establishing synthetic populations, the first step is to allow as many seeds as possible to germinate and to produce seedlings. When a sufficient number of adult plants is reached to ensure progenies, it is important to let the populations build up their own soil seed bank. If sufficiently large, this reserve of seeds will buffer the poor seed production that could occur in some years and enhance the demographic stability of the population. After successful germination, a seedling, within its first year, is destined either to die or to reach the juvenile or the lignified stage. Reducing

the mortality of seedlings is an important challenge for *in situ* conservation of wild Lima beans in the Central Valley, as most individuals die at this stage in natural populations (Degreef 1998). In synthetic populations, death rates can be decreased by appropriate management practices, in particular using a mulch to maintain soil moisture during the dry season.

Another challenge is to facilitate the transition from the juvenile to the lignified stage within a year. Lignification strengthens the plant and allows better tolerance to water stress during the dry season, and good seed production. In the Central Valley, a rapid transition from the seed stage to the lignified stage is encouraged by such management practices as applying insecticide to limit the attack of beetles and caterpillars on the leaves, supplying water at critical periods during the dry season, and maintaining good soil moisture by mulching. Finally, another key parameter is the fecundity of individuals, i.e. the number of well-developed dry seeds they produce. The data showed a very large variability in seed production within and among populations, which could not easily be related to critical limiting factors of genetic and/or environmental origin. Further investigations are needed to design interventions to improve this demographic parameter.

6. CONCLUSIONS AND RECOMMENDATIONS

This project has developed a uniquely detailed and comprehensive dataset of ecogeographic, genetic, demographic and phenological information on a crop wild relative. We have then used this dataset to develop an *in situ* conservation strategy for *P. lunatus* in the study region based on a two-pronged approach: the selection of key natural sites and the establishment of synthetic populations. Management interventions have been designed, also based on the results of project activities, and their demographic and genetic effects monitored, albeit over a short period. Finally, the elements of a collecting strategy for *ex situ* conservation to complement the *in situ* activities have been developed.

In order to build on the scientific achievements of the project, future efforts should be focused on the further implementation and monitoring of the *in situ* conservation strategy that has been developed. At the same time, it is important to improve and refine the strategy, and the methodology used to reach it, through further investigation. The following research activities could be highlighted for future work:

- ***In situ* management and monitoring of wild *P. lunatus*.** Two types of populations are involved in the *in situ* conservation effort: existing natural populations and 'synthetic', or artificially established, populations. Management, monitoring (through genetic characterization as well as phenological and demographic observations) and refinement of the management strategy should be carried out to ensure long-term conservation. In particular, plant growth and development, extent of gene flow among patches within micro-conservation sites, and appearance of novel multilocus genotypes through genetic hybridization should be followed.
- **Maintenance of genetic variability in the Central Valley.** GIS tools should be used to examine in more depth any relationship between the distribution of genetic variation in the Central Valley and ecogeographic factors. For the conservation objective, it is essential to identify those populations that best represent ecological and genetic diversity. Data from various genetic markers (biochemical and molecular) could be tested against micro-scale environmental data to highlight the combinations of factors which best suit the choice of individuals or populations for inclusion in conservation programmes. Such data will also be relevant for the determination of the minimum sample size required for maintaining a given level of allelic diversity.
- **Better understanding of the dynamics of wild populations.** A 'carrying capacity' component should be added to the demographic model developed by the project, with a view to determining the effects of plant density on mortality and growth rates of Lima bean individuals. A study should be carried out to further evaluate the impact of the 'extinction–recolonization' process on the genetic structure of populations. As the soil seed bank plays a significant role in population survival, it would be interesting to compare the genetic diversity between populations established from the soil seed bank after a local extinction and populations established with previously collected seeds from the same original populations.

- **Assessment of gene flow.** As gene flow is a key element in determining the genetic structure of the wild populations, it is essential to follow up a study started in 2000 in three selected regions of the Central Valley. This study is using microsatellite markers to measure the impact of gene flow on a very large scale through the sampling of individuals located at large distances from a central population.
- **Improvement of computer simulation software.** Software has been developed to simulate the genetic behaviour of wild Lima bean populations. However, some issues which are important for *in situ* conservation have not yet been considered in this simulation program. Refinements to this software could be made, integrating, for example, the carrying capacity of natural sites and long-distance gene dispersal. The validity of the system could also be tested with other *Phaseolus* species with different breeding systems, demographic and phenological strategies, and floral biologies.
- **Wider application of the conservation model to the *Phaseolus* genepool.** Costa Rica is part of the Mesoamerican centre of diversity of the *Phaseolus* genus, harbouring wild and cultivated populations of several species, such as *P. lunatus*, *P. coccineus*, *P. polyanthus*, *P. vulgaris* and *P. costaricensis*. In some areas of the Central Valley, wild populations of *P. lunatus* are in fact growing together with wild populations of *P. vulgaris* and *P. costaricensis,* an interesting situation for *in situ* conservation. The different breeding systems found in these species will affect the conservation model used. Investigations should be undertaken to study the genetic structure, genetic population parameters, phenology, demography and gene flow of other *Phaseolus* species. Data obtained from such investigations could help to widen the application of the simulation model. Special attention should also be given to the interaction between wild and cultivated materials of *P. lunatus*. Such information could help in elaborating some elements of on-farm conservation programmes. Taking into account the whole area of distribution of *P. lunatus*, it would also be interesting to compare the dynamics of populations in the two major genepools of the species, i.e. the Mesoamerican and Andean genepools. Materials from the Andean genepool are probably distinct due to their higher natural outcrossing rates. Therefore, it would be valuable to assess dynamics of *in situ* populations of Andean Lima beans (e.g. in Peru) and to identify the modifications in the conservation strategy developed in the Central Valley of Costa Rica which will be necessary.
- **Training and extension.** Several UCR and FUSAGx students carried out research and benefited from training during the course of the project in various aspects of genetic resources management, including taxonomy, ecology, molecular population genetics, plant demography and reproductive biology. This process should continue. Workshops and publications could be used to transfer the tools, methodologies and results described here to a diverse audience of conservation managers in Latin America, including those from research institutes, protected areas, universities and non-governmental organizations (NGOs). This will strengthen *in situ* conservation of genetic resources of crop wild relatives in the region, and stimulate collaboration among the disparate actors who have a stake in this important field.

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