SPSD II

CONSERVATION AND RESTORATION OF FRAGMENTED BIODIVERSITY HOT SPOTS:CALCAREOUS GRASSLANDS OF SOUTH-BELGIUM (BIOCORE)

O. HONNAY, M. BAGUETTE, I. ROLDÁN-RUIZ

PART 2

GLOBAL CHANGE, ECOSYSTEMS AND BIODIVERSITY

ATMOSPHERE AND CLIMATE

MARINE ECOSYSTEMS AND BIODIVERSITY

TERRESTRIAL ECOSYSTEMS AND BIODIVERSITY

NORTH SEA

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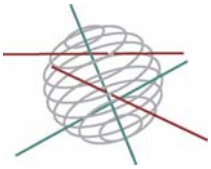
SCIENTIFIC SUPPORT PLAN FOR A SUSTAINABLE DEVELOPMENT POLICY
(SPSD II)



Part 2:
Global change, Ecosystems and Biodiversity

FINAL REPORT

CONSERVATION AND RESTORATION OF FRAGMENTED BIODIVERSITY HOT SPOTS:CALCAREOUS GRASSLANDS OF SOUTH-BELGIUM (BIOCORE)

EV/26

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September 20th 2006

Prof. dr. ir. Olivier Honnay

Project coordinator

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I. CONTEXT

One of the most species rich plant communities in the world at a small scale are calcareous grasslands (up to 60 plant species / m²) (Peet *et al*., 1983). Moreover, a very high arthropod diversity is associated with this extraordinary plant species richness (WallisDeVries *et al*., 2002). These grasslands were once widespread in the hilly calcareous regions of Western Europe but due to changes in agricultural use (i.e., abandonment of grazing by sheep and cattle and/or fertilization) their extent has decreased dramatically. In Belgium, the calcareous grasslands of the Viroin valley are the country's most species rich ecosystem and they can be described as so called biodiversity hot spots. As in the rest of Europe, since World War II, urbanization, abandonment of grazing practice and fertilization resulted in a dramatic decrease of the grassland area and an increase of the isolation of the remaining grassland fragments.

Habitat fragmentation can have severe effects on the persistence and viability of plant and insect populations. Small patches often contain small populations (Agren, 1996; Jacquemyn et al., 2002; Bruun, 2005; Krauss et al., 2005), which are at greater risk of decline or eventually extinction (Shaffer, 1981; Pimm et al., 1988; Ouborg, 1993; Menges and Dolan, 1998; Eisto et al., 2000) as they are much more sensitive to genetic, demographic and environmental stochasticity (Shaffer, 1981; Holsinger, 2000). Isolation can further affect population viability. A higher degree of isolation can hamper individuals from other populations reaching the patch. Such recolonization may prevent the population from going extinct, the so-called rescue-effect (Brown and Kodric-Brown, 1977). Additionally, this also impedes recolonization after extinction (Hanski, 1998).

The major aim of the project is to provide guidelines for the conservation and the remediation of the biodiversity of the system of fragmented calcareous grasslands of the Viroin Valley. We focus on the conservation and restoration of plant species richness and of butterfly species richness. Plant species are the basic components of each ecosystem as they provide food and shelter for heterotrophic organisms. Butterflies were selected because they are relatively easy to study and they respond quickly to environmental changes and to changes in plant composition. Hence they are very suitable as indicator species.

We study the effects of grassland fragmentation on the three primary descriptors of the community structure of plants and butterflies. This means that we focus on the effects of fragmentation on 1) the species number of the fragments, 2) their

community composition and 3) the performance of a selection of individual species. Most attention goes to the latter, i.e. the population level. The performance of individual species is quantified by their demographics, population genetics and phenotypic fitness. Special attention is paid to the integration of the plant and butterfly data. Secondly, using our insights in the ecological consequences of the habitat fragmentation process, we will provide clear guidelines to conserve the present biodiversity, especially with respect to minimal population sizes and the connectivity between habitat fragments. We will also provide a general methodological framework to deal with the remediation of habitat fragmentation.

The added value of this research project lays in the first place in the quite unique multidisciplinary approach of a representative fragmentation problem at the landscape scale. The close co-operation of molecular geneticists, plant ecologists and entomologists generates an important added value. There is growing a consensus among scientists that the conservation of biodiversity requires an integrated approach covering the requirements of a large number of different species. The limited exchange of information between scientists is constraining successfully integrated conservation management.

II. STUDY AREA: THE CALCAREOUS GRASSLANDS OF THE VIROIN VALLEY

This study focuses on the calcareous grasslands in the Viroin Valley in southern Belgium. The Viroin Valley is part of the Calestienne region, which is situated between the Fagne-Famenne region in the north and the Ardennes region in the south. Geologically, it is characterized by the occurrence of Devonian limestone hills. These hills often originate from former coral reefs. The formation is about 4 km wide and stretches from northeast to southwest (Fig. 1). Historically, these limestone hills were covered with calcareous grasslands, which were mostly grazed by sheep. Since 1850, many of these grasslands have entirely, or at least partially, been lost due to the abandonment of the traditional grazing regime, afforestation and urbanization (Decocq et al., 2004). The remaining grasslands occur as small, isolated patches in the landscape. A total of 71 calcareous grassland fragments can be distinguished along the whole of the Viroin Valley, ranging in size from 0.02 to 8.46 ha. Although these grasslands are known for their extremely high biodiversity, many of them have been restored and managed from a nature conservation point of view only for a few years.

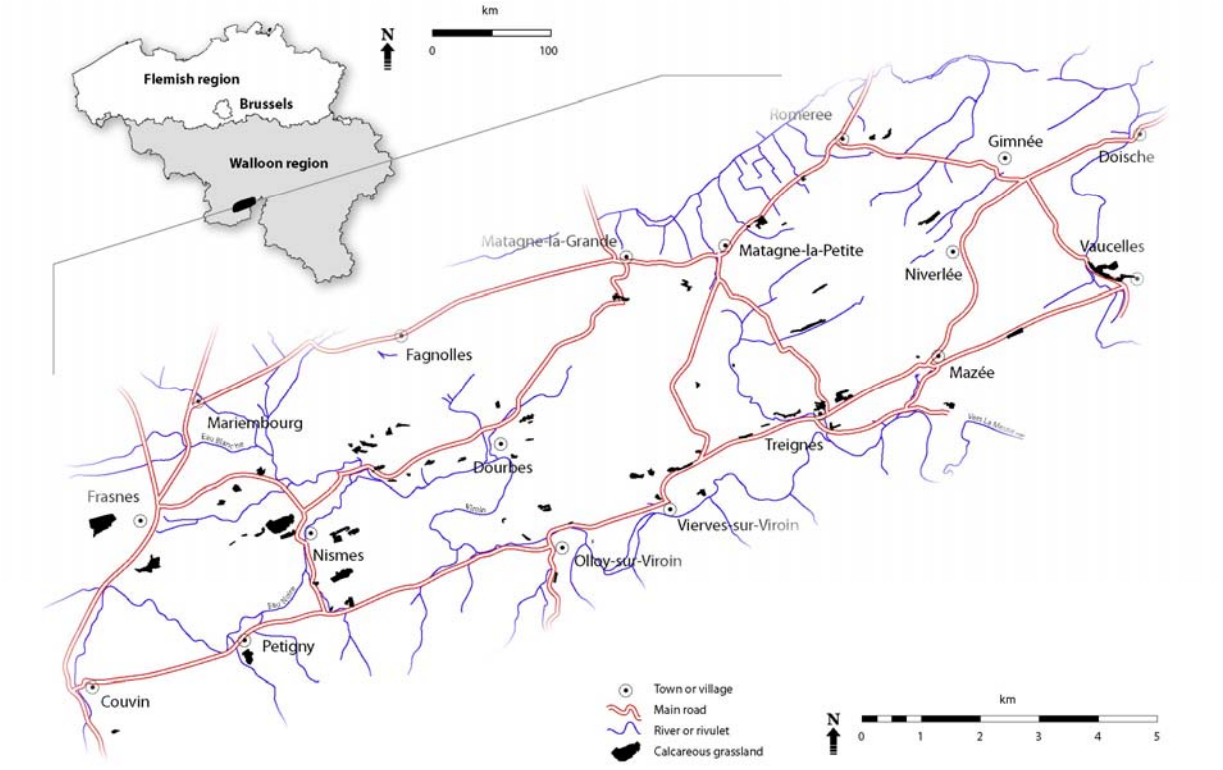

Fig. 1 *Location of the study area*

III. PLANTS

A. COMMUNITY LEVEL

1. PHYTOSOCIOLOGY AND PHYTOGEOGRAPHY OF THE CALCAREOUS GRASSLANDS OF THE VIROIN VALLEY

Despite their high biodiversity value, a thorough and detailed phytosociological description of calcareous grasslands in Belgium, or more specifically of the Viroin Valley, was still lacking. Therefore, an extensive vegetation survey (401 vegetation relevés) was performed in 54 calcareous grasslands in the Viroin Valley during the summer of 2003. The cover (%) of all plant species was recorded in 1m² plots. A total of 237 higher plant species was found. Based on classification (TWINSPAN) and ordination techniques (DCA) seven vegetation types were distinguished (Table 1). Three of these can be classified as xerophilous, while the other four are characteristic of mesophilous conditions.

Table 1 *Frequency (%) table of the derived vegetation types based on the TWINSPAN classification*

Vegetation type	I	II	III	IV	V	VI	VII
Number of relevés	7	28	51	167	94	44	10
Xerobromion							
Arabis hirsuta	29	46	29	1	-	2	-
Buxus sempervirens	57	25	2	2	-	-	-
Carex humilis	14	46	-	5	-	-	-
Melica ciliata	57	82	33	1	-	-	-
Lactuca perennis	-	4	-	-	-	-	-
Allium sphaerocephalon	-	71	16	2	-	-	-
Veronica prostrata	-	50	10	2	-	-	-
Sesleria caerulea	-	36	51	31	3	16	-
Orobanche teucrii	-	4	2	1	-	-	10
Anemone pulsatilla	-	7	-	1	-	-	-
Linum leonii	-	-	-	1	-	-	-
Linum tenuifolium	-	-	-	1	-	-	-
Fumana procumbens	-	-	4	2	-	-	-
Sedo-Scleranthetea							
Arenaria serpyllifolia	-	14	14	2	-	-	-
Satureja acinos	14	61	24	2	1	-	-
Sedum acre	-	32	16	4	-	-	-
Sedum album	14	75	63	7	-	-	-
Sedum rupestre	-	11	24	9	-	2	-
Taraxacum section erythrocephala	-	18	10	8	-	-	-
Erophila verna	-	-	4	-	-	-	-
Teucrium botrys	-	-	8	1	-	-	-
Cerastium brachypetalum	-	-	-	2	-	-	-
Cerastium pumilum	-	4	2	1	-	-	-
Cerastium semidecandrum	-	-	-	1	3	-	-
Poa compressa	-	4	8	3	1	-	-
Echium vulgare	-	-	14	2	-	-	-
Thlaspi perfoliatum	-	11	2	1	-	2	-
Veronica arvensis	-	7	8	-	-	-	-
Saxifraga tridactylites	-	-	-	1	-	-	-
Festuco-Brometea							
Helianthemum nummularium	29	89	65	72	46	25	-
Hippocrepis comosa	43	43	20	11	41	11	-
Polygala vulgaris	29	32	-	5	30	9	10
Thymus pulegioides	29	4	18	17	46	-	-
Inula conyzae	43	39	10	5	2	16	-
Carex caryophyllea	14	-	22	59	35	11	10
Carex flacca	43	-	14	55	96	86	10
Festuca ovina	-	32	45	88	90	18	40
Sanguisorba minor	-	7	39	92	93	36	10
Potentilla neumanniana	-	75	75	89	65	20	10
Scabiosa columbaria	-	11	8	41	45	2	-
Thymus praecox	-	11	20	60	19	7	-
Teucrium chamaedrys	-	86	88	85	17	25	-
Koeleria macrantha	-	7	18	43	35	7	-
Brachypodium pinnatum	-	-	51	96	93	98	50
Allium oleraceum	-	-	10	3	-	5	10
Polygala comosa	-	-	2	9	6	-	-
Eryngium campestre	-	-	-	1	-	-	-
Phleum bertolonii	-	-	-	1	2	-	-
Euphorbia cyparissias	-	-	2	1	-	2	-
Platanthera bifolia	-	-	-	-	1	-	-
Mesobromion							
Galium pumilum	-	7	4	53	54	18	-
Pimpinella saxifraga	-	-	4	34	56	43	30
Galium verum	-	-	4	16	18	18	20
Genista sagittalis	-	-	-	18	6	2	10
Centaurea scabiosa	-	-	2	10	22	9	-
Cirsium acaule	-	-	-	20	55	9	-
Medicago lupulina	-	-	8	10	30	25	-
Carlina vulgaris	-	-	2	12	17	2	-
Platanthera chlorantha	-	-	-	6	3	5	-
Primula veris	-	-	2	4	6	2	-
Trifolium campestre	-	-	-	1	6	2	-
Aceras anthropophorum	-	4	-	1	-	-	-
Carex montana	-	-	4	-	1	2	-
Ophrys fuciflora	-	-	-	3	1	-	-
Ophrys insectifera	-	-	-	1	1	-	-
Gymnadenia odoratissima	-	-	-	-	1	-	-
Himantoglossum hircinum	-	-	-	1	-	2	-
Koeleria pyramidata	-	-	-	-	1	-	-
Onobrychis viciifolia	-	-	-	1	1	-	-
Anthyllis vulneraria	-	-	2	19	6	-	-
Asperula cynanchica	-	4	2	11	1	-	-
Leontodon hispidus	29	-	2	14	69	5	-
Ononis repens	-	-	-	5	37	11	-

Vegetation type	I	II	III	IV	V	VI	VII
Number of relevés	7	28	51	167	94	44	10
Gymnadenia conopsea	-	-	2	4	21	9	-
Epipactis atrorubens	-	-	-	2	10	7	-
Genista tinctoria	-	-	-	4	20	11	-
Bromus erectus	-	-	-	1	16	2	-
Prunella laciniata	-	4	-	3	22	-	-
Ranunculus bulbosus	-	4	-	2	16	-	-
Gentianella germanica	-	-	-	2	16	-	-
Plantago media	-	-	-	7	19	-	-
Trifolio-Geranietea							
Viola hirta	71	4	12	66	74	77	-
Fragaria viridis	14	54	12	19	18	9	-
Origanum vulgare		4	16	8	7	23	-
Vincetoxicum hirundinaria	-	11	29	7	1	9	-
Anthericum liliago	-	4	4	1	-	9	10
Clematis vitalba	29		4	2	3	7	-
Geranium columbinum	14	14	6	1	-	-	-
Thlaspi montanum	-	11	-	-	-	-	-
Aster linosyris	-	7	10	-	-	-	-
Cotoneaster integerrimus	-	7	8	-	-	-	-
Geranium sanguineum	-	54	4	1	-	-	-
Polygonatum odoratum	-	46	12	1	-	2	-
Silene nutans	-	7	16	1	-	2	-
Seseli libanotis	-	11	12	2	2	-	-
Verbascum lychnitis	-	4	12	1	2	2	-
Poa angustifolia	-	7	4	4	9	41	-
Bupleurum falcatum	-	4	8	26	11	5	-
Agrimonia eupatoria	-	-	-	1	10	14	-
Calamintha clinopodium	-	-	-	1	1	5	-
Picris hieracioides	-	-	-	2	4	-	-
Orchis mascula	-	-	-	2	-	-	-
Lithospermum officinale	-	-	-	-	-	2	-
Orchis purpurea	-	-	-	-	1	2	-
Helleborus foetidus	-	-	-	2	-	9	-
Lathyrus sylvestris	-	-	-	-	-	5	-
Astragalus glycyphyllos	-	-	-	-	-	7	-
Malva alcea	-	-	-	-	1	-	-
Malva moschata	-	-	-	-	-	2	-
Molinio-Arrhenatheretea							
Hypericum perforatum	14	46	47	48	20	20	40
Hieracium pilosella	29	-	27	65	70	2	30
Agrostis capillaris	14	11	-	3	5	5	100
Taraxacum officinale	71	18	4	18	22	18	-
Plantago lanceolata	14	-	4	19	46	-	20
Senecio jacobaea	-	7	2	5	15	9	10
Leucanthemum vulgare	-	7	2	4	22	5	-
Linum catharticum	-	4	6	44	77	27	-
Briza media	-	4	2	39	54	5	-
Avenula pubescens	-	4	4	34	18	9	-
Vicia hirsuta	-	7	4	2	14	18	-
Knautia arvensis	-	-	-	11	44	16	-
Centaurea jacea	-	-	8	34	39	7	-
Lotus corniculatus	-	-	6	73	95	39	10
Dactylis glomerata	-	-	4	3	2	2	10
Achillea millefolium	-	-	-	3	20	23	-
Arrhenatherum elatius	-	-	-	4	2	11	-
Colchicum autumnale	-	-	-	1	3	18	-
Succisa pratensis	-	-	-	-	6	2	-
Carex tomentosa	-	-	-	-	5	-	-
Stachys officinalis	-	-	-	5	2	7	20
Anthoxanthum odoratum	-	-	-	1	-	-	-
Cerastium fontanum	-	-	-	-	1	2	-
Rhinanthus alectorolophus	-	-	-	-	1	-	-
Cirsium vulgare	-	-	-	1	-	-	-
Daucus carota	-	-	-	1	5	7	-
Geranium molle	-	-	2	-	-	-	-
Heracleum sphondylium	-	-	-	-	-	2	-
Hieracium maculatum	-	-	-	1	-	-	-
Holcus lanatus	-	-	-	1	-	-	-
Lathyrus pratensis	-	-	-	-	-	7	-
Ornithogalum umbellatum	-	-	-	-	-	2	-
Potentilla reptans	-	-	-	-	3	5	-
Tragopogon pratensis	-	-	-	1	2	7	-
Trifolium medium	-	-	-	1	5	2	-
Vicia sativa	-	-	-	1	-	7	-
Trifolium repens	-	-	-	1	-	-	-
Trisetum flavescens	-	-	-	1	7	2	-
Vicia cracca	-	-	-	-	9	9	-
Prunella vulgaris	14	-	-	2	14	-	-

Vegetation type	I	II	III	IV	V	VI	VII
Number of relevés	7	28	51	167	94	44	10
Molinio-Arrhenatheretea							
Trifolium pratense	-	-	-	-	1	2	10
Crepis biennis	-	-	-	-	-	-	10
Rumex acetosa	-	-	-	-	-	-	20
Rumex acetosella	-	-	-	1	-	-	40
Ornithopus perpusillus	-	-	-	-	-	-	10
Leontodon autumnalis	-	-	-	-	-	-	30
Nardo-Callunetea							
Calluna vulgaris	-	-	-	-	-	2	10
Cerastium arvense	-	4	-	1	-	-	-
Cytisus scoparius	-	-	-	1	-	5	70
Holcus mollis	-	-	-	-	-	-	30
Potentilla erecta	-	-	-	1	1	-	10
Sieglingia decumbens	-	-	-	1	2	-	-
Coeloglossum viride	-	-	-	1	-	-	-
Genista anglica	-	-	2	-	-	-	-
Cuscuta epithymum	-	-	-	1	1	-	-
Querco-Fagetea							
Crataegus monogyna	14	-	10	30	46	30	10
Prunus spinosa	-	7	14	30	18	64	40
Quercus robur	-	7	2	14	53	20	-
Rosa canina	43	7	14	10	17	25	-
Rubus sp.	43	-	4	2	5	20	-
Carpinus betulus	29	-	-	1	7	5	-
Cornus sanguinea	14	-	-	2	10	16	-
Rhamnus cathartica	-	7	4	1	-	-	-
Fraxinus excelsior	-	-	-	2	-	2	-
Viburnum lantana	-	-	-	1	-	5	-
Corylus avellana	-	-	-	1	3	7	-
Quercus petraea	-	-	-	2	3	-	-
Sorbus torminalis	-	-	-	-	1	-	-
Fagus sylvatica	-	-	-	-	3	-	-
Acer campestre	-	-	-	-	1	7	-
Acer pseudoplatanus	-	-	-	-	2	2	-
Euonymus europaeus	-	-	-	-	-	5	-

Vegetation type	I	II	III	IV	V	VI	VII
Number of relevés	7	28	51	167	94	44	10
Other species							
Fragaria vesca	86	4	-	1	4	18	-
Atropa bella-donna	57	-	-	-	-	-	-
Cirsium arvense	57	-	-	-	1	-	-
Veronica officinalis	57	-	-	1	1	2	-
Myosotis arvensis	29	-	4	-	1	11	-
Stachys alpina	14	-	-	-	-	-	-
Stachys sylvatica	14	-	-	-	-	-	-
Solanum dulcamara	14	4	-	-	-	-	-
Galium aparine	14	-	-	-	-	-	-
Hypochoeris radicata	14	-	-	4	1	-	-
Galium mollugo	-	14	16	2	-	14	-
Ligustrum vulgare	-	7	4	1	2	9	-
Teucrium scorodonia	-	-	2	1	-	5	40
Campanula rotundifolia	-	-	4	40	47	7	20
Asplenium ruta-muraria	-	-	16	1	1	2	-
Reseda luteola	-	-	2	-	-	-	-
Asplenium adiantum-nigrum	-	-	2	-	-	-	-
Bromus sterilis	-	-	2	-	-	-	-
Senecio erucifolius	-	-	2	-	-	-	-
Carex muricata	-	-	2	1	-	-	-
Polygonum convolvulus	-	-	2	1	-	-	-
Rosa micrantha	-	-	2	1	-	-	-
Asplenium trichomanes	-	-	2	1	-	-	-
Lepidium campestre	-	-	-	1	-	-	-
Trifolium arvense	-	-	-	1	-	-	-
Viola canina	-	-	-	1	-	-	-
Convolvulus arvensis	-	-	-	1	-	-	-
Plantago major	-	-	-	1	-	-	-
Rosa pimpinellifolia	-	7	-	1	-	-	-
Cotoneaster horizontalis	-	-	-	1	-	-	-
Allium vineale	-	-	-	2	-	-	-
Rubus idaeus	-	-	-	1	-	-	-
Luzula multiflora	-	-	-	1	-	-	-
Listera ovata	-	-	-	-	6	-	-
Euphorbia stricta	-	-	-	-	2	-	-
Centaurium erythraea	-	-	-	-	3	-	-
Euphrasia stricta	-	-	-	-	5	-	-
Hieracium laevigatum	-	-	-	-	1	-	-
Populus tremula	-	-	-	-	5	-	-
Carex panicea	-	-	-	-	3	2	-
Solidago virgaurea	-	-	-	1	2	5	-
Agrostis stolonifera	-	-	-	2	5	-	-
Sonchus asper	-	4	-	-	2	7	-
Carduus crispus	-	-	-	-	1	2	-
Digitalis lutea	-	-	-	2	-	2	-
Mentha arvensis	-	-	-	-	-	2	-
Sedum telephium	-	-	-	-	-	2	-

I = *Fragaria vesca-Atropa bella-donna* community; II = *Carex humilis-Geranium sanguineum* community; III = *Sesleria caerulea* community; IV = *Teucrium chamaedrys-Thymus praecox* community; V = *Brachypodium pinnatum* dominated community; VI = *Leontodon hispidus-Cirsium acaule* community; VII = *Agrostis capillaris-Cytisus scoparius* community.

The xerophilous communities have a characteristically open turf which is often interrupted by fractured rock outcrops and small patches of bare soil and which is often disposed in a more fragmentary fashion over narrow ledges and in crevices. Soil depth seldom adds up to more than 5 cm. Most of these grasslands are situated on relatively steep slopes with a southerly aspect. The mesophilous grasslands typically comprise rich mixtures of grasses and herbaceous dicotyledons in a continuous, closed sward. These vegetations form a plagioclimax which is largely dependent on grassland management practices and especially grazing. When they are abandoned, their stability is disrupted and *Brachypodium pinnatum* starts to dominate, resulting in a higher vegetation and large amounts of litter.

Three xerophilous communities can be distinguished:

I. *Fragaria vesca-Atropa bella-donna* community
This community is typical of recently deforested sites. Most of these sites were ancient calcareous grasslands which were covered with *Buxus sempervirens*. The community is characterized by a combination of relict species from the former forest habitat, such as *Clematis vitalba* and *Fragaria vesca*, and many early-successional species such as *Cirsium arvense*, *Rubus fruticosus*, *Taraxacum officinale*, *Atropa bella-donna* and *Myosotis arvensis*. The occurrence of these species can be explained by nearby propagule sources emerging from either a disturbed long-living seed bank or from gaps in the former *Buxus sempervirens* canopy. Furthermore also some characteristic calcareous grassland species can already be found here, like for example *Polygala vulgaris*, *Thymus pulegioides*, *Helianthemum nummularium* or *Hippocrepis comosa*.

II. *Carex humilis-Geranium sangineum* community
This is the most characteristic xerophilous grassland community. It is present on rocky, relatively steep sites with high direct insolation. Many of the differential species are almost exclusively restricted to this community, like for example *Globularia punctata*, *Veronica prostrata*, *Allium sphaerocephalon* and *Geranium sangineum*. Also some of the rare species in the region, such as *Anemone pulsatilla*, *Orobanche teucrii* and *Thlaspi montanum* are present in this community.

III. *Sesleria caerulea* community
The species composition of this community forms the transition towards more mesophilous communities, but xerophilous characteristics clearly dominate, which is expressed by the presence of *Sedum*-species such as *Sedum album*, *Sedum rupestre* and *Sedum acre*. However, on the deeper soils around the uncovered rock surfaces and patches with stones very near or above the surface species such as *Sesleria caerulea*, *Brachypodium pinnatum*, *Koeleria macrantha* and *Sanguisorba minor*, which are species typical of less extreme conditions, are present. Locally, on very shallow soils, *Globularia bisnagarica*, *Aster linosyris* and *Silene nutans* reach their optimum. On soils which suffer from compactation *Echium vulgare* can be a prominent species.

Within the mesophilous grasslands, four communities can be discerned:

IV. *Teucrium chamaedrys-Thymus praecox* community
This is the most xerophilous community within the mesophilous grasslands, which is indicated by the high frequency of occurrence of *Teucrium chamaedrys*, *Helianthemum nummularium* and *Potentilla neumanniana*. Other less frequently found typical xerophilous species are *Sesleria caerulea* and

Globularia bisnagarica. Species like *Genista sagittalis*, *Asperula cynanchica* and *Anthyllis vulneraria* have their highest frequency of occurrence in this community.

V. *Leontodon hispidus-Cirsium acaule* community
This is the most species rich community within the calcareous grasslands of the Calestienne region. It is an intimate mixture of grasses and dicotyledonous species. Dominant grass species are *Brachypodium pinnatum*, *Koeleria macrantha*, *Briza media*, *Avenula pubescens* and *Festuca ovina*. Dicotyledonous species that constantly occur throughout the community are *Lotus corniculatus*, *Linum catharticum* and *Leontodon hispidus*. *Teucrium chamaedrys*, a species characteristic for more xerophilous calcareous grasslands, is very rare.

VI. *Brachypodium pinnatum* dominated community
When grazing pressure is lowered, or even completely absent, mesophilous grasslands evolve from species rich to species poor communities dominated by *Brachypodium pinnatum*, and, to a lesser degree, *Carex flacca*. Species that are relatively resistant against this abandonment of grazing are *Sanguisorba minor*, *Viola hirta* and *Lotus corniculatus*. *Colchicum autumnale* and *Origanum vulgare* even reach their highest frequency in this community. The local encroachment of *Rosa canina*, *Crataegus monogyna* and *Prunus spinosa* is indicative for the start of succession towards forest.

VII. *Agrostis capillaris-Cytisus scoparius* community
When soil conditions become more acid, a vegetation with *Agrostis capillaris*, *Cytisus scoparius*, *Holcus mollis*, *Leontodon autumnalis* and even *Calluna vulgaris* develops. Strictly speaking, these grasslands do not form part of the calcareous grasslands, although they are present in the Calestienne region. The soils of sites where this community is present are characterized by a mixture of calcareous rock and acid schist or are locally decalcified by the relatively acid precipitation. This community is closely related to the *Nardo-Callunetea*.

In a European context, the mesophilous calcareous grasslands in the study area belong to the *Mesobromion* and show an intermediate position between the central European and the Atlantic calcareous grassland communities. The xerophilous calcareous grassland communities on the other hand show strong affinities with the calcareous vegetation of central and southern Europe.

2. NO EVIDENCE OF A PLANT EXTINCTION DEBT IN HIGHLY FRAGMENTED CALCAREOUS GRASSLANDS IN BELGIUM

The calcareous grasslands in the study region at present are highly fragmented. To quantify the current degree of fragmentation and to reconstruct the fragmentation process, several detailed historical maps (dating from 1775, 1850, 1875, 1905, 1968 and 1984) were digitized in a geographical information system (GIS). The present-day situation was obtained after digitizing recent topographic maps and aerial photographs, complemented with terrain surveys. Overlay of the current fragments allowed for calculation of the corresponding historical area and connectivity.

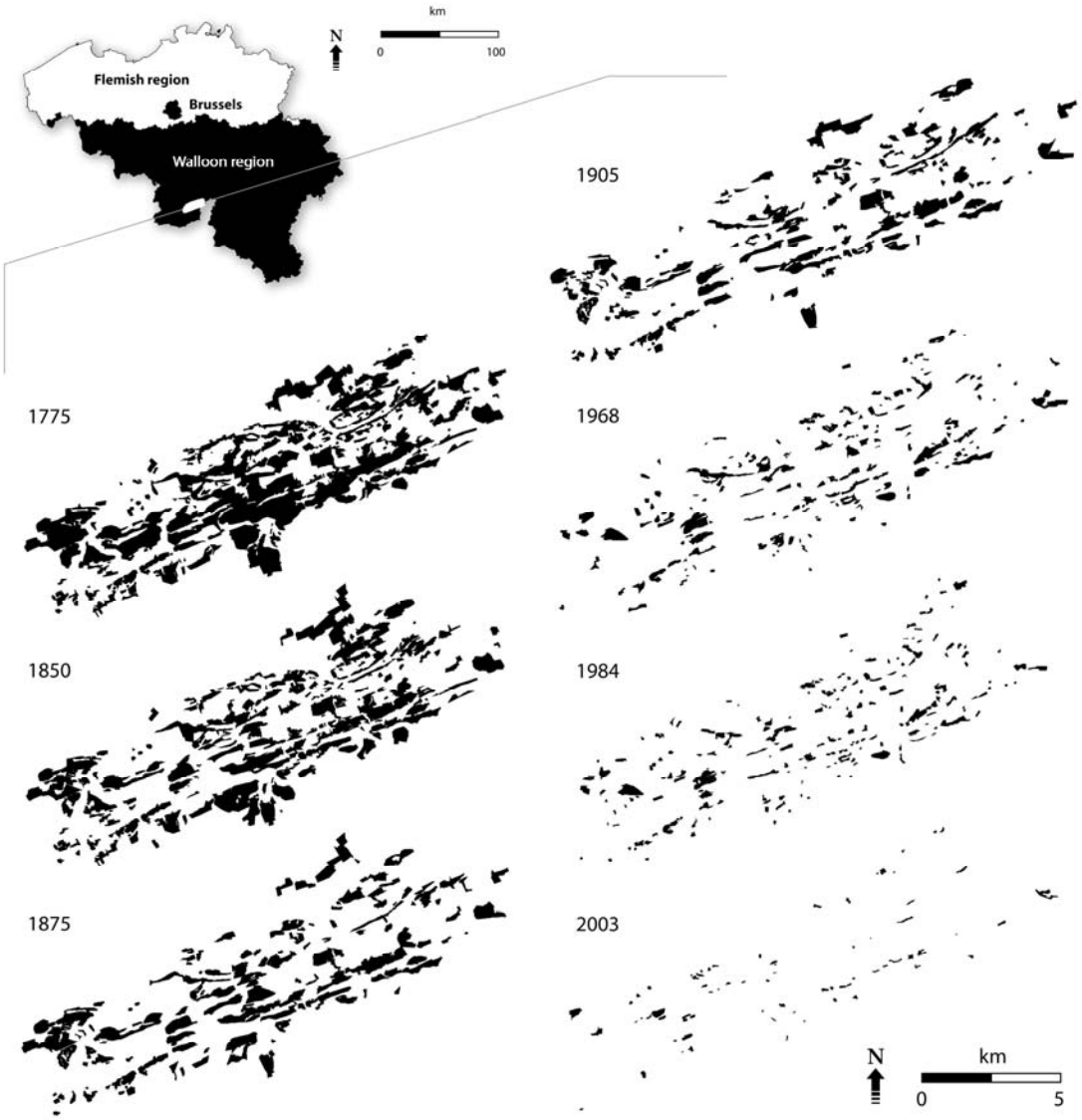

***Fig. 2** Historical development of the extent of calcareous grassland fragments in the study area.*

The development of the extent of calcareous grassland area during the last two centuries clearly demonstrates the process of habitat fragmentation (Fig. 2). With a steadily steep decrease in total area from more than 4000 ha in 1775 to the current area of 73.1 ha, the pure loss of habitat is evident (98%) (Fig. 3). Average fragment area fell down from 28.2 ha (range: 0.1-1154.1 ha) in 1775 to 1.0 ha (range: 0.02-8.5 ha) nowadays. Average interfragment distance to the five nearest fragments increased from 260 m in 1775 to a current distance of 547 m. Hence, average fragment connectivity decreased from 471.79 in 1775 to 3.23 in 2003. The number of fragments increased from 146 in 1775 to 201 in 1968, and finally collapsed to the current number of 71.

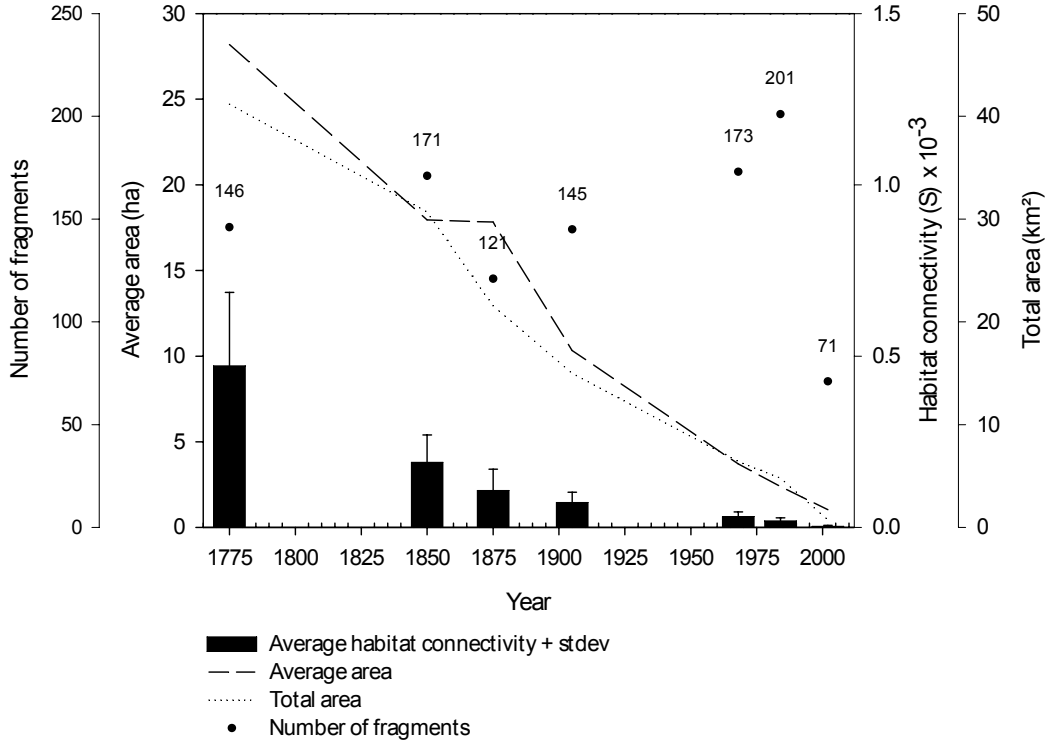

Fig. 3 *Development of several landscape configuration related descriptors of calcareous grassland fragments in the study area during the last centuries.*

In 64 of the 71 remaining calcareous grassland fragments, presence of all higher plant species was surveyed. Species were added to the pre-existing species list acquired from the vegetation relevés obtained for the phytosociological study. Specialist species were determined based on this phytosociological classification (Butaye *et al.*, 2005; part III A.1.)). Altogether, 340 herbaceous plant species were identified, with an average of 70 and standard deviation of 20 species per fragment and a minimum and maximum of 24 and 119 species, respectively. Specialist species accounted for 36% (n=122) and generalist species for 64% (n=218) of total

species richness (n=340). A minimum of 14% and a maximum of 57% specialist species was found in a single fragment. On average, 33% of the specialist and 13% of generalist species were present in a single fragment.

In addition to species composition, also a number of biotic and abiotic variables describing local environmental conditions were measured. A principal component analysis (PCA) was then performed on these variables to reduce the number of variables to be used in further analyses. This resulted in two axes, of which the first can be described as a main productivity gradient, while the second is highly associated with local inclination and hence the amount of insolation.

Next, effects of fragmentation (both present and historical landscape configuration) and local conditions on the number of species present in the remaining calcareous grassland patches were investigated. As not all species respond similarly to fragmentation, species were first grouped into functional groups. A functional group can be defined as a set of species that have similar morphological, physiological and phenological life history trait combinations (Lavorel et al., 1997). Sixteen traits were selected for emergent group delineation. Traits were chosen to represent the key processes shaping plant communities: dispersal, establishment and persistence (Weiher et al., 1999). Only specialist species were included, since incorporating generalist species would obscure fragmentation effects. Clustering of the data resulted in the delineation of four emergent groups: (1) Orchids, characterized as summergreen, short flowering, iteroparous perennial half-rosette plants, that produce ample low mass seeds that are wind dispersed, (2) Perennial rosette plants that are not vegetatively reproducing and have no long distance dispersal mode associated, (3) Early and long (repeatedly) flowering small autogamous annuals, exclusively reproducing by seeds that are highly spherical, low mass and lacking long distance dispersal adaptations, (4) Both summer- and wintergreen perennial half-rosette plants with fruity dispersules as well as seeds, with optional vegetative reproduction mechanisms.

Neither historical area, nor historical connectivity were significantly related to the number of species in any group. Pearson's correlation coefficients revealed that the species richness of the species groups was related to PCA2, present fragment area and present connectivity. Therefore, only PCA2 and present connectivity and fragment area were included in a GLM-analysis. There were no significant correlations between the independent variables.

Species number increased with increasing habitat area for both specialist ($r=0.614$; $p<0.001$) and generalist species ($r=0.371$; $p=0.018$), and hence, also for all species

combined (r=0.633; p<0.001). The z-values (slope of log-log regression) for specialist and generalist species were 0.135 and 0.201, respectively, and did not differ significantly (comparison of regression lines, F=1.52, p=0.220). Significant relationships with present connectivity were only observed for habitat specialist species (r=0.372, p=0.003, Fig. 4) and EG 1 (r=0.302, p=0.019), EG 2 (r=0.420, p=0.001) and EG 4 (r=0.336, p=0.009).

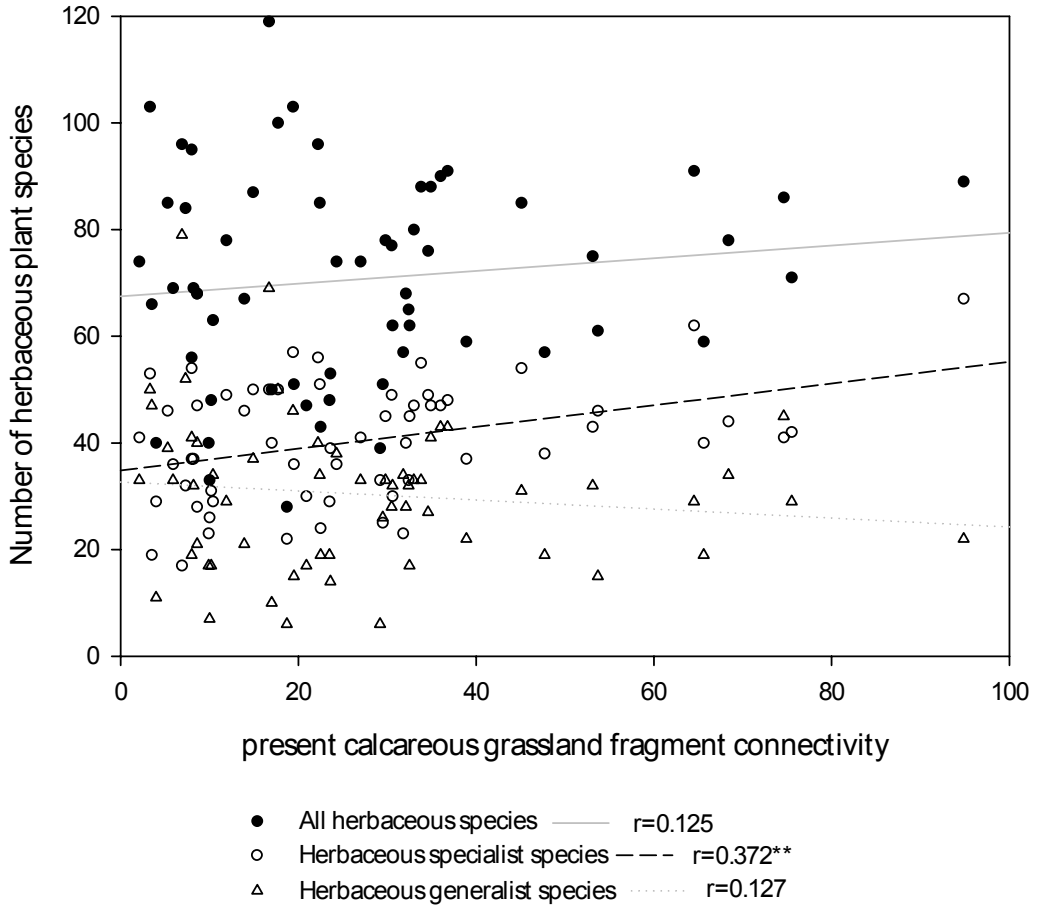

***Fig. 4**. Relationship between total, generalist and specialist herbaceous species number and present connectivity of calacareous grassland fragments (n=63).*

The general linear models demonstrated a significant relationship with the second environmental ordination axis for both total and generalist species richness, overruling the area factor (Table 2). Specialist species responded significantly to present habitat area, even when controlled for major environmental conditions. This relationship with current habitat area persisted for most of the emergent specialist species groups. Current habitat connectivity and the scores for the second environmental ordination axis tended to be only marginally related to total specialist species number. For EG 2 present connectivity was more important than habitat area in explaining species number. Environmental gradients did not influence any of the

emergent groups, except for group 4. Emergent group 3 was responding neither to area and connectivity, nor to environmental conditions.

Table 2 *F values of GLMs for species number against present habitat area and connectivity (S), and the second principal component (PC2) of environmental variable ordination (n=63).*

	Species number						
	All	Generalist	Specialist				
			All	EG 1	EG 2	EG 3	EG 4
Model	7.80 ***	4.83 **	9.27 ***	5.58 **	6.30 ***	1.95	7.79 ***
Intercept	349.54 ***	137.76 ***	289.89 ***	23.30 ***	132.49 ***	31.35 ***	325.17 ***
$Area_{2003}$	2.33	0.00	7.58 **	4.45 *	2.88 °	5.51 *	4.99 *
S_{2003}	0.11	2.79	2.98 °	1.57	6.92 *	0.80	2.03
PC2	13.42 ***	11.95 **	3.03 °	2.16	0.49	0.06	4.47 *

***P ≤ 0.001; **0.001 < P ≤ 0.01; *0.01 < P ≤ 0.05; °0.05 < P ≤ 0.1

The differences in response between generalist and specialist species and between the different emergent specialist species groups highlight the importance of not merely relying on broad community measures (species richness), but investigating specific and ecologically relevant subgroups. Otherwise, effects seen at the emergent group level can be obscured at the whole community level.

Our results gave no evidence of an extinction debt. Neither total species number, nor generalist, specialist or emergent group species number was related to any of the historical landscape structure measures. Only present area and connectivity tend to explain the variation in species richness among the remaining habitat fragments. Therefore, species seem to have responded rather quickly to fragmentation that occurred throughout history in our study area. Hence species richness of the habitat remnants seems to be in quasi-equilibrium with current landscape configuration. This implies that the remnant populations may be rather short living and species may be quickly lost in response to new fragmentation events. To conserve species richness it is therefore vital that the current state of habitat fragmentation is at least maintained. Furthermore, the importance of local environmental conditions indicates that also safeguarding habitat quality through accurate management is necessary. The re-establishment of traditional grazing practices seems to be the best option.

3. SEED BANK COMPOSITION OF OPEN AND OVERGROWN CALCAREOUS GRASSLAND SOILS IN SOUTHERN BELGIUM

The former part of the study illustrated the serious decline in calcareous grassland area in the study region. This points at the need to prevent further fragmentation and to recreate these grasslands at sites where they have been abandoned and have

undergone succession to scrub or forest. Populations of target species can establish through seed dispersal from other grassland fragments or through germination of seeds present in the soil seed bank. Since spontaneous seed dispersal is unlikely to occur because of the large spatial isolation of the remaining patches and the difficulty to graze these small and isolated patches by cattle or sheep, which could act as dispersal vectors, recreation success will be mainly determined by the species composition of the seeds present in the soil. Therefore, the change in seed bank composition along a successional gradient with increasing time since grassland abandonment was studied.

The seed bank and vegetation of five existing calcareous grasslands were sampled to provide reference plant communities. These were compared with the seed bank composition of three sites which had recently been abandoned (scrub vegetation less than 15 years old) and seven forest sites abandoned between 40 and 225 years ago.

A total of 419 seedlings of 42 plant species germinated from the soil samples, corresponding to a seed density of 559 seeds/m² over all sites. The seed bank was largely composed of rather common, non-target species of nutrient poor grasslands (e.g. *Hypericum perforatum* and *Arenaria serpyllifolia*). In contrast, 102 species were recorded in the intact grassland, of which 31 were also found in the seed bank. Consequently, 11 species that germinated from the soil samples were not present in the vegetation. However, typical calcareous grassland species, abundantly present in the vegetation, were poorly represented as seeds in the soil.

Species composition of the seed bank under grassland that was abandoned 15 years ago was similar to the species composition in the soil of grassland. In contrast, similarity in species composition with the seed bank under grassland strongly decreased after 40 years of grassland abandonment. There was a significant decline in the number of seeds of species with age of abandonment for species associated with calcareous grassland (r_s=-0.82, $p<0.001$), nutrient rich grasslands (r_s=-0.60, $p=0.019$) and nutrient poor grasslands (r_s=-0.73, $p=0.002$). Total seed density and seed density for these three ecological groupings were also significantly different between grassland and forest sites (Fig. 5). Furthermore, both total seed density and number of species significantly decreased with increasing time since abandonment (r_s=0.79, $p<0.001$; r_s=-0.88, $p<0.001$ respectively) (Fig. 6). Seed density ranged from an average of 930 seeds/m² in grassland soils to over 760 seeds/m² in scrub vegetation to 214 seeds/m² in forest. This indicates that only few calcareous grassland species produce long term persistent seeds.

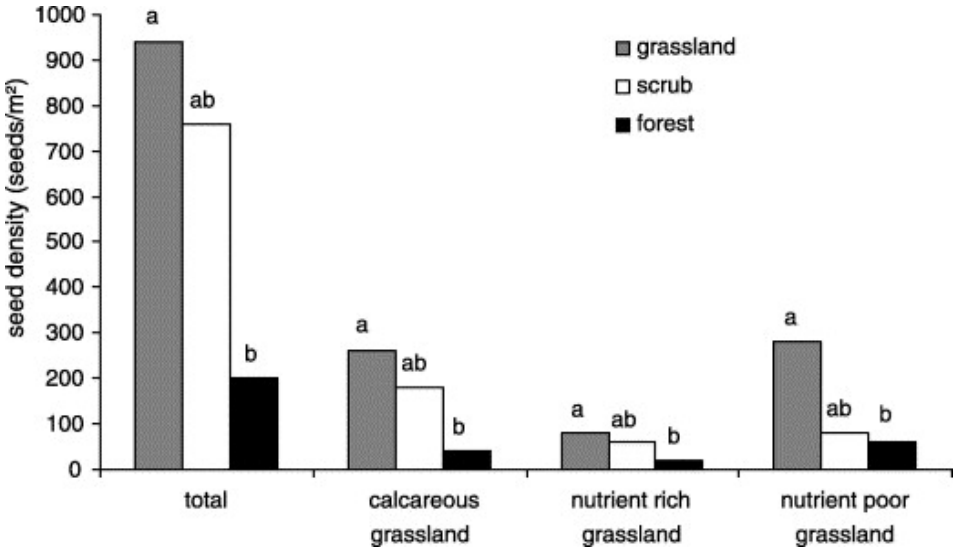

***Fig. 5**. Total seed density (seeds/m²) of species within ecological groupings (i.e. calcareous grassland, nutrient-rich grassland and nutrient-poor grassland on acid to neutral soils) in calcareous grassland, scrub and forest soil seed banks. Significant differences between vegetation types are indicated with different letters (Kruskal Wallis test).*

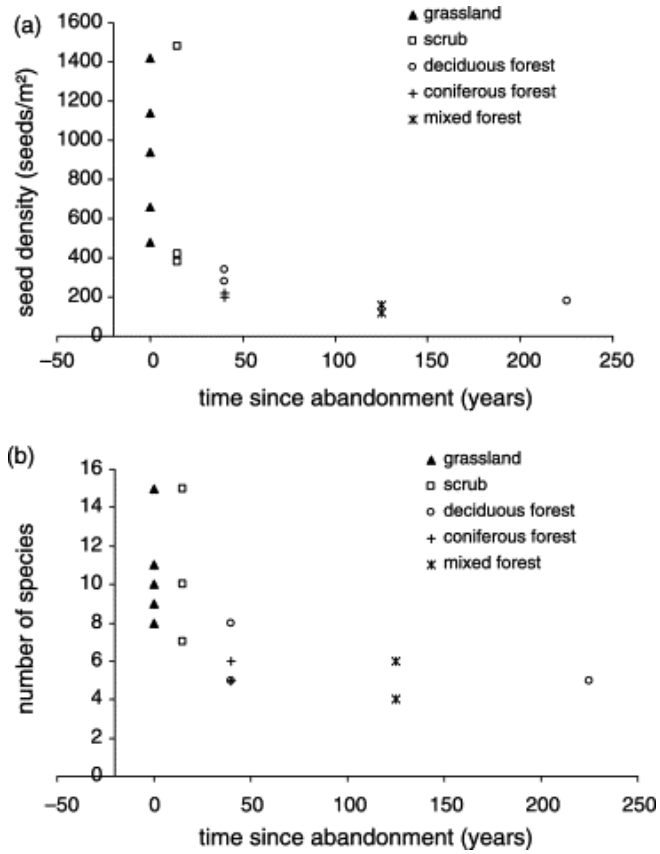

***Fig. 6** Seed density (a) and species richness (b) in the seed bank versus time since abandonment.*

The results indicate that the recreation of species rich calcareous grassland on sites abandoned more than 15 years cannot rely on germination from the seed bank alone. Taking into account the isolation of the sites, natural dispersal and colonisation from other grassland fragments is very unlikely unless traditional management practices, which might transport propagules between sites, are reinstated or restored (e.g. grazing and mowing). Therefore, when such grasslands are to be restored, other possibilities will need to be considered, including the artificial introduction of species as seed mixtures, plug-plants, green hay or turf transplants (Rosén and van der Maarel, 2000; Walker *et al.*, 2004) combined with the re-introduction of traditional practices, such as sheep grazing and mowing, which transport propagules between grassland fragments (Poschlod *et al.*, 1998).

B. POPULATION LEVEL

Three focal plant species have been selected to study at the species and population level, being *Anthyllis vulneraria*, *Anemone pulsatilla* and *Globularia bisnagarica*.

1 ANTHYLLIS VULNERARIA

1.1 STUDY SPECIES

The first study species is *Anthyllis vulneraria* (*Fabaceae*) (Fig. 7). It is a rosette-forming legume of 15-30cm height. In the study area, it is restricted to calcareous grasslands. It is the unique host plant of the butterfly *Cupido minimus* (Krauss *et al*., 2004a). *A. vulneraria* starts flowering in June and each plant develops between 1 and 22 flower stalks with 1 to 14 flower heads. In natural habitats, individuals are iteroparous perennials which remain vegetative during the first year and flower after one to five years (Sterk, 1982). Fruit and seed set are initiated a few weeks after flowering and the fruits generally contain one seed. Seeds are not persistent in the soil (Sterk, 1982). The most vital populations in terms of flowering are located in the grazed grassland fragments. *A. vulneraria* is a mainly selfing species. Although cross-fertilization can be obtained experimentally, under natural circumstances pollen are deposited on the style before opening of the flowers (Couderc, 1971).

Fig. 7 *Anthyllis vulneraria*

1.2 LOW IMPACT OF PRESENT AND HISTORICAL LANDSCAPE CONFIGURATION ON THE GENETICS OF FRAGMENTED *ANTHYLLIS VULNERARIA* POPULATIONS

To study effects of current and historical landscape configuration and management on the population genetic structure of *A. vulneraria*, 20 calcareous grassland patches where the species was present were randomly selected. These are all situated in the western part of the study area (Fig. 8). For these patches current and historical (1850, 1905, 1968 and 1984) area and isolation were calculated. In summer 2003 a leaf sample was taken of 20 randomly chosen flowering individuals per population. If less than 20 flowering individuals were present, all plants were sampled. Furthermore, the total number of individuals in each population was estimated. Leaves were lyophilized for 48h and homogenized with a mill (Retsch MM 200) to fine powder.

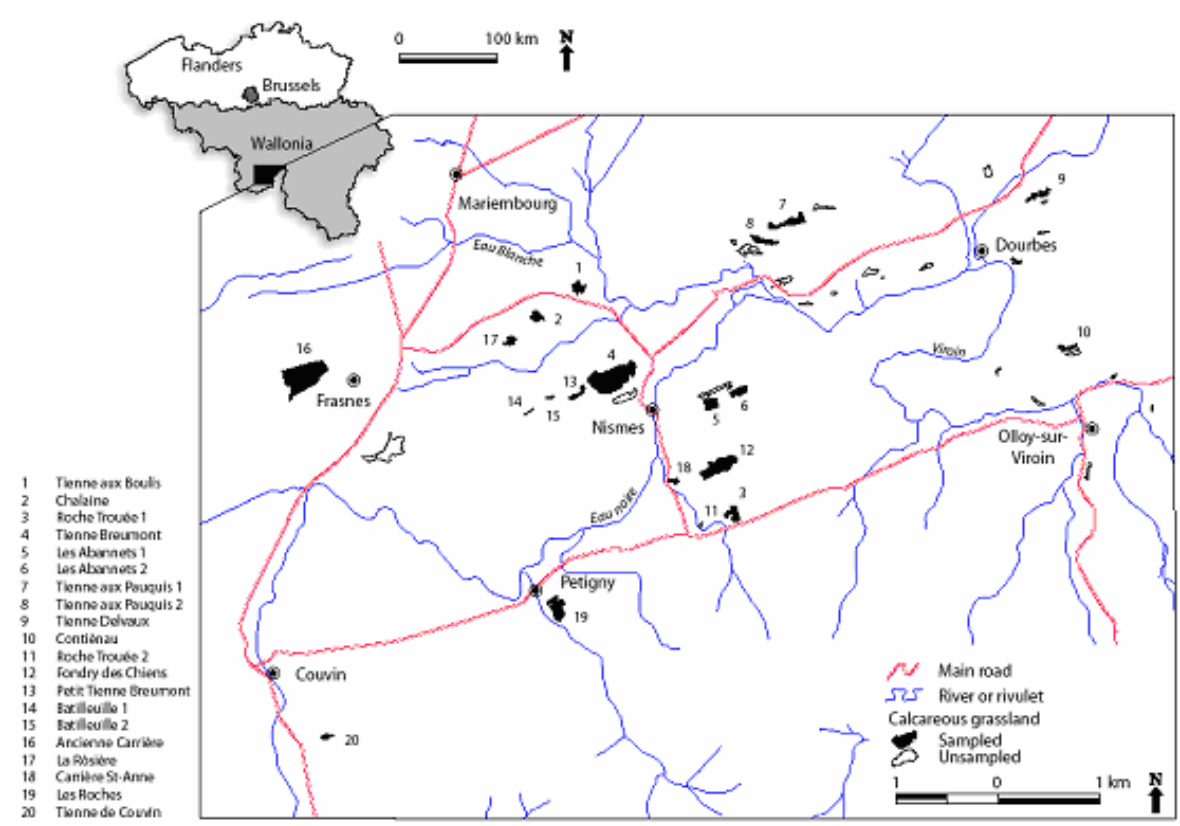

Fig. 8 *Fragments sampled in the A. vulneraria study.*

AFLP protocol

Two different CTAB-based DNA-extraction protocols were tested, following Lefort and Douglas (1999) and Dumolin *et al*. (1995), as was the DNeasy Plant Minikit (Qiagen). The protocol described by Lefort and Douglas (1999) was chosen for DNA-

extraction because it yielded DNA of good quality and it is less expensive than the Qiagen kit. DNA quality and concentration were estimated on 1.5% agarose gels. The AFLP (Amplified Fragment Length Polymorphism) protocol (Vos *et al*., 1995) was chosen to screen the genetic diversity of our samples. Time and cost efficiency, replicability and resolution of AFLP markers are superior or equal to those of other markers except that AFLP techniques generate dominant markers, making it difficult to assess gene frequencies without prior knowledge of the level of inbreeding (F_{is}) (Lynch and Milligan, 1994; Mueller and Wolfenbarger, 1999). Selective amplifications with a total of either 5 or 6 selective nucleotides were carried out on 5 selected samples (Table 5).

Table 5 *Summary of the primer combinations tested. The finally selected primer combinations are marked in bold (combinations number 10, 13 and 14).*

PC Nb.	***Eco*RI primer**	***Mse*I primer**
1	AAA	CC
2	AAA	CG
3	AAA	CGG
4	AAC	CGC
5	AAG	CAA
6	AAG	CT
7	AAT	CA
8	ACA	CG
9	ACG	CA
10	**ACG**	**CGA**
11	ACT	CC
12	ACT	CG
13	**ACT**	**CTA**
14	**AGC**	**CGT**
15	AGG	CAC
16	AGG	CT

In general, primer combinations containing 6 selective nucleotides resulted in AFLP-fingerprints of better quality than the primer combinations in which only 5 selective nucleotides were added. Three primer combinations were selected: *Eco*RI-ACT/*Mse*I-CTA, *Eco*RI-AGC/*Mse*I-CGT and *Eco*RI-ACG/*Mse*I-CGA (MWG biotech, Ebersberg, Germany). AFLP analysis was carried out according to the protocol described in Roldán-Ruiz *et al.* (2000). Fragments were separated on an ABI Prism 377 DNA sequencer on 36 cm denaturing gels using 4.25% polyacrylamide (4.25% acrylamide/bisacrylamide 19/1, 6M Urea in 1X TBE). GeneScan 500 Rox labelled size standard (Perkin Elmer) was loaded in each lane.

The fluorescent AFLP patterns were scored using Genotyper 2.0 (Perkin Elmer, 1996). Each marker was coded as 1 or 0 whether present or absent in an individual to form a binary data matrix. Each sample was thus represented by a vector of 1s and 0s.

The three AFLP primer combinations used resulted in 140 scorable markers. Reproducibility of the methodology was tested by repeating the complete protocol three times for five randomly chosen samples. As average, 94% of all markers were scored identically when repeated samples were compared. Only samples exhibiting a clear banding pattern for all three primer combination were used. This resulted in a data set containing information about 140 AFLP loci for 290 samples. However, for populations 14 and 15 (Fig. 8), only 3 and 4 samples remained respectively. These populations were omitted from further analysis, resulting in 283 samples from 18 populations. Genetic and other population characteristics are presented in Table 6.

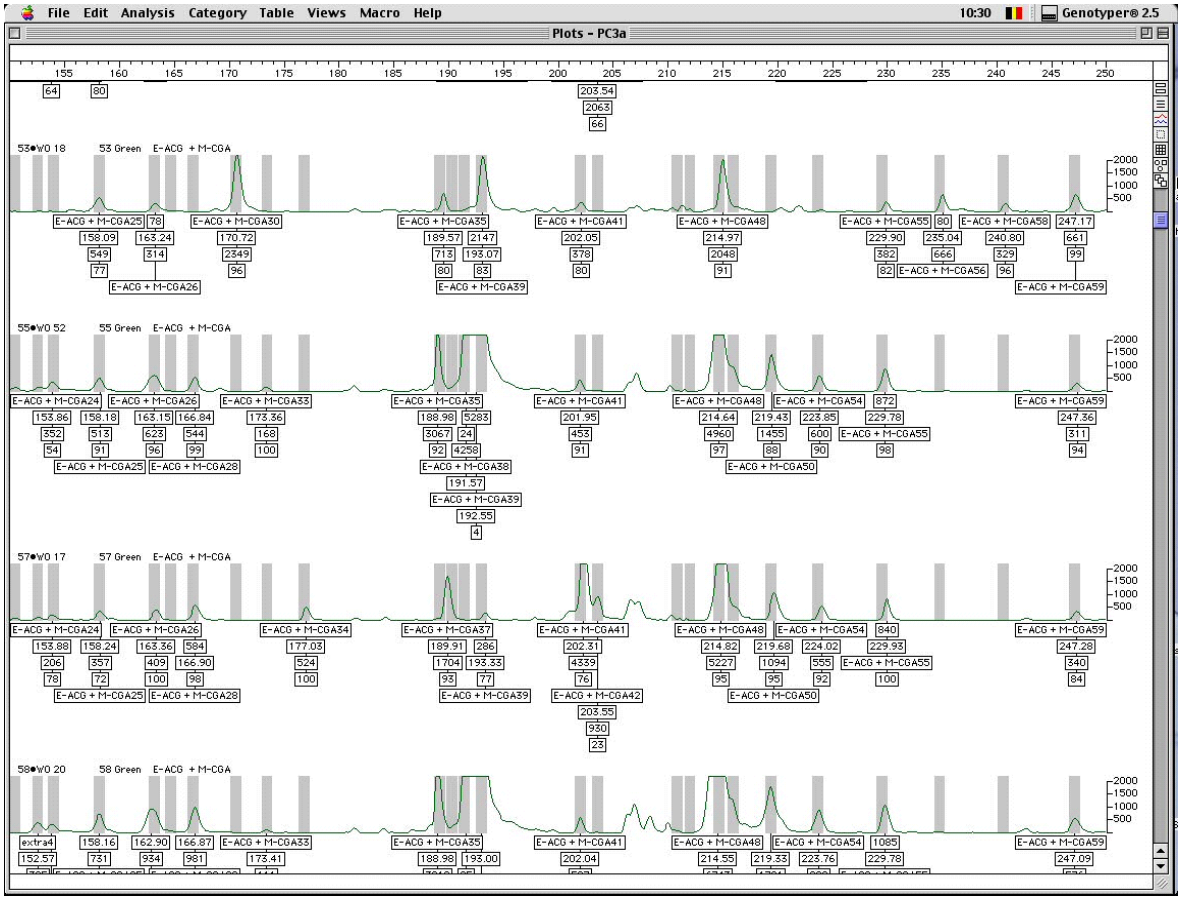

Fig. 9 *AFLP patterns of four Anthyllis vulneraria samples generated with primer combination E-ACG + M-CGA. Each row represents a sample with its unique peak pattern (in green). Each grey block represents a selected marker; if a peak in a block is labelled, this particular individual contains this particular marker.*

Table 6 *Genetic and population characteristics of the 18 sampled Anthyllis vulneraria populations.*

Fragment name	Manage-ment	Pop. $Size_{2003}$	Fragment $area_{2002}$ (ha)	S_{2002}	Sample size	H_j	Mol. Var.	Br
Tienne aux Boulis	G	22	0.88	7.05	16	0.249	14.371	1.560
Chalaine	G	46	0.70	7.71	19	0.276	16.634	1.628
Roche Trouée 1	G	130	0.94	6.60	19	0.284	17.127	1.635
Tienne Breumont	G	130	8.05	5.68	19	0.315	18.274	1.636
Les Abannets 1	G	90	1.09	9.85	20	0.264	16.190	1.595
Les Abannets 2	G	300	0.99	8.88	20	0.292	17.160	1.597
Tienne aux Pauquis 1	G	27	1.01	3.84	9	0.293	15.469	1.611
Tienne aux Pauquis 2	G	18	0.70	4.93	11	0.300	16.652	1.611
Tienne Delvaux	NG	250	0.86	1.09	18	0.301	17.524	1.635
Contieneau	NG	40	0.07	0.99	17	0.272	15.830	1.599
Roche Trouée 2	NG	40	0.21	7.15	12	0.254	14.229	1.558
Fondry des Chiens	NG	500,000	4.08	6.58	15	0.305	18.457	1.662
Petit Tienne Breumont	NG	10	0.29	11.98	7	0.270	13.775	1.571
Ancienne Carrière	NG	1000	7.19	1.24	14	0.298	17.923	1.645
La Rosière	NG	21	0.53	7.58	19	0.300	18.850	1.671
Carrière St-Anne	NG	40	0.25	9.12	18	0.266	15.830	1.592
Les Roches	NG	35	2.15	2.80	11	0.301	16.867	1.608
Tienne de Couvin	NG	50	0.33	0.74	19	0.257	15.160	1.578

S_{2002}: present fragment connectivity, for calculation: see text. Higher values imply less spatial isolation. H_j: Gene diversity for F_{is} = 0.5; Molecular variance: AMOVA SS/n-1; Br: Band richness standardized for sample size 7 (see text for explanation). Management: G: currently grazed; NG: not grazed.

Analyses and results

Total genetic diversity was partitioned among and within populations by carrying out a hierarchical analysis of molecular variance (AMOVA) on Euclidean pairwise genetic distances. The Φ_{st} analog for G_{st} was also calculated based on Euclidian genetic distances, and its significance was determined using a Monte Carlo procedure. A first measure of genetic variance, the Molecular variance, for each population was calculated by dividing the obtained AMOVA Sum of Squares for each population by the number of sampled individuals in that population minus 1 (Fischer and Matthies, 1998). Gene flow among populations was then calculated as $N_em = 0.25(1/\Phi_{st}-1)$ (Wright, 1951).

Next we estimated AFLP allele frequencies for each population from the observed AFLP-fragment frequencies using a Bayesian approach (Zhivotovski, 1999). Allele frequencies were then used to calculate expected heterozygosity or gene diversity

(H_j) and genetic differentiation between populations (F_{st}) (Lynch and Milligan, 1994). Genetic variation was recalculated for different values of F_{is} (0, 0.5 and 1), as no data regarding the level of inbreeding within the populations were available. As a final measure of within-population genetic diversity we calculated band richness (Br) for a standardized sample size (7), according to the rarefaction method of Petit *et al.* (1998). The band richness is the number of phenotypes expected at each locus (i.e. each scored AFLP fragment) and can be interpreted as an allelic richness analogue, ranging from 1 to 2 (R. Petit, pers. com.; Coart *et al*., 2005).

Populations were characterized by low but significant genetic differentiation (Φ_{st} = 0.056) (Table 7). AFLP-SURV-derived genetic differentiation measures were: $F_{st}^{(Fis = 0)}$ = 0.028; $F_{st}^{(Fis = 0.5)}$ = 0.042 and $F_{st}^{(Fis = 1)}$ = 0.050. All F_{st} values were significantly different from zero ($p<0.01$). Average Expected heterozygosity ($H_j^{(Fis = 0.5)}$) over all fragments was 0.28, average Molecular variance was 16.46. Estimated gene flow between populations per generation (N_em) was high (4.21).

Table 7 *AMOVA results for 18 fragmented A. vulneraria populations.*

Source	df	SS	MS	Est. Var.	Φ_{st}	p
Among populations	17	616.02	36.24	1.11	**0.056**	0.01
Within populations	265	4987.26	18.82	18.82		

Spatial structure was examined using a Mantel test. This indicated that there was no isolation by distance relation (Mantel statistic for matrix correlation = 0.17, p = 0.17, Fig. 10). Finally, the relation between present and historical landscape configuration (area and connectivity) on the one hand and the different measures for population genetic diversity (Molecular variance, H_j, and Br) on the other hand, was quantified using a General Linear Model (GLM). For each date (1850, 1905, 1968, 1984 and 2002) a different GLM was run. For 2002 also management (grazing or not) was introduced in the GLM as a fixed factor. We only found a significant effect of current landscape structure (fragment area) on genetic diversity for 2002 (Table 8). No effects of historical landscape structure were present.

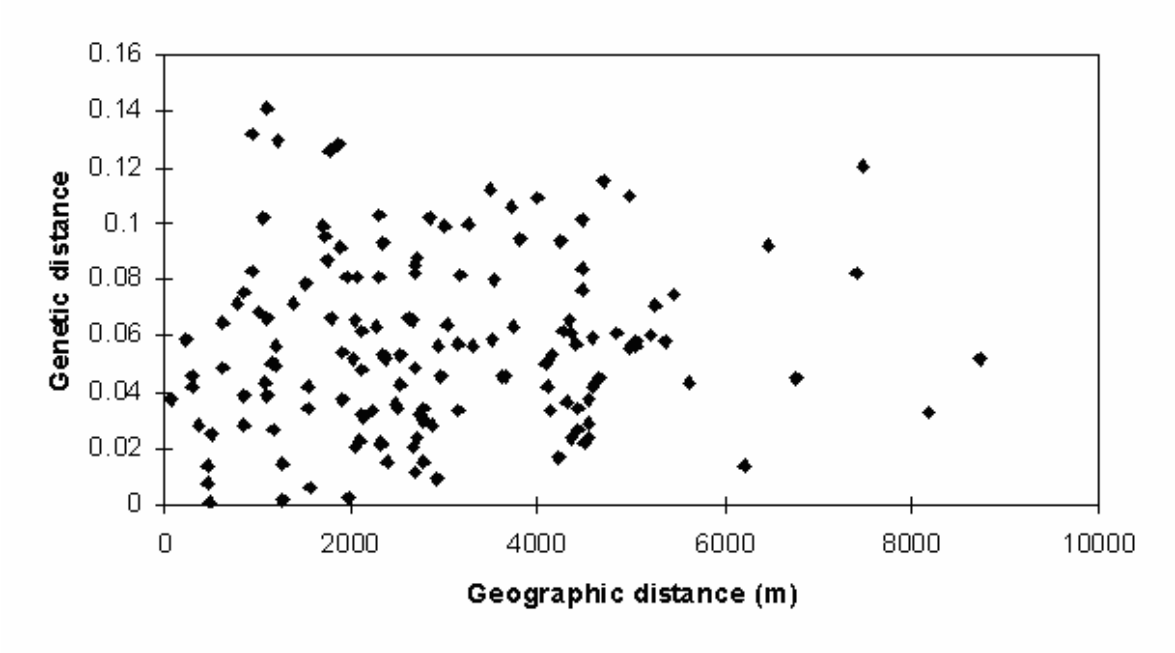

Fig. 10 *Isolation-by distance relation for 18 Anthyllis vulneraria populations in calcareous grasslands in the western part of the Viroin valley. There is no significant relation (p>0.1).*

Table 8 *F-values of GLMs for three measures of population genetic diversity of 18 A. vulneraria populations (dependent variables) against present fragment connectivity (S), fragment area and management (grazing or not) (independent variables).*

	H_j	Molecular variance	Br
Model	2.66 (*)	2.43 (*)	1.50
Intercept	669.63 ***	388.04***	6828.97 ***
S 2002	0.32	0.31	0.11
AREA 2002	6.30 *	5.04*	3.67 (*)
Management	0.00	0.18	0.04

H_j: Gene diversity for F_{is} = 0.5; Molecular variance: AMOVA SS/n-1; Br: Band richness standardized to sample size 7 (see text for explanation). S: fragment connectivity.
*** : $p \leq 0.001$; * : $0.01 < p \leq 0.05$; (*) : $0.05 < p \leq 0.1$

Finally, Φ_{st} values between grazed and other fragments were compared using an ANOVA-like Mantel test on the pairwise Φ_{st} values. This analysis revealed that populations in fragments which were regularly grazed were not different in terms of genetic differentiation compared to the abandoned populations (ANOVA-like Mantel test, p>0.1).

The most conspicuous result of this part of the study is the very low genetic differentiation among the sampled populations. Although gene flow between the sampled populations through pollen cannot be completely excluded, the predominantly selfing breeding system of *A. vulneraria* suggests that genetic erosion

in the study area was mitigated by a rather permanent seed flow among populations. This seed flow prevented genetic differentiation and loss of genetic diversity. It is very likely it predominantly occurred through roaming and grazing livestock. Therefore, this study provides indirect evidence that grazing management not only positively affects *A. vulneraria* viability through increased habitat quality but that it might also mitigate the effects of fragmentation on genetic structure. However, further genetic differentiation and reduction of genetic variability should be avoided. Therefore, management should aim to re-establish seed flow among all grassland fragments as soon as possible. This can be accomplished by including all fragments in the grazing scheme and by using a restricted number of flocks of grazing animals that should be regularly transported among grassland fragments. The conclusions reached for *A. vulneraria* are possibly also applicable to other plant species typical of calcareous grasslands, but further research is necessary before the conclusions of this study can be extrapolated to other species.

1.3 ISOLATION AND CHARACTERIZATION OF POLYMORPHIC MICROSATELLITE MARKERS IN *ANTHYLLIS VULNERARIA*

As described above, average within fragment genetic diversity measured as Molecular variance and Expected heterozygosity, were relatively high for the studied populations (16.46 and 0.28, respectively). Estimated values for these diversity parameters were close to reported estimates for outcrossing species (e.g. Jacquemyn *et al*., 2004) or species with mixed breeding systems (e.g. Schmidt and Jensen, 2000; Auge *et al.,* 2000). We therefore hypothesized that the reproductive system of *A. vulneraria* might be variable under different ecological conditions and that its selfing character should be reconsidered. In this respect, the estimation of heterozygosity and inbreeding statistics (Lowe *et al*., 2005) for these *A. vulneraria* populations using fully informative co-dominant markers, in combination with controlled pollinations should provide useful information. As no co-dominant markers were yet available for *A. vulneraria*, a set of eight SSR markers was developed for this species and applied to two *A. vulneraria* populations. Both populations were included in our AFLP based survey of genetic diversity (III B 1.2) and are located in two fragmented calcareous grasslands of the Belgian Viroin region. Population 'Tienne Breumont' (TB) consists of 130 plants and population 'Les Abannets 2' (Ab2) consists of 300 plants (Honnay *et al*., 2006).

SSR-markers were developed using the sequence tagged microsatellites profiling technique (STMP, Hayden and Sharp, 2001). The STMP-method generates short characteristic sequence tags for SSR-fragments, which are ligated to form concatemers for cloning and sequencing. Based on these tags, primers for the

amplification of specific microsatellite loci are developed. The protocol described by Hayden and Sharp (2001) was followed, with minor modifications (Kyndt *et al*., 2005).

In total, 100 unique sequence tags could be identified and 28 STM-primers were tested on pre-amplified *Pst*I-*Mse*I fragments, in combination with one adapter primer to amplify specific loci. Amplified fragments were cloned and sequenced to check for unique repeats and to obtain sequence information on both sides of the repeat in order to develop a specific primer pair for each locus. Nine of the cloned fragments contained repetitive sequences and were used to develop specific primer pairs. Thirty-five plants (16 of TB and 19 of Ab2 respectively) were typed for these eight loci. One primer pair (WO28) revealed only one monomorphic band, amplified with the primer pair TAAATACCTCCTAACACCTCTGTCCCA and CTCGGTACTCCCGC GGGGAGAAAACAG. Eight primer pairs amplified polymorphic SSR loci (Table 9).
DNA-extractions were carried out as described in 1.2. Genomic DNA (0.5 ng) was used as template for PCR, performed in a total volume of 15 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.01% (w/v) gelatine, 10.67-46.67 nM of each primer (Table 9), 0.2 mM dNTPs and 0.75 U AmpliTaq DNA polymerase (Applied Biosystems). The forward primers were 5' labelled with either 6-FAM, HEX or NED fluorescent dyes (Applied Biosystems). Amplifications were carried out using an Applied Biosystems 9700 Thermal Cycler according to the following reaction profile: one hold for 2 minutes at 94 °C followed by 30 cycles at 92 °C for 1 min, 53 °C for 1 min, 72 °C for 90 sec and a final extension at 72 °C for 30 min. Different dye-labelled PCR products were pooled for capillary electrophoresis after optimisation of primer concentrations (WO2+WO3+WO7; WO8+WO12+WO23; WO10+WO14 +WO28). PCR products were separated using capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems), using GeneScan-500 ROX (Applied Biosystems) as internal size standard. GeneMapper 3.7 (Applied Biosystems) was used for allelic scoring. Exact tests for linkage disequilibrium between pairs of loci and Hardy-Weinberg equilibrium were performed and diversity statistics were estimated.

The number of alleles per locus ranged from 2 to 5. For population TB, observed and expected heterozygosities ranged from 0.125 to 0.688 and from 0.121 to 0.631 respectively. For population Ab2, observed and expected heterozygosities ranged from 0.053 to 0.667 and from 0.053 to 0.625 respectively. Significant deviations from HWE ($P<0.05$) were detected for loci WO10 and WO12 in population TB. Also the multilocus inbreeding coefficient for population TB showed a slightly significant deficiency of heterozygotes (F_{is} = 0.122, P = 0.049). No significant linkage disequilibrium was observed between any pair of loci (with $P < 0.05$; Fisher exact test for genotypic disequilibrium between pairs of loci across all populations).

Table 9 *Description of the developed microsatellite primers and their characterization in 35 Anthyllis vulneraria genotypes, including observed (H_0), expected heterozygosity corrected for small sample sizes(H_E) and inbreeding coefficients over all alleles with correction for small sample sizes (F_{is}). All parameters are calculated with GEN-SURVEY (Vekemans and Lefèbvre, 1997)*

Locus	Repeat motif	Primer sequences (5'-3')	Dye	Primer Conc (nM)	PLP	Tienne Breumont		Les Abannets		Size range (bp)	No. of alleles		
						H_O-H_E	F_{is}	H_O-H_E	F_{is}		TB1	Ab2	Total
WO2	$(CT)_{10}$	F: ACTATATAAAACCTTACCACTTTCCCC R: CAGCAGCATGCAATAATCAAAAACTGA	FAM	18.67	100	0.438-0.573	0.235	0.389-0.475	0.180	121-129	3	2	3
WO3	$(CT)_9$	F: TAAATTACATCGACCTAGTC R: TGTGCAGTTTGTTGTTCTTTTTTGTGT	HEX	17.33	100	0.688-0.526	-0.308	0.200-0.191	-0.049	66-79	2	3	4
WO7	$(CT)_6$	F: TCTGATGGGCTGACCCGTCGGGTCAGG R: CAGAATCGCATCAATTGCTTC	FAM	26.67	100	0.125-0.121	-0.034	0.053-0.053	-0.001	156-164	2	2	2
WO8	$(CT)_5$	F: ACCCTTACTGTCACCCTCCACGTTCCC R: AGAACTGGAAGGGTCATCGTTCACCGT	HEX	10.67	100	0.313-0.331	0.054	0.438-0.486	0.099	238-264	3	3	3
WO10	$(CT)_6$	F: TAAACATGATTGGCAATGTGTGAAGTG R: ACTTATAGCAGTATTCGAAGAGGAATC	NED	46.67	100	0.357-0.577	0.380*	0.526-0.570	0.077	227-234	4	4	4
WO12	$(CT)_{10}$	F: TAACTACTACTCACACCAAGCTCTTAC R: CAGATGTCTCTTCCTTGTGGGCTCAAC	FAM	12.00	100	0.563-0.631	0.108*	0.579-0.505	-0.147	85-93	5	3	5
WO14	$(CT)_6$	F: TAAATGTTTACAGATGAGATTCTCTAT R: CCGTACATGCATGAATAACTTTTGATT	HEX	33.33	100	0.188-0.179	-0.046	0.167-0.208	0.198	223-227	3	3	3
WO23	$(CT)_5$	F: TAAAATCTCTCTGTCTCTGTCT R: AATACGGATTAGTGACAGAAAAAAGAT	NED	26.67	100	0.357-0.553	0.353	0.667-0.625	-0.067	104-108	3	3	3
			Mean over loci		100	0.3783-0.4363	0.1217*	0.3772-0.3891	0.0315		3,13	2,88	3,38

*: estimates of Hardy-Weinberg exact P-values with 0.01 < P < 0.05. All other P-values > 0.05.

The data obtained here further support our AFLP-results (1.2), and the studied *A. vulneraria* populations seem to consist of mainly outcrossing plants. The largest population (Ab2, 300 individuals) even showed allelic frequencies consistent with a random mating population in Hardy-Weinberg equilibrium, whereas in population TB (130 individuals) a slight deficit of heterozygotes was detected. When comparing the estimated F_{is} with values obtained for species with known reproductive systems (Hamrick and Godt, 1996), it can be concluded that the reproductive system of *A. vulneraria* in the fragmented calcareous grasslands studied is mainly outcrossing. It would therefore be interesting to analyze a larger number of populations with the SSR-markers developed and to perform pollination experiments in the area of Southern Belgium studied, in order to compare with the results of Couderc (1971), who found that allogamy was only possible in male sterile individuals.

2 ANEMONE PULSATILLA

In 2003 three DNA-extraction protocols were tested for *A. pulsatilla* (Fig. 11). Both CTAB procedures tested failed in extracting DNA of appropriate quality to carry out restrictions. The Dneasy Plant Minikit (Qiagen) resulted in sufficient quantities of better quality DNA.

Fig. 11 *Anemone pulsatilla*

Table 10 *AFLP primer combinations tested for A. pulsatilla*

Selective forward primer	Selective reverse primer	Label forward primer
Primers with 5 selective bases		
E-AGC	M-CA	Joe
E-AAG	M-CG	Joe
E-ACC	M-CT	Hex
Primers with 6 selective bases		
E-AGG	M-CAA	Joe
E-ACA	M-CAT	Fam
E-ACT	M-CAC	Fam
E-ACC	M-CTG	Joe
E-ACG	M-CTT	Hex
E-AAG	M-GCC	Hex
E-ACC	M-GAT	Hex
E-ACT	M-CAA	Fam
E-ACT	M-CTA	Fam
E-AAC	M-CTG	Fam
E-AGG	M-CTC	Hex
E-ACC	M-CTG	Hex

Primers with 6 selective bases (continued)		
E-ACG	M-CTA	Hex
E-AGG	M-CAA	Hex
E-AGG	M-CCA	Hex
E-AAG	M-CAC	Hex
E-AAG	M-CTA	Hex
E-AAG	M-CGG	Hex
E-ACC	M-CAG	Hex
E-AGC	M-CTC	Hex
E-ACA	M-CAA	Fam
E-ACA	M-CTT	Fam
E-AAC	M-CAG	Fam
E-AAC	M-CGG	Fam
E-ACT	M-CGA	Fam
E-AAT	M-CGG	Fam
E-AAA	M-CGC	Fam
E-AAA	M-CGT	Fam
E-AGG	M-CAA	Hex
E-AGG	M-CCA	Hex
E-ACC	M-CAG	Hex
E-AGC	M-CTC	Hex
E-AGG	M-CAA	Hex
E-ACC	M-CAG	Hex
H-TCG	M-CTA	Fam
P-AGG	M-CCA	Fam

Primers with 7 selective bases		
E-ACG	M-CCAT	Joe
E-AAC	M-CTTG	Fam
E-ACG	M-CTTG	Hex
E-ACT	M-CTCA	Fam
E-ACT	M-CTAA	Fam
E-AAC	M-CTGC	Fam
E-AGG	M-CTCC	Hex
E-ACC	M-CTGT	Hex
E-AAC	M-CAGT	Fam

However, when AFLP patterns were generated in January 2004 following the protocol described in Roldán-Ruiz *et al.* (2000), the AFLP patterns were of visibly low reproducibility. In order to overcome this problem, different approaches were followed. First, different restriction enzymes were tested besides the usually applied *Mse*I and *Eco*RI. The enzymes *Hin*dIII and *Pst*I were applied and also restriction on restriction was performed to make sure that all restriction sites were cut. Secondly, the number of selective bases added to the pre-amplification and selective amplification primers was changed to clarify the patterns. A total of 48 primer combinations were tested on different restriction digests (see Table 10).

None of the procedures tested however did result in clear and reproducible AFLP patterns. At this point it was decided to end the laboratory analyses for *Anemone pulsatilla* and start working on the optimisation of the AFLP protocol for a newly selected species, *Globularia bisnagarica*.

3 GLOBULARIA BISNAGARICA

3.1 STUDY SPECIES

Globularia bisnagarica L. (Globulariaceae) (Fig. 12) is a nectar producing, rosette forming long-lived perennial (Höllander and Jäger, 1998). Both outcrossing and selfing are common in this diploid species (2n = 16) (Klotz *et al*. ,2002). Typical pollinators are butterflies, long tongued bees and syrphids (Klotz *et a*l., 2002). The species flowers between May and July. Each plant develops between 1 and 10 flower stalks carrying one flower head. Fruits (<1 mg) have an appendage, suggesting epizoochory. In Belgium, the species is restricted to xerophilous rocky outcrops of the calcareous grasslands of the Calestienne region and the Haute-Meuse.

Fig. 12 *Globularia bisnagarica*

3.2 GENETIC DIVERSITY WITHIN AND BETWEEN REMNANT POPULATIONS OF THE ENDANGERED CALCAREOUS GRASSLAND PLANT *GLOBULARIA BISNAGARICA L*.

To study the genetic diversity of the remnant *Globularia bisnagarica* populations, 27 of the 35 *G. bisnagarica* populations occurring in the study area were randomly selected (Fig. 13). The number of flowering individuals was determined for each population. Furthermore, within each population, 20 flowering individuals were randomly selected, or all individuals if less than 20 were present. From all selected individuals a leaf sample was taken for AFLP analysis. Around each selected plant a 40 x 40 cm plot was established in which several environmental variables were measured. Furthermore, present and historical area and connectivity were calculated for each patch.

AFLP protocol

Before the collection of *G. bisnagarica* samples was started, optimization of AFLP for this species was started on four test samples collected from one population on a calcareous grassland fragment. All sampled leaves were immediately frozen in liquid nitrogen, freeze-dried for 48 h and homogenized with a mill (Retsch MM 200) to fine powder. DNA extractions were carried out on fresh and lyophilized material using the DNeasy Plant Mini kit (Qiagen). For both types of material, this protocol resulted in DNA of good quality and for further extractions 20 mg of lyophilized material was

used. DNA quality and concentration were estimated on 1.5% agarose gels. We chose for the AFLP (Amplified Fragment Length Polymorphism) protocol (Vos *et al*., 1995) to screen the genetic diversity of our samples. Restriction digests were made with the enzymes *Eco*RI and *Mse*I. Fingerprints were generated with primers with a different number of selective bases.

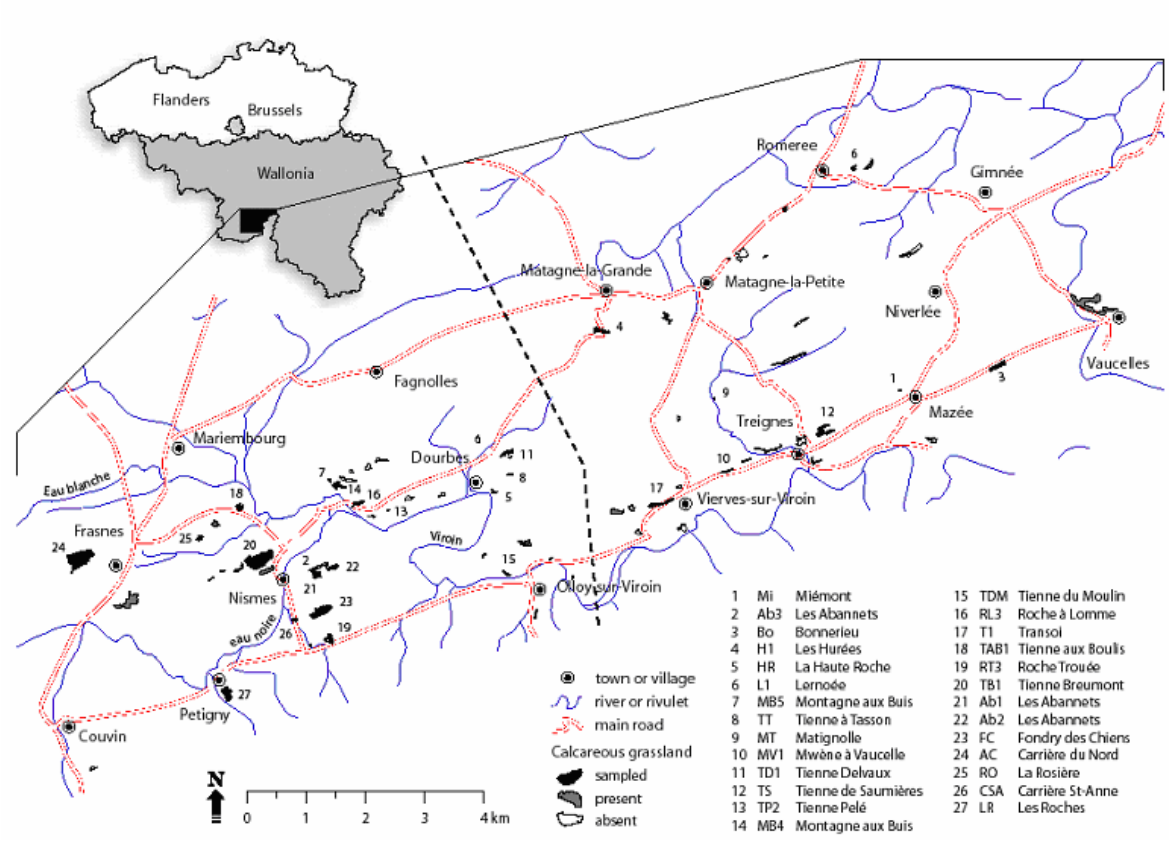

Fig. 13 *Location of the study area. Sampled grassland fragments (27) are indicated in black. Not sampled fragments where G. bisnagarica is occurring are grey. The dashed line separates the Western and Eastern cluster as indicated in Fig. 16.*

In a first step, three primer combinations were tested for the pre-amplification (E-A/M-C, E-A/M-CT, E-A/M-CA) and four primer combinations for the selective amplification (E-AAG/M-CT, E-AAG/M-CTG, E-AAG/M-CTGA, E-AGG/M-CAGT). The primer combination with six selective nucleotides resulted in a clear and reproducible pattern and additional primer combinations with six selective nucleotides were tested for this species. In total 88 additional primer combinations were tested in order to obtain fingerprints with sufficient polymorphisms among the samples. This was realized by combining eight *Eco*RI-primers (E-ACG, E-AGG, E-AAG, E-ACC, E-AGC, E-ACA, E-ACT, E-AAC) with 11 *Mse*I-primers (M-CAA, M-CAC, M-CAG, M-CAT, M-CTC, M-CGT, M-CTG, M-CGC, M-CTT, M-CGG, M-CGA). Finally, six primer combinations

were selected and applied for the study of genetic diversity: *Eco*RI-AAC/*Mse*I-CTG, *Eco*RI-AAC/*Mse*I-CAC, *Eco*RI-ACG/*Mse*I-CAG, *Eco*RI-ACT/*Mse*I-CGT, *Eco*RI-AGC/*Mse*I-CGC and *Eco*RI-AGG/*Mse*I-CAC (MWG biotech, Ebersberg, Germany). AFLP analysis was carried out according to the protocol described in Roldán-Ruiz *et al*. (2000). Size separation of the PCR-products was carried out using capillary electrophoresis on an automated ABI 3130 genetic analyzer with the polymer Pop 7 (Applied Biosystems). Mixes of 1 µL of each of three differently fluorescent labeled PCR-products, 0.03 µl Genescan^{TM} 500 Rox Size standard (Applied Biosystems) and 0.97 Hidi^{TM} Formamide (Applied Biosystems) were injected. Size scoring of AFLP-fragments was performed in a semi-automated way using Genemapper version 3.7.

Table 11 Population genetic characteristics of the 27 sampled *G. bisnagarica* populations.

code	Name	Sample size	Population size	H_j
Ab1	Les Abannets	17	33	.122
Ab2	Les Abannets	20	32	.099
Ab3	Les Abannets	16	92	.044
AC	Carrière du Nord	19	211	.067
Bo	Bonnerieu	15	930	.072
CSA	Carrière St-Anne	17	42	.101
FC	Fondry des Chiens	20	90,000	.089
H1	Les Hurées	18	496	.052
HR	La Haute Roche	19	55	.078
L1	Lernoée	20	35	.051
LR	Les Roches	17	97	.043
MB4	Montagne aux Buis	17	65	.102
MB5	Montagne aux Buis	20	530	.051
Mi	Miémont	20	106	.040
MT	Matignolle	14	43	.096
MV1	Mwène à Vaucelle	19	680	.072
RL3	Roche à Lomme	20	68	.102
RO	La Rosière	19	27	.075
RT3	Roche Trouée	20	342	.098
T1	Transoi	18	64	.072
TAB1	Tienne aux Boulis	19	542	.083
TB	Tienne Breumont	18	1780	.109
TD1	Tienne Delvaux	18	25	.100
TDM	Tienne du Moulin	14	610	.061
TP2	Tienne Pelé	5	7	.132
TS	Tienne de Saumières	17	553	.063
TT	Tienne à Tasson	17	117	.105

H_j: Gene diversity for F_{is} = 0.5.

The fluorescent AFLP patterns were scored using GeneMapper 3.7 (Applied Biosystems). Each marker was coded as 1 (present) or 0 (absent) in an individual to form a binary data matrix. Each sample was thus represented by a vector of 1s and 0s.

The six AFLP primer combinations resulted in 77 scorable polymorphic markers. Only samples exhibiting a clear banding pattern for all six primer combinations were used. This resulted in the omission of a number of samples from further analysis and yielded a data set with 473 samples from 27 populations (Table 11).

Analyses and results

Total genetic diversity was partitioned among and within populations by carrying out a hierarchical analysis of molecular variance (AMOVA) on Euclidean pairwise genetic distances, and genetic differentiation between populations (F_{st}) and expected heterozygosity or gene diversity (H_j) (Lynch and Milligan, 1994) were calculated as described in III B 1.2.

Populations were characterized by very high and significant genetic differentiation (AMOVA Φ_{st} = 0.53, p<0.001) (Table 12). AFLP-SURV-derived genetic differentiation measures were: $F_{st}^{(Fis = 0)}$ = 0.42 (SE 0.07); $F_{st}^{(Fis = 0.5)}$ = 0.44 (SE 0.06) and $F_{st}^{(Fis = 1)}$ = 0.48 (SE 0.05). All F_{st} values were significantly different from zero (p<0.01). Gene diversities for the three different F_{is} values were highly correlated (>95%) and we continued with the H_j values for F_{is} = 0.5. Average gene diversity ($H_j^{(Fis = 0.5)}$) over all populations was 0.081 (SE 0.025) (Table 11). This strong genetic differentiation between the remnant populations of G. bisnagarica indicate that conservation management should focus on the conservation of all populations.

Table 12 *Analysis of Molecular Variance of the 27 sampled G. bisnagarica populations.*

Source	df	SS	MS	Est. Var.	Phi_{st}	p
Among Pops.	26	1251.68	48.14	2.61		
Within Pops.	446	1034.47	2.31	2.31	0.53	<0.001

Recorded habitat variables were averaged across all sampled individuals for each population and grouped using a Principal Component Analysis (PCA). Two PCA axes were retained. PCA1 was positively correlated with vegetation height and cover of *B. pinnatum* and PCA 2 was positively correlated with soil depth. Next, we performed a stepwise linear regression with H_j as the dependent variable, and connectivity (CON), distance to the nearest population (DIST), PCA 1, PCA2 and population size as the independent variables. All level two interactions between independent variables were also tested. Only the isolation measures (CON and DIST) were significant. The

model with DIST (B=-0.51, $F_{1,27}$=9.28, p< 0.01, r=0.26) (B=standardized regression coefficient) was most significant (Fig. 14). This relation provides evidence for increased gene flow into less isolated *G. bisnagarica* populations. The fact that genetic diversity is not correlated with population size indicate that even currently small populations can contain considerable genetic diversity while large populations may be impoverished.

Spatial genetic structure of the 27 populations was first examined using a Mantel test. To detect geographical clustering we used the unweighted pair group method with arimetric mean (UPGMA) on pairwise population F_{st} values. The obtained clusters of genetically similar populations were visually compared with the historical landscape configuration in 1775 and 1875. We found a highly significant isolation-by-distance relation (Mantel statistic for matrix correlation = 0.61, p < 0.001, Fig. 15). Moreover, there was a clear geographical clustering of the populations, with one cluster encompassing the 8 most eastern populations and one cluster encompassing the 18 most western populations (Fig. 16). Only population AB3 behaved idiosyncratically. With the exception of the most peripheral populations (Bo, L1, H1 in the east and RO, TAB1, RC, TB, RT3, LR in the west), all populations of the eastern and the western cluster were connected on the 1775 map (Fig. 16). On the 1875 map, grassland fragmentation has increased to a degree at which only neighboring populations were connected.

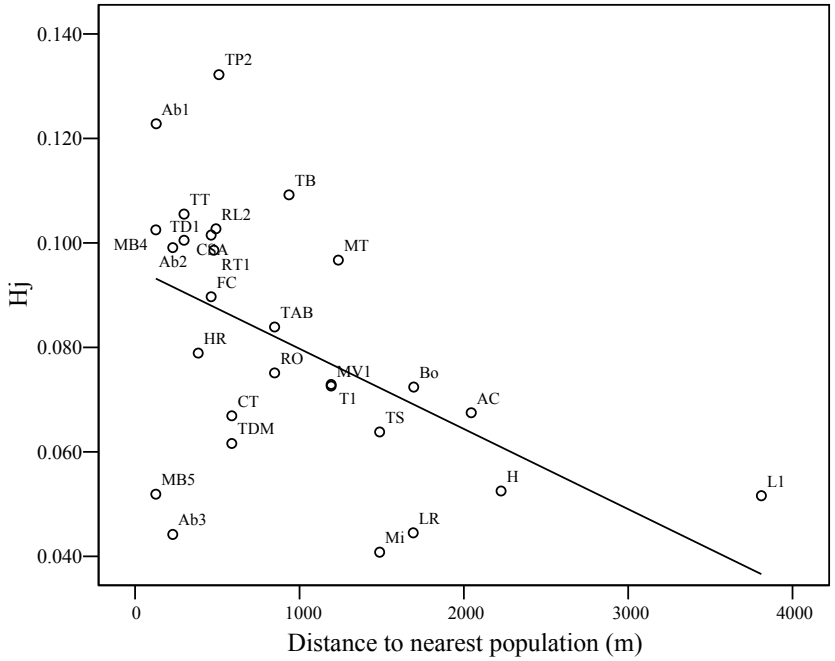

Fig. 14 *Relation between gene diversity (H_J) and isolation for 27 populations of G. Bisnagarica. $R^2_{(Pearson)}$ = 0.20 (p<0.01). See Figure 13 for fragment codes.*

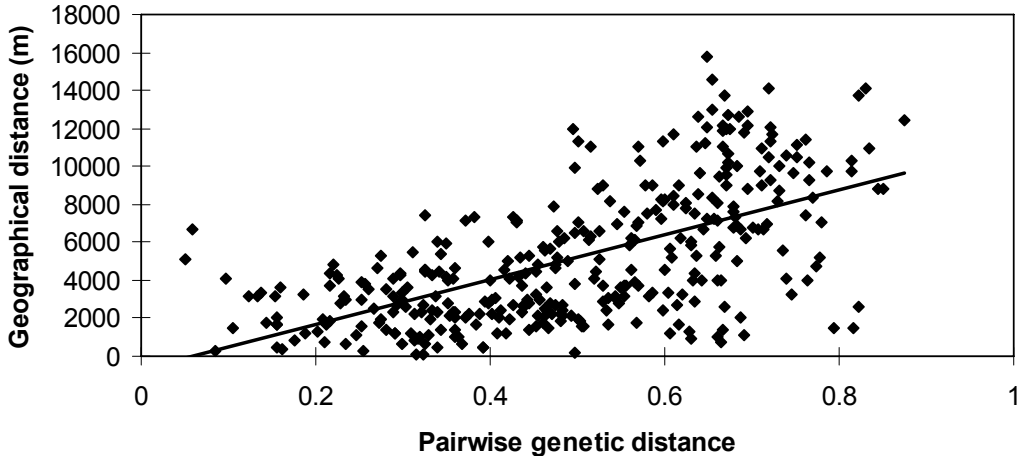

Fig. 15 *Relation between pairwise genetic distance and geographical distance for 27 G. bisnagarica populations.*

Because of the clear clustering of the populations in two groups, we finally performed a hierarchical AMOVA revealing that 28% of all variance was among populations within regions while 38% was among regions. F_{st} $^{(Fis = 0.5)}$ for the western cluster was 0.33 (SE 0.16) and for the eastern cluster 0.29 (SE 0.16).

These results suggest that after the fragmentation of the grassland, individual populations started to differentiate from each other through genetic drift and/or the effect of bottlenecks. The differentiation, however, was not (yet) strong enough to erase the genetic similarity between fragments that historically belonged to the same large grassland fragment.

It is important to note that the results obtained for *G. bisnagarica* are very different from those obtained for the 18 populations of *Anthyllis vulneraria* studied in the former part. Very low genetic differentiation (Φ_{st}=0.05) was attributed to frequent seed dispersal between populations through endo- and epizoochory by roaming sheep flocks. *A. vulneraria* however is characterized by both a much higher grassland fragment occupancy and average population size than *G. bisnagarica*, partly because the latter species is confined to xerophilous rock outcrops within grassland fragments. Therefore, the chance that seeds reach suitable sites attached to, or digested by, roaming sheep is rather low, especially because sheep tend to avoid the xerophilous grassland parts when grazing.

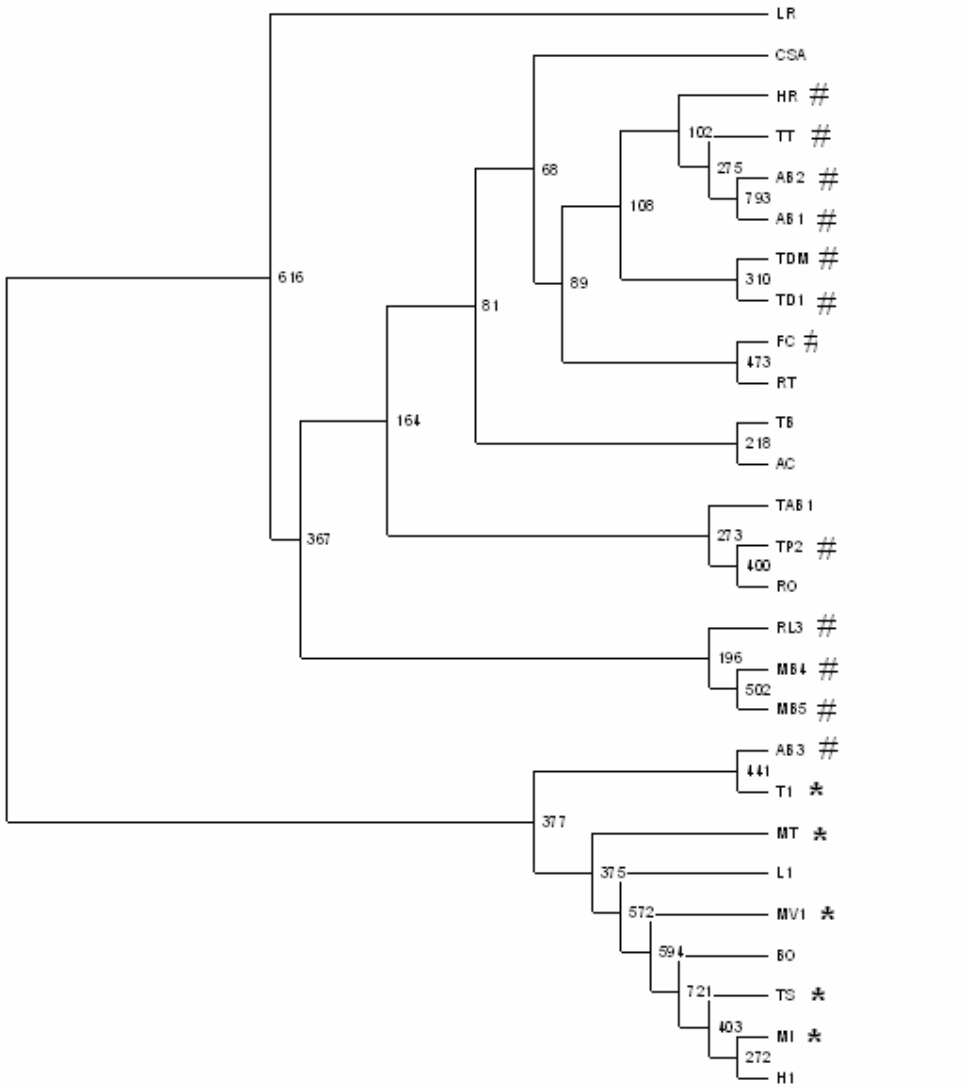

Fig. 16 *Results of UPGMA clustering for 27 G. bisnagarica populations. Populations that were connected in 1775 are indicated with '*' for the eastern cluster, and with '#' for the western cluster. Bootstraps values are indicated at each node. See Figure 13 for fragment codes.*

IV. BUTTERFLIES

A. COMMUNITY LEVEL

The objectives of this project at the community level include: 1) a survey of the presence/absence of butterfly species in the fragments, 2) calculate measures of species diversity and community composition, and 3) relate species richness and community composition with fragment features.

To realize these objectives, we began by tracking the effects of one century of habitat loss on the diversity of butterfly communities (1). For this, we combined data collected by ourselves and data available through past records / literature. Next, based on field data collected (by transects) during the three year period, butterfly community composition and structure were modelled (2) so as to extract those parameters that are essential to the conservation of butterfly diversity in the Viroin Valley. A new technique for ordering asymmetrical data was tested using the transect data (3) indicating that there is a temporal stability in species composition among the studied calcareous grassland butterflies communities.

1 TRACKING THE EFFECTS OF ONE CENTURY OF HABITAT LOSS AND FRAGMENTATION ON CALCAREOUS GRASSLAND BUTTERFLY COMMUNITIES

Since the beginning of the 20th century, agropastoral practices have been progressively abandoned due to a lack of economical interest and land use intensification, consequently endangering calcareous grasslands and their species communities (Balmer and Erhardt, 2000). Butterflies were used as models to track the effects of habitat loss and fragmentation on the long-term because (1) it has already been shown that the amount of modifications in butterfly community structure and composition is proportional to the degree of fragmentation (Thomas, 1991; New, 1997), and (2) they react quickly to environmental changes (Erhardt and Thomas, 1991; Bourn and Thomas, 2002), and (3) they are considered to be umbrella species (sustaining habitat to conserve them will also conserve many other taxa) and indicators of habitat quality (New, 1997). Moreover, specialist (monophagous) butterfly species are more affected by environmental changes than generalist species (Erhardt and Thomas, 1991; New, 1997; Steffan-Dewenter and Tscharntke, 2002; Tscharntke *et al*., 2002). We took advantage of the simultaneous availability of (1) one century of surveys of the butterfly community in calcareous grasslands and (2) maps which allow us to track the changes in the distribution of calcareous grasslands in the landscape.

Approximately 1150 hectares, about 10% of total regional area, were analysed over a timeframe of about 100 years. The 1905 situation was based on an army map (scale 1/20000), whereas the 1948, 1965, 1982, 1987 and 2000 landscapes were based on aerial photographs. The current situation (2004) was derived from field surveys (J. Butaye, personal communication). All the maps were imported in GIS. The change in calcareous grassland number, area and connectivity over the six time periods were computed. Due to the lack of adequate data before the 1900's, the proportion of existing calcareous grasslands in 1905 is fixed as the maximum (100%).

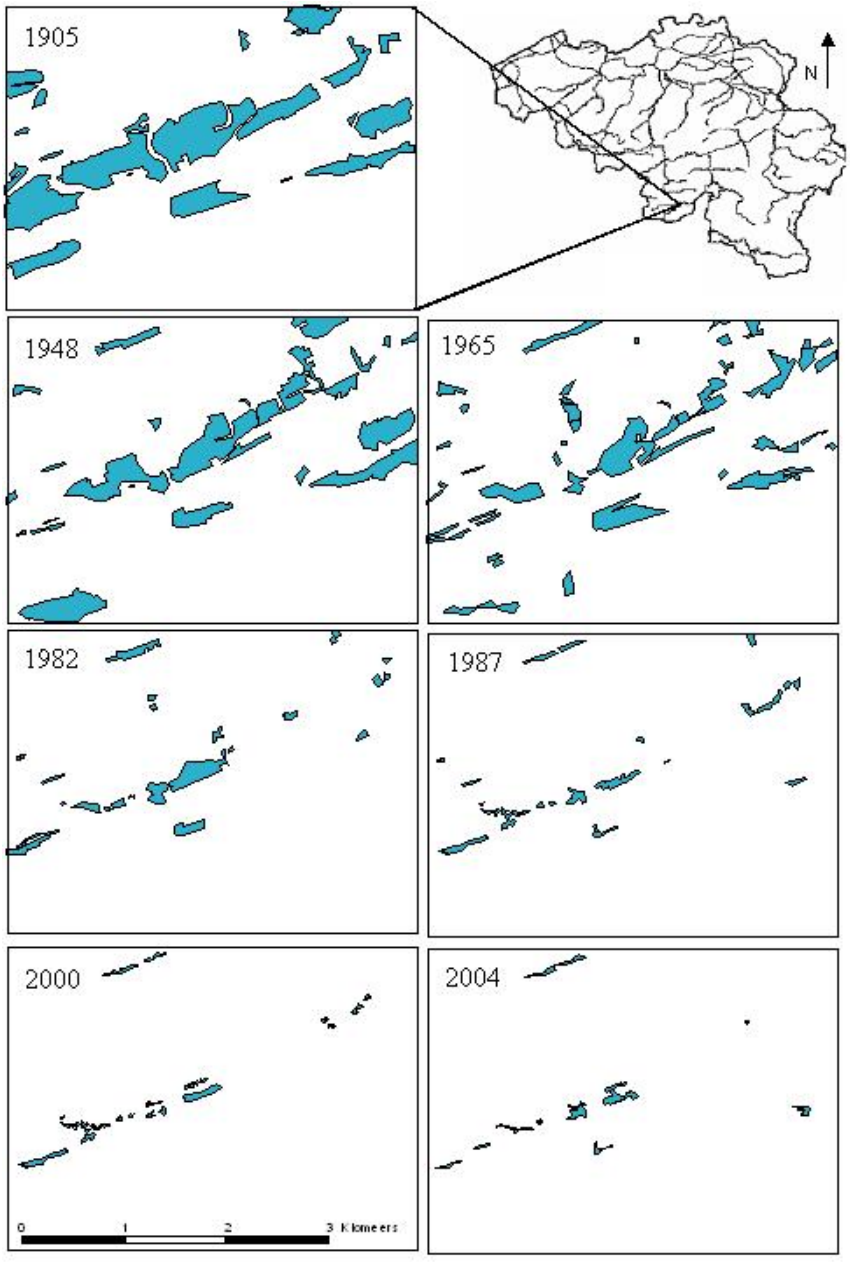

Fig. 17 *Temporal development (1905 to 2004) of the calcareous grassland fragments in part of the Calestienne landscape.*

Whereas calcareous grasslands represented about 14.9% of the studied landscape in 1905, less than 0.7% still remains today (Fig. 17). Consequently, the average area of habitat patches has also continuously decreased, i.e. from 7.79 hectares in 1905 to 0.82 hectares in 2004. Figure 17 shows that the number of fragments in the Viroin Valley continuously increased from 1905 until 1965. Subsequently, the number of fragments has decreased until present day. The average connectivity of a habitat patch, S_i, has continuously decreased from 0.323 at the beginning of the 20th century to only 0.021 at the beginning of the 21st century.

We examined butterfly species incidence (presence/absence) in the Calestienne region using a series of published surveys (Lambillion, 1903; Lhomme, 1923; Lameere, 1940; Van Schepdael, 1963; Fontaine *et al*., 1983; Goffart *et al*., 1992; Lafranchis, 2000). Species listed during one time lag and not afterwards were regarded as extinct, which does not signify that they are or were not present elsewhere in Belgium. The composition of the butterfly communities of 1903, 1923, 1940, 1950, 1970, 1980, 1990 and 2000 was identified and was related to the landscape parameters listed above. In addition, 16 calcareous grasslands in part of this landscape were visited every two weeks during the butterfly's flight season of 2003, 2004 and 2005.

Based on literature dating back to 1900's, 83 species of butterflies have been observed in the Calestienne and a total of 69 species were identified during the 2003, 2004 and 2005 field seasons. The data in this study suggest that, since the beginning of the century, the proportion of both habitat specialists and generalists with a 'common' observed frequency decreased (Fig. 18), i.e. more species were 'rare' in 2004 than in 1903. Common butterflies and common specialist/generalist species decreased linearly with decreasing average patch area and connectivity, whereas the number of rare butterflies and rare generalist species increased. Although no habitat generalists went extinct during this time period, nine specialist species did. Over the entire 20th century, 64.8% habitat generalists and only 34.4% habitat specialists maintained their initially observed frequencies. For a given species, being a habitat specialist or habitat generalist significantly influenced its observed frequency. Indeed, habitat specialists were more likely to be rare or extinct than are habitat generalists. This trend is all the more significant with the passing of time. Rare and extinct specialist species were sensitive to average patch area; their numbers increased with decreasing area.

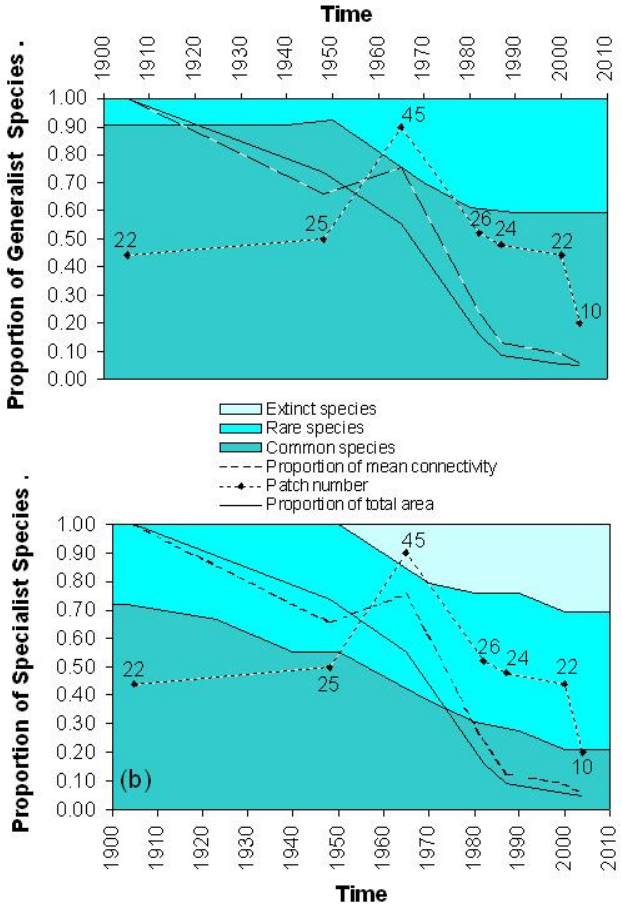

Fig. 18 *Development of the generalist (a) and specialist (b) species communities since 1903. The proportions of common, rare, and extinct species are represented respectively in average grey, light grey, and white. The proportion of total area of remaining calcareous habitat (continuous line), the proportion of mean connectivity (discontinuous line), and the number of remaining calcareous fragments (dotted line) are superposed on both figures (a) and (b).*

Our results indicate that the extinction and rarefaction phenomena observed for the specialist species are rather linked to the fragmentation and loss of calcareous grasslands, whereas for generalist species, these processes appear to be linked to important changes observed in the landscape structure as of the 1950's. Indeed, a decrease in habitat fragment number increases the isolation between habitats, rendering the habitat specialist persistence dependent on their dispersal capacities. This is less so for habitat generalists, which can occupy several different habitats and will consequently be less susceptible than specialists to habitat fragmentation and habitat loss. The effects of the calcareous grassland restoration and management campaign have yet to be detected. This has been observed elsewhere as well and has been coined as 'restoration lag' by Huxel and Hastings (1999). Some clues indicate, however, that these restoration efforts are not fruitless, like the return in the Calestienne region of the butterfly *Mellitaea cinxia* during the exceptional warm flight season in 2003, after more than 30 years of absence.

2 MODELING COMMUNITY STRUCTURE AND COMPOSITION: EXAMPLE OF CALCAREOUS GRASSLAND BUTTERFLIES IN A HIGHLY FRAGMENTED LANDSCAPE

Understanding which factors determine the presence of a series of species has intrigued scientists for a long time. As already discussed above, the decline in insect species is largely due to a decrease in habitat quality. Consequently, modeling species diversity enables conservation biologists to extract the most important factors influencing species diversity, and elaborate conservation management plans accordingly. Here we model community diversity, specialist and generalist species diversity using both biotic and abiotic factors as predictive variables.

Therefore, butterflies were sampled by conducting a standardized line transect method (Pollard, 1977). All butterflies were recorded within a ten meter width along the transect routes in suitable weather conditions (Pollard, 1977). Butterflies were identified in flight and if not possible were caught with a net and released immediately after identification. All sites were visited once every two weeks from April to September during the 2003 field season. Furthermore, plant species abundance and diversity were inventoried for a total of 125 one meter squared plots within the *P. coridon* study system. Microclimatic conditions, including light (L), temperature (T), humidity (F) and nutrient acidity (N) (inferred by pooling knowledge on the vegetation composition with Ellenberg values of individual plant species) and litter depth, bryophytes coverage, soil depth and percentage of bare soil, were summarized using the principal component analyses (PCA). For every patch, patch area, edge length and distances between patches were calculated using aerial photographs (2000) using ArcMap.

Simple and multiple regressions were used to assess habitat quality on (i) community diversity as a whole, (ii) specialist species diversity, (iii) generalist species diversity and (iv) guilds. We found that the total species richness of butterflies is particularly affected by patch size and altitude and connectivity, although all subgroups and species are not affected in the same way. For example, while the predictive model for specialist groups always contains the isolation factor, the predictive model for generalist species never does (Table 13). Altitude most likely represents a degree of isolation from human activity (with higher altitudes related to lower human disturbance). Host plant species number only intervenes in the majority of selected models for the GRA guild.

Table 13 *Appearance frequency of the most often appeared explanatory variables (columns) in models describing species diversity indices (lines) (total diversity, generalist and specialist species diversity and several associations defined by the caterpillars host plants (FLR, GRA, LEG, LGX)).*

	Area	Altitude	Connectivity	PCA1	PCA2	Light	#Host plants	# Models
Total	100%	100%	55%	33%	44%	22%	22%	9
Generalists	100%	100%	16%	100%	33%	16%	66%	6
Specialists	100%	100%	100%	20%	60%	20%	40%	5
FLR guild	100%	100%	100%	100%	33%	33%		3
GRA guild	100%	100%	100%	100%	100%	100%	100%	1
LEG guild	100%	100%	100%	100%	100%	33%	33%	3
LGX guild	100%	100%	43%	86%	57%	57%		7

PCA1: *Mesobrometum erecti* / *Alysso* / *Sedion albi*, PCA2: *Xerobrometum erecti* / *Geranion sanguinei*
FLR: *Apiaceae, Asteraceae, Boraginaceae, Geraniaceae, Marlvaceae, Orobanchaceae, Plantaginaceae, Polygonaceae, Primulaceae, Rosaceae, Violaceae*; GRA: *Cyperaceae, Poaceae*; LEG: *Brassicaceae, Fabaceae, Tropeolaceae*; LGX: *Betulaceae, Caprifoliaceae, Cornaceae, Fagaceae, Grossulariaceae, Rhamnaceae, Rosaceae, Salicaceae, Ulmaceae*.

We also found that species diversity (community, generalist and specialist) increases exponentially until a maximum patch area of 2 ha (Fig. 19). Furthermore, we found that, in small patches, specialist species diversity significantly decreased when the amount of vegetation at the edge of the patch increases. This is in accordance with theoretical hypotheses. Indeed, in small sites, more edge vegetation changes the microclimatic conditions through a decrease in temperature and an increase in humidity. These conditions are unfavorable for specialist grassland butterfly species. Additionally, the specialist species have to compete with generalist and often invasive species coming from the edge vegetation. However, this relationship was not significant when large sites were included in the analyses.

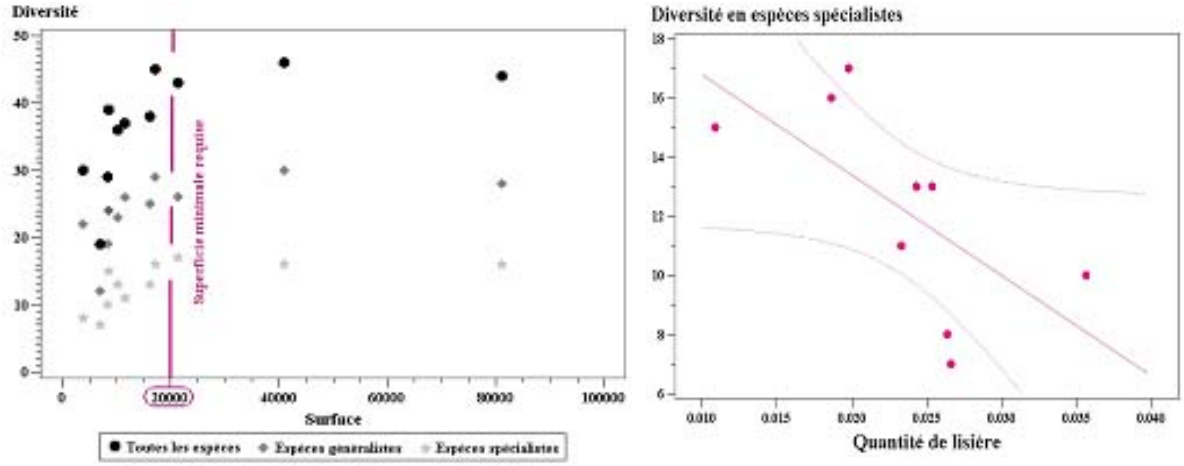

Fig. 19 *(a) Community, generalist and specialist diversity in relation to patch size. Two hectares is the ideal patch area that conservation managers should stride to attain. This is the minimal surface providing habitat for a maximum number of butterfly species. (b) Specialist species diversity decreases significantly with the amount of edge vegetation.*

3 USE OF BUTTERFLY COMMUNITY DATA TO ILLUSTRATE A NEW TECHNIQUE FOR ORDERING ASYMMETRICAL THREE-DIMENSIONAL DATA SETS IN ECOLOGY

The aim of this part of the study is to tackle the problem which arises from asymmetrical cubes formed by two crossed factors fixed by the experimenter (factor#A and factor#B, e.g. sites and dates) and a factor which is not controlled for (the species). The entries of this cube are densities in species. We approach this kind of data by the comparison of patterns, that is to say by analyzing first the effect of factor#B on the species-factor#A pattern and second the effect of factor#A on the species-factor#B pattern. The analysis of patterns instead of individual responses requires a correspondence analysis. We use a method we call Foucart's correspondence analysis to coordinate the correspondence analyses of several independent matrices of species * factor#A (resp. B) type, corresponding to each modality of factor#B (resp.A). Such coordination makes it possible to evaluate the effect of factor#B (resp.A) on the species-factor#A (resp.B) pattern. The results obtained by such a procedure are much more insightful than those resulting from classical single correspondence analysis applied to the global matrix which is obtained by simply unrolling the data cube, juxtaposing for example the individual species * factor#A matrices through modalities of factor#B. This is because a single global correspondence analysis combines three effects of factors in a way that cannot be determined from factorial maps (factor#A, factor#B and factor#A * factor#B interaction) whereas the applications of Foucart's correspondence analysis clearly discriminate two different issues.

We illustrate that this technique proves to be particularly powerful in the analyses of ecological convergence which include several distinct data sets and in the analyses of spatio-temporal variations in species distributions by using the transect butterfly data collected in 2003, 2004 and 2005. We studied (i) the differences in the site-species pattern during the three years (we evaluate whether the pattern of differences in species composition among sites remains steady with time); and (ii) the differences in the year-species pattern among the fifteen sites (we test whether the dynamics of species composition is similar in all sites). We found a common pattern for the three years with some discrepancies. We found that some sites are characterized by high temporal variations whereas others were more stable than on average. This may be related to patch size and / or conservation management efforts during the study period.

B. POPULATION LEVEL

At the population level focal butterfly species were selected 1) to monitor the dynamics of demography and dispersal and to model these dynamics within structured population models and 2) to investigate the genetic structure of calcareous grassland butterfly species.

1 REGULAR AND FREQUENT TRANSECTS CORRECTLY ESTIMATE POPULATION ABUNDANCE

Butterflies are often used as model species to evaluate the diversity of a region. Estimating the population sizes of endangered butterflies is of vital importance to their conservation. Transects, counting the number of individuals along a fixed path, enable the evaluation of the abundance of a particular species. When they are carried out during several years, transects enables us to evaluate the evolution of the abundance of the species in question. This can then be used to evaluate for example the impact of climate change on the long term, or the effects of particular conservation efforts on the short term. Transect counts are much more efficient than capture-mark-recapture (CMR) techniques in that they necessitate less intensive sampling. Although there does not yet exist a consensus on the ideal number of transects during a butterfly's flight season, they are at the most carried out once every week. On the other hand, when one carries out a CMR study, sites have to be visited as often as possible, and ideally every day. Additionally, marking every encountered butterfly and detailing supplementary information (sex, behaviour, age, …) is much more time-consuming than counting the number of individuals encountered. Many important conservation organizations, such as Butterfly Conservation (United Kindom) and Vlinderstichting (The Netherlands) use transect counts to evaluate the diversity and abundance of their butterflies. They implicitly make the assumption that transect counts are highly correlated to the census population sizes. Here we aim at confirming this relation statistically. Therefore, both transect and CMR data were collected.

To obtain transect data, butterflies were sampled by conducting a standardized line transect method (Pollard, 1977). All butterflies were recorded within a ten meter width zone along the transect routes in suitable weather conditions (Pollard, 1977). Butterflies were identified in flight and if not possible were caught with a net and released immediately after identification. Each site was visited once every two weeks from April to September during the 2003 field season. In this study, only the sites of Abannets, Montagne au Buis, Fondry des Chiens, Roche à Lomme and Tienne Breumont are compared with data collected though the CMR method for the butterfly species *Polyomatus coridon*. To obtain the abundance the numbers of individual

butterflies observed during each transect were summed. This sum was then standardized between sites by taking into account 1) the number of transects during the butterfly's flight period and 2) the length of the transects. This was then multiplied by the area of each site.

CMR data were collected during the entire flight period (end of June to beginning of September) by visiting sites as often as possible (weather permitting). Every encountered imago was individually marked with a permanent pen and immediately released. For each (re)capture, the following data were recorded: tag number, sex, age (estimated though wings wear), date and hour, and site and patch.

A very strong correlation between butterfly abundance based on transect data and CMR based population size estimates is observed (Fig. 20). This suggests that transect counts correctly reflect population sizes, but only when the number of visits is frequent enough. Indeed, in this study between four and six transect counts were carried out during the butterfly's flight season. It is also important that at least one transect count falls during the peak of the butterfly's population size. It should also be mentioned that transect based abundance values can not be used per se, but that they are only useful when comparing with other such abundances. Therefore, transect based abundances are very useful to follow the trend of butterfly population abundances. They are especially recommended for biodiversity hot spots, where several species should be monitored at the same time. To conclude, transect counts (when carried out at a sufficiently high frequency) are a valuable tool for conservation managers. They are not too labor-intensive and give a good indication, in comparison with other populations, of the population size.

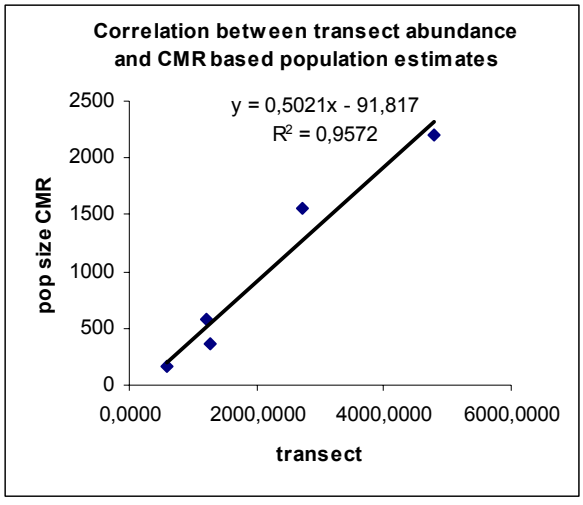

Fig. 20 *Correlation between transect abundance and CMR based population estimates.*

2 POSITIVE GENETIC DIVERSITY- FITNESS CORRELATIONS NOT LIMITED TO SMALL, ISOLATED POPULATIONS

Theory predicts that loss of genetic diversity generates adverse consequences on fitness, and subsequently on demography, in small, isolated populations, which is indeed confirmed by empirical evidence (e.g. Saccheri *et al*., 1998; Madsen *et al*., 1999). Here we demonstrate this effect in a well-connected natural butterfly metapopulation with high population densities. We show that lower genetic diversity was coupled to a sharp decrease in mean adult lifetime expectancy, a key component of individual fitness. Our findings indicate that large and well-connected populations are not immune to the negative effects of inbreeding. Our results also suggest that the ratio of effective over census population sizes may be much lower than previous studies have suggested.

So far, the interaction between genetic diversity and fitness has only been revealed when effective population size becomes low. Although for most species the effective population size remains unknown (Frankham, 1995), previous experimental results suggest that the census population size (N) is about twice as large as the effective population size (Ne). Consequently, rule of thumb conservation measures, such as the maintenance of populations of 50/500 individuals to hinder inbreeding effects, have been applied. The fact that we detect a high correlation between genetic diversity and fitness (and no correlation between genetic diversity and census population size) in large well-connected populations suggests that census population sizes may be tens of times as large as effective population sizes. This may be particularly true at the edge of a species distribution where increased inter-patch habitat quality variation leads to a disparity in the number of reproducing individuals, which can then result in a skewed N/ Ne ratio.

We simultaneously carried out a demographic (Capture-Mark-Recapture, CMR) and a genetic study in a metapopulation of the chalk-hill blue butterfly *Polyommatus coridon* situated at the Northern edge of the species European distribution range (Fig. 21). Five populations were studied during the entire 2003 flight season. Demographic parameter estimates were obtained for each population by analysing the huge CMR data set (7228 captures of 2789 individuals) using Jolly-Seber type model selection with MARK software (procedure nearly identical from the one described in details with references in Schtickzelle *et al*., 2002). Sexes were pooled to enable comparisons with the genetic results. Daily lifetime expectancy (LTE_t) was computed from a virtual life table constructed from estimates of daily survival rate (φ_t) using formula:

$$LTE_t = \sum_{t=1}^{k} S_t \Big/ S_t$$

with $S_t = S_{t-1} \cdot \varphi_{t-1}$ and S_1 being an arbitrarily fixed initial population size. The mean *LTE* of butterflies at birth was computed by combining values of LTE_t and daily number of births (B_t) using formula:

$$LTE = \sum_{t=1}^{k} LTE_t \cdot B_t \Big/ \sum_{t=1}^{k} B_t$$

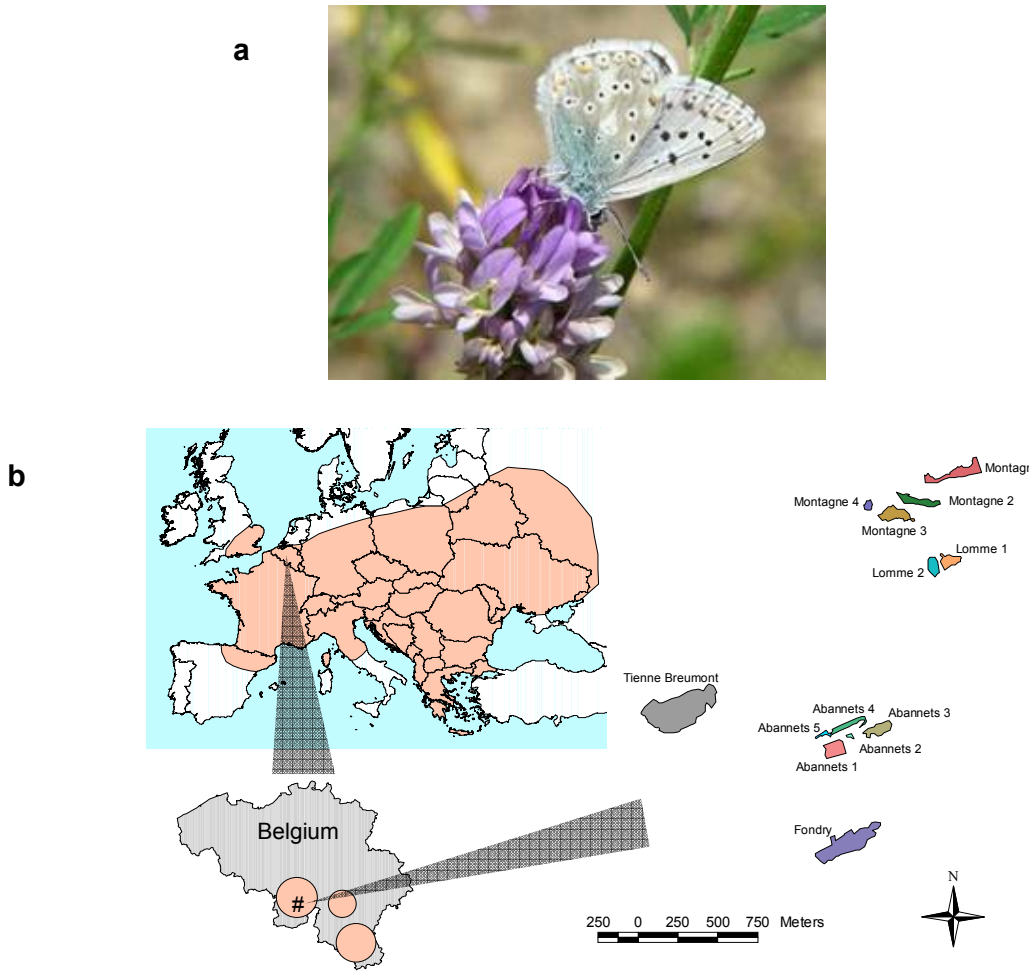

Fig. 21 *(a) photo of P. coridon, (b) geographic distribution range of P. coridon in Europe, distribution and location of studied calcareous grasslands in Belgium, and limit of patches studied.*

A total of 144 individuals (between 25 and 31 per population) were collected using a non-invasive sampling technique and analysed using dominant neutral markers, i.e. intersample sequence repeat (ISSR). Predictably, preliminary trials with microsatellites failed (Neve and Meglecz, 2000) and we subsequently chose a highly

polymorphic molecular marker which did not require much DNA (i.e. non-invasive). The successful application of this type of molecular marker is of vital importance in conservation biology where more time- and cost-efficient options enable the study and conservation of a larger number of species. To evaluate genetic diversity, expected heterozygosity (calculated from the null allele frequency and based on Hardy-Weinberg equilibrium, with allele frequencies calculated from null homozygote frequencies assuming panmixia and corrected for dominance) and average gene diversity (mean number of pairwise haplotype differences) were estimated based on 34 unambiguously scorable and polymorphic loci.

Statistical significance of the *He*-*LTE* correlation (Fig. 22) was assessed from the distribution of Pearson correlation under the hypothesis of no correlation obtained by simulation: the observed *He* values for each locus were randomly shuffled between sites (five groups of 34 values) and the mean computed for each site; one value of *LTE* per site was randomly generated using variance-covariance matrices of φ_t and B_t, and the five values shuffled between sites; the *He*-*LTE* correlation between site means was then computed. This procedure was repeated 50000 times to construct the distribution and the *p* value classically computed as the probability of correlation higher or equal to the observed one. Despite the uncertainty on both *He* and *LTE* estimates, the probability of obtaining a correlation as high as 0.995 is only 0.0002.

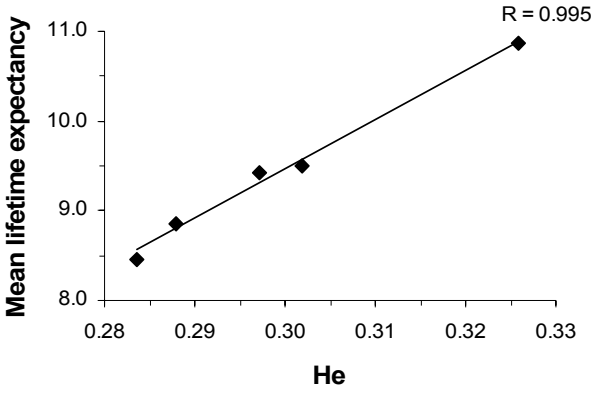

***Fig. 22 Fitness dramatic decrease**. Butterflies from local populations with the highest genetic diversity have a mean lifetime expectancy ca. 25% higher.*

Expected heterozygosity and gene diversity were significantly correlated (R^2 = 0.9892, < 0.0001) even though the former is based on allele frequencies and the latter on haplotype identity. Both expected heterozygosity and gene diversity were positively and significantly correlated to average life expectancy (R^2 = 0.9898, p = 0.0005 and R^2 = 0.94513, p = 0.0153). Average adult life expectancy is a major component of individual fitness in this species since both males and females can

mate more than once, and hence a longer life span will allow a greater number of matings. Moreover, females are single-egg layers and consequently the number of eggs laid is highly dependent on life span. These results signify that positive genetic diversity – fitness correlations are certainly not limited to small and isolated populations.

Neither population size (or log of population size) nor habitat area (or log of patch area) were significantly correlated with expected heterozygosity or gene diversity. Although it is generally accepted that genetic variation is related to population size and area (Frankham, 1996), their independence at range margins has been observed in other species (Rowe *et al*., 1999; Krauss *et al*., 2004b). A likely explanation for a skewed N/ Ne ratio in *P. coridon* is the increase in inter-patch habitat quality variation near the range margins of the species. This variation in habitat quality may lead to a disparity in the number of reproducing individuals, which consequently affects the Ne. The absence of a significant correlation between population size / area and genetic diversity accentuates all the more our result that census population sizes are likely to be a great deal larger than effective population sizes.

It is generally accepted that inbreeding depression contributes to extinction risk in most wild populations of naturally outbreeding species through a decrease in individual fitness (Frankham, 1995). However, so far this has only been demonstrated in a few studies focusing on small (census population size) low-density populations. A significant relationship between genetic diversity and mean adult lifetime expectancy shows that large high-density populations interconnected by frequent dispersal events (consistent dispersal rate of 3%) are not sheltered from inbreeding depression. Additionally, the absence of a significant correlation between genetic diversity and population size suggests that the effective population sizes of the studied populations is only a tiny fraction of the census population sizes. Consequently, conservation guidelines should not be blindly based on census populations sizes as this example clearly demonstrates.

3 POPULATION DEMOGRAPHY, DISPERSAL AND POPULATION GENETICS OF *POLYOMMATUS CORIDON*

The chalk-hill blue *Polyommatus coridon* (Lycaenidae, Lepidoptera) (Fig. 21) is one of the six most characteristic butterfly species of European calcareous grasslands (Van Swaay, 2002), where it can reach very high densities (Bink, 1992). It is however relatively rare outside of these favorable habitats. Its unique larval foodplant,

Hippocrepis comosa L. (Fabaceae), has exacting requirements and is also a species in need of conservation (Asher *et al*., 2001).

Lepidoptera are often considered to be effective umbrella species (New, 1997) for biodiversity conservation. Consequently, conservation guidelines obtained from the study of this butterfly species should benefit the fauna and flora of the calcareous grasslands in general. Additionally, quite a few genetic population studies have been carried out on *P. coridon* at different scales, i.e. local (Krauss *et al*., 2004b), regional (Schmitt *et al*., 2002) and continental (Schmitt *et al*., 2002) where decreasing genetic diversity and decreasing gene flow were respectively detected in populations of smaller size and landscapes with a higher degree of fragmentation. Habitat isolation and quality appear to contribute to population occurrence and density of this species only when fragmentation is extreme (Krauss *et al*., 2004b). These large-scale studies have demonstrated that *P. coridon* clearly suffers from habitat destruction and fragmentation. However, to our knowledge, no studies have concentrated on the demographic and dispersal processes important to the population dynamics of this species. A better understanding of local factors influencing the processes responsible for the spatial distribution and density of a species will enable the effective application of conservation measures.

We studied five *P. coridon* sites (Montagne au Buis, Roche à Lomme, Tienne Breumont, les Abbanets and Fondry des Chiens, further subdivided into patches (Fig. 21)) using the capture-mark-recapture (CMR) method to estimate various demographic (survival, population size) and dispersal parameters. In addition, the same populations were analyzed with genetic markers in order to 1) confront demographic and genetic population structures (for example, observed versus effective dispersal) and 2) to evaluate the long-term viability of this group of populations in relation to their genetic diversity.

During the entire flight period (end of June to beginning of September), sites were visited as often as possible (weather permitting). Every encountered imago was individually marked with a permanent pen and immediately released. For each (re)capture, the following data were recorded: tag number, sex, age (estimated though wings wear), date and hour, and site and patch. Demographic analyses of CMR data were carried out as mentioned above. Dispersal was measured at the "patch" level (Fig. 21). We calculated emigration and immigration rates as follows: $E_i = ER_i/(SPR_i + ER_i)$ where E_i is the emigration rate, ER_i the number of recapture events involving an emigration movement from patch i to another patch, and SPR_i the total number of same-patch recaptures in patch i; $I_i = IR_i/(SPR_i + IR_i)$ where I_i is the immigration rate and IR_i the number of recapture events involving an immigration

movement to patch i from another patch. Philopatry was calculated as the percentage of individuals that were uniquely recaptured within their natal patch.

Intersample sequence repeat (ISSR) markers (Zietkiewicz *et al*., 1994) were used to describe the genetic population structure of *L. coridon* in this study. DNA was extracted using the same phenol-chloroform protocol as the one described in Vandewoestijne and Baguette (2002). Similar PCR and revelation techniques were applied as those in Vandewoestijne and Baguette (2002). To evaluate genetic diversity, expected heterozygosity (calculated from the null allele frequency and based on Hardy-Weinberg equilibrium, with allele frequencies calculated from null homozygote frequencies assuming panmixia and corrected for dominance) and average gene diversity (mean number of pairwise haplotype differences) were estimated based on 34 unambiguously scorable and polymorphic loci. A Mantel test was used to assess the association between Nei's unbiased (1978) genetic distance matrix and the geographical distance matrix. An UPGMA (i.e. unweighted pair-group method using an arithmetic average) tree was constructed based on Nei's unbiased (1978) genetic distance, to further examine the relationship between geographic and genetic distances. Bootstrap values were obtained by performing 1000 permutations.

The following predictive variables were calculated to explain the variations in population size, dispersal rates, and genetic diversity: area, isolation, edge length, bare rock abundance, soil depth, one variable describing microclimatic and topographic conditions, abundance of host plant, and abundance of nectar sources.

Daily survival and catchability are likely to differ between sexes due to the patrolling behaviour of males. Indeed, males constantly patrolling in search of receptive females are more easily caught and predated than females. Population sizes increased with increasing vegetation height around the patch perimeter, increasing area and more xeric, drier, hotter and rockier microclimatic conditions. Surprisingly host plant abundance was never an explicative variable. For conservation purposes it may therefore be more important to concentrate on rehabilitating a certain type of microhabitat, in this case warm, dry and rocky, rather than focussing on maximising host plant abundance for example. In this case, favourable microhabitat for *P. coridon* is also typical *H. comosa* habitat.

At the very local scale, we observed no differences in dispersal measurements (total distance moved, distance moved per day) between *P. coridon* males and females. However, males were significantly more often recaptured outside their natal patch than females were (82% versus 47%), i.e. males are less philopatric than females are. The resident rate of *P. coridon* is surprisingly high (89.38%) compared with

those of *Aporia crataegi* (58%) and *Melanargia galathea* (64%) but similar to that of *Cupido minimus* (91%) (Baguette et al. 2000).

Little or no genetic differentiation and no effect of isolation by distance were detected in this study. These results indicate either 1) that a few dispersal events are amply sufficient to maintain high gene flow at the landscape scale and/ or 2) that the sampled sites once formed a unique or highly connected group of populations and that large population sizes have limited genetic drift and subsequent genetic differentiation.

4 DISPERSAL, DISTRIBUTION PATTERNS AND POPULATION STRUCTURE IN THE BUTTERFLY *MELANARGIA GALATHEA*

Fig. 23 *Melanargia galathea*

Dispersal is a key ecological process linking metapopulation dynamics in the landscape to distribution patterns at larger spatial scales. In this study, we investigated the distribution pattern and genetic population structure of the butterfly *Melanargia galathea* (Fig. 23). Several landscapes differing in composition and structure were sampled as well as populations at different spatial scales. We found that *M. galathea* occupied 91.3 % of all habitat patches available within a particular landscape, probably due to a dominance of landscape scale processes such as rescue effect and recolonisation. A high level of genetic polymorphism within the sampled populations and a very low amount of genetic differentiation between populations was observed (G_{st} = 0.034), characteristic of species with high dispersal capacity and/or high density. High dispersal rates ensured considerable gene mixing at the landscape scale while the influence of distance on dispersal success was detected at the regional and continental scales by a significant amount of isolation by distance. We also found that, at the landscape scale, the dispersal of this butterfly species was influenced by the spatial distribution of its habitat patches.

5 FURTHER RESEARCH

Statistical analyses on the second focal butterfly species, *Cupido minimus*, are still under way. We are also in the process of integrating CMR data from 1996. Indeed,

during the summer of 1996, *C. minimus* was studied in three of the chosen sites (Montagne au Buis, Abannets and Fondry des Chiens). These sites have undergone major management actions and have, amongst others, greatly increased in size. Comparing data from 1996 with current data will enable us to determine the effects of the conservation management efforts during this time period. Populations of the third focal butterfly species, *Colias alfacariensis,* were only present in two of the sites within the study area, even though the species was recaptured in several sites. No demographic analyses will be performed on this data. Rather we will concentrate on the dispersal movements observed.

V. PLANT-BUTTERFLY BIOTIC INTERACTIONS

Fig. 24 *Cupido minimus* on its host plant *Anthyllis vulneraria*

Many species of higher trophic levels depend for their survival and reproduction on the presence of one specific plant species. Both on theoretical and empirical grounds it has been suggested that species of higher trophic levels may be more affected by habitat fragmentation than species of lower trophic levels (Zabel and Tscharntke, 1998; Holt *et al.*, 1999; Steffan-Dewenter and Tscharntke, 2002). The butterfly *Cupido minimus* is a habitat specialist of calcareous grasslands (Van Swaay, 2002). Its unique larval hostplant is *Anthyllis vulneraria* (Fig. 24). Here we studied how this specialized insect herbivore *C. minimus* is affected by the fragmentation of the populations of its host plant *A. vulneraria* and how the butterfly reacts on changes in host plant population size. More specifically we studied how *C. mimimus* reacted on the collapse of the *A. vulneraria* populations in the study area as a result of the extremely hot and dry summer of 2003.

The same 20 populations sampled to study the genetic structure of the *Anthyllis* populations (see III.B.1.2; Fig. 8) were surveyed for this part of the research. These populations cover a wide gradient of the number of flowering *A. vulneraria* plants. All populations were able to survive the extremely hot and dry summer of 2003, but population size was largely affected (Table 14). On average, population size in 2004 was reduced to about a quarter of the 2003 population size. In 6 populations less than 10 flowering individuals remained. In the following year most populations could recover, except for the smallest populations (Table 14).

Table 14 *Overview of the 20 studied Anthyllis vulneraria populations.*

Population	Connectivity	Area	Population size *Anthyllis vulneraria*			Population size *Cupido minimus*		
		(ha)	2003	2004	2005	2003	2004	2005
Tienne aux Boulis	4.15	0.81	22	23	16	56	33	15
Chalaine	4.24	0.70	46	-	52	160	-	0
Roche Trouée 1	4.36	0.90	130	35	700	228	296	511
Tienne Breumont	10.00	8.03	130	38	4198	656	412	4198
Les Abannets 1	6.68	1.09	90	17	129	936	128	286
Les Abannets 2	5.99	0.99	300	82	269	1515	637	89
Montagne aux Buis 1	2.33	1.71	27	8	1	49	0	0
Montagne aux Buis 2	3.09	1.02	18	8	4	2	10	0
Tienne Delvaux	1.08	0.86	250	58	273	950		0
Contieneau	0.93	0.59	40	23	-	56	25	-
Roche Trouée 2	4.36	0.21	40	19	8	100	201	0
Fondry des Chiens	7.44	3.65	500000	413	12000	250000	2180	0
Petit tienne Breumont	5.10	0.41	10	6	0	9	37	0
Batilleuille 1	1.88	0.03	38	8	26	61	31	0
Batilleuille 2	3.00	0.11	7	8	31	9	6	0
Ancient Carrière	1.73	8.46	700	210	310	3129	1912	333
Rosières	3.74	0.53	21	1	1	71	0	0
Carrière St-Anne	3.80	0.25	40	28	60	116	59	94
Les Roches	2.26	2.15	35	21	45	170	66	0
Tienne de Couvin	0.28	0.33	50	35	300	30	96	0

In each of the selected *A. vulneraria* populations also the presence of the butterfly *C. mimimus* was studied. A random selection of 20 *A. vulneraria* plants within each population was investigated for the presence of eggs of this butterfly, and the number of eggs present was counted. Total *C. minimus* population size was consequently estimated as the average number of eggs per plant multiplied by the proportion of plants with eggs in the subsample and the host plant population size.

In 2003 *C. minimus* was present in each of the surveyed *A. vulneraria* populations, and hence the butterfly distribution pattern perfectly reflected that of its host plant (Table 14). The proportion of flowering individuals with eggs varied between 5% and 85% of the surveyed plants (mean 54%). The average number of eggs per plant varied between 1 and 10 (mean 3.8). Patches which were regularly grazed and consequently were characterized by a low vegetation with a limited cover of the competitive grass species *Brachypodium pinnatum* proportionally had a higher number of plants with eggs. Moreover, these plants carried more eggs. This was also the case for large *A. vulneraria* populations (Fig. 25): large *A. vulneraria* populations harbored significantly larger *C. minimus* populations.

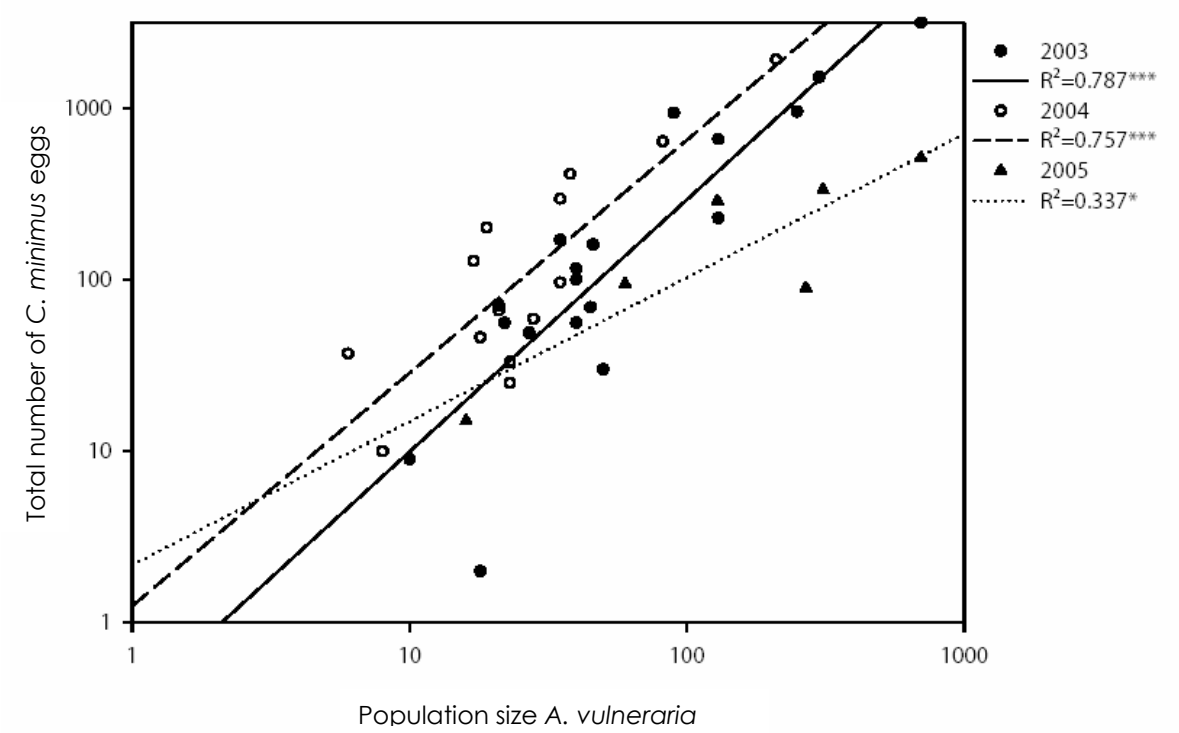

Fig. 25 *Relation between number of eggs of C. minimus per patch and A. vulneraria population size.*

Butterfly populations in fragments with small *A. vulneraria* populations turned out to be most sensitive to extinction. After 3 years of study, 12 of the 20 butterfly populations initially present had gone extinct. These were all present in calcareous grasslands which initially contained small *A. vulneraria* populations (Table 14).

Consequently, it can be concluded that the small, very specialized *C. minimus* populations are extremely sensitive to extinction. The presence and abundance of *C. minimus* is primarily affected by its host plant population size and the management of the calcareous grassland fragments. A collapse in host plant population size as a result of extreme climatological events is immediately reflected in an even stronger decline of the butterfly in the study area. No relation was found between the distances between *A. vulneraria* populations and the local extinction of *C. minimus*.

The importance of host plant population size and management indicate that the prime action to protect and conserve *C. minimus* is to increase *A. vulneraria* population size through adequate management. Grazing is the most appropriate management type to conserve the calcareous grassland flora as a whole. However, specifically for the conservation of *C. minimus*, grazing immediately before or during the flowering period of *A. vulneraria* should certainly be avoided, especially in small populations. In larger populations at least part of the *A. vulneraria* population should

be shielded form grazing during flowering. Furthermore, it is important to increase the number of *A. vulneraria* populations through calcareous grassland restoration.

VI. CONSERVATION GUIDELINES

Our results indicate significant consequences of calcareous grassland fragmentation both on plant and butterfly species (Table 15). Especially reduced patch area resulted in important effects on both species groups. At the community level, specialist plant and butterfly species richness in particular was significantly reduced by decreasing calcareous grassland fragment area. Effects on generalist plant species were very limited, while generalist butterfly species did show significant area and, to a lesser extent, isolation effects.

Table 15 *Overview of fragmentation effects*

	Response to decreased fragment size	**Response to increased isolation**
Community		
Plants		
Species richness	- -	0
# Specialist species	- - -	-
# Generalist species	-	0
Butterflies		
Species richness	- - -	- -
# Specialist species	- - -	- - -
# Generalist species	- - -	-
Individual species		
Plants		
Anthyllis vulneraria		
Genetic diversity	-	0
Population size	-	0
Globularia bisnagarica		
Genetic diversity	0	- -
Population size	0	0
Butterflies		
Polyomatus coridon		
Genetic diversity	0	0
Population size	- -	0
Melanargia galathea		
Genetic diversity	not studied	- -
Cupido minimus		
Population size	- (host plant population)	0

At the individual species level, significant differences in fragmentation sensitivity could be observed, both for plant and butterfly species. Again, for most species studied area effects turned out to be most pronounced. However, the study of

fragmentation on plant-biotic interactions, with the example of the butterfly *Cupido minimus* and its single host plant *Anthyllis vulneraria*, indicated that in this case the main determinant for presence and abundance of the butterfly was host plant population size, which again is largely affected by calcareous grassland fragmentation.

Although isolation effects are more limited than area effects, our results pointed at the importance of management, especially grazing or mowing, in increasing propagule flow, and consequently also gene flow, between calcareous fragments by exchanging grazing animals or mowing equipment between calcareous grassland sites. Moreover, the significant effects of habitat quality on both plant and butterfly species further indicate the importance of adequate management to preserve the environmental conditions typical for calcareous grasslands.

Both plant and butterfly species show strong area effects. These effects hold both at the community and at the individual species level (Table 15). Therefore, the first priority to assure conservation of species rich calcareous grassland is to increase grassland area (Fig. 26). The absence of an extinction debt indicates that species react very quickly to changes in patch area. Hence, further area reduction will result in the subsequent loss of characteristic calcareous grassland species. Small populations are indeed much more vulnerable to demographic, stochastic and genetic stochasticity (Shaffer, 1981; Pimm *et al*., 1988; Holsinger, 2000), and consequently to extinction. Consequently, future area losses should certainly be prevented.

However, in many situations enlargement of remaining calcareous grassland patches is not a realistic option. Therefore, the second priority is to increase grassland connectivity through connecting isolated grasslands by intermediate grassland strips (Fig. 26). This results in a decreased isolation of the remaining grasslands. As isolation effects were present both for plant and butterfly species, although less important than area effects, a reduced isolation of the remaining grassland patches can enhance gene flow between populations to increase species fitness and, in the long term, species survival.

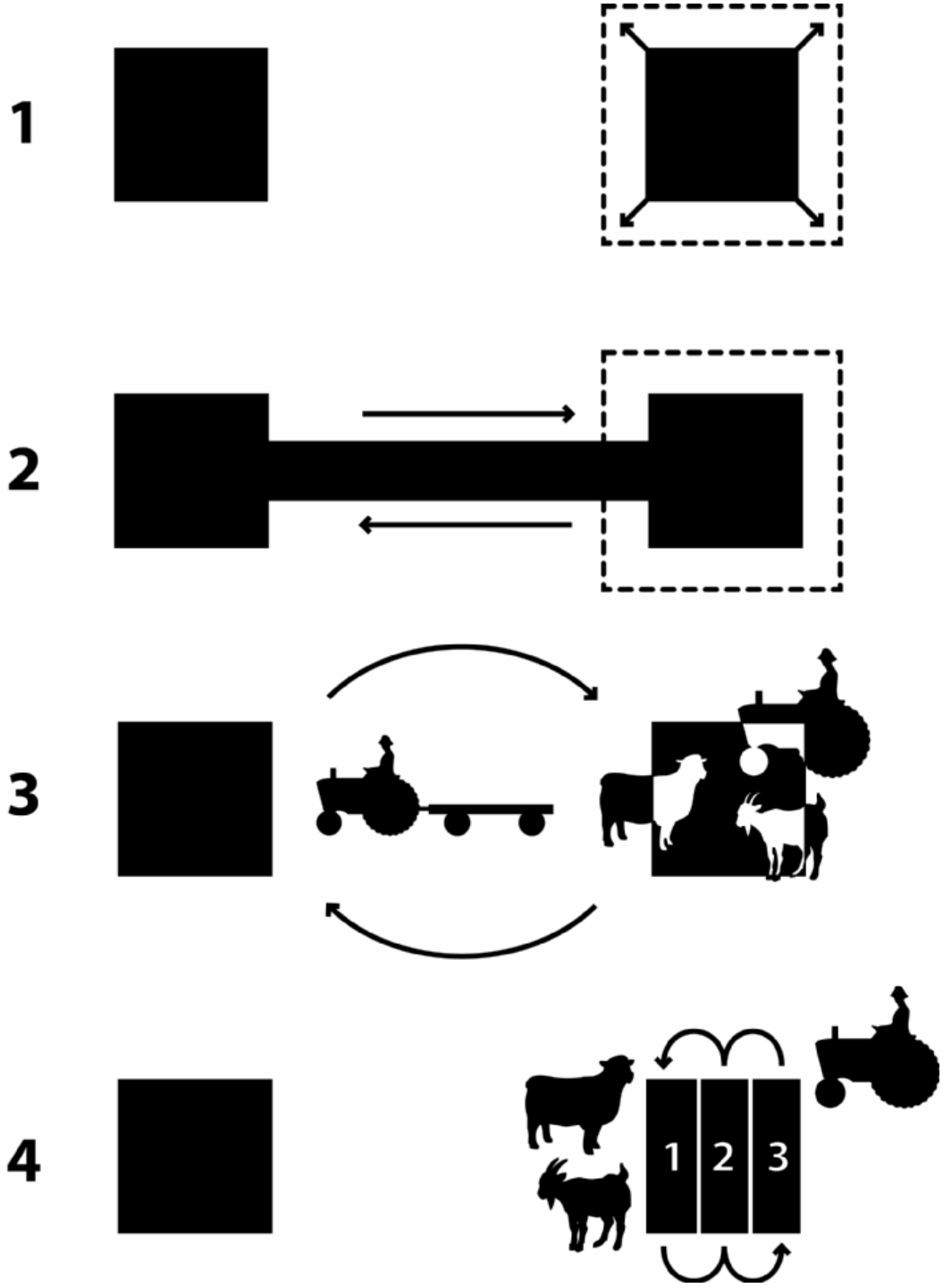

Fig. 26 *Priority actions to undertake to assure calcareous grassland conservation: (1) enlargement of patch area; (2) connecting isolated calcareous grassland patches by grassland strips; (3) exchange grazing animals or mowing equipment between isolated grassland patches; (4) rotation of management within fragments (maximum of 1/3 of the area managed each year).*

The much larger area of calcareous grassland present in the past indicates that suitable abiotic conditions for calcareous grassland were certainly present in the area. Hence, enlargement of calcareous grassland fragments or connecting isolated

fragments with grassland strips through calcareous grassland recreation on former grassland sites by using an adequate restoration management is probably feasible in many cases. Especially for calcareous grasslands surrounded by forest (either afforested sites or sites where grasslands spontaneously evolved to forest through succession) or scrub, enlargement of the grassland fragment is relatively easy to achieve, at least when legal or ownership impediments do not obstruct this option. Our results showed that on scrub vegetations, resulting from the abandonment of calcareous grassland, at least up to 15 years old, calcareous grassland recreation is possible through regeneration from the seed bank (Bossuyt *et al*., 2005; part III A3). Also many other studies indicate that at least up to 20 years after reforestation grassland recreation relying on the seed bank should be achievable (Dutoit and Allard, 1995; Milberg, 1995; Bakker et al., 1996; Maccherini and De Dominicis, 2003). However, when these remnants are situated in agricultural areas, surrounding abiotic conditions probably are too different, and consequently calcareous grassland is more difficult to restore. Also for fragments located in the vicinity of built up areas this option is not feasible.

When intermediate land use makes both options unfeasible, a third priority is to interconnect the remaining grasslands by exchanging grazing animals among the different grassland fragments (Fig. 26). These animals can consequently act as propagule transporters between the different sites, as the high isolation of the different patches often hampers natural dispersal and colonization. This can also be achieved by mowing, in which the mowing machinery can act as dispersal vector. As a result, population extinction is prevented by the addition of new individuals, a process called rescue effect. Furthermore, pollen or seed dispersal from other patches increases genetic diversity, thereby increasing population fitness and consequently population survival in the long run.

As mentioned above, calcareous grasslands need adequate management to be able to sustain. Therefore, it should be stressed that when grasslands are enlarged or connectivity is increased, it is also necessary, next to taking measures to mitigate fragmentation effects, to properly manage the individual grassland patches to conserve calcareous grassland diversity. Most calcareous grasslands, except for the most xerophilous types, situated in the most extreme environmental conditions (very shallow and/or rocky soils, steep southern facing slopes) are semi-natural grasslands. Consequently, they concern a plagioclimax vegetation type where management is necessary to prevent succession to forest or scrub and to prevent dominance of competitive grass species like *Brachypodium pinnatum* or *Bromus erectus*, leading to an excessive built up of litter. Grazing and mowing are the two

most frequently used management types in this kind of grasslands (Duvigneaud and Saintenoy-Simon, 2004).

The best option, when fragments are large enough, is to apply a spatial and temporal rotational management by grazing or mowing (Verbeke and Lejeune, 1996; Delescaille, 2005) and graze or mow a maximum of 1/3 of the area each year (Delescaille *et al.*, 1991) (Fig. 26). This seems to be the best option, both for plant and animal species (Delescaille, 2005). This way habitat heterogeneity is increased, creating optimal habitat conditions for a very broad range of species. Especially for several faunal taxa the presence of zones with a higher or denser vegetation can be important. Species can survive there and recolonize the other parts of the fragment. Furthermore, as it is impossible to determine a grazing period ideal for every species, rotational grazing management enables to create optimal conditions for the largest possible number of species. For example, to conserve *Cupido minimus,* grazing immediately before or during flowering of *Anthyllis vulneraria* can be detrimental because together with the flowerheads, the grazers eat the eggs and larvae of the butterfly. This can consequently result in the extinction of the butterfly in that patch the next year. Additionally, the combination of mowing and grazing often gives the best results (Delescaille, 2005). Grazing effects can be improved by mowing the same part of the patch the following year or after the grazing period to remove the remaining scrub (especially thorny species like e.g. *Crataegus monogyna* or *Prunus spinosa*) and tree seedlings which are not eaten by the grazing animals or to limit excessive *Brachypodium pinnatum* dominance.

The above described conservation priorities relate to the calcareous grassland plant and butterfly community at the whole. Most characteristic species will benefit from these actions. However, since species differ in fragmentation sensitivity and habitat requirements, conservation priorities should be reviewed when the preservation of one specific species is at stake. In this case species-specific measures are needed, taking into consideration the characteristic habitat requirements and fragmentation sensitivity of that species.

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