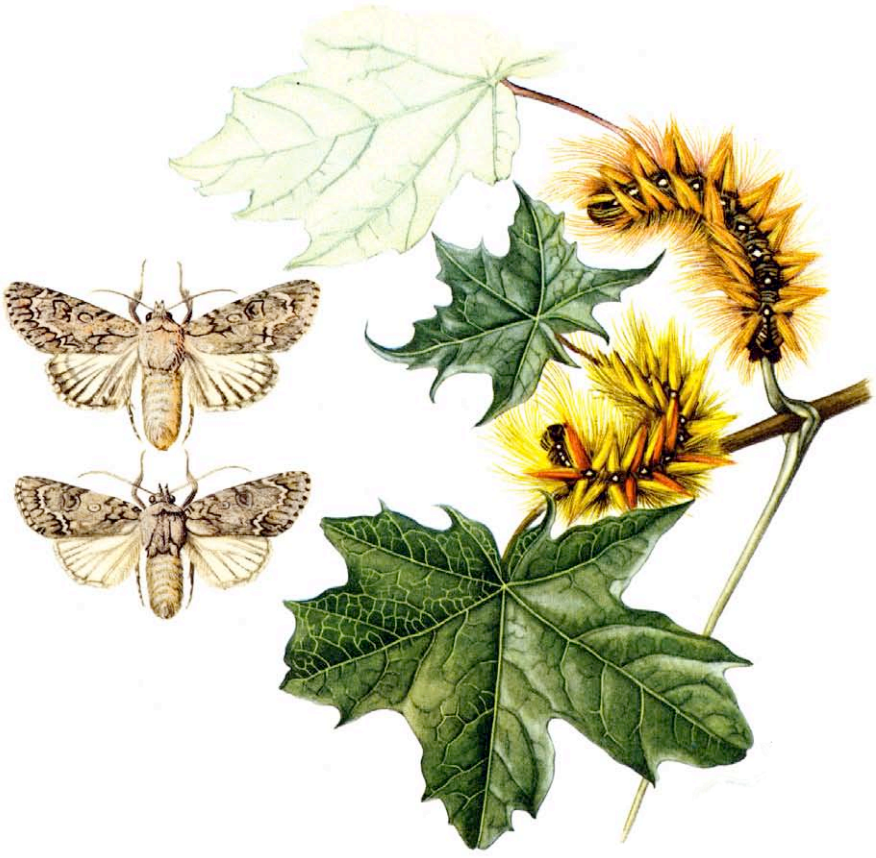
2 Tree phenology, genetic variation, and herbivory



Contents

2.1 Reproductive biology of the main tree species at the canopy crane investigation site

Ophir Tal[1] & Wilfried Morawetz

The reproductive processes of trees are a key component in the forest ecosystem, as they support the persistence of the trees and are the basis for their numerous interactions with other organisms. This chapter presents general findings about the sexual systems, flowering phenology and fruit and seed production of the four dominating tree species in the stand. *Fraxinus excelsior* is polygamous, *Acer* spp. heterodichogamous, *Tilia cordata* andromonoecious, and tree gender correlates with tree size, manifesting an individual based variability. *Fraxinus excelsior* flowers every year in a high intensity and its flowering phenology is sensitive to climate whereas the flowering phenologies of *Acer* spp. and *Tilia cordata* are quite constant in duration but strongly change intensity between years. This variability implies a differentiated pattern of floral resources in space and time. Corresponding to flowering phenology, *Fraxinus excelsior* is also the major fruit producer in the stand, followed by *Tilia cordata*. *Acer pseudoplatanus* is the least productive species. The results demonstrate the high complexity of the reproductive biology of the tree species and the large spatial and temporal variability in resources they present to other organisms. The paper enhances the high conservation value of the Leipzig floodplain forest, and the importance of relating to the individual trees when studying interacting organisms.

INTRODUCTION

Trees are the largest organisms of a forest, and as such they are the basis for most of the biological interactions in it. The reproductive biology of the trees determines on the long range the constitution of the forest and on the short range it is the basis to different biotic interactions around the flowers and fruit (Röhrig & Bartsch 1992).

The LAK project concentrates on studying biological diversity in the forest and the ecological processes in it (Morawetz & Horchler 2003). The diversity of tree species is relatively high, and is considered a basis for the diversity of smaller organisms in the forest. In this chapter, we present some further, intraspecific diversity of the trees that is observed by examining their reproductive biology.

Reproductive biology includes the study of the sexual system, flowering phenology, pollination and fruit and seed production (Richards 1997):

(1) The sexual system of a species concerns the gender distribution of flowers on the plant and their functionality (Richards 1997). The genera *Fraxinus* and *Acer* are both renown for the large diversity of sexual systems they present, and were thus an object for at least ten taxonomical studies each, most of which however in artificial or immature stands, and without canopy accessibility to large trees (e.g. De Jong 1976; Wallander 2001, respectively).

(2) Flowering phenology, especially in early spring, is among the most climate sensitive biological processes (Fitter & Fitter 2002). As such it is strongly connected to microclimate (Stoutjesdijk & Barkman 1992) and may be used to indicate climate change (Roetzer *et al.* 2000). It also has a strong influence on reproductive success and genetic diversity of the offspring (Fox 2003).

(3) Pollination is an intriguing contact zone between plants and insects (Faegri & van der Pijl 1966; Proctor *et al.* 1996). Plants may depend on different insects and in different measures for successful reproduction, or they may rely predominantly on wind pollination (Culley *et al.* 2002). All three studied genera present a diversity of strategies in this respect (Eisenhut 1957; Ogata 1967; De Jong 1994; Fromm 2001; Wallander 2001). Insects depend on floral resources for their own reproductive processes.

(4) Fruit and seed production are the result of the above-mentioned characters, and represent the future growing potential of the trees. At the same time they are a resource for different animals. Masting (fluctuating synchronous fruit production) is found in forest trees (Herrera *et al.* 1998) and is supposed to

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be linked with fluctuating weather (Kelly & Sork 2002), satiation of predators (Ostfeld & Keesing 2000), and wind pollination (Smith *et al.* 1990).

These topics were studied in the last four years in the main tree species in the plot - *Fraxinus excelsior* L. (Oleaceae, common ash, Esche), *Acer pseudoplatanus* L. (Sapindaceae, sycamore maple, Bergahorn), *Tilia cordata* Mill. (Malvaceae, small-leaved lime, Winterlinde), and *Acer platanoides* L. (Norway maple, Spitzahorn), in practically all canopy trees in the plot (Seele and Rohrschneider, this volume).

The aim of this paper is to present the intraspecific variability and overview data on the reproductive characteristics of these species.

MATERIALS AND METHODS

The study site is the investigation plot of the Leipzig Canopy Crane, located at the edge of the Leipzig floodplain forest (see details in Morawetz & Horchler 2003). The crane enables access to ca. 1.6 ha of canopy, up to the height of 32 m. Most canopy trees of the studied species were inspected during 2002 to 2005 (Table 1) and their gender distributions, flowering phenologies, pollination levels, insect in inflorescences and fruit and seed production were recorded and analysed (Tal 2006). Data of tree height and stem diameter was taken from Seele (2004) and the crown area (included in the canopy's upper surface) was measured by outlining the tree crowns on an aerial photo. Data on individual trees is available from the corresponding author. Table 1 presents the number of studied trees after study year and topic.

F. excelsior is a polygamous species, trees were categorised as: **(1)** Males, with no or less than 1% hermaphrodite flowers. **(2)** Male-biased hermaphrodites with mostly male flowers but more than 10% hermaphrodite flowers (with large anthers and small but functional pistils). **(3)** Balanced hermaphrodites with all flowers hermaphrodite (balanced morphology). **(4)** Female-biased hermaphrodites with reduced but mostly functional anthers and large pistil. **(5)** Females with almost no stamens (when existing rudimentary).

Inflorescences of *A. pseudoplatanus* and *A. platanoides* usually include male and female flowers but in anthesis only one gender is presented at a time, synchronously in the tree (De Jong 1976; Cruden 1988). Trees are either protandrous (male then female, commonly followed by a second male phase), protogynous (female then male), or male.

Tilia cordata is considered hermaphrodite (Pigott 1991).

The intensity of flowering for each tree was categorised after the proportion of buds with inflorescences as full (> 50%, usually over 80%), partial (10–50%, usually around 30%), scant (less than 10%, usually 1–5%, on single twigs), or no flowering. We gave efforts to scrutinize each tree both during flowering and at fruit ripening.

Fruit amount was quantitatively assessed by direct counting of infructescences or twigs in a part of the crown and assessing the number of subunits in the whole canopy (in large trees subunits were counted at two levels). The estimated error is 50%, i.e. an assessment of total fruit on a tree of 100 000 means the actual number is between 50 000 and 200 000 (Tal 2003). Samples of ripe fruits were checked for seeds (details in Tal 2006).

Table 1 – Number of studied trees after species, year of study and theme. Intensity of flowering and number of fruit include non-flowering and non-fruiting trees, respectively.

	Theme of study			
Species and year	**Tree gender**	**Intensity of flowering**	**Flowering time**	**Number of fruits**
F. excelsior 2002	68	69	38	21
F. excelsior 2003	64	64	48	21
F. excelsior 2004	66	71	63	21
F. excelsior 2005	91	97	90	26
A. platanoides 2004	8	8	8	8
A. platanoides 2005	6	10	1	10
A. pseudoplatanus 2004	53	50	53	47
A. pseudoplatanus 2005	60	65	52	74
T. cordata 2004	9	30	9	30
T. cordata 2005	9	30	9	30

RESULTS

In *F. excelsior*, about a half of the trees in the stand were predominantly male, the other half were mostly different types of hermaphrodite trees, with only 6% of the trees being purely female. Most of *A. pseudoplatanus* were protandrous and most *A. platanoides* were protogynous. *T. cordata* trees had a large proportion of male flowers beside the hermaphrodite flowers, a sexual system termed andromonoecy (Fig. 1).

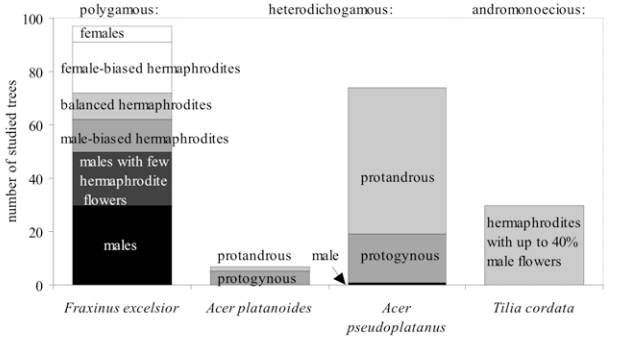

Figure 1 – Gender distribution in the studied species and number of studied trees.

The largest *F. excelsior* trees in the stand were male. Males (and male-biased hermaphrodites) were significantly larger than other hermaphrodites and females (10% in stem diameter, t-test p = 0.03 and 3% in tree height, Mann-Whitney rank sum test, p = 0.007) among the larger canopy trees (stem diameter greater than 40 cm, tree height greater than 28 m, 68% of all canopy trees). However, in respect to all canopy trees, there was no significant difference between the gender groups (Mann-Whitney rank sum test, p = 0.22 for stem diameter, p = 0.10 for tree height, in total 97 trees).

However, taking only canopy trees with stem diameter larger than 30cm, no significant difference was found (t tests for stem diameter and tree height p=0.2 and 0.4 respectively). Protogynous *A. pseudoplatanus* trees were larger than protandrous trees (25% in stem diameter, t-test p = 0.007; 10% in tree height, Mann-Whitney rank sum test p = 0.045, 18 protogynous, 50 protandrous trees). However, taking only canopy trees with stem diameter larger than 30 cm, no significant difference was found (t-tests for stem diameter and tree height; p = 0.2 and 0.4 respectively).

F. excelsior flowered in a constant high intensity in four years whereas the other three species fluctuate in the years of study (Fig. 2). 80% of *F. excelsior* trees at least flowered fully. All male trees flowered fully every year, whereas some of the fruit producing trees flowered at a low intensity in some of the years. 2004 was a full flowering year for the other three species, whereas 2005 a weak flowering year for them. The *Acer* species suffered increased herbivore damage (mainly caused by aphids) to the flowers in 2005, the year of weak flowering.

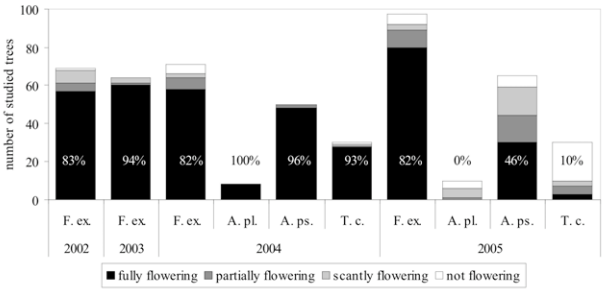

Figure 2 – Flowering intensities of the studied species. The number of trees is separated after four intensity levels, and the percent of fully flowering trees from total is noted. F. ex.: *Fraxinus excelsior*, A. pl.: *Acer platanoides*, A. ps.: *Acer pseudoplatanus*; T. c.: *Tilia cordata*

T. cordata trees flowering in 2005 were significantly higher and with thicker stems than trees not flowering in 2005 (Mann-Whitney rank sum test, $p < 0.001$, $p < 0.001$ respectively, 35 trees). The large flowering trees are all in the northern part of the plot, whereas the central and southern parts of the plot had exclusively small non-flowering *T. cordata*.

Flowering duration was most variable in the early flowering *F. excelsior* and quite constant in the later flowering *Acer* spp. and *T. cordata*. The former flowered in the end of March to mid April during 3–8 weeks in different years, *A. platanoides* in mid April for three weeks, *A. pseudoplatanus* in May for 4–5 weeks and *T. cordata* in mid June to mid July for 3–4 weeks in different years.

Table 2 – Correlations of fruit production per tree and tree dimensions (Pearson product moment coefficients); ns: not significant, *: p<0.05, **: p<0.01, ***: p<0.001.

Species	Correlated crop	Tree height	Stem diameter	Crown area
Fraxinus excelsior	maximal per tree	ns	ns	0.44 *
Acer pseudoplatanus	2004	0.32 *	0.38 *	0.55 ***
Tilia cordata	2004	0.52 **	0.58 **	0.76 ***

F. excelsior produced the largest number of fruit in the plot. Both the annual seed production and the year-to-year constancy were superior to *A. pseudoplatanus* and *T. cordata*. The latter followed in fruit quantity, and *A. pseudoplatanus* produced the smallest crop, with a large proportion of seedless fruit (Fig. 3).

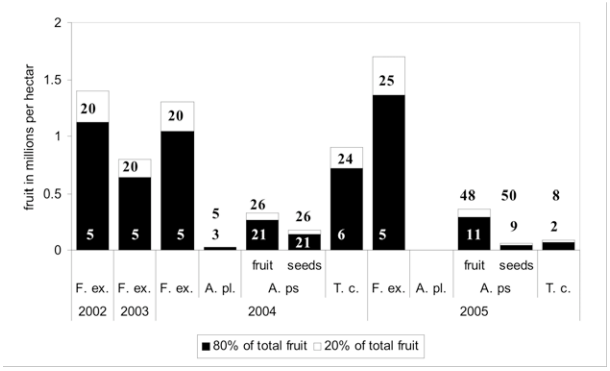

Figure 3 – Overall fruit production in the stand in millions per hectare for studied years and species. The lower numbers are the number of trees producing 80% of total fruit (trees with greatest number of fruit) and the upper numbers are of trees producing the rest 20%. F. ex.: *Fraxinus excelsior*, A. pl.: *Acer platanoides*, A. ps.: *Acer pseudoplatanus*; T. c.: *Tilia cordata*

In the major fruit producers, *F. excelsior* and *T. cordata*, the crop was produced by a small proportion of the trees. In these species the number of seeds is equal to the number of fruit, whereas in the least productive *A. pseudoplatanus* less than one seed per fruit are produced.

Large fruit production is correlated with tree size, and is especially well explained by crown area, as presented in Table 2.

DISCUSSION

This paper presents the characteristics of reproductive biology of the main species in the stand, encompassing ca. 200 canopy trees (Table 1) and applying intensive observations using the high canopy accessibility provided by the LAK crane. The results exemplify the large interspecific diversity of the species in the forest, implying a high conservation value (Primack 2002, Brown *et al.* 1997). Some of the complexity arises from the mature status of the forest, thus underlining its value (Brown *et al.* 1997).

The gender distributions of *F. excelsior* and *Acer* spp. (Fig. 1) are similar to those described by Wallander (2001) and DeJong (1974) respectively, whereas andromonoecy in *T. cordata* is described for the first time (Pigott 1991; Fromm 2001), cryptic andromonoecy was reported for the close relative *T. japonica* by Ito & Kikuzawa (1999) and Ito (2002).

The correlation of tree gender types with vegetative characteristics (Table 2, see also Parolin *et al.*, this volume) as well as their effects on sex-dependent biotic interactions (Wardle 1961; Hargasim 1977; Delph 1999; Verdú 2004; Tal 2006), and possibly on variability in vegetative tissue in respect to herbivores (Årgen *et al.* 1999; Verdú *et al.* 2004; Ruhnke *et al.* this volume) implies that tree gender should be taken into account in the study of inter- and intraspecific variability in biotic interactions.

The flowering and fruiting of *F. excelsior* in a more or less constant intensity and constant high flowering intensity of males (Fig. 2) contrast other studies of *F. excelsior* which report a two-year cycle (Tapper 1996; Wallander 2001), and may result from the maturity of the forest. The contrast between wind pollinated species producing a large crop every year and insect pollinated species producing fluctuating crop (Fig. 3) counters the idea that masting is associated with wind pollination (Kelly & Sork 2002). Fluctuating flowering intensity may have strong effects on flower visitors as well as on flower and fruit foragers (Ostfeld & Keesing 2000), especially if these are not very mobile or if these phenomena are synchronised in the forest.

The flowering duration in *F. excelsior* is the most variable, reflecting the sensitivity of this early flowering species to macro- and microclimate. Flowering duration in *Acer* spp. is relatively constant and may be related to its heterodichogamy, which requires synchronisation within and among trees (Renner 2001). Flowering duration has important implications to the degree of synchronicity between individual trees and thus to the gene flow in the population (Willson & Burley 1983; Primack & Kang 1989) which are discussed for *F. excelsior* and *A. pseudoplatanus* in Tal (2006). The finding that in *F. excelsior* and *T. cordata* most yield is produced by the few largest trees in the stand has implications both to the genetic variability of the stand and demonstrates the importance of the struggle for space among canopy trees (Frech *et al.* 2003).

F. excelsior is the most prolific fruit producer in the plot (Fig. 3). Both the maximal fruit production and the constancy of yield are much larger than the ones of the other species. *A. pseudoplatanus* produces a lower fruit crop and a still lower seed crop than *F. excelsior* and *T. cordata*, whereas its seedlings are the most abundant on the forest floor (Schöne & Jentsch, this volume). This indicates that vegetative competition is more important than reproductive competition in determining the species composition of the stand.

ACKNOWLEDGEMENTS

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2.2 Phenotypical and genetic variation of Fraxinus excelsior L. at the LAK site

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In the forest of the "Leipzig Canopy Crane Project" (LAK), the common ash (*Fraxinus excelsior*) is the dominant tree species. This ash population is most likely the result of different phases of plantings after 1800. In the LAK study plot, 64 adult individuals exceeding 22 m in height and a large number of seedlings and saplings show a high phenotypic variation. In the present study, the genetic variation of the semi-natural population of *F. excelsior* is analysed. The main questions are whether phenotypic variation is reflected by a genotypic variation, and how large the genetic variability of the population is. The establishment of microsatellite and AFLP markers for analyses of the LAK ash population is described as well as first results. One main result is that the ash population possesses an astonishingly high level of genetic variation within this population, especially regarding the putative origin and strong selection of this forest during the last centuries. Because of this high variability in microsatellite markers all individuals analysed in this first screening could be identified via individual DNA fingerprinting. After a first test of utility of microsatellite and AFLP markers a screening of a set of 77 individuals of the LAK plot containing 220 samples is in progress. Additionally to the comparison of the geno- and phenotypic variability of the ash population – especially in floral phenology – in future studies it is planned to compare this semi-natural with a natural population, and based on the high genetic variability, investigate the role of somatic mutations in genetic diversity.

INTRODUCTION

The ash trees in the Leipzig Canopy Crane Project (LAK) are the dominant species in the tree canopy (see Seele and Rohrschneider, this volume). They show great phenotypical variation: the sex distribution and floral phenology of *Fraxinus excelsior* was studied in detail by O. Tal within the context of a master thesis (Tal 2003 and this volume). Results of this work show that among the 64 adult individuals (> 22 m in height), a large variation in the development of floral sex (female, male, androgynous, female-androgynous, male-androgynous) was predominant (Fig. 1, 2). Even considering various levels – single flowers, florescence, tree, subpopulation or stand – a high variability was revealed and interesting distribution patterns emerged; but these appear to be typical for ashes (Binggeli & Power 1991, Wallander 2001). As Darwin wrote in 1877: "As far as the sexual relations of flowers are concerned, Linnaeus long ago divided them into hermaphrodite, monoecious, dioecious, and polygamous species. This fundamental distinction, with the aid of several subdivisions in each of the four classes, will serve my purpose; but the classification is artificial, and the groups often pass into one another". This last case also applies for the common ash. Functionally, trees have a defined sex; yet morphologically they are characterised by many deviations.

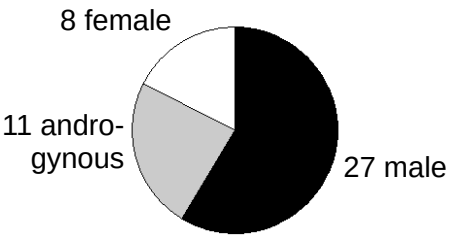

Figure 1 – Quantitative distribution of flower gender in the analysed LAK ash population (Tal 2003).

On the other hand, it is as yet unknown how large the genetic variability of the investigated population is. As early as the 19^{th} century, *F. excelsior* was not an uncommon species in Leipzig (Gläser 2001), but little is known about the origin of the LAK and neighbouring forest. The ash trees of the LAK may possibly have been planted: diameter and height distribution reveal two groups (Fig. 3, 4; Seele, this volume), one

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group with individuals which are approx. 100–200 years old, and a second with 50–60 year-old trees. 200 years ago, the ash tree was presumably rare in the Leipzig floodplain forest (Gläser 2001). Being promoted (directly by planting and indirectly by river adjustment), most of the trees probably reproduced from local populations, as well as domestic gardens (Gläser pers. comm.).

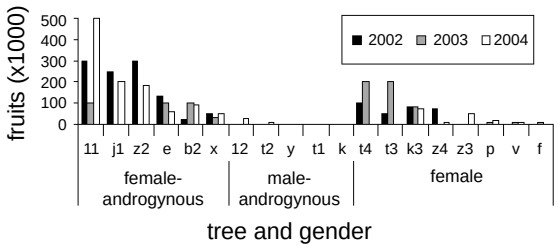

Figure 2 – Sex of the tree, defined by the produced fruits; mean of the years 2002–2004 from fruits of 19 ash trees (in thousand) (Tal 2003).

Further, there is one adult and five younger trees of *F. pennsylvanica* in the investigated floodplain plot, being planted and introduced from the USA, which also possibly reproduce. This could lead to a hybridisation between the species, which is however improbable according to Wallander (pers. comm.), since *F. excelsior* and *F. pennsylvanica* belong to two different sections of the genus (Wallander in preparation).

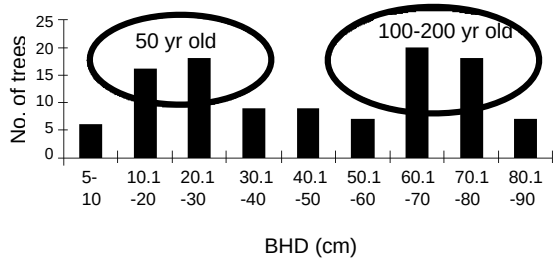

Figure 3 – Distribution of diameter at breast height (dbh) of the LAK trees: two phases of planting become evident (Seele 2004; see also this volume).

However, membership of various sections of a species or various genera must not be an exclusion criterion for hybridisation events, as examples of natural hybrids in Asteraceae (examples: Molloy 1995, Okada *et al.* 1997), Caricaceae (van Droogenbroeck *et al.* 2004) Brassicaceae (Heenan 1999) or Orchidaceae (Abele *et al.* 2005) have shown. "Consideration of Proposals for Amendments of Appendices I and II"[2] alone shows a multitude of intergeneric hybrids in *Cattleya*, *Cymbidium*, *Dendrobium*, *Oncidium*, and *Vanda* (all Orchidaceae).

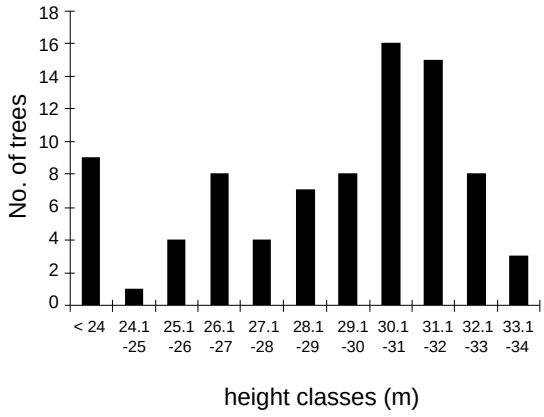

Figure 4 – Height distribution of the LAK trees (Seele 2004; see also this volume).

The aim of the current investigations is to examine the genetic variability of the LAK ash population. For this purpose, the genetic differences within the overall adult population are first investigated and the genotypic and phenotypic variations (Tal 2003) were compared. The *F. excelsior* population in the LAK was examined on the basis of a combination of microsatellite markers (SSR: simple sequence repeat) (Litt & Luty 1989, Weber & May 1989, Jeandroz *et al.* 1995, 1996; Brachet *et al.* 1999, Lefort *et al.* 1999a, b, Morand-Prieur *et al.* 2002) and amplification fragment length polymorphisms (AFLP markers) (Vos *et al.* 1995). In order to correlate genotypical and phenotypical variations, phenological and floral-ecological data from the master thesis by O. Tal (2003) were included.

MATERIALS AND METHODS

Plant material

In order to establish microsatellite and AFLP markers for population genetic estimations of the ash trees in the LAK, a total of 25 leaf samples were collected from September until November 2003. This set comprises 11 individuals of *F. excelsior* from the Leipzig floodplain forest experimental plot, as well as one individual of *F. pennsylvanica* from the LAK plot. In order to check whether the populations from various regions can be distinguished using this marker type, five individuals from the Hamburg area were included in this set. This involved two adult trees and three young trees from a presumably planted private garden in Hamburg Othmarschen. In addition, the reproducibility of the markers was checked

[2] www.cites.org/eng/cop/12/prop/E12-P51.pdf

via double sampling from 8 ash individuals from the LAK. The characteristics (dbh, tree height, position coordinates, origin, etc.) of the sampled individuals are indicated in Table 1. From this set, the four *F. excelsior* 2312/j, HHd, 1101/b and 2101/j2 were used for establishing the AFLP method for estimations of *Fraxinus* populations (see Table 1).

Genetic analyses

The molecular marker method for population analyses on *Fraxinus* was established at the Plön Max-Planck-Institute for Limnology and at the Biocenter Klein Flottbek at the University of Hamburg. A time-consuming development of microsatellite markers was avoided, since microsatellite primers for analyses on *Fraxinus* have already been published (Lefort *et al.* 1999a, b; Morand-Prieur *et al.* 2002). The methods for the outstanding investigations were optimised according to Rudolph 2001. For the AFLP analyses, suitable combinations of restriction enzymes and primers were selected. Here, various primer combinations first had to be tested for their usability. In order to optimise the SSR marker analysis, initial molecular biological examinations of the ash tree population were carried out on the basis of a set of 25 leaf samples (Table 1).

DNA isolation

Fresh ash leaves were dried using silica gel. The DNA isolation was carried out according to Dumolin *et al.* (1995) with some modifications. This protocol based on the CTAB method by Doyle & Doyle (1990). For the extraction, approx. 50 mg of leaf material was used. Each sample was ground to a fine powder in a Retsch mill at a frequence of 30/sec for 2 min.

The samples were lysed at 55 °C for 1 h, shaken gently, in 1 ml extraction buffer (2% (w/v) ATMAB, 1% (v/w) PVP 40 000, 20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl) with 50 mM DTT. After cooling for approx. 10 min at room temperature, a precipitation in 400 μl dichloromethane and adjacent centrifugation at 13 000 rpm, (17949 rzb, Eppendorf centrifuge 5804R) for 10 min took place. To the upper phase 400 μl ice-cold isopropanol was added and centrifuged for 10 min at 13 000 rpm. The pellet was washed with 1 ml 76% ethanol and centrifuged (see above). The cleaned DNA was dried and resuspended overnight at 4 °C in TE buffer containing RNase A (10 μg RNase A/ml TE). RNA was digested at 37 °C for 30 min. The concentration of the extracted DNA was measured via OD determination in a biophotometer (Eppendorf). Working solutions were diluted to 100 ng/μl DNA.

Table 1 – Analysed *Fraxinus excelsior* from the LAK plot and from Hamburg, and one *F. pennsylvanica* in the LAK plot.

No.	Tree	DBH	Tree height	Sample height	Direction	Comment	Date	Site
1	2312/j	76,4	30,50	—	—	—	12.09.2003	LAK
2	3007/b2	43,9	29,40	—	—	—	12.09.2003	LAK
3	3309/p1	37,6	27,00	—	—	*F. pennsylvanica*	12.09.2003	LAK
4	2701/f	70,0	30,40	—	—	—	12.09.2003	LAK
5	HHa	—	—	—	—	old growth	20.10.2003	Hamburg
6	HHb	—	—	—	—	old growth	20.10.2003	Hamburg
7	HHc	—	—	—	—	young 1	20.10.2003	Hamburg
8	HHd	—	—	—	—	young 2	20.10.2003	Hamburg
9	HHe	—	—	—	—	young 3	24.10.2003	Hamburg
10	3208/r1	38,2	30,40	20 m	W	—	03.11.2003	LAK
11	3208/r1	38,2	30,40	29 m	O	—	03.11.2003	LAK
12	4310/l	64,0	28,80	26 m	S	—	03.11.2003	LAK
13	4310/l	64,0	28,80	27 m	NO	1. twig	03.11.2003	LAK
14	2322/i	89,1	32,60	30 m	NW	—	03.11.2003	LAK
15	3210/t	60,5	30,40	30 m	O	—	03.11.2003	LAK
16	1101/b	53,2	31,00	29 m	S	main branch	03.11.2003	LAK
17	1101/b	53,2	31,00	29 m	NO	—	03.11.2003	LAK
18	2101/j2	48,1	29,00	25 m	SO	main branch	03.11.2003	LAK
19	1206/a	79,6	32,50	26 m	SO	side branch	03.11.2003	LAK
20	1206/a	79,6	32,50	29 m	SW	main branch	03.11.2003	LAK
21	2312/j	76,4	30,50	25 m	central	—	03.11.2003	LAK
22	3007/b2	70,0	33,70	30 m	cental	—	03.11.2003	LAK
23	2101/j2	48,1	29,00	20 m	S	twig S	03.11.2003	LAK
24	3219/t3	63,7	31,40	27 m	W	—	03.11.2003	LAK
25	3219/t3	63,7	31,40	27 m	W	fruits	04.11.2003	LAK

SSR screening

The PCR reaction took place in a 10 μl total volume containing 25 ng DNA, 1.5 mM $MgCl_2$, 1x buffer, 2% DMSO, 0.2 mM dNTPs, 0.5 μM Cy-5 labelled forward primer, (Metabion), 0.5 μM reverse primer (Metabion), 2.5 U Biotherm DNA polymerase (Invitek). The sequences of the primers used are described in Table 2. A touchdown PCR was carried out in a PTC 200 gradient cycler (Biozym) with the following program: an initial denaturation at 94 °C for 2 min; followed by cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 30 sec, elongation at 72 °C for 45 sec. The annealing temperature was reduced by 1 °C every second cycle. On reaching the annealing temperature of 55 °C, 20 repetitions were carried out. The reaction was completed with a 10-minute elongation at 72 °C (Rudolph *et al.* 1999, Rudolph 2001). The PCR products were separated on an ALFexpress II (GE Healthcare) using a Reprogel High Resolution Gel (GE Healthcare) in 0.5x TBE. As an internal standard, 71 bp, 140 bp and 300 bp PCR products (Rudolph 2001) were added to the loading buffer (5 mg dextran blue/ml deionised formamide). The samples were mixed 1 : 1 with the loading buffer containing the internal standard and denatured at 94 °C (4 min). From each sample 0.8 to 2 μl were analysed on the automated sequencing gel.

AFLP analyses

For the establishment of AFLP analyses for ash populations, samples from four different ash trees were used. This DNA was applied in four different concentrations. The AFLP analyses were carried out with 100 ng, 250 ng, 500 ng and 1 000 ng DNA respectively. Double restriction was effected in 25 μl total volume with 5 U Pst I and 5 U Tru I (Mse I) (MBI Fermentas) and 1x tango buffer. The samples were incubated for 2 h at 37 °C. For the adapter ligation, adapter mix PstI (Adapter 1 CTCGTAGACTGCGTACATGCA and 20 pM PstI Adapter 2 CATCTGACGCATGT) (Metabion) together with 20 pM Tru I (MseI) (Adapter 1 GACGATGAGTCCTGAG) and TruI (MseI) (Adapter 2 TACTCAGGACTCAT) (Metabion), 5 U T4 ligase (MBI Fermentas) and 1x ligase buffer were used in a total volume of 25 μl. The ligation was incubated at 16 °C for 12 h. For the preamplification, the adapter ligations were diluted 1 : 10 using HPLC water. For the PCR, 5 μl of the adapter ligation was used. The pre-amplification itself was carried in a total volume of 50 μl with the following components: 5 μl template, 1 U Biotherm DNA polymerase (Invitek). 1x PCR buffer, 3 mM $MgCl_2$, 0.1 mM dNTP (Roche); 0.3 μM primer-mix (PstI + X: GACTGCGTACATGCAGX and TruI (MseI) + X: GACGATGAGTCCTGAGTAAX) (Metabion). The PCR was carried out in a PTC 200 gradient cycler (Biozym) as follows: an initial denaturation step at 94 °C for 5 min, followed by 20 cycles with denaturing at 94 °C for 30 sec, annealing at 60 °C for 30 sec, elongation at 72 °C and 1 min, followed by a final elongation step at 72 °C for 10 min. The samples were likewise diluted 1 : 10 using HPLC water and 5 μl was used for the selective PCR. The reaction conditions were: 20 μl total volume with 5 μl template, 0.5 U Biotherm DNA polymerase (Invitek), 1x PCR buffer, 3 mM $MgCl_2$, 0.25 mM dNTP (Roche), 0.6 μM primer mix: PstI + XXX (GACTGCGTACATGCAGXXX) and TruI (MseI) + XX (GACGATGAGTCCTGAGTAAXX) (Metabion). The PCR was carried out in the gradient cycler according to the following touchdown program: an initial denaturation at 94 °C for 5 min, followed by 3 cycles: denaturation at 94 °C for 30 sec, annealing at 65 °C for 30 sec and an elongation at 72 °C for 1 min. The annealing temperature was then reduced for one degree every four cycles, until a final temperature of 56 °C was reached. This cycle was repeated 24 times. The reaction was completed by a 10-minute elongation step at 72 °C. The samples were separated on the ALFexpress, as described for the SSR analyses.

Data analysis

Size determination of the fragment lengths of the AFLPs and microsatellites was carried out using ALFwin Fragment Analyser Version 1.03 (GE Healthcare). The fragment lengths obtained were transferred manually into a binary matrix, whereas the presence of a PCR product was coded as 1 and the absence of a PCR product as 0. In so doing, the assumption was that fragments of identical size correspond to homologous sequences and are not of the same size by coincidence. Dubious PCR products were not taken into account. Although SSR analyses enable a co-dominant evaluation and these should also be evaluated co-dominantly in future, in the initial analyses of these markers a dominant evaluation method was chosen during which every allele of a locus is evaluated as an independent marker.

In order to check the reliability of the SSR markers, for 8 examined individuals, two samples per individual were examined in parallel and distances or, respectively, similarities were calculated in pairs. This matrix was used for cluster algorithms such as UPGMA (unweighted pair group method) were used. For these calculations, the PAUP program, version 4.0b10 for Macintosh (Swofford, D.L. 2002, Sinauer Associated Inc. Publishers, Sunderland, MA, USA) was used in order to produce distance trees.

Relations with the phenological variation

In order to compare the phenotypical and the genetic variation within the ash population of the LAK, the trees were grouped according to phenotypical features. Subsequently these are compared with the genotypical evaluations.

RESULTS

Establishment of SSR markers for analysis of the LAK ash population

In total, 10 microsatellite markers were tested. Seven of these resulted in reproducible PCR products. These microsatellites showed the typical profile of two alleles each per locus. However, more than two bands per marker were also identified.

The number of alleles per SSR locus was between 11 and 19, with an allele diversity (A) of 13.9 (Table 2). Allele frequencies were between 0.016 and 0.319 with an average value of 0.076. Average heterozygocity was 0.54. In a separate analysis of the LAK populations – excluding the Hamburg population and *F. pennsylvanica* – 9 and 19 alleles per locus were scored. A total of 310 polymorphic markers were identified, which could be used for an initial calculation of genetic distances.

Initial population analyses using SSR markers

The UPGMA distance analysis showed that all double samples cluster with one another (Fig. 5, 6). The Hamburg population, however, can be separated from the LAK population only partially. One ash tree from the Leipzig floodplain forest is integrated in the Hamburg cluster, while one ash tree from the Hamburg population shows a smaller distance to the LAK population than to the rest of the Hamburg population. The *F. pennsylvanica* of the LAK is also integrated in the Hamburg population. It cannot be genetically separated from the *F. excelsior* population as a basic group. In total, four clades can be differentiated within this examined set, where the Hamburg ash trees are integrated within the overall population and were not divided basally.

Table 2 – Overview of the SSR primer pairs used. Forward primer was labelled with Cy5 for the detection on an the ALFexpress sequencer. – no reproducible PCR-products (these primer pairs were not analysed further). A: allele diversity, H: mean heterozygosity.

Primer name	Sequence	Reference	A	H
FEMSATL1	forward: 5' AGC AGC ATT TAT GAA TGT TC 3' reverse: 5'ATC AAC TGA AGA TGA CGA CG	Lefort *et al.* 1999a, b	19	0,72
FEMASTL2	forward: 5' TCT TTA TCA TCA AAA AAT AA 3' reverse: 5 TAC AAG GTG ATA TCA CTT CT 3	Lefort *et al.* 1999a, b	12	0,48
FEMSATL4	froward: 5' TTC ATG CTT CTC CGT GTC TC 3' reverse: 5' GCT GTT TCA GGC GTA ATG TG 3'	Lefort *et al.* 1999a, b	13	0,68
FEMSATL5	forward: 5' GGA TTG AGA TTC AAT TTG CA. 3' reverse: 5' TCC GAG TGA TGC CTA CTC TA 3'	Lefort *et al.* 1999a, b	11	0,24
FEMSATL8	forward: 5' TGT AGC TCA GGA TTG GCA AT 3' reverse: 5' AGC GTT GTC CTT AAC CTT TT 3'	Lefort *et al.* 1999a, b	–	–
FEMSATL10	forward: 5' TTG AGC AAC ATG TAA TTA TG 3' reverse: 5' AAA TAT CCG GTG CTT GTG TA 3'	Lefort *et al.* 1999a, b	14	0,16
FEMSATL11	forward: 5' GAT AGC ACT ATG AAC ACA GC 3' reverse: 5' TAG TTC TAC TAC TTC AAG AA 3'	Lefort *et al.* 1999a, b	–	–
FEMSATL12	forward 5' TTT TTG GAA CCC TTG ATT TT 3' reverse: 5' GAT GGA CGG GCA TTC TTA AT 3'	Lefort *et al.* 1999a, b	–	–
FEMSATL16	forward: 5' TTT AAC AGT TAA CTC CCT TC 3' reverse: 5' CAA CAT ACA GCT ACT AAT CA. 3'	Lefort *et al.* 1999a, b	11	0,64
FEMSATL19	forward: 5' CTG TTC AAT CAA AGA TCT CA. 3' reverse: 5' TGC TCG CAT ATG TGC AGA TA 3'	Lefort *et al.* 1999a, b	17	0,88
			Ø 13,86	Ø 0,54

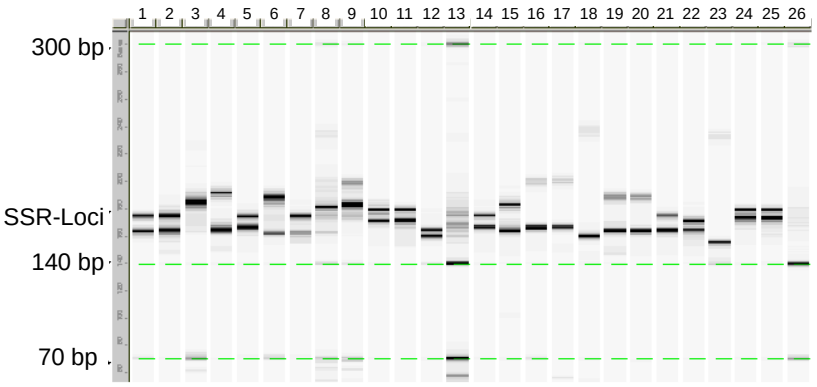

Figure 5 – Cut-out of one microsatellite analysis on an ALFexpress. Samples 1 to 25 see Table 1, green: internal standard 300 bp, 140 bp und 71 bp.

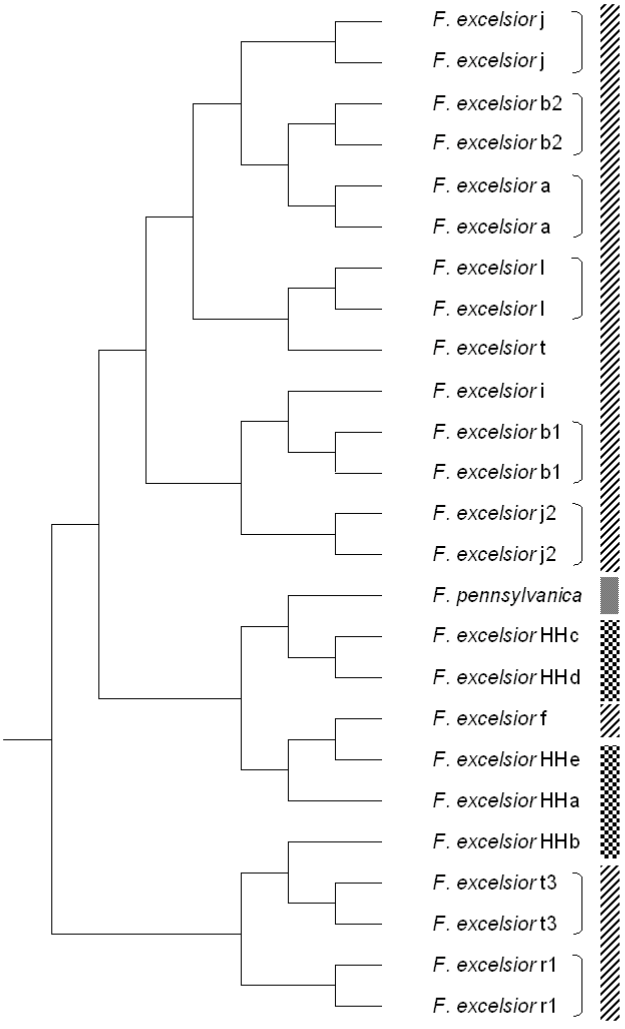

Figure 6 – UPGMA tree calculated using 310 SSR markers: 16 individual trees of *F. excelsior*, fasciated: population of the LAK plot, checkered: samples from Hamburg, black: *F. pennsylvanica*, parentheses show dual samples from one individual.

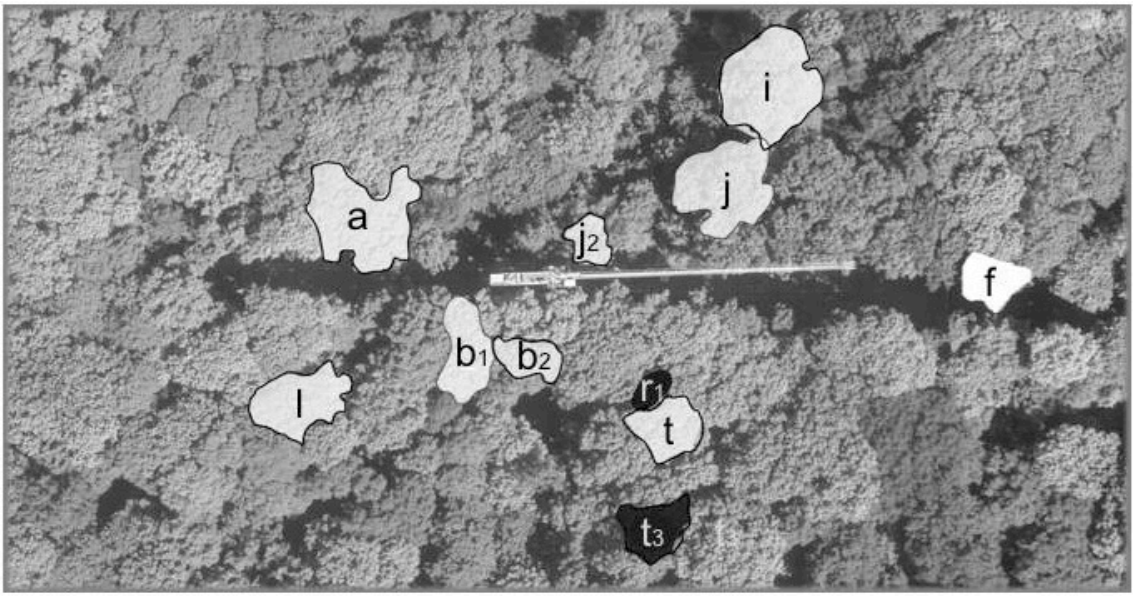

Figure 7 – LAK plot with 64 adult ash trees with more than 22 m height (Tal 2003) combined with results of the UPGMA analysis.

DNA fingerprint

Due to the high genetic variability of the SSR markers in the LAK ash tree population, all trees examined in this set could be identified by individual alleles of one or two SSR primer combinations and thereby were provided with a genetic fingerprint.

Establishment of the AFLP analyses

Since the establishment of AFLP marker analyses for various taxa is, by our experience, more time consuming, a test set consisting of 16 samples was used. This set comprised four individuals (three from the LAK plot and one individual from the Hamburg population) in four different concentrations to identify the optimal template composition and to test the reproducibility and variability of the AFLP marker method within certain parameters. Initial analyses in this test set indicate the same high extent of polymorphism as was already demonstrated in the SSR analyses. Due to the low number of individuals in this test, a population-genetic evaluation of the AFLP markers has been omitted so far.

Connections with phenological variation

Due to the low sample number, no statements concerning the comparison of genotypical and phenotypical groupings could be made so far. Also, no geographical division of the clusters could be found yet (Fig. 7). In this connection, an analysis of a total of 77 ash trees using 130 leaf samples from the LAK plot with microsatellite and AFLP primers is currently underway at the MPI Plön.

DISCUSSION

Genetic variability of the LAK ash tree population

The main questions – a) genetic differences within the overall adult population, b) relations between the genotypical and phenotypical variations, i.e. are there similarities between phenotypical and genetic characteristics of individuals, are there genetic/phenological similarities between the two planting phases – cannot yet be answered using the database examined so far. Only the establishment of two different marker types for the analyses is described here, and the reproducibility of these markers proven via double sampling. All examined ash trees in the LAK could also be provided with a DNA fingerprint by means of SSR markers.

The results presented here, however, provide an initial insight into the astonishingly high genetic variability of the adult semi-natural ash tree population of the LAK. The high genetic diversity can be seen clearly from the high allele diversity as well as from the high value of the average heterozygocity of the SSR markers. More than half of the individuals examined show heterozygous microsatellite loci.

The intention is to examine the overall population, including the regeneration occurring naturally in the

forest as well. Here, evidence of paternity and maternity will be aspired to (Wagner 1996, Rudolph 2001, Ziegenhagen *et al.* 2003). The comparison of adult trees with the regeneration should deliver further information concerning the dispersal strategy of domestic trees. To this end, a combined evaluation of relatedness analyses of young and adult trees, of genetic distances, of geographical data and, in particular, of floral phenology is also to be carried out. For this purpose, a total of 130 leaf samples were collected between September and November 2003, and August and September 2004. Molecular biological analyses are currently being carried out using this sample set.

Influence of invasive species

Furthermore, the significance of the introduced ash species is to be examined. Therefore, 6 individuals from *F. pennsylvanica / F. americana* from the LAK plot were integrated into the sample set described above.

The intention is to investigate the possibility of spreading out of *F. pennsylvanica / F. americana* or even hybridising *F. excelsior* with *F. pennsylvanica* and/or *F. americana*. This should clarify the potential endangering of the species *F. excelsior* by the introduction of putatively invasive species (see also Volk 2002). The spread out of the introduced species and possible displace of *F. excelsior*, or crossbreed with it, is to be investigated. There are no larger-scale plantings of *F. pennsylvanica* to be found in Germany, only in parks and botanic gardens, meaning that supersession by this species appears to have been of no significance so far. On the other hand, in the case of *F. angustifolia* near Darmstadt, repression of *F. excelsior* (C. Niers, pers. comm.) resulted after seed was obtained from southern Europe. Later on it was noticed that it was a different species. With the establishment set of 25 samples used up to now, the integrated *F. pennsylvanica* could not be basally separated from *F. excelsior*. In this case, further investigations with a larger number of individuals need to be described.

Planning comparison of the LAK with natural populations

The plan is to complete a comparison with a natural ash tree population, in view of genetic diversity in planted and natural tree populations. Suitable locations for natural stands exist, for example, in the Storman and Lauenburg regions (Poppendieck, pers. comm.). Analyses using the establishment set have so far been unable to separate the Hamburg population from the LAK population completely; this could also not be expected due to the low sample number. 20 to 40 individuals therefore need to be sampled when further natural locations are analysed.

Do somatic mutations increase the genetic variability of the ash tree population?

Leipzig Canopy Crane Project provides us with a golden opportunity to take samples at any random point within the canopy. Particularly in the case of older trees, this makes it possible to investigate genetic differences within a tree and discover somatic mutations. Whitham & Slobodchikoff were able to detect somatic mutations in plants as early as 1981. Moreover, it could be demonstrated that somatic mutations occur more frequently in plants than in animals. Klekowski Jr. & Godfrey (1989) even mention an accumulation of mutations in perennial, long-lived plants and demonstrated this in mangroves. This accumulation of mutations, particularly in the apical meristems, result from an open growth system of plants where most of the mutations within the plant do not directly threaten the plant's life, and are therefore fatal, or necessarily lead to reduced fitness of the plant. Moreover, plants do not have – and this is also not the case with animals – a fixed germline. Flowers, which are responsible for sexual reproduction, develop on the apical meristems. Particularly in plants which predominantly reproduce via inbreeding, or reproduce more or less vegetatively, this higher variability appears to have benefits, for example during adaptation between shoots of one individual (Whitham & Slobodchikoff 1981), in the changing of a population's allele frequencies (Orive 2001) or in plants' defence system and evolution (O'Connell & Ritland 2004).

For morphological mutations of an individual, the mutation rate in this context is about 1 x 10^{-5} per locus. Similar rates are also expected for AFLP markers. The mutation speed of microsatellites is higher by one decimal power, because they tend to cause so-called hot spots of spontaneous mutations due to their repetitive motifs in the DNA sequence. The mutation rate is 1 x 10^{-4} (Frankham *et al.* 2004).

Ash trees live approx. 150 years long and it can be expected that somatic mutations will occur during these periods and therefore accumulate at the apical points. The mutation rate in ash trees during their lifetime is to be calculated in this additional study. On the one hand, it is interesting to see the extent to which these somatic mutations have an effect on the reproducibility of the AFLP and microsatellite markers as marker system for relatedness analyses and DNA fingerprinting. On the other hand, the intention is to use these investigations to clarify whether the high genetic and phenotypical variability can be explained by additional accumulation of somatic mu-

tations. For this reason, in August to September 2004, ten further old trees were sampled with ten leaves each at principal branches located as far apart as possible (the order of branches was noted) within the tree canopy. Somatic mutations within the trees are to be investigated using these samples.

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2.3 Leaf fall in the Leipzig riparian forest

Anja Homscheid & Peter J. Horchler[1]

SHORT COMMUNICATION

All trees shed their leaves or needles in order to rejuvenate photosynthetic tissue. In temperate deciduous forest this process is cyclic and strongly linked to the seasonal climate change. Collecting falling leaves in a systematic way allows to calculate a rough estimate of the forests annual net primary production of leaf mass. From October 2003 to February 2004 we collected litter fall (leaves, fruits and twigs) in an area of 5 500 m^2 by means of 79 litter traps (buckets) with an total area of 5.58 m^2. The collected material was air-dried at 35 °C for two days. All leaves and fruits were sorted according to the species they belonged and were counted and weighed subsequently.

The overall leaf mass we obtained was 2.32 kg which corresponds to 4.16 tons per hectare. The number of collected leaves amounted for 9 467 (16 965 950 / ha). Fruit mass was 160 g (0.3 t / ha). Most leaves fell between mid October and mid November with some differences between species. The sequence was *Tilia cordata*, *Fraxinus excelsior*, followed by *Carpinus betulus*, and finally *Quercus robur*.

The value obtained for the overall leaf mass is similar to other values. But we believe that it has been underestimated because a notable number of leaves were shed during the extraordinarily dry and warm summer 2003. Hence, the relatively high leaf mass of supposedly more than 4.2 tons per ha reflects the high productivity of the Leipzig forest.

Since it is known that the net primary production of plant biomass changes from year to year, more litter collections would be useful.

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Table 1 – Comparison of leaf mass from different forest sites.

Source	LAK (this study)	Sitte *et al.* (2002)	Ellenberg (1986)	Körner (pers. comm)
Forest type	Mixed deciduous	Mixed deciduous	Beech	Mixed deciduous
Leaf mass (t/ha)	4.16 +	4.0	3.72 (S.D. 0.4)	3.0

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2.4 Spatial patterns of folivory at Acer pseudoplatanus L. in the Leipzig forest canopy

Jan Mitscherling & Peter J. Horchler[1]

Herbivory is a key process in most ecosystems and is functionally linking ecosystem compartments and trophic levels across space and time. Yet, this process is insufficiently understood. We conducted two studies addressing folivory, i.e. leaf tissue 'eaten' by herbivorous insects, in a temperate, mixed deciduous forest. One study aimed to assess stand-level folivory, i.e. the overall proportion of herbivory in a forest, the second, more intensive study, dealt with spatial aspects of folivory at Sycamore Maple (*Acer pseudoplatanus* L.). In both studies leaves (n = 570/2 913) were collected in October 2002/2003, using a canopy crane system. Folivory was measured by scanning the leaves and 'counting' the pixels of the leaf area removed by herbivorous insects using a graphical software. The results are: *First study*: Stand-level folivory in 2002 amounted for 1.13% (Median = 0.21%) with a very high variation (SD = 2.55%). The distribution of folivory rates to area classes revealed an extremely right-skewed distribution, i.e. most leaves did not show any or only minor damage by folivory. *Second study*: The overall degree of folivory was 1.71% (Median = 0.59%, SD = 2.84%). The frequency distribution of the degree of folivory to area classes is also extremely right-skewed. The degree of folivory was significantly different between most (73%) of the individual trees. Significant overall differences of folivory could be detected (tested by GLM) between understorey and canopy as well as between midstorey and canopy. The differences between understorey and midstorey were not significant. The highest degrees of folivory were found at upper canopy leaves. However analysing individual trees resulted in highly contrasting patterns. The results are discussed.

INTRODUCTION

Herbivory is a key process in almost all ecosystems (e.g. Schowalter 2000) but its controlling factors are still poorly understood. Herbivores are primary consumers that feed on the primary food resource, green organic matter. At the same time herbivores serve as prey for higher order consumers like spiders, parasites, birds, and bats. But they also nourish the forest soil and its organisms by dropping leaves, green frass and faeces (Rinker & Lowman 2004). Hence, the process herbivory links ecosystem compartments as well as trophic levels across space and time. Especially in forests where the access to the biggest part, the canopy, is strongly restricted, herbivory is insufficiently studied. Most studies performed so far were case studies with a sampling at one moment in time (Rinker & Lowman 2004). Yet, it is known that herbivory varies considerably in space and time (Lowman 1992). Hence, clearly long-term studies are needed for a better understanding of this process. Herbivory studies also offer a peculiar chance because they easily enable to gain insight to an important ecosystem process while many other processes remain difficult to study because patience and/or expensive equipment is needed.

We conducted a study on the herbivory in a central European mixed deciduous forest using a crane system to access the canopy (Morawetz & Horchler 2003). In two different approaches we studied **(1)** stand-level herbivory and **(2)** herbivory at one tree species. We selected *Acer pseudoplatanus* L. since this is the most frequent and abundant tree species in the study area. We decided to study folivory, i.e. leaf damages by herbivorous insects since this can easily be done in a quantitative way.

Besides addressing the question of the overall, so called stand-level folivory, we concentrated on its spatial distribution. The research question were: **(1)** What is the overall degree of folivory in the forest? **(2)** Are there differences in the degree of folivory between different woody species? **(3)** Are there differences in the degree of folivory at different heights in the canopy? **(4)** What is the overall degree of folivory at *Acer pseudoplatanus*? **(5)** Are there differences in the degree of folivory between the individuals of *A. pseudoplatanus*? **(6)** Are there differences in the

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degree of folivory at *A. pseudoplatanus* at different heights in the canopy?

MATERIALS AND METHODS

Field work

To study stand-level folivory, we followed a proposal for a standard protocol developed by K. Ernest and collaborators (Ernest 2004). We took advantage of the existing canopy crane, which enables to access almost all parts of the forest canopy (Morawetz & Horchler 2003). For spatial reference the area accessible by the crane was subdivided using a three-dimensional coordinate system. This allowed us to assign 100 sampling sites completely at random. Based on this design we collected 570 leaves at 57 sites in the canopy in late summer 2002. Since many sites that had been chosen were empty spaces only collections at 57 sites could be realised. At each collection site 10 leaves were randomly harvested.

For the second study we randomly selected 10 trees of *Acer pseudoplatanus* in the study area. At these trees ca. 100 leaves were collected each at three heights in the canopy (0–3 m, 12–16 m, 25–30 m) summing up to a total of 2 913 leaves. The leaves were collected in October 2003. Light conditions at each collection site were estimated subjectively using a five-score ordinal scale ranging from very shady to very sunny.

Data processing

The collected leaves were scanned with a flatbed scanner in black/white mode (300 dpi) and stored to files readable by a graphical software (Adobe Photoshop™). Thus, the leaf area appears as black area, the area removed by herbivorous insects as white dots. In case of area removed at leaf margins we tried to reconstruct and outline the original margin with a thin black line. With the help of the 'histogram' function of Adobe Photoshop it was possible to count black and white pixels and compare them to the number of pixels of an area (square) with a known size. In this way leaf area and folivory area were measured for each leaf and the percentage of folivory was calculated. Note that only a complete removal of the leaf tissue can be detected by this method. Damages like leaf stippling (German: Fensterfraß), which only comprises a part of the leaf's cross section (parenchyma), cannot be detected in that way.

Data analysis

The data of the first study were analysed taking all 570 samples as one sample supposedly representative for the whole forest stand. For this sample the mean, standard deviation and median of the area removed by herbivores (in cm^2 and %) were calculated. Furthermore the distribution of those values to area classes was represented as histogram.

The same procedure was carried out for all leaf collections specific to the woody species. A graphic representation of folivory at different heights in the forest was done for *Acer pseudoplatanus* since only for this species sufficient leaves were collected. To check for a correlation of folivory at this species to light levels, we could use height specific data of light (PAR) measurements (Horchler, this volume). We plotted folivory rates against relative PAR values and performed a regression analysis.

The data of the second study focussing on *Acer pseudoplatanus* were analysed in a similar way. Additionally, the samples of the 10 tree individuals were analysed separately. For those, as well as for the complete sample, differences in folivory at the three different heights were tested for significance in pair wise comparisons using a Mann-Whitney test as well as a Generalised Linear Model (GLM). Most data were also represented graphically. All analyses were carried out with the software MS Excel™, Analyse-It™(`www.analyse-it.com`) and R (`www.r-project.org`).

RESULTS

The results are presented separately for the two studies.

Study of stand-level folivory

The total area of the collected leaves summed up to 4.74 m^2. The leaf area removed by herbivorous insects was 531.2 cm^2 (0.053 m^2), resulting in an average stand-level degree of herbivory of 1.13% (Median 0.21%) with a very high variation (SD = 2.55%). The most striking feature of the folivory data is that its distribution to area classes was extremely right-skewed, i.e. most leaves did not show any or only minor damage by folivory. There were notable differences between the leaves of different species. *Tilia cordata*, *Fraxinus excelsior*, and *Ulmus* sp. for example showed higher degrees of folivory than *Carpinus betulus* and *Acer pseudoplatanus*.

Only for the most abundant tree species (*Acer pseudoplatanus*) sufficient leaves (194) were collected to analyse differences in folivory at different heights. The result showed higher degrees of folivory at upper canopy leaves except the uppermost outer canopy leafs. Regression analysis (Fig. 1) of these average values of folivory and the average percentage of light showed a rather close and significant positive cor-

relation, except the uppermost outer leaves. This sample consisted of just 10 leaves of one tree and is regarded as statistical outlier since further studies mostly showed high degrees of folivory at canopy leaves of *Acer pseudoplatanus*.

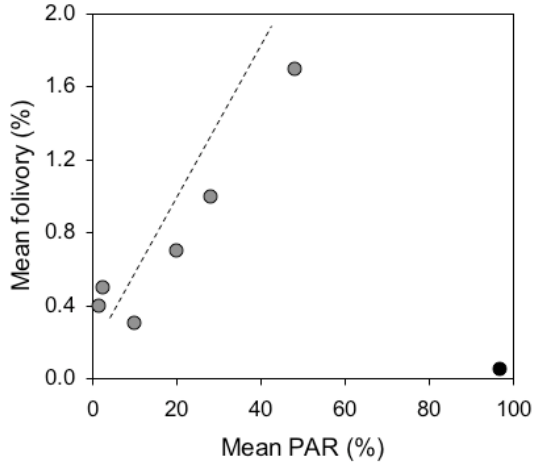

Figure 1 – Mean folivory [%] at leaves of *Acer pseudoplatanus* L. plotted against the mean values of photosynthetically active radiation (PAR) at different heights in the Leipzig Canopy Crane plot (n = 194). The average folivory rates linearly increase up to a medium rate of PAR. The data point at ca. 100% PAR is likely to be a statistical outlier.

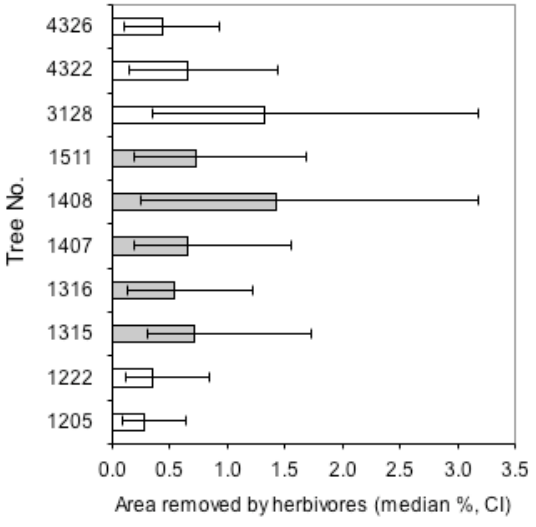

Figure 2 – Folivory rates (median and 95% confidence intervals) at leaves (n = ca. 300) at 10 trees. Note the high variation (CI lines). The grey shaded bars represent the adjacent trees shown in Fig. 4 (see below).

The overall degree of folivory is on average 1.71% (Median = 0.59%). Again the variation is high (SD = 2.84%). The frequency distribution of the degree of folivory to area classes is also extremely right-skewed. The degree of folivory as tested by a Mann-Whitney test was significantly different between most (73%) of the individual trees (Fig. 2). Taking all leaves as one sample supposedly representative for the whole forest plot, significant differences of folivory could be detected (tested by GLM) between the understorey and canopy as well as between the midstorey and canopy. The differences between understorey and midstorey were not significant. The highest degrees of folivory were found at upper canopy leaves (Fig. 3).

Figure 3 – Folivory rates (% removed area, median and 95% confidence intervals) at leaves (n = ca. 1 000 for each level) from different heights in the canopy. A test (GLM) revealed significant differences in folivory rates between understorey and canopy, and midstorey and canopy but no significant difference between understorey and midstorey.

However analysing individual trees resulted in highly contrasting patterns even for trees standing next to each other (Fig. 4). Estimates of light conditions at the sites of leaf collections did not show any correlation to the observed patterns.

DISCUSSION

Stand-level herbivory

Most studies on herbivory in forests were carried out in the understorey. If data from the forest canopy were taken into account they often originated from litter trap collections. Visual estimates of stand-level herbivory in temperate deciduous forests range from 7–10% (e.g. Nielson 1978). Exact measurements by Schowalter *et al.* (1981) lowered the range to 1–5%. Near ground level Bray (1964) found 3–10% of annual losses by herbivore defoliation.

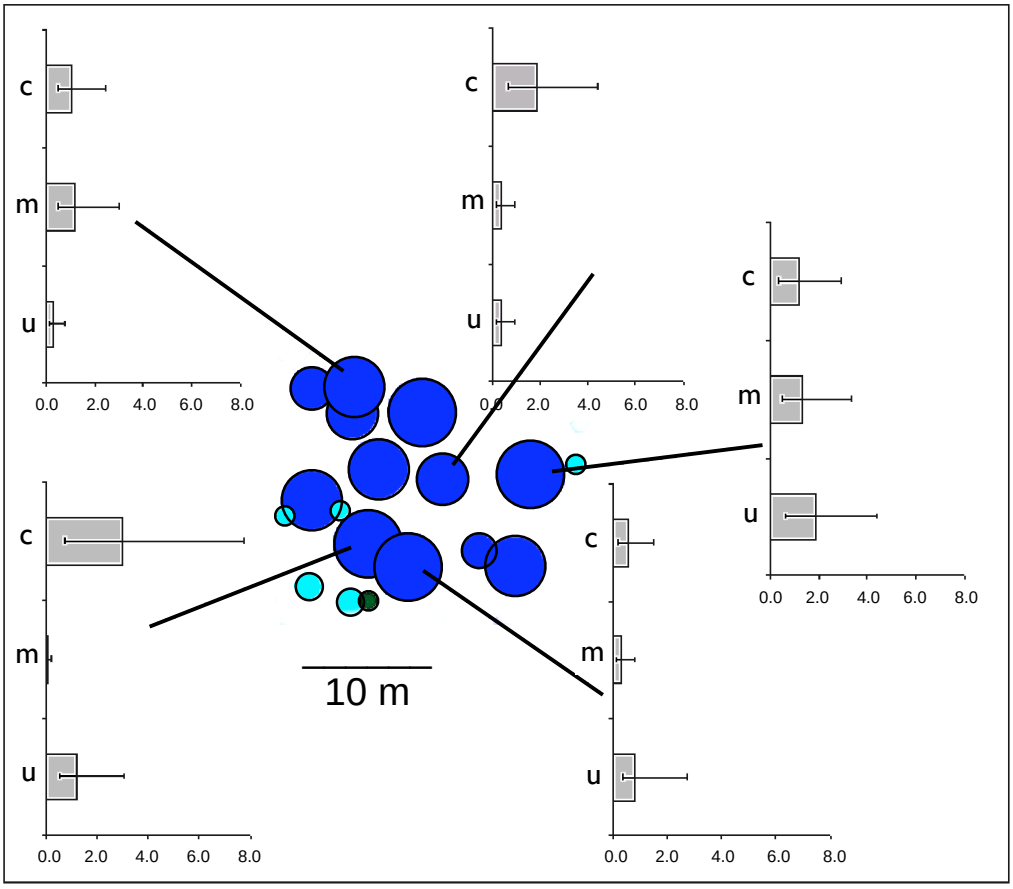

Figure 4 – Folivory rates (% removed area, median and 95% confidence intervals) at leaves (n = ca. 100 for tree at each height level) from different heights in the canopy (c = canopy, m = mid storey, u = under storey) and from five trees which are situated very close to each other. Note the striking differences. Dark blue dots represent trees of *Acer pseudoplatanus*. The diameter corresponds roughly to the stem diameter. Light blue dots represent tress of *Acer platanoides* and the green dot a tree of *Ulmus* cf. *laevis*.

For evergreen tropical forests Lowman & Heatwole (1992) and Schowalter (1994) reported values ranging from 2–15% or higher. The value obtained for the Leipzig forest of 1.13% is quite low and in the range of coniferous forests (Schowalter 1989). Of course 570 leaves might be too low to provide a good estimate, but a similar study of leaves of three tree species in the forest under storey at the same site in 2003 also revealed a low overall level of herbivory (unpublished). The large sample of almost 2 913 leaves of *Acer pseudoplatanus* obtained by the second study also showed a fairly low level of 1.7%. It is known that herbivory rates can differ considerably between sites (Lowman 1995, Schowalter 2000). Among the potential causes for this Rinker & Lowman (2004) lists variation in phenology, leaf age, vegetation stratum, forest type, as well as differences in natural history and demography among local arthropods including predators and parasites of the herbivores.

In conclusion, the low degree of folivory might be normal for the Leipzig forest. But there are also various facts that may explain this low value.

As stated above we may have missed the 'right time' to detect higher degrees of folivory. Given that we always sampled leaves by the end of summer, we might have missed bigger folivory damages in early summer. In early June 2003 for instance, we observed a notable degree of folivory at leaves of *Quercus robur*, with a subsequent dropping of those leaves, followed by a second leaf flush. Therefore we may have underestimated the 'true' degree of folivory at least for the annual calculation.

The climatic situation, especially a fairly wet summer 2002 followed by an extremely dry summer 2003 may have caused a considerably lower abundance of Lepidopteran herbivores than 'normal', which in turn caused less folivory (Fröhlich 2004 and Fröhlich *et al.* this volume). The same unusual climatic factors might have led to a shift in herbivore/predator and/or herbivore/parasite ratio causing a higher pressure on herbivores.

Finally, the forest, situated in close vicinity to the city, may still suffer of the decades of environmental pollution in GDR times, which ended just a decade

ago. This also might have led to a rather unbalanced situation of ecological processes.

Clearly, more long-term studies at all seasons are needed to get further insight to this process.

Folivory at different heights

The trend for the average folivory was similar in both studies and revealed a higher percentage of removed plant tissue in the upper canopy. In the first study one sample of 10 leaves from the upper canopy did not show a notable damage. This is surely due to a sampling outlier. In the second study most of the upper canopy leaves did show high degrees of folivory, which led to the general trend mentioned above. These findings suggest a general herbivores' preference of *Acer pseudoplatanus* leaves in the upper canopy with higher light levels. This might be due to a higher photosynthetic activity and hence a better food quality (e.g. higher sugar content) of leaves of *Acer pseudoplatanus* in the upper canopy. The most interesting finding in this study appears to be the striking differences in folivory between individual trees and at different canopy heights. There are various potential reasons for this observation.

Microclimatic preferences of the herbivorous insects along with predation avoidance may lead to a preference of certain sites in the canopy with higher feeding intensities.

The trees may differ genetically in their resistance or defence to herbivore attacks, just like humans differing in the performance of their immune system.

A varying biochemical composition of the leaf tissue due to slight differences in the tree's growing sites (soil chemicals, light conditions) might also be responsible for these patterns.

Finally the scale of the studies may have simply been inappropriate, i.e. the sampling area might be to small to detect clearer patterns or trends.

Again it can be concluded that only more and long-term studies including the whole forest space enable to get more insight in this complex ecosystem. Such studies accompanied by detailed surveys of the spatial and temporal distribution of herbivores and other important organisms of the food web (e.g. birds, bats, arthropods, parasites) may lead to a much better understanding of the forest ecosystem. This is essential to deal with future requirements and developments for a sustainable forest management and conservation.

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2.5 Plant-animal interactions in the canopy: intraspecific variability in herbivory on sycamore (Acer pseudoplatanus L.)

Haike Ruhnke[1], Martin Brändle, Stefan Klotz, Roland Brandl & Martin Schädler

The quality of leaf tissue differs not only among plant species, but may also show considerable variability among and within individuals of a given species. Insect populations may respond to this resource heterogeneity by forming ecologically and genetically distinct groups (adaptive deme formation; ADF, Edmunds & Alstad 1978). To evaluate central assumptions of the ADF-hypothesis, we analysed variability in herbivore attack, palatability and leaf utilization in a feeding experiment as well as leaf traits among and within three sycamore individuals in two consecutive years on the crane plot in Leipzig. Herbivore attack in the field, palatability and leaf utilization by a generalist herbivore as well as leaf traits differed among the investigated individuals of sycamore. Only in 2003 did herbivore attack differ significantly between the upper and the lower tree layer. In the feeding experiments, larvae of *Spodoptera littoralis* showed differences between tree layers only in terms of conversion efficiency in the first year of our study. All measured variables point to considerable heterogeneity of leaf quality among the investigated individuals, with only little variation within trees. Palatability, the relative growth rate and the conversion efficiency of the larvae of *S. littoralis* correlated negatively with the carbon/nitrogen-ratio of the leaf tissue. Herbivore attack in the field, however, was not related to palatability, relative growth rate and conversion efficiency of *S. littoralis* or to the measured leaf traits. Due to confounding environmental effects, levels of herbivory do not always follow the patterns of palatability and leaf traits in space and time. Overall, our analyses support two basic assumptions of the ADF-hypothesis. Firstly, there is considerable variability of quality among individual trees and, secondly, the variability is to some extent predictable across time.

INTRODUCTION

Leaves of trees may differ in palatability to insect herbivores. Several authors showed that concentration of leaf nitrogen and secondary compounds may vary among individual trees within species (Howard 1990; Suomela & Ayres 1994; Laitinen *et al.* 2000; Osier *et al.* 2000b) as well as between sun and shade leaves within individuals (Hollinger 1989; Dudt & Shure 1994). These variations may affect attack, feeding behaviour and development of associated insect herbivores (Ayres *et al.* 1987; Howard 1990; Strauss 1990; Osier & Lindroth 2001; Fortin & Mauffette 2002). Ultimately, differences in the quality of leaves may translate into genetic differentiation between populations of phytophagous insects living on different individuals. The adaptive deme formation hypothesis (hereafter called ADF-hypothesis) predicts the evolution of distinct groups (demes) within species of herbivorous insects in response to differences of resource quality among individuals. Demes are adapted to a particular individual (Edmunds & Alstad 1978). Although some studies have demonstrated genetic variation among populations of phytophagous insects, the underlying mechanisms are still poorly understood (Mopper 1996).

In our study, we investigated the intraspecific variability of herbivore attack, of palatability and leaf utilization as well as of leaf traits among and within individuals of sycamore (*Acer pseudoplatanus* L.) in two consecutive years. We approached the following questions: **(1)** Is there any difference in herbivore attack, palatability and leaf utilization as well as of leaf traits among and within individual trees? **(2)** If such differences exist, is there any correlation between herbivore attack in the field and palatability as well as of leaf traits? **(3)** Do the differences among individual trees show a consistent pattern across the two consecutive years?

MATERIALS AND METHODS

We estimated herbivore attack in the field. Palatability and related variables of leaves were measured with

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laboratory experiments using a polyphagous moth. Furthermore, we measured two leaf traits known to be important for herbivores. For our work we sampled three mature sycamore trees (*Acer pseudoplatanus* L.) in two consecutive years. The maximum distance between individual trees was about 90 m. Within trees we distinguished between an upper (sun leaves) and a lower layer (shade leaves).

Herbivore attack in the field

At the end of growing season in August 2002 and 2003, we quantified the levels of herbivory by estimating the amount of removed leaf material. From each tree individual we selected four branches in each layer. We collected 25 leaves from each branch (200 leaves per tree). Consumed leaf area [mm^2] was estimated after digitising and measuring leaf area (SigmaScan-Pro5). The specific weight of leaf material differed between the two layers. Therefore, we corrected the consumed leaf area of the upper layer by tree-specific correction factors. These correction factors were the ratio of leaf fresh mass to dry mass of 20 samples from each tree layer collected in July of 2002 and 2003.

Feeding experiments

For our feeding experiments we used larvae of the African cotton leafworm (*Spodoptera littoralis* (Boisduval), Lepidoptera: Noctuidae) a polyphagous herbivore. Larvae originated from a laboratory stock and were reared on artificial diet.

The experiments were carried out in July 2002 and July 2003, when the foliage was fully mature. In every experiment, we used different branches within each tree layer to avoid possible confounding effects of induced plant defence. The branches selected for the feeding experiments were near to those selected to estimate herbivore attack in the field. We collected leaves from short shoots of each branch. Leaves were sprayed with deionised water to keep them turgescent and leaves were stored at 4 °C. The experiments started a few hours after sampling. From six leaves of each branch we punched leaf discs (diameter 23 mm). Discs were weighed and placed individually in Petri dishes lined with moist filter paper. For each Petri dish we used one larva of *S. littoralis* (third instar). Prior to the experiments, larvae were weighed. The Petri dishes were placed in a climate chamber (26 °C and 12 h light) for 24 h. At the end of the experiment larvae were killed by freezing. Dead larvae and remaining leaf material were dried at 60 °C to weight constancy. Initial larval mass was converted to dry mass using a linear regression equation for each year (30 larvae in each year). The initial fresh mass of leaf discs was converted to dry mass by using the mean water content of leaves for each selected branch (see below). Leaf consumption was expressed as mg consumed leaf dry mass in 24 h.

Osier *et al.* (2000a) showed that the growth of caterpillars reared in bags on trees was highly correlated to the growth of larvae reared in the laboratory on leaves of the same tree. Hence, we expect that the feeding experiments in the lab reflect leaf quality in the field (see also Kleiner 1991). Lab experiments have the advantage that all experiments are performed under identical climatic conditions. In the field microclimatic differences between trees and layers may influence the results.

Leaf traits

Leaf water, nitrogen and carbon contents are known to be closely related to functional leaf traits and to palatability (Schädler *et al.* 2003). Therefore, we measured these traits for the foliage of each branch used during the feeding experiments and to the same time as in the bioassays. Water content was determined by the ratio of leaf dry mass to fresh mass of five leaf discs per branch. To estimate the carbon/nitrogen-ratio a sample of leaves from every branch was vacuum-dried for 48 h, milled with a high-speed rotor mill and analysed for carbon and nitrogen with an element analyzer (Vario EL, Elementar Analysensysteme GmbH, Hanau, Germany).

Data analyses

For the analysis of consumed leaf area in the field we averaged the 25 individual values of each branch. These means were square-root transformed to normalize the distribution of residuals. The effects of year, tree individual and tree layer were tested using a three-way ANOVA (Proc GLM [Version 8.02]; SAS Institute).

In the analyses of the feeding experiments, for every trait we used the average across the six values of each branch. The effects of year, tree individual and tree layer on consumed leaf material, final larval dry mass, and increase of larval mass during the experiment were analysed using an ANCOVA (see Raubenheimer & Simpson 1992, Horton & Redak 1993). For the analysis of consumed leaf material and final larval dry mass, initial larval dry mass was used as a covariate. By using type I sums of squares, we analysed the effects after removing confounding effects of initial larval dry mass from the analysis. Thereby, we standardized leaf consumption to herbivore mass, and adjusted means are a measure of palatability. Similarly, adjusted means of final larval dry mass measure relative growth rates of larvae; for this analysis we log-transformed initial and final larval dry mass. For the

analysis of increase in mass, the consumed leaf material was used as covariate. Thereby, we estimated an equivalent to the efficiency of conversion of ingested food into body substance (see WALDBAUER 1968).

Effects of year, tree individual and tree layer on leaf C/N-ratio and water content were analysed using a three-way ANOVA. The relationships between variables measured during the feeding experiments (adjusted means from the ANCOVA for every layer of each tree individual), the herbivore attack in the field, and leaf traits were tested by a Spearman's rank correlation using the mean of every trait per layer, tree and year.

RESULTS

Herbivore attack in the field

In the field, the consumed leaf area differed significantly between the two consecutive years (Table 1). We observed a significant interaction between year and tree individual indicating different levels of herbivory on the individuals between years (Fig. 1a). Separate statistical analyses for the two years showed, however, significant differences among individual trees only in 2003 (Fig. 1a, ANOVA results not shown). Herbivore attack was larger in the upper tree layer in the second year only (significant year x tree layer interaction, Table 1, Fig. 1b). Furthermore, the effect of tree layer varied among individual trees (significant tree individual x tree layer interaction, Table 1).

Table 1 – Results of the ANOVA of effects of year, tree individual, and tree layer on the consumed leaf area by herbivores (herbivore attack). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, df: degrees of freedom, MS: Mean square.

Source	df	F values
Year	1	15.37 ***
Tree individual	2	1.56
Tree layer	1	15.08 ***
Year x tree individual	2	3.73 *
Year x tree layer	1	25.10 ***
Tree individual x tree layer	2	4.82 *
Year x tree individual x tree layer	2	1.89
Residual	36	[MS = 8.26]

Feeding experiments

Palatability, relative growth rate, and conversion efficiency of *Spodoptera littoralis* differed significantly between the two years (Table 2). All traits differed among individual trees (Fig. 2). We found no general difference between the upper and lower tree layer for all three variables. Overall, we found few significant interactions between factors. For the conversion efficiency we found a significant interaction between year and tree layer; in 2002 we found higher conversion efficiency for the lower layer (Fig. 3). For the growth rate we found a three-way interaction.

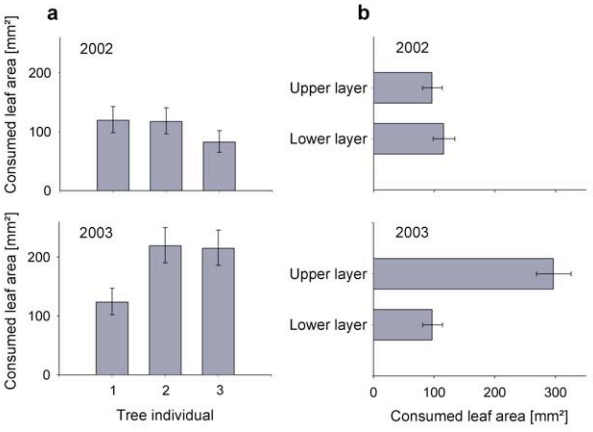

Figure 1 – Herbivore attack measured by the consumed leaf area on sycamore by herbivores in the field. The graphs present means of tree individuals (a) and tree layer (b) for each year (back-transformed means ± 1 SE).

Leaf traits

The C/N-ratio of leaves differed between the two years as well as among individual trees (Table 3). We found a significant interaction of year and tree individual as well as a significant three-way interaction (Table 3). The water content of leaf tissue was similar in the two years but differed among individual trees and between tree layers. Generally, water content was significantly lower in leaves of the upper tree layer. However, the strength of this effect differed between years and individual trees (significant year x tree layer and tree individual x tree layer interaction, Table 3). Palatability ($P = 0.052$), relative growth rate ($P = 0.019$), and the conversion efficiency ($P = 0.003$) of larvae of *S. littoralis* were all negatively correlated to the C/N-ratio of the leaf tissue, although we could only use the mean of the trait per layer and tree and year. In contrast, we could not detect correlations with the water content of leaves (all $P > 0.3$). We found no relationship between herbivore attack in the field and variables measured during the feeding experiments as well as with the measured leaf traits (all $P > 0.3$).

Table 2 – Results of an ANCOVA of effects of year, tree individual, and tree layer on palatability, growth rate and conversion efficiency of the larvae of *S. littoralis*. *: P < 0.05, **: P < 0.01, ***: P < 0.001, df: degrees of freedom, MS: Mean square.

Source	df	F values		
		Palatability	**Growth rate**	**Conversion efficiency**
Covariate	1	52.16 ***	204.23 ***	424.69 ***
Year	1	6.65 *	76.03 ***	52.07 ***
Tree individual	2	23.94 ***	41.98 ***	15.55 ***
Tree layer	1	3.02	0.93	3.48
Year x tree individual	2	0.62	1.19	1.35
Year x tree layer	1	0.88	0.17	6.59 *
Tree individual x tree layer	2	0.76	1.65	1.19
Year x tree individual x tree layer	2	2.58	3.48 *	0.98
Residual	35	[MS = 3.27]	[MS < 0.01]	[MS = 0.06]

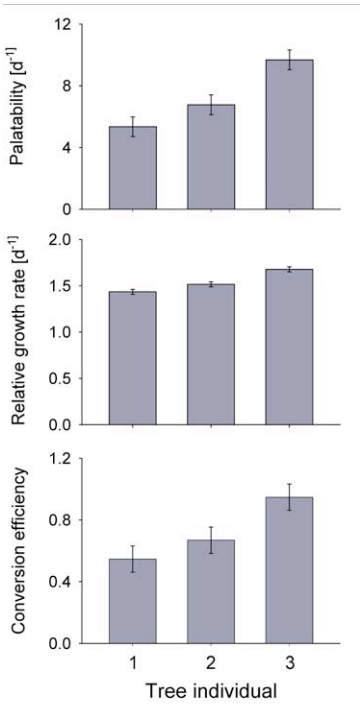

Figure 2 – Effects of tree individual on palatability, relative growth rate, and conversion efficiency of larvae of *S. littoralis* on sycamore (adjusted means of both years ± 1 SE).

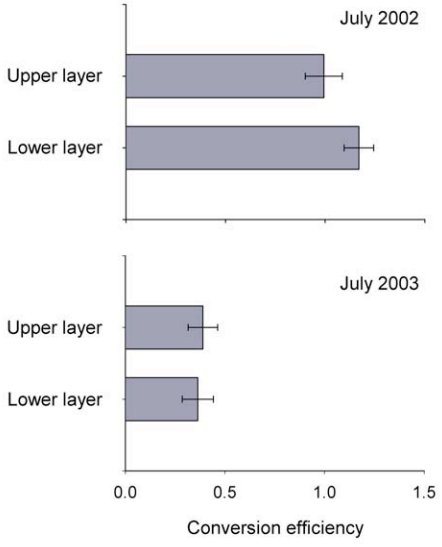

Figure 3 – Effects of tree layer on the conversion efficiency of larvae of *S. littoralis* for 2002 and 2003 (adjusted means ± 1 SE).

Table 3 – Results of an ANOVA of effects of year, tree individual, and tree layer on the carbon/nitrogen-ratio and water content of leaves. *: P < 0.05, **: P < 0.01, ***: P < 0.001, df: degrees of freedom, MS: Mean square.

Source	df	F values	
		C/N-ratio	**Water content**
Year	1	235.26 ***	1.07
Tree individual	2	17.00 ***	19.83 ***
Tree layer	1	2.86	411.99 ***
Year x tree individual	2	6.97 **	1.72
Year x tree layer	1	0.50	19.85 ***
Tree individual x tree layer	2	0.95	3.93 *
Year x tree individual x tree layer	2	8.38 **	1.00
Residual	36	[MS = 0.908]	[MS = 0.001]

DISCUSSION

One may answer the three questions posed in the introduction as following: **(1)** We found significant differences among individual trees in herbivore attack in the field, variables derived from feeding experiments as well as measured leaf traits. **(2)** We found no correlation between herbivore attack in the field and variables derived from the feeding experiments or measured leaf traits. However, variables derived from the feeding experiments and leaf traits were correlated **(3)**. Although we found significant interactions for the leaf traits, the results from the feeding experiments provided a consistent ranking of tree individuals across the two years.

A number of studies have shown that the content of certain compounds differs among tree individuals (Howard 1990; Suomela & Ayres 1994; Laitinen *et al.* 2000; Osier *et al.* 2000b). Further, their utilization by herbivores varied among host-plant individuals (Ayres *et al.* 1987; Howard 1990; Strauss 1990; Osier & Lindroth 2001). We found significant differences in herbivore attack, palatability and related variables as well as simple leaf traits among three sycamore individuals. Note that these individuals grow close to each other. However, the variation in herbivore attack in the field was not correlated to the results of the feeding experiments and measured leaf traits. This lack of correlation between field patterns and lab experiments has been reported by a few other authors (Rowe & Potter 1996; van Nouhuys *et al.* 2003). Rowe & Potter noticed "...there is no a priori expectation for whether leaves in the upper or lower canopy will be preferred on the basis of foliar chemistry". This points to fundamental differences between patterns of herbivory in the field and lab studies. In the field the variation in herbivore pressure within and among trees is not only influenced by leaf quality but by a plethora of factors such as microclimate (Stamp & Bowers 1990), predation, parasites (Stamp & Bowers 1988) or migration (Batzer *et al.* 1995; Magalhães *et al.* 2002).

Leaf traits like water content, C/N-ratio, or concentration of secondary compounds influence leaf palatability (Mattson 1980; Scriber & Slansky 1981; Hartley & Jones 1997; Schädler *et al.* 2003). Nevertheless, several studies found that palatability is sometimes not affected by those traits (Dudt & Shure 1994; Rowe & Potter 1996; Osier & Lindroth 2001; Shibata *et al.* 2001). Furthermore, the details of the relationship may depend on the specific plant-insect species combination considered (Howard 1990; Hemming & Lindroth 1995), and the relationship may change with environmental factors (Jansen & Stamp 1997). In our study, palatability, relative growth rate and the conversion efficiency measured with the larvae of *S.littoralis* showed the expected negative relationship to the C/N-ratio of the leaf tissue.

We found significant differences in herbivore attack as well as water content of leaves between tree layers. However, we were not able to demonstrate general differences in palatability, growth rate and conversion efficiency between layers. Fortin & Mauffette (2002) found that leaves from the upper layer of sugar maple were more palatable to larvae of a generalist moth, with positive effects on pupal mass and number of eggs. In addition, these leaves were preferred in feeding tests. Only our results from the second year support these findings. In general, little information is available on the variability of herbivore attack as well as leaf palatability within individual trees (Howard 1990; Rowe & Potter 1996; Kause *et al.* 1999; Fortin & Mauffette 2002). Some authors suggested that differences in leaf quality within plants are directly or indirectly related to the effects of solar irradiation (references in Fortin & Mauffette 2002). However, many published experiments used sun and shade leaves from different plants respectively. Our finding of considerable differences among individuals suggests that results in the literature are confounded by those differences.

The ADF-hypothesis suggests that monophagous herbivorous insects may form distinct adaptive groups in response to resource heterogeneity among host individuals. However, the formation of demes requires temporal predictability of host plant quality for the insects. Mopper *et al.* (2000) showed for a leafminer that ten generations may be necessary to form demes. At least across such temporal scales host individuals should have predictable properties to which the insect can respond. Leaf traits, however, show considerable seasonality (McKinnon *et al.* 1998; Osier *et al.* 2000b; Riipi *et al.* 2004) and leaf traits differ between years (Laitinen *et al.* 2000; Covelo & Gallaro 2001). Thus, insect herbivores have to deal with considerable variability in leaf quality within (Kause *et al.* 1999; Haukioja *et al.* 2002) and between years (McPheron *et al.* 1988; Cronin *et al.* 2001). Cronin *et al.* (2001) even showed fluctuations in host-plant preferences and performance between successive years. Although in our study we found significant interactions between tree individuals and year in their effect on leaf traits, few interaction terms were significant for variables measured during the feeding experiments. The relative ranking among individuals was the same in the two years (see also Riipi *et al.* 2004). Leaves of some tree individuals may be in general a better food source than leaves of other individuals. Hence, our study suggests that resource heterogeneity is predictable between years. But remember that about ten or even more generations are required to form demes. Hence more long-term stud-

ies are badly needed to draw safe conclusions (see also Cronin *et al.* 2001).

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