SCREENING OF COMMON *PLANTAGO* SPECIES IN HUNGARY FOR BIOACTIVE MOLECULES AND ANTIOXIDANT ACTIVITY

S. GONDA [1] *, L. TÓTH [2], P. PARIZSA [1], MÓNIKA NYITRAI and G. VASAS [1 *]

*: Corresponding author, e-mail: gondasandor@gmail.com, vasasg@tigris.unideb.hu

[1] University of Debrecen, Department of Botany, Division of Pharmacognosy; H-4010 Debrecen, Egyetem tér 1, Hungary.

[2] University of Szeged, Faculty of Pharmacy, Department of Pharmacognosy; H-6720 Szeged, Eötvös u. 6, Hungary.

ABSTRACT

Five species of *Plantago*, namely, *P. lanceolata*, *P. major*, *P. media*, *P. altissima* and *P. maritima* were screened for iridoid content (CE-MEKC), total caffeoyl phenylethanoid glycoside (CPG) content and antioxidant activity (CUPRAC assay). The five species could be distinguished by TLC pattern analysis in a single run in a system commonly used for quality management of *P. lanceolata* leaves, as shown by cluster analysis of major bands; with the exception, that *P. altissima* and *P. lanceolata* did not show enough pattern difference to be fully separated. *P. maritima* was shown to have the highest antioxidant capacity (0.42 μmol ascorbic acid equivalent (AAE) / g DW), and the highest level of CPGs (4.29%). *P. altissima* was shown to be chemically indistinguishable from *P. lanceolata* with repsect to iridoid content (aucubin 0.55±0.04% 0.68±0.23%, catalpol 0.66±0.13% and 0.89±0.22%, respectively), CPG content (2.40±0.38% and 2.54±0.56%, respectively) and antioxidant capacity (0.2206±0.0290 and 0.2428±0.0191 μmol AAEAC / g DW). The presented data show the potency of medicinal use of Hungarian wild populations of the studied five species, especially in the case of *P. maritima*, and that *P. altissima* can be a potential replacement of *P. lanceolata* in herbal mixtures.

Keywords: iridoid glycosides - capillary electrophoresis - CUPRAC antioxidant capacity - *Plantago* L. - caffeoyl phenylethanoid glycoside.

Abbreviations: AAEAC, ascorbic acid equivalent antioxidant capacity; AUC, aucubin; CAT, catalpol; CE-MEKC capillary electrophoresis - micellar electrokinetic chromatography; CPG, caffeoyl phenylethanoid glycoside; CUPRAC, cupric reducing antioxidant capacity; DW, dry weight; IG, iridoid glycoside, NP, Natural Product reagent A; TLC, thin layer chromatography; TEAC, Trolox equivalent antioxidant capacity.

*Suggested running title***:** Bioactive molecule screening of *Plantago* species

INTRODUCTION

Plantago species, especially *P. lanceolata* and *P. major* are frequently administered herbal medicine against a variety of common diseases, which inculde including common cold, cough, bronchitis, fever, inflammation of the mouth and pharynx in the case of *P. lanceolata* [3], uses of *P. major* are also associated with immunmodulatory and anti-infective properties [13].

Several studies have already been published on chemical composition of *Plantago* species, [10] analysed seven *Plantago* species for quantitative iridoid glycoside (IG) content with hot water extraction (HWE) including *P. altissima*, *P. lanceolata* and *P. maritima.* [4, 5] have studied flavonoid pattern, total flavonoid content (with a spectrophotometric assay) and antioxidant activity of five *P.* species (including *P. major*, *P. media* and *P. maritima*) and also the correlation between the above mentioned parameters was established. They concluded that there is strong correlation between flavonoid content and antioxidant activity from various assays, but no estimation of caffeoyl phenylethanoid glycosides (CPGs) was mentioned. Luteolin-7-O-glucoside was found to be a chief flavonoid of *P. major*, while luteolin in *P. maritima*. Detailed chemotaxonomic investigation of the genus is described by [12] who gave qualitative information on occurence/absence of metabolites in many *Plantago* species.

Fortunately, some *Plantago* species and bioactive molecules have well established uses. Scientific evidence for traditional applications includes bioactivity data *in vitro* and in animal studies. Acteoside and plantamajoside (Figure 1) were shown to have antispasmodic properties on isolated guinea-pig ileum [8] and to protect endothelial cells from oxidative damage [7]. Effects of acteoside and plantamajoside are in part associated with their ability to scanvenge free radicals. Iridoids are rather associated with antibacterial effects: activity of AUC (aucubin) (Figure 1) against several strains was shown by [14], this molecule was also found effective in accelerating healing of

oral wounds, and anti-inflammatory effect was also demonstrated [16].

However, some other possible uses of the pure major metabolites of the studied *Plantago* species have no respective counterparts in traditional uses of *Plantago* species. Novel evidence has shown, that aucubin can prevent hippocampal neuron loss [18], or has the ability to protect the pancreas B-cells in animal studies [9].

No comparative quantitative data are available on total CPG content of the less frequently used species, though qualitative data showed, that acteoside or plantamajoside can be present as the major CPGs all of the mentioned ones [12]. Comparative quantitative analysis of species official and unofficial in the pharmacopoeias and compendia can support new theoretical data on their usefulness in therapy. It is also important to exclude similar or heavily distinguishable, yet useless species from medicinal practice. Possible misidentification of *P. altissima* (similar to *P. lanceolata*) and *P. media* (similar to *P. major*, when not flowering) also claims for more knowledge on levels and patterns of bioactive molecules in these species. As possible sources of these useful metabolites, Hungarian populations of *Plantago* species have to be examined.

Different populations of five members of the genus, *P. lanceolata, P. altissima, P. major, P. maritima* and *P. media* were screened for iridoid, total caffeoyl-phenylethanoid ester contents and total antioxidant to assess potential medicinal uses. TLC patterns were also examined in a qualitative assay to provide additional data that can help distinguishing these species on the chemical level, as a part of essential quality assurance. To obtain more representative data, specimens are collected from distinct populations.

MATERIALS AND METHODS

Plant material

Leaves of the five *Plantago* species were collected on different sampling sites (populations) to obtain data on within-species variation. All samples were collected during flowering to aid proper species identification, in the summer of 2008 and were dried at temperatures not exceeding 50 ºC. Samples were obtained from the following sites: Hajdúsámson, Nádudvar, Debrecen, Budapest, Mátraháza, Jászapáti, Tépe. Each species is represented by at least 4 samples.

Determination of iridoids

The method of [17] was used for the determination of aucubin (AUC) and catalpol (CAT) (Figure 1.) from the plant samples, with minor modifications. Accurately weighed 50 mg of plant material (dry weight) was extracted with 1000 µl of 10% PrOH for 60 minutes at 100 ºC, after allowed to stand at room temperature for 20 min. The unsoluble part was removed by centrifugation at 13000 rpm, the supernatant was used for CE analysis directly. A PrinCE-C 700 capillary electrophoresis instrument with a diode array detector was used. Five-point standard calibration curves were used from both AUC and CAT (Sigma-Aldrich), dissolved in 10% PrOH. Background electrolyte contained 25 mM sodium tetraborate, 100 mM sodium dodecyl sulphate, pH was set 9.35, applied voltage was +30kV.

Thin layer chromatography

Accurately weighed 100 mg of plant material was extracted with 1000 µl of MeOH for 60 minutes at 65 ºC. After centrifugation, supernatants were used for TLC analysis. TLC was accomplished on silica gel layers (Macherey-Nagel 200 µm thick Silicagel 60 on glass or aluminium foil). For pattern recognition purposes, the system of [6] was used, eluent composed a mixture of EtOAc + HCOOH + AcOH + water, 100+11+11+27. Visulalisation was accomplished by

spraying with 1% Natural Product Reagent A (NP; 2-aminoethyl diphenyl borate; Carl-Roth) in ethanol, heating at 105 ºC for 10 min, and subsequently sprayed with 1% PEG 6000 (50% EtOH), also followed by the same heating procedure. For visualization of TLC, a CAMAG CABUVis chamber was used, using visible light and 356 nm UV illumination modes. Rf values of TLC plates were measured with the aid of CP Atlas 2.0 software, applied to photographs of TLCs acquired with commercial digital cameras.

Determination of total phenylethanoid content

Quantification of caffeoyl phenylethanoid glycosides is done according to the monographs of the official European Pharmacopoeia 6.2. (Monograph No. 1884, Plantaginis lanceolatae folium) in a minor-scale version. Briefly, accurately weighed 50 mg of plant material was extracted with 1000 µl of 50% EtOH, centrifuged and the supernatant is subjected to analysis. Total CPG's are given as equivalent % (on BW basis) total ortho-dihydroxicinnamic acid derivates expressed as acteoside.

Total antioxidant capacity assay

Detailed background of the assay is described in [1]. Briefly, an Cu^{2+}-neocuproine (Nc) complex is added to the diluted plant extracts in a solvent system buffered by NH_4OAc (pH 7.4). The reagent oxidizes polyphenolic substances and other antioxidants in stochiometric reactions, and Cu^{+}-Nc complexes are formed, which are measured at 425 nm. The plant material is diluted to such extent, that no absorbance is detected versus water at the λ_{max} of Cu^{+}-Nc, making measurement of individual blind samples unnecessary. In *Plantago* extracts, caffeic acid derivates (Figure 1.) as well as flavonoids are measured by the assay under the described conditions [1]. The 50% EtOH extracts were used for ascorbic acid equivalent antioxidant capacity (AAEAC) determination, after 100-fold dilution with the solvent to fit the linear range of the assay. Data are given as AAEAC in µmol / g

DW plant drug. Calibration curve was constructed from absorbance data of a serial dilution of ascorbic acid.

Statistical analyses

One-way ANOVA models are used to test differences between species, with respect to any of the measured parameters. ANOVAs were followed by Tukey's HSD test for multiple comparison. Runned tests were implemented in R [11].

RESULTS

Iridoid glycoside content

IGs AUC and CAT (Figure 1.) were measured by CE-MEKC, after extraction with hot 10% PrOH. Data on IG content is shown in Table 1. Only samples of *P. lanceolata* and *P. altissima* were found to contain catalpol, while *P. media*, *P. major* and *P. media* contained only aucubin. *P. lanceolata* samples were found to contain the most iridoids, significantly more than that of *P. major* and *P. media* ($p < 0.05$). No significant difference could be outlined between the heavily distinguishable *P. altissima* and *P. lanceolata* with respect to iridoid content ($p > 0.05$), similar data were obtained by [10].

TLC pattern

NP and PEG was successfully applied to the TLCs, producing various colors to be observed in UV 356 illumination. Bands that appeared in most samples (in 3 of 4, or in 4 of 5 samples of a sepcies) of a single species were given in the detailed pattern description in Table 2. The occurrence

and relative intensities of these bands (0-3, arbitrary scale) were also subjected to cluster analysis (Ward method, using Euclidean distances) in order to accurately define and quantify differences among species. Result is plotted in Figure 2. Bands that allow the fast recognition of the species are the following: *P. major* can easily be distinguished from the other species in TLC, as is characterized by many orange bands in the region Rf 0.60-0.76, and an intensive blue/green one at 0.50 and a dark adsorbing zone at 0.70. The other species showed an intensive blue/green band at 0.65. *P. media* can be distinguished from others by lack of orange bands, and a light blue band at Rf 0.17. *P. maritima* has similar pattern to *P. altissima* and *P. lanceolata* (Table 2), but is rich in orange bands in the region of 0.61-0.81. *P. altissima* and *P. lanceolata* could not be distinguished under the current conditions, the pattern results were very similar. The other three species could be easily recognized, as it is also shown by cluster analysis results (Figure 2).

Antioxidant capacity and CPG content

P. maritima was shown to contain the most CPGs (4.29±0.91%), significantly ($p < 0.05$) more than any other species, the same was the case for AAEAC values (0.4124±0.7071 μmol / g AAEAC). *P. major* was shown to contain the least antioxidants and CPGs (0.1722±0.0573 μmol AAEAC / g and 1.81±0.56 % CPGs). The other species were shown to have more or less equivalent values of AAEAC and CPG content. Results from the antioxidant capacity assay and total CPG estimation are summarized in Table 2.

DISCUSSION

Iridoid glycoside content

Presence and absence of AUC and CAT in different species is in accordance with the previous works of [12] in *Plantago* chemotaxonomy. Measured iridoid content was much more in the case of

P. maritima, *P. lanceolata* and *P. altissima*, than in the study of [10], which can be the consequence of differences in sampling site (thus, genetical abilities), drying conditions. MeOH extracts of different *Plantago* species were analysed for AUC content by [5], and found data similar to ours, if we take into account, that MeOH extracts about 50% less AUC and CAT, than HWE [17]. We have shown, that 10% PrOH extracts as much as IGs as HWE (p<0.05). It is important to note, that the misidentifiable *P. altissima* and *P. lanceolata* showed similar quantities of both IGs, supporting the sense therapeutic use of *P. altissima.* IG contents of the frequently administered *P. major* and the similar *P. media* leaves were significantly lower than that of *P. lanceolata (*p<0.05, Table 1.). Within-species variation was not high (mean RSD being 17% among samples from different collection sites).

Data support rationality of folk medicinal use of *P. altissima*, as it contains the same amount (and pattern) of iridoids, like *P. lanceolata* of officially established uses. With respect to the therapeutic uses that can be linked to IGs, used of all species studied makes sense.

Antioxidant capacity and CPG content

The CUPRAC assay was successfully applied for the examination of *Plantago* species. It was shown, that *Plantago* herbal teas and preparations can exert high antioxidant potential, the AAEAC values of the 50% EtOH extract of *Plantago* species (especially *P. maritima*) are comparable to CUPRAC TEAC values of many commercial herbal teas, like thyme and sage infusions, and so on [2] (ascorbic acid has molar TEAC of 0.96 in CUPRAC assay [1]). *P. maritima* extracts showed the highest activity (significantly higher than all other species, $p < 0.05$), and the highest content of CPGs ($p < 0.05$) was also measured from *P. maritima* samples. These data support the statement by [4], that *Plantago* species are a rich source of natural antioxidants. All wild population samples would have passed the Pharmacopoeial lower limit of CPG content (1.5% of DW, expressed as acteoside).

It is visible from Table 1, that there is a correlation between CPG content and AAEAC values, established Pearson correlation coefficient was found 0.9202. It seems likely, that CPGs are involved in the antioxidant activity of the plant drug, an unsurprising fact if one looks at their structure (Figure 1.). Deviation from linear correlation may be caused by fluctuating flavonoid quantities and patterns (and also other polyphenolic and other antioxidants), and metabolite-metabolite interactions, though the latter seems to be negligible in the CUPRAC assay, as it was found approximately additive for real mixtures [19].

Based on these findings, *P. altissima* can possibly be used as an anti-inflammatory herbal medicine as a replacement of *P. lanceolata* (also TLC pattern is virtually identical, see below). *P. maritima* is shown to be a rich source of CPGs, and probably is a potent candidate for herbal medicinal products. The widely used *P. major* was shown to be less potent from this point of view.

TLC pattern

NP / PEG visualization was proven useful for identification of *Plantago* species. Orange/brown fluorescent bands are likely to be flavonoids (in particular, ones with for example quercetin or myricetin aglyca [15]), as are reported to give such signal with the NP specific reagent. It is important to note, that the method given in the European Pharmacopoeia 6.2. as official TLC method (visualization only by heating) for pattern determination did not distinguish the above mentioned species (except *P. major*), all the extracts showed the same pattern. Identification of species was successfully accomplished, cluster analysis (Figure 2.) revealed, that all species could be separated, except *P. lanceolata* and *P. altissima.* Existence of real clusters containing only samples from a single species is shown by high AU values (see Figure 2.).

REFERENCES

1. Apak, R., Güçlü, K., Demirata, B., Ozyürek, M., Celik, S. E., Bektaşoğlu, B., Berker K. I., Ozyurt, D. (2007) Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the cuprac assay. *Molecules*, *12*, 1496-1547.

2. Apak, R., Güçlü, K., Ozyürek, M., Esin Karademir, S., Erçağ, E. (2006) The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. *Int. J. Food Sci. Nutr.*, *57*, 292-304.

3. Awang, D. V. (2009) *Tyler's herbs of choice: the therapeutic use of phytomedicinals, third edition*. CRC Press, p. 84.

4. Beara, I. N., Lesjak, M. M., Jovin, E. D., Balog, K. J., Anackov, G. T., Orcic, D. Z., Mimica-Dukic N. M. (2009) Plantain (*Plantago* L.) species as novel sources of flavonoid antioxidants. *J. Agric. Food Chem.*, *57*, 9268-9273.

5. Beara , I. N. (2010) Phytochemical screening and evaluation of antioxidant and anti-inflammatory potential of secondary metabolites of *Plantago* L. species , PhD thesis, Novi Sad, University of Novi Sad, Serbia

6. Biringanine, G., Chiarelli, M. T., Faes, M., Duez, P. (2006) A validation protocol for the hptlc standardization of herbal products: application to the determination of acteoside in leaves of *Plantago palmata* Hook. f.s. *Talanta*, *69*, 418-424.

7. Chiou, W. F., Lin, L. C., Chen, C. F. (2004) Acteoside protects endothelial cells against free

radical-induced oxidative stress. *J. Pharm. Pharmacol.*, *56*, 743-748.

8. Fleer, H., Verspohl, E. J. (2007) Antispasmodic activity of an extract from *Plantago lanceolata* L. and some isolated compounds. *Phytomedicine*, *14*, 409-415.

9. Jin, L., Xue, H. Y., Jin, L. J., Li, S. Y., Xu, Y. P. (2008) Antioxidant and pancreas-protective effect of aucubin on rats with streptozotocin-induced diabetes. *Eur. J. Pharmacol.*, *582*, 162-167.

10. Jurisic, R., Debeljak, Z., Vladimir-Knezevic, S., Vukovic, J. (2004) Determination of aucubin and catalpol in *Plantago* species by micellar electrokinetic chromatography. *Z.Naturforsch.(C)*, *59*, 27-31.

11. R Development Core Team. (2009) *R: a language and environment for statistical computing*. Vienna, Austria. Site: http://www.R-project.org

12. Ronsted, N., Franzyk, H., Molgaard, P., Jaroszewski, J. W., Jensen, S. R. (2003) Chemotaxonomy and evolution of *Plantago* L. *Plant Syst. Evol.*, *242*, 63-82.

13. Samuelsen, A. B. (2000) The traditional uses, chemical constituents and biological activities of *Plantago major* L. a review. *J. Ethnopharmacol.*, *71*, 1-21.

14. Senatore, F., Rigano, D., Formisano, C., Grassia, A., Basile, A., Sorbo, S. (2007) Phytogrowth-inhibitory and antibacterial activity of *Verbascum sinuatum*. *Fitoterapia*, *78*, 244-247.

15. Sherma, J., Fried, B. (2003) *Handbook of thin-layer chromatography*. CRC Press. pp. 719-720.

16. Shim, K. M., Choi, S. H., Jeong, M. J., Kang, S. S. (2007) Effects of aucubin on the healing of oral wounds. *In Vivo, 21*, 1037-1041.

17. Suomi, J., Siren, H., Hartonen, K., Riekkola, M. L. (2000) Extraction of iridoid glycosides and their determination by micellar electrokinetic capillary chromatography. *J. Chromatogr. A, 868*, 73-83.

18. Xue, H. Y., Jin, L., Jin, L. J., Li, X. Y., Zhang, P., Ma, Y. S., Lu Y. N., Xia Y. Q., Xu Y. P. (2009) Aucubin prevents loss of hippocampal neurons and regulates antioxidative activity in diabetic encephalopathy rats. *Phytother. Res.*, *23*, 980-986.

19. YIldIz, L., Başkan, K. S., Tütem, E., Apak, R. (2008) Combined HPLC-CUPRAC (cupric ion reducing antioxidant capacity) assay of parsley, celery leaves, and nettle. *Talanta*, *77*, 304-313.

TABLES

Table 1. Measured chemical parameters of leaves of five different *Plantago* species.

Every parameter is separately subjected to one-way ANOVA, and subsequently, Tukey's HSD test. Samples not sharing the same letter are statistically significant at $p<0.05$. CPG content values (%w/w) are given as acteoside equivalent.; n.d., not detected.

	Catalpol content (% w/w)	Aucubin content (%w/w)	AAEAC (µmol / g DW)	total CPG content (%w/w)
P. altissima	0,66 ± 0,13% [b]	0,55 ± 0,04% [ab]	0,2206 ± 0,0290 [a]	2,40 ± 0,38% [a]
P. lanceolata	0,89 ± 0,22% [b]	0,68 ± 0,23% [a]	0,2428 ± 0,0191 [a]	2,57 ± 0,56% [a]
P. major	n.d. [a]	0,34 ± 0,02% [b]	0,1722 ± 0,0573 [a]	1,81 ± 0,56% [a]
P. maritima	n.d. [a]	0,47 ± 0,08% [ab]	0,4124 ± 0,0701 [b]	4,29 ± 0,91% [b]
P. media	n.d. [a]	0,34 ± 0,05% [b]	0,2368 ± 0,0480 [a]	2,57 ± 0,73% [a]

Table 2. TLC pattern of MeOH extracts of different *Plantago* species in the system EtOAc + HCOOH + AcOH + water : 100+11+11+27 on silica gel stationary phase. Colors are detected under 356 nm UV illumination after spraying with NP / PEG 6000 and heating. Abbreviations: V, various (not detected in all samples), P, present, (detected in all samples from the given species), no mark, detected in less than 50% of total samples. Intensive bands are marked by bold letters.

Rf	Color	*P.major*	*P.media*	*P. altissima*	*P. lanceolata*	*P. maritima*
0,84	light blue					V
0,81	orange					P
0,78	orange	V				
0,76	orange	P				P
0,73	grey	P				
0,7	dark (black)	P				
0,68	orange				V	V
0,66	orange	P				
0,65	blue/green		**P**	**P**	**P**	**P**
0,61	orange	**P**		P	P	P
0,57	light blue		V	P	V	
0,55	light blue					P
0,5	blue/green	**P**	V	P		
0,44	blue/green					P
0,39	blue/green		**P**	P	P	
0,34	blue/green				V	
0,27	blue/green		V	P	V	
0,23	dark (black)			V		
0,21	blue/green			V	V	
0,18	blue/green					P
0,17	light blue	V	P			
0,16	dark (black)			V	P	

FIGURE CAPTIONS

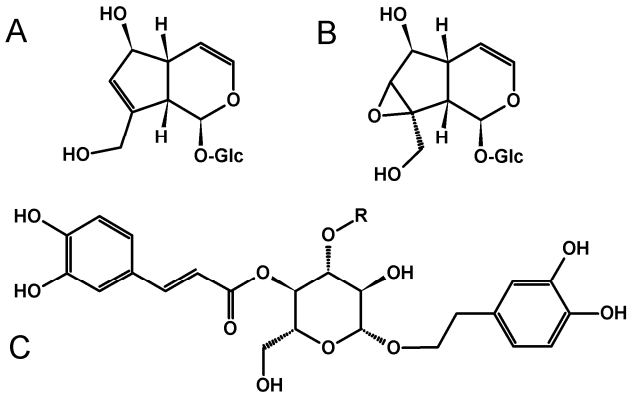

Figure 1. Structures of selected chief metabolites from the studied *Plantago* species. a., aucubin b., catalpol c., caffeoyl phenylethanoid glycosides: plantamajoside (R = Glc) and acteoside (R = Rha).

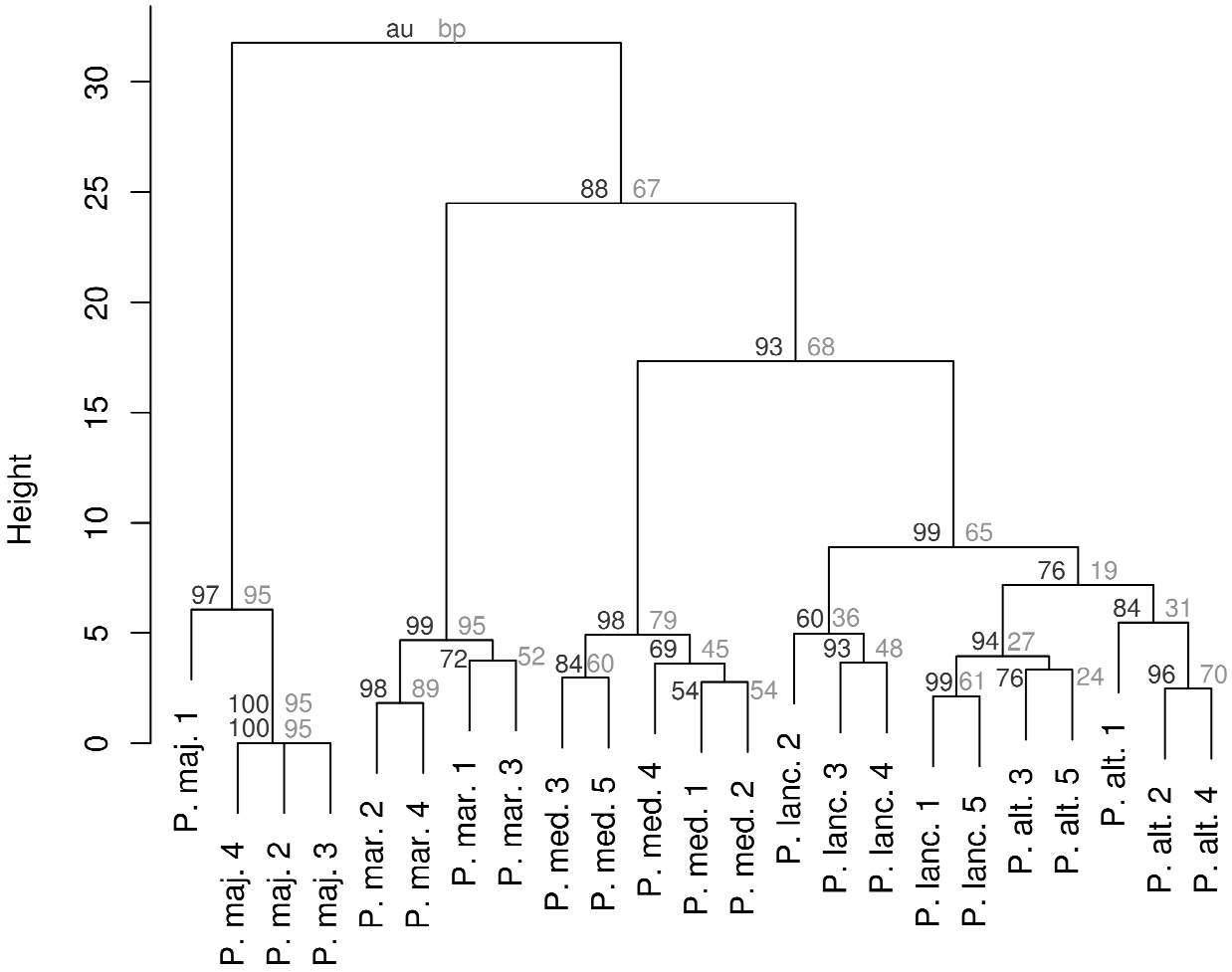

Figure 2. Results of cluster analysis of selected, intensive TLC bands (details given in text). Values presented are approximately unbiased p-value ("AU", left), bootstrap probability ("BP", right).