Artificial intelligence as a potential tool for micro-histological analysis of herbivore diets

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Abstract
Different non-invasive techniques have been used to determine herbivore diet composition from faecal samples, including micro-histological analysis of epidermal fragments. This method can provide reliable semi-quantitative data through the identification of plant cell structures visualized under an optical microscope. However, this method is highly time-consuming and it requires significant expertise in microscopic identification. Since micro-histological analysis is based on pattern recognition, automated identification and counting of epidermal fragments using artificial intelligence (AI) could be used to make this method more time efficient. We developed a software application based on an AI model that, appropriately trained, can identify and count epidermal fragments from photographed microscope slides. We compared the performance of this model to that of visual identification by a trained observer using in vitro mixtures of fragments from two plant species, *Arbutus unedo* and *Rubia peregrina*, with very different epidermal characteristics. Both the human observer and the AI model estimated proportions of plant fragments very close to those of the original mixtures. In addition, once trained the AI model was over 350 times faster in identifying and counting fragments compared to a human observer. Our study highlights the potential of AI to be applied to the study of herbivore diets for labour intensive pattern recognition tasks.

Keywords
machine learning; deep learning; epidermal fragments; fecal samples; microscope slides; cuticle analysis

Introduction

Knowledge of diet composition is often required in studies on wild herbivores. Diet composition is most efficiently estimated non-invasively from fecal samples. Several methods have been used for such purposes, with micro-histological analysis of epidermal fragments being one of the most useful (e.g. Stewart, 1967; Bartolomé et al., 1995). This well-established method provides reliable semi-quantitative data through the identification of plant cell structures, mainly the epidermis and trichomes, visualized under an optical microscope. However, this method requires significant expertise in microscopic identification, and it is also highly time-consuming (Holecheck and Gross, 1982). Other methods, such as near infrared reflectance spectroscopy (NIRS) (e.g. Coates and Dixon, 2008), quantification of n-alkanes (e.g. Ferreira et al., 2007), isotope stability analysis (e.g. Codron and Brink, 2007) and genetic meta-barcoding (e.g. Pegard et al., 2009; Espunyes et al., 2019) have also been used for herbivore diet estimation. However, many of these techniques are costly, require advanced laboratory instrumentation, and may not provide direct quantification of dietary proportions. No method has managed to provide more accurate information of the dietary proportion of herbivore diets than micro-histological analysis (Pareja et al., 2021). Therefore, automating the reading of microscopic slides could represent a major advance in the analysis of herbivore diet composition.

Since micro-histological analysis is based on pattern recognition, artificial intelligence (AI), in particular deep learning networks, could be used automate the process of identifying and counting epidermal fragments in microscope slides. Deep learning has been used to develop efficient models for object classification, such as Alexnet (Krizhevsky et al., 2012). Although deep learning has seen a broad range of recent uses in ecological research (Christin et al. 2019, Høye et al 2021), the only studies we are aware of that have used deep learning algorithms for micro-histological determination of herbivore diets are both over ten years old (Larcher and Costaguta, 2004; Larcher et al., 2008). With recent advances in image processing, deep learning algorithms and computing power (Bochkovskiy et al. 2020), we believe it is time to re-evaluate the possibility of using deep learning methods improve the efficiency of micro-histological analysis of herbivore diets.

The aim of this work was to evaluate the speed and accuracy of a deep learning algorithm to automatically identify and count epidermal plant fragments from photographed microscope slides. For this purpose, we developed a software application based on the YOLO (You Only Look Once) algorithm (Redmon et al. 2016), which is a fast neural network-based algorithm that has been widely used for object detection and classification. We used the current version YOLOv5 (https://github.com/ultralytics/yolov5), which has models of great accuracy and speed compared to other deep learning algorithms. We developed our software application to, once appropriately trained, automatically identify and count epidermal fragments in images from microscope slides. We evaluated how accurate this software application was in correctly identifying the proportions of epidermal fragments in samples with mixed cell proportions using in vitro mixed samples of two plant species selected because of their clear epidermal differences. We also compared the accuracy and time efficiency of the software to that of a human observer. We used this simple in vitro design since it provided great control of the original mixtures, which is a requirement for a rigid assessment of identification accuracy.

Methods

We performed the trial using two plant species, *Arbutus unedo*, which has polygonally shaped epidermal cells, and *Rubia peregrina*, which has lobularly shaped epidermal cells (Fig. 1). The two species were mixed in different proportions to simulate mixed herbivore diets. An amount of 5 g of

leaves of each species were dried in an oven at 60ºC until constant weight was obtained, after which the dried leaf material was ground in a 1 mm cutting mill. With the ground material, four mixtures were made with different percentage of weight used of each species: *Arbutus* 5% – *Rubia* 95% (Mix 1); *Arbutus* 25% – *Rubia* 75% (Mix 2); *Arbutus* 60% – *Rubia* 40% (Mix 3) and *Arbutus* 90% – *Rubia* 10% (Mix 4).

We prepared 10 microscope slides from each mixture following the method of Stewart (1967) and modified by Bartolomé et al. (1998). From each mixture, 0.5 g of material was suspended in 3 ml of concentrated HNO_3 to allow non-epidermal tissue digestion. The samples were placed in a water bath at 80˚C for 2 minutes and then diluted with 200 ml of distilled water. This suspension was sieved through 0.25 mm filter, and the solid filtered material recovered and suspended in 3 ml of NaClO for 15 minutes, in order to bleach the epidermal fragments. The samples were diluted again with 200 ml of distilled water and the solid material collected from the 0.25 mm filter. A sample of the recovered material was spread in a 50% aqueous solution of glycerin over ten microscope glass slides, at a density that prevented any significant overlapping of fragments. Cover slips (24 x 60 mm) were fixed to the slides with DPX varnish (Herter Instruments, Spain).

We used a Nikon Eclipse Ci-L microscope take photographs of the microscope slides at 100x magnification. The photographs had a resolution of 5400 x 3958 pixels, and each contained between 1 and 5 epidermal fragments. We took a total of just over 100 photographs of each mixture (101 - 115 per slide), including 170-219 fragments per mixture. Our data consisted of 604 photographs and 1124 epidermal fragments used for training the AI model and an additional 423 photographs taken to compare the efficiency of the AI algorithm against a human observer (J. Bartolomé Filella). For the training data, a trained observer identified all epidermal fragments from the photographs and labeled each fragment to species using annotation tools in LabelImg (v1.8.0, https://github.com/tzutalin/labelImg). Both the the human observer and the AI model identified and classed the fragments in each of the 423 evaluation photographs photograph, and the time required for these tasks were recorded for each method.

The AI model included a set of deep learning algorithms developed for object detection and classification implemented in the YOLOv5 architecture (https://github.com/ultralytics/yolov5). This is a Python implementation of the YOLO system using the Pytorch framework (https://pytorch.org/). Both YOLOv5 and Pytorch are available as open source software. The YOLOv5 architecture consists of three major components; a "backbone" which extract features from images, a "neck" which mixes and combines feature maps from the backbone and make them available for classification, and finally a "head" which takes prepared features from the neck and applies box and class prediction steps. The model was based 21.2 million parameters and 2 classes of objects (i.e. *A. unedo* and *R. peregrina*). For ease of use we attached the AI model to a graphic interface for the Windows operating system.

The training of the AI model was carried out using classifiers based on deep learning features targeting micro-anatomical plant traits in the images. This method has a very high accuracy, even with high morphological variation of structures and the presence of noise from the image acquisition (Aono et al., 2021). To further improve the accuracy of the AI model and its robustness in the image acquisition process, as well as to minimize over fitting in the training process, we used data augmentation to generate to expand the original 604 images to an augmented data set of 2,416 images. Data augmentation was conducted using the Albumentation libraries proposed by Buslaev et al. (2020). It consisted of constructing 3 additional versions of each original image where we randomly adjusted blur, contrast, hue, brightness, horizontal and vertical orientation. The augmented datasets was split into a training bin with 2,174 images and a validation bin with 242

images. The model was trained in 250 epochs and achieved a mean average precision (mAP) of 0. 993. The training was done on a virtual machine in Google Colab Pro with a GPU NVIDIA V100.

Once trained, the accuracy and efficiency of the software was compared to a human observer using the additional datasets of 423 images that were novel to the model. The AI model predictions for this comparison were done using a computer with a Intel(R) Core (TM) i7-9750H processor with a base frequency of 2.6 GHz and a maximum clock speed of 4.50 GHz (6 cores and 12 threads), a NVIDIA GTX 1660 Ti GPU and 12 GB of RAM.

For both the AI model and the human observer the number of fragments identified for each species was transformed into a percentage. To provide an estimate of the accuracy of the human observer and the AI model, we correlated percentages of *A. unedo* estimated from each of these two methods to those of the original mixtures using Pearson correlation coefficients. To provide an estimate of how agreement in the estimates provided by the human observer and the AI model we also correlated the percentages derived from the two methods. We did not correlate the percentages of *R. peregrina* since it would have derived identical results. For each mixture, we used two proportion z-tests to evaluate pair-wise differences between the percentage of fragments of *A. unedo* estimated by the AI model and the percentage in the original mixture, in the percentage of fragments of *A. unedo* estimated by the human observer and the percentage in the original mixture, as well as in the percentage estimated by the AI model and by the human observer. For all statistical tests, alpha error was set to 0.05.

Results and discussion

Estimates of the proportion of *A. unedo* were highly correlated with the original mixtures for both the human observer ($r>0.99$, $p<0.001$) and the AI model ($r>0.99$, $p=0.002$). There was also a high level of agreement between estimates from the human observer and the AI model ($r>0.99$, $p<0.001$). The error compared to the original mixtures ranged between 1.5% to 9.0% for the AI model and between 2.0% to 7.0% for the human observer (Table 1). For neither detection method, none of the estimated mixtures deviated significantly from the original ones, nor did the two detection methods deviate significantly from each other. The maximum difference between methods was 2.6%, observed with the mixture of 90% *Arbutus unedo* and 10% *Rubia peregrina*. The time required for counting and classifying 200 fragments was over 350 times faster for the AI model (35 seconds) compared to the time required for the human observer (3 hours).

Our AI model achieved great accuracy in the identification and counting of epidermal plant fragments in microscope slides of mixed samples, and gave almost identical results to those from a human observer. Although we acknowledge that this method would still require the time consuming steps of sample preparation and taking photographs, which in our case took approximately 4 hours to prepare the 10 microscope slides, the increased time efficiency of the AI model for object detection and identification was substantial, using approximately 0.3% of the time required by the human observer. Although we did observe some errors in the estimated proportions for both methods, those errors were probably due to the fact that the original mix was made on a weight basis, while the results of the micro-histological analysis were based on fragment counts. In addition, the erodability of both species could have been slightly different, which could have accounted for the observed differences between the estimated and real proportions of plants in our artificial mixtures. However, estimations of the proportions in mixtures of plants with less distinct cell structures than those used here may obviously give lower accuracy that what we observed.

Although this design did not include plant fragments that had passed animal digestion tracts we regard it appropriate for two reasons. First, it allowed us to test the method with very high

precision since it permitted full control of the original plant mixtures. Second, although the plant fragments themselves may be influenced by animal digestion, the method rests on identification of the shape of the cellulose cell walls. These do not generally change shape after passing animal digestion (Bartolomé et al. 1995). Therefore, we believe that our experimental design provides a robust and accurate test of the suitability for using AI to determine animal diet using micro-histology.

Artificial intelligence has previously been used for plant identification based on digital leaf images, employing the combination of shape and texture modeling methods (Pankaja and Suma, 2020), although not from micro-anatomical characters. Using neural networks for the identification of epidermal fragments of four different species, Larcher and Costaguta (2004) achieved an accuracy of between 62.5 and 87.5%. Our results show that precision can be increased from these earlier estimates using modern deep learning algorithms. Data augmentation procedures provide an efficient method to achieve such high precision by allowing the model to be trained on a larger and more diverse data set that what would be practically possible to generate through physical samples (Buslaev et al. 2020). Our study clearly shows that this methodology offers great potential to automatically identify and quantify the diversity of plant fragments that appear in fecal samples improving the study of herbivore diets.

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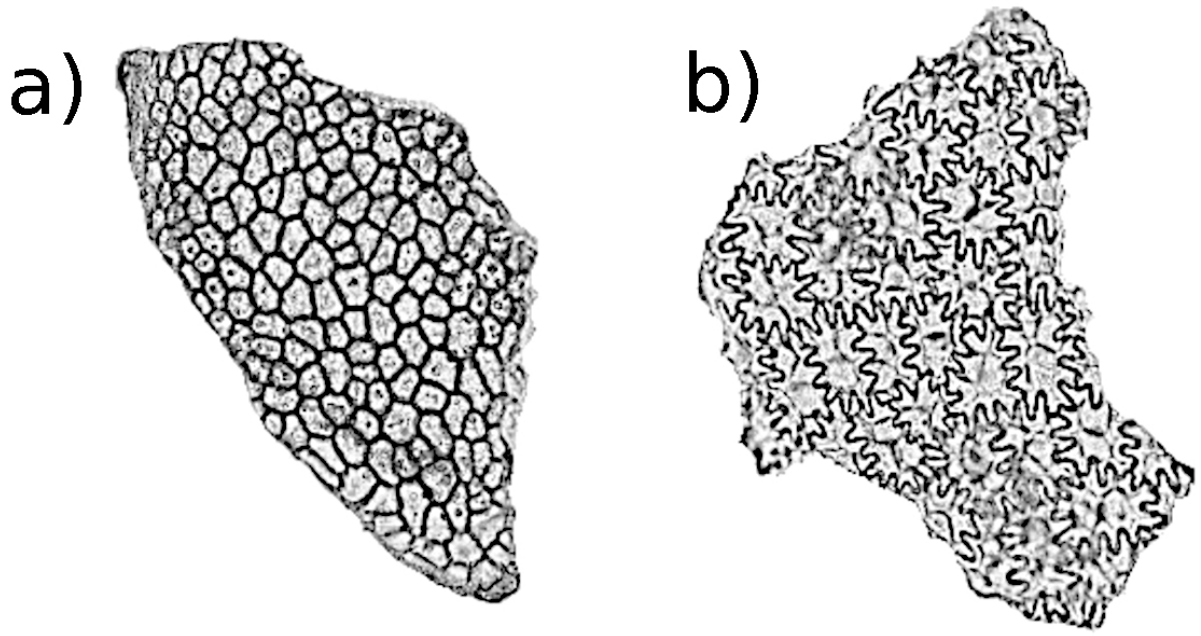

Fig. 1 Fragments of *Arbutus unedo* (a) and *Rubia peregrina* (b) at 100 times magnification, with the cell structures used for classification clearly visitble.

Table 1 Percentages of *Arbutus unedo* and *Rubia peregrina* in the original mixes (OM), percentages of *A. unedo* estimated from micro-histological analysis by a human observer (Human) and using the AI model (AI), number of identified fragments by the human observer and the AI model in 423 images used for methodological evaluation, pair-wise comparisons of the deviations from the original mixtures for the human observer (Human-OM) and the AI model (AI-OM), as well as pair-wise comparisons between the two estimation methods (AI-Human).

	Percentage*			N**		Human-OM			AI-OM			AI-Human		
	OM	Human	AI	Human	AI	Diff***	Z	P	Diff***	Z	P	Diff***	Z	P
Mix 1	5.0% / 95.0%	9.0% / 91.0%	8.2% / 91.8%	149	155	4.0%	0.12	0.406	3.2%	0.04	0.531	-0.8%	1.54	>0.999
Mix 2	25.0% / 75.0%	28.4% / 71.6%	29.5% / 70.5%	170	178	3.4%	0.26	0.701	4.5%	0.10	0.578	1.1%	1.12	>0.999
Mix 3	60.0% / 40.0%	59.0% / 41.0%	58.5% / 41.5%	193	200	-1.0%	1.54	>0.999	-1.5%	0.79	0.943	-0.5%	1.54	>0.999
Mix 4	90.0% / 10.0%	83.6% / 16.4%	81.0% / 19.0%	*219*	221	-6.4%	0.33	0.250	-9.0%	0.62	0.108	-2.6%	0.39	>0.999

* Percentages of each species in the original mixtures as well as those estimated by the AI model and the human observer.
** Number of fragments identified by the two quantification methods in 423 photograps used for model verification.
***Difference between the AI model and the human observer in estimated percentages of *Arbutus unedo*.