INTERNATIONAL BIOLOGY OLYMPIAD e. V. IBO

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Student Code:

24th International Biology Olympiad

14th-21st July, 2013

Bern, Switzerland

BERN 2013 International Biology Olympiad

Practical Exam 1

Molecular Cell Biology

Total points: **100**

Duration: **90 minutes**

Dear participants,

This test consists of two tasks:

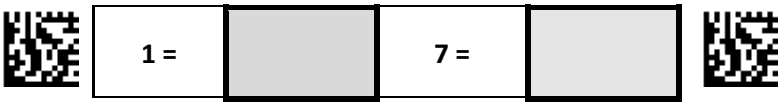
Introduction [2 points] **4**
Task 1: Presence of the β-glucuronidase [12 points] **5**
Part 1.1: Determine the presence of the β-glucuronidase [12 points] 5

Task 2: Presence of the procyclin protein [86 points] **6**
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Part 2.2: Washing the magnetic beads 7
Part 2.3: Density of trypanosomes not binding to magnetic beads [37.5 points] 7
Part 2.4: Total trypanosome density [25.5 points] 7
Part 2.5: Success of the deletion of the procyclin gene [9 points] 8
Part 2.6: Verification of binding to beads [9 points] 10
Part 2.7: Interpretation of your results [4 points] 10

Please write your student code into the box on the title page.

There is no separate answer sheet. Please fill in your answers into the specific answers boxes indicated with a gray background. **Only answers given inside these boxes will be evaluated.**

The answers have to be given either with a tick (√) or with Arabic numbers. The numbers "1" and "7" can look very similar in handwriting. To make sure that those two numbers can be well distinguished by the IBO staff, please write them as you normally would into the following box.



Stop answering and **put down your pen IMMEDIATELY** when the bell rings at the end of the exam. Put the entire protocol with all the answers back into the exam envelope.

Material and equipment

Make sure that you have received all the materials and equipment listed for each task. If any of these items are missing, please raise your hand.

Equipment

- Water bath at 37°C (used in common)
- 1 Micropipette P1000
- 1 Micropipette P200
- 1 Micropipette P20
- 1 Box of pipette tips for P1000
- 1 Box of pipette tips for P200 and P20
- 1 Eppendorf holder
- 1 Tube holder
- 1 Solid waste container
- 1 Liquid waste tube **[LW]**
- 1 Polystyrene (styrofoam) box filled with ice
- 1 Timer
- 1 Marker
- 1 Microscope
- 3 Cell counting chambers
- 3 Microscope slides
- 3 Cover slips
- 21 Eppendorf tubes
- 1 Magnetic Eppendorf holder
- Blank paper
- 1 Flag to call an assistant
- 1 Yellow sheet labeled with your student code

Chemicals

- 1 Eppendorf tube with magnetic beads **[MB]**
- 1 Tube with phosphate buffer **[PBSB]**
- 1 Eppendorf tube with Fixation Buffer **[FB]**
- 1 Eppendorf tube with Substrate Buffer **[SB]**
- 1 Eppendorf tube with Substrate (X-gluc) **[S]**

Trypanosome suspensions

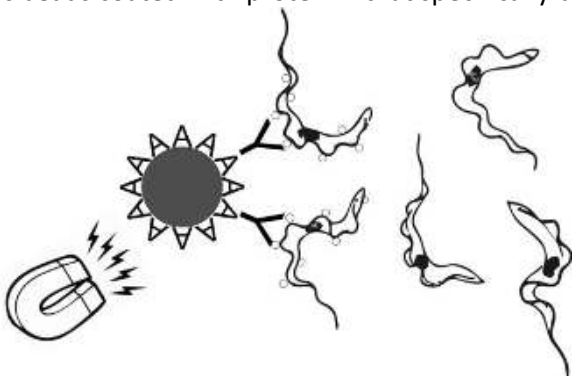
- 1 Eppendorf tube with suspension of Strain 1 **[T1]**
- 1 Eppendorf tube with suspension of Strain 2 **[T2]**
- 1 Eppendorf tube with suspension of Strain 3 **[T3]**

Introduction [2 points]

Trypanosoma brucei is a parasite causing sleeping sickness in humans and nagana in animals. It is transmitted between individuals via the tsetse-fly and is almost exclusively found in Africa south of the Sahara. To better understand the function of different proteins implicated in the life cycle and infection pathway of *T. brucei*, it is the goal to create mutant strains that lack procyclin, but express another protein of interest instead. Procyclin is a surface protein found in *T. brucei* but not in other trypanosome species and is hypothesized to have an effect on the infection pathway. Different trypanosome species rely on different surface proteins for their infectivity. For example, *T. congolense* relies on a surface protein called GARP.

In this practical you will work with strains of the subspecies *T. brucei brucei*, which can infect domestic and wild animals, but is not dangerous for humans. In a first step, cells were transfected or not with a single construct coding for both GARP and β-glucuronidase and grown as pure strains. β-glucuronidase, which is absent in wild-type *T. brucei*, is a protein that can cleave X-gluc, an artificial substrate, into a blue product that can easily be observed by eye. In this setting, β-glucuronidase serves as a convenient reporter gene that will allow you to recognize the strains carrying a successfully introduced construct by simply incubating the strains with X-gluc (Task 1).

The strains were then subjected to a protocol to delete the gene coding for the protein procyclin. This would allow verifying whether procyclin is indeed important for the infectivity cycle and whether GARP can compensate for the procyclin function. Since the deletion efficiency is not 100%, you will separate the cells where the procyclin gene was successfully deleted from the ones in which the deletion did not work. To achieve this, trypanosomes pre-incubated with antibodies specific against procyclin will be separated with magnetic beads coated with protein A that specifically binds to the Fc part of antibodies,



as is illustrated below.

Before starting the practical work, indicate for each of the following statements if it is true or false with a tick (√). **[2 points]**

Q1

	true	false
If the suspension of a strain incubated with X-gluc turns blue, the β-glucuronidase gene was successfully introduced.		
Inferring the successful introduction of the GARP gene based on the presence of the reporter gene (β-glucuronidase) may result in false positives or negatives if only one gene of the construct was successfully inserted.		
The location in the genome where the construct is introduced affects the level of gene expression of the introduced genes.		
A similar approach with magnetic beads and specific antibodies can be used to separate cells, with successful deletion of a gene coding for an intracellular protein, from cells where the deletion did not work.		

Task 1: Presence of β-glucuronidase [12 points]

Part 1.1: Determine the presence of β-glucuronidase [12 points]

Prepare the following reaction mix for each of the three trypanosome strains T1, T2 and T3 in a separate Eppendorf tube and mix by pipetting up and down:

1. 20 µl of the trypanosome suspension. Since the trypanosomes sink to the bottom of the tube, make sure to mix the tubes by inverting prior to pipetting.
2. 100 µl substrate buffer **(SB)**
3. 10 µl substrate **(S)**

Label each tube with the strain you used as well as with your three letter country code (as indicated on your badge).

The reaction mixes must be incubated for at least 1 hour at 37°C. Place your flag into the tube on your partition wall to call an assistant who will put your tubes in a water bath. Also, use your flag to indicate to the assistant that you want to get your tubes back from the water bath. Consider working on the other task during the incubation.

Put your tubes on the yellow sheet with your student code in the corresponding box, they will be photographed and evaluated.

Indicate with a tick (√) for each of the three strains if the sample turned blue or remained colourless after incubation. **[12 points]**

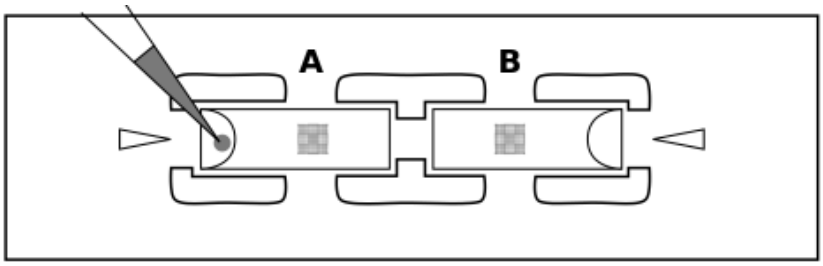
Q2

	Strain T1	**Strain T2**	**Strain T3**
Blue			
Colourless			

Task 2: Presence of the procyclin protein [86 points]

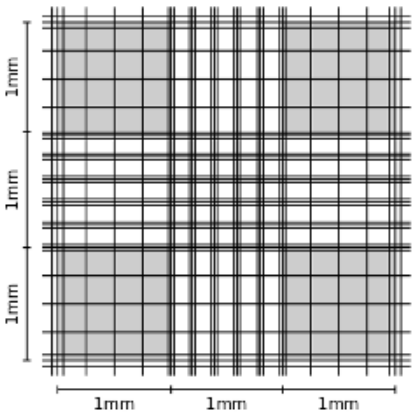
Part 2.1: How to use the counting chamber [1 point]

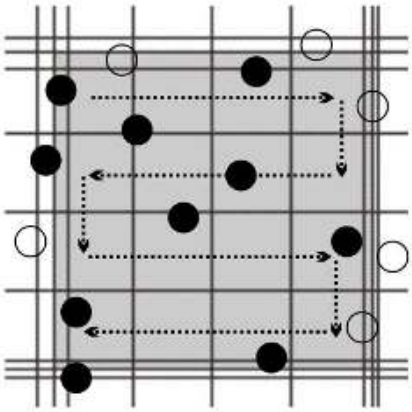
You will use counting chambers to determine the density of trypanosomes in parts 2.3 and 2.4. Two counting chambers that can be individually filled are organized on a single slide. **Please be aware that these chambers cannot be cleaned and that no extra counting chambers will be provided. Also, these counting chambers do not need a cover slip.**



The following steps should be performed when determining the density of trypanosomes in a suspension:

1. Pipette 10 µl of the suspension into a counting chamber.
2. Wait at least 2 minutes for the cells to sink to the bottom.
3. Put the slide under the microscope and count the number of trypanosomes individually in three of the four larger squares highlighted in gray.





You may use either the 10x or the 40x objective, whichever you prefer. It is advised to follow a serpentine (snake-like) route to go through each of the smaller squares to avoid losing orientation. In order to prevent a potential bias, count trypanosomes within the square and those crossing the left or bottom limit (filled circles), but not those outside the square or crossing the right or top limit (open circles).

To obtain the trypanosome density from the number of counted trypanosomes, first determine the volume in which the cells are counted and indicate it in the table below. Note that the height of the counting chamber is exactly 0.1 mm and each of counting cell is exactly 1 mm wide (see figure above) **[1 point]**.

Q3

Volume of 1 counting square (mm^3)	
Volume of 1 counting square (ml)	

Part 2.2: Washing the magnetic beads

Wash the magnetic beads **(MB)** twice as follows:

1. Add 1 ml cold phosphate buffer **(PBSB)** to the tube and mix by pipetting up and down.
2. Place the Eppendorf tube in the magnetic holder. Wait at least 1 minute for the magnetic beads to get pulled down.
3. Pipette the supernatant into the liquid waste tube **(LW)**.

Finally, resuspend the magnetic beads in 35 µl PBSB buffer and put them on ice.

Part 2.3: Density of trypanosomes not binding to magnetic beads [37.5 points]

Pull down trypanosomes expressing procyclin in a sample of each of the three trypanosome strains T1, T2 and T3 as follows:

1. Pipette 190 µl of the trypanosome suspension in a fresh Eppendorf tube. Mix the trypanosome suspension by inverting prior to pipetting.
2. Add 10 µl of washed magnetic beads. Make sure the beads are resuspended prior to pipetting.
3. Incubate 30 minutes on ice. Resuspend the magnetic beads very gently every 3-5 minutes by inverting and finger-flicking the tube. Consider working on other parts of Task 2 during the incubation.
4. Pull down the magnetic beads using the magnetic holder.
5. Transfer the entire supernatant, while the tube is still in the magnetic holder, into a fresh Eppendorf tube and put on ice.
6. Immediately resuspend the magnetic beads in 50 µl phosphate buffer **(PBSB)** very gently and put on ice.
7. Prepare 100 µl of a 1:10 dilution of the supernatant in phosphate buffer **(PBSB)** in a fresh Eppendorf tube.
8. Pipette 36 µl of this dilution into a separate Eppendorf tube and add 4 µl of Fixation Buffer **(FB)**. Mix well by pipetting up and down.
9. Count the number of trypanosomes according to the protocol in Part 2.1 and enter the values in the table below.
10. Calculate the mean of the number of trypanosomes per square (precision: 3 positions after the decimal point). You will use these numbers in Part 2.5. [37.5 points]

Q4

	Strain T1	**Strain T2**	**Strain T3**
Square 1			
Square 2			
Square 3			
Mean counts per square			

Part 2.4: Total trypanosome density [25.5 points]

To determine the total trypanosome density in the original suspension, perform the following steps for each of the three trypanosome strains T1, T2 and T3:

1. Prepare 100 µl of a 1:10 dilution of the original suspension in phosphate buffer **(PBSB)** in a fresh Eppendorf tube. Mix the trypanosome suspension by inverting prior to pipetting.

2. Pipette 36 µl of this dilution into a fresh Eppendorf tube and add 4 µl of Fixation Buffer **(FB)**. Mix well by pipetting up and down.
3. Count the number of trypanosomes according to the protocol in Part 2.1 and report the values in the table below.
4. Calculate the mean and the standard deviation of the number of trypanosomes per square (precision: 3 positions after the decimal point). You will use these numbers in Part 2.5. **[25.5 points]**

The formula for the standard deviation (SD) is given below with being the number of replicates, the value of the replicate and the mean.

Q5

	Strain T1	Strain T2	Strain T3
Square 1			
Square 2			
Square 3			
Mean counts per square			
Standard deviation of counts per square			

Part 2.5: Success of the deletion of the procyclin gene [9 points]

The ultimate goal is to calculate and compare the density of trypanosomes not binding to magnetic beads to the total trypanosome density in the starting suspension from the average counts observed. However, you will first have to determine the standard error of the mean (SE_{mean}) to decide on the correct number of significant digits. Under the assumption that the counts are normally distributed, the SE_{mean} is given by

Calculate SE_{mean} for the strain for which you observed the largest standard deviation among the counts of trypanosomes per cell from Part 2.4 and enter your result in the table below (precision: 3 positions after the decimal point). **[0.6 points]**

Q6

SE_{mean}	

The SE_{mean} tells you the accuracy with which you estimated the mean number of trypanosomes per square. Use this estimate to decide the correct number of significant digits (all digits including the first for which you are uncertain) by comparing the estimated mean plus the SE_{mean} with the estimated mean minus SE_{mean}. For instance, if the mean is 1234.567 and the SE_{mean} 98.765, you will have to compare 1234.567 + 98.765 = 1333.332 with 1234.567 - 98.765 = 1135.802. In this case, there are two significant digits and the mean should be reported as $1.2x10^3$. Indicate the number of significant digits you should use with your data. **[1.5 points]**

Q7

Number of significant digits	

Report in the table below the mean counts per square with and without pull-down for all three strains using the number of significant digits you indicated above. **[0.6 points]**

Q8

	Strain T1	**Strain T2**	**Strain T3**
Mean counts per square of trypanosomes not binding to magnetic beads (from Part 2.3)			
Mean counts per square of total trypanosomes (from Part 2.4)			

Now use these values to estimate the density of trypanosomes in the dilutions used for counting, and report your values in the table below with the same number of significant digits. **[3.7 points]**

Q9

	Strain T1	**Strain T2**	**Strain T3**
Trypanosomes not binding to magnetic beads /ml in dilution used for counting (from Part 2.3)			
Total trypanosomes / ml in dilution used for counting (from Part 2.4)			

Finally, calculate both the density of trypanosomes not binding to magnetic beads as well as the total trypanosome density in the original suspension, and report your values in the table below with the same number of significant digits. **[1.1 points]**

Q10

	Strain T1	**Strain T2**	**Strain T3**
Trypanosomes not binding to magnetic beads /ml in original suspension (from Part 2.3)			
Total Trypanosomes / ml in original suspension (from Part 2.4)			

In order to assess the success rate of the gene deletion experiment, calculate the percentage of trypanosomes that did not bind to magnetic beads for each of the three strains. Use the estimates for the densities in the original suspension for your calculations and indicate your results in the table below (precision: only full percentages). **[1.5 points]**

Q11

	Strain T1	Strain T2	Strain T3
Percentage of trypanosomes **not** binding to beads			

Part 2.6: Verification of binding to beads [9 points]

You will next verify under the microscope if a reduction in trypanosomes observed after pull down is indeed due to binding of trypanosomes to the magnetic beads. To do so, perform the following steps for each of the three strains:

1. Pipette 10 µl of the beads, you resuspended in Part 2.3, on a microscope slide.
2. Cover the drop with a cover slip.

Make a rough assessment of the fraction of trypanosomes that are attached to a magnetic bead. A good indication that a trypanosome is bound to a bead is when the bead wiggles as the trypanosome moves.

In the table below, indicate with a tick (√) for each of the three strains which description best fits your observation. **[9 points]**

Q12

	Strain T1	Strain T2	Strain T3
Practically no bound trypanosomes in the sample			
<50% of the trypanosomes present in the sample are bound			
>50% of the trypanosomes present in the sample are bound			

Part 2.7: Interpretation of your results [4 points]

Indicate with a tick (√) the statement best describing the reduction in trypanosomes in the supernatant after pull down for each strain. **[3 points]**

Q13

	Strain T1	Strain T2	Strain T3
A reduction, at least in part due to binding to beads			
No or only a purely stochastic change			

Indicate with a tick (√) the strain where the deletion was most efficient. **[1 point]**

Q14

	Strain T1	Strain T2	Strain T3
Highest deletion efficiency			

End of the Practical Exam

Student Code:

24th International Biology Olympiad

14th-21st July, 2013

Bern, Switzerland

BERN 2013 International Biology Olympiad

Practical Exam 2

Plant Physiology, Morphology and Ecology

Total points: **88**

Duration: **90 minutes**

Dear participants,

This test consists of three tasks:

Task 1: Determination of glucose content in plant extracts [44 points]

- Part 1.1: Calibration curve [13.5 points]
- Part 1.2: Glucose content [15 points for measured raw values]
- Part 1.3: Data analysis [15.5 points]
  - 1.3.1 NADH calibration curve
  - 1.3.2 Glucose concentration in plant extracts
  - 1.3.3 Interpretation of your results

Task 2: Staining of transitory starch [9 points]

Task 3: Floral morphology and pollination ecology [35.5 points]

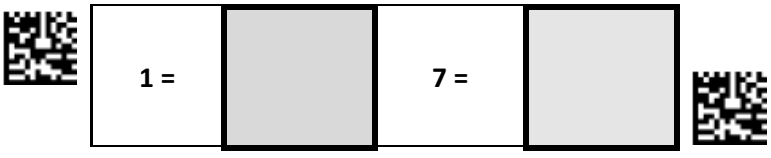
- Part 3.1: Floral morphology
  - 3.1.1 Type of inflorescence
  - 3.1.2 Number of floral parts
  - 3.1.3 Fusion of floral parts
  - 3.1.4 Ovary position
  - 3.1.5 Floral symmetry
- Part 3.2: Pollination ecology
  - 3.2.1 Floral shape
  - 3.2.2 Pollinators

Please write your student code into the box on the title page.

You are strongly advised to start working with Task 1. During this task, you will have to incubate your probes for 20 minutes and then allow for some time for the lab assistants to perform the necessary measurements. During this waiting time you may work on Tasks 2 and 3.

There is no separate answer sheet. Please fill in your answers into the specific answers boxes indicated with a gray background. **Only answers given inside these boxes will be evaluated.**

The answers have to be given either with a tick (√) or with Arabic numbers. The numbers "1" and "7" can look very similar in handwriting. To make sure that those two numbers can be well distinguished by the IBO staff, please write them as you normally would into the following box.



Stop answering and **put down your pen IMMEDIATELY** when the bell rings at the end of the exam. Put the entire protocol with all the answers back into the exam envelope.

Material and equipment

Make sure that you have received all the materials and equipment listed for each task. If any of these items are missing, please raise your hand.

Task 1

Plant material:

- 6 x 250 µl plant extracts **[WT light, WT dark, sex1 light, sex1 dark, pgm1 light, pgm1 dark]**

Solutions and reagents:

- 1.5 ml "Master mix" **[MM]**
- 1 ml NADH solution 500 µM **[NADH]**
- 200 µl G6PDH **[G6PDH]**
- 10 ml H_2O **[H2O]**

Technical material:

- 1 micropipette 50 µl-200 µl (can be used down to 20 µl)
- 1 96-well Microplate (**don't touch the bottom of the plate!**)
- 1 Timer
- Container for used materials
- 2 blank paper sheets for notes
- Flag to call the lab assistant

Task 2

Plant material:

- 8 tubes with de-pigmented plants **[A, B, C, D, E, F, G, H]**

Solutions and reagents:

- 10 ml Lugol's solution **[Lugol]**

Technical material:

- 8 plastic dishes
- Plastic Pasteur pipette
- Waterproof pen for writing on plastic dishes

Task 3

Plant material:

- 5 tubes with floral specimen in 70% ethanol **[V, W, X, Y, Z]**
- Color print with photos of the flowers V-Z

Technical material:

- 1 dissecting microscope
- 1 razor blade
- 1 forceps
- 2 toothpick
- 1 plastic dish filled with water

Task 1: Determination of glucose content in plant extracts [44 points]

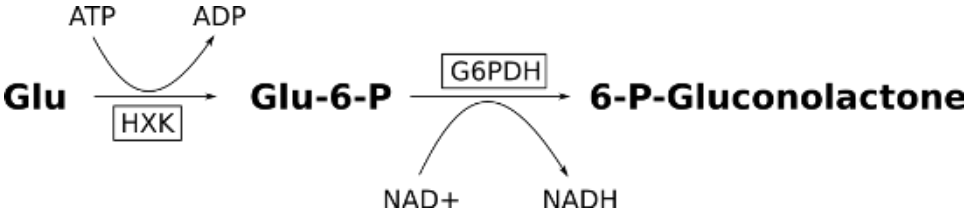
Under sufficient light, plants synthesize carbohydrates from atmospheric CO_2 by photosynthesis. While a fraction of these photoassimilates is exported to the cytosol and then transformed to soluble glucose, another fraction is retained within the chloroplasts and stored transiently as starch. This starch is subsequently degraded and mobilized to provide a supply of carbon and energy during dark periods.

Several mutants of *Arabidopsis* have been identified that are unable to either synthesize or degrade starch. In this practical, you will work with plant extracts from two of them, along with extract from wild type **[WT]** plants:

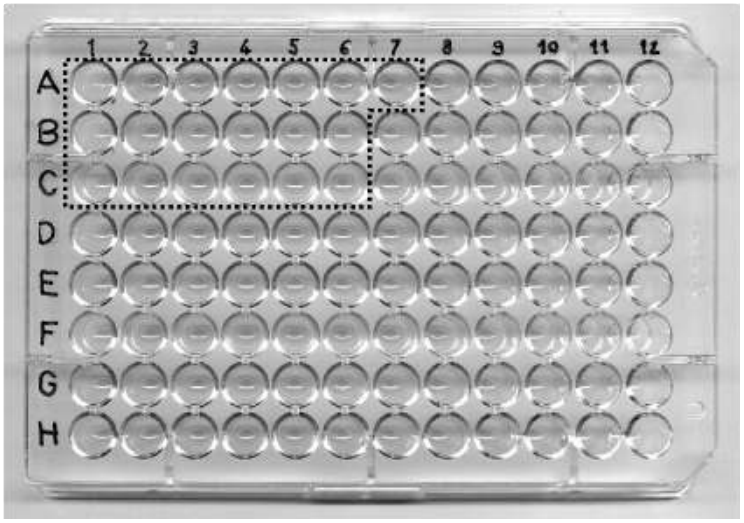
1. **WT** : unmutated wildtype plants
2. **sex1** : starch degradation mutants
3. **pgm1** : starch synthesis mutants

All plants were grown for 4 weeks with 8 hours light and 16 hours of darkness per day and then either exposed to complete darkness for 48 hours (dark-incubated or "dark") or to 10 hours of light (light-incubated or "light") immediately prior to harvesting.

You are going to quantify the glucose content in those plant extracts by measuring the absorbance of NADH. As shown below, one molecule of NAD+ is converted into one molecule of NADH per molecule of glucose in a two-step reaction that is catalyzed by the enzymes hexokinase (HXK) and glucose-6-phosphate dehydrogenase **[G6PDH]**. The "master mix" **[MM]** contains NAD+, HXK and ATP.



You will work with a 96-well microplate (see figure below). **Only touch its edges**! Positions on this plate are indicated by a number (1-12) and a letter (A-H) specifying columns and rows, respectively. **Use only the wells which are listed in the protocol** (contained within the area indicated by the dotted line).



Part 1.1: Calibration curve [13.5 points]

In order to quantify glucose by measuring the absorbance of NADH, you will first have to make a calibration curve with different concentrations of NADH. In the table below, indicate the required volumes of the 500 µM NADH stock solution and H_2O that are needed to achieve the desired concentrations of NADH in a total volume of 200 µl. Then pipette the required volumes into the corresponding well on the microplate (A1-A7) and mix by pipetting up and down three times. **[3 points for calculations + 10.5 points for measured raw values]**

Q 1

Well	A1	A2	A3	A4	A5	A6	A7
[NADH] (µM)	0	50	100	150	200	250	300
500 µM NADH solution (µl)							
H_2O (µl)							

Part 1.2: Glucose content [15 points for measured raw values]

To determine the concentration of glucose in the different plant extracts, you will now prepare two solutions for each plant extract. The Wells B1-B6 will serve as a blank to quantify the base absorption and differ from the Wells C1-C6 which will contain a solution of G6PDH. Begin by pipetting the following into Wells B1-B6 and C1-C6:

1. 100 µl "Master mix" into the Wells B1-B6 and C1-C6
2. 20 µl H_2O into the **Wells B1-B6**
   20 µl G6PDH solution into the **Wells C1-C6**
3. 80 µl of the corresponding plant extract **as indicated in the table** below and mix by pipetting three times up and down

Wells	B1 and C1	B2 and C2	B3 and C3	B4 and C4	B5 and C5	B6 and C6
Plant extract	WT light	WT dark	sex1 light	sex1 dark	pgm1 light	pgm1 dark

Incubate for at least 20 and up to 60 minutes at room temperature. After incubation, call an assistant by placing your flag into the tube on your left partition wall. The assistant will measure the absorbance at 340 nm and bring you a printout of the absorbance values measured for each well. You will need these values for the analysis in Part 1.3.

NOTE: Due to the limited number of microplate readers, you might have to **wait up to 15 minutes** to have your microplate measured after putting up your flag. During incubation time and waiting time you may work on Tasks 2 and 3.

Part 1.3: Data analysis [15 points]

Important: Label the printout with your name and your student code. At the end of the exam, put it into your exam envelope.

1.3.1 NADH calibration curve

Calculate the scaled extinction coefficient (ε_s) for all measured NADH concentrations according to

$$\varepsilon_s = \varepsilon \cdot l = \frac{A_C - A_0}{c_{NADH}}$$

where A_0 is the background absorbance value at 0 μM NADH (Well A1) and A_c the absorbance value at concentration c_{NADH}. ε_s is equivalent to the extinction coefficient ε multiplied with l, the path length of light through the solution. Write the calculated values in the answer fields (precision: five positions after the decimal point). **[3 points]**

Q 2

Well	A2	A3	A4	A5	A6	A7
[NADH] (μM)	50	100	150	200	250	300
ε_s (μM^{-1})						

Calculate the mean value of all calculated scaled extinction coefficients $\bar{\varepsilon}_s$ and write your result in the corresponding field below (precision: five positions after the decimal point). **[1 point]**

Q 3	$\bar{\varepsilon}_s$ (μM^{-1})	

1.3.2 Glucose concentration in plant extracts

Calculate the glucose concentration $c_{glucose}$ in each well (diluted plant extract) measured as

$$c_{glucose} = \frac{A_{G6PDH} - A_{H2O}}{\bar{\varepsilon}_s}$$

where A_{G6PDH} is the absorbance measured for the sample incubated with G6PDH (Wells C1-C6), A_{H2O} is the absorbance measured for the sample incubated with H_2O only (Wells B1-B6), and $\bar{\varepsilon}_s$ is the mean scaled extinction coefficient you have calculated above. Then, use these values to calculate the initial glucose content that was present in the fresh leaves, indicated as mmol/g. Each plant extract was made of 25 g leaf material per liter. Report your values in the table below (precision: one position after the decimal point). **[7 points]**

Q 4

Sample	Concentration in well (μM)	Content in leaves (mmol/g)
WT light		
WT dark		
sex1 light		
sex1 dark		
pgm1 light		
pgm1 dark		

1.3.3 Interpretation of your results

Indicate with a tick (√) for each of the following statements if it is true or false based on **your** measurements. **[4 points]**

Q 5

	true	false
Plants light-incubated prior to extraction contain more glucose than those incubated in the dark.		
Dark-incubated plants have used up all their carbohydrate energy sources in the dark period.		
Glucose levels in light-incubated wild-type plants are lower than in dark-incubated wild-type plants.		
Both "pgm1 light" and "sex1 light" samples contain more glucose than "WT light" samples.		
The "sex1 light" sample contains more glucose than the "pgm1 light" sample.		
The difference in glucose concentrations between dark- and light-incubated plants is bigger in wild type plants than in mutant plants.		
sex1 plants are likely to grow faster than pgm1 plants.		
Measuring samples without addition of G6PDH is needed to correct the effect of the background concentration of 6-P-Gluconolactone.		

Task 2: Staining of transitory starch [9 points]

Transitory starch can be easily visualized in leaves by staining it with "Lugol's solution" containing iodine in ethanol-destained leaves. On your lab bench you find two groups of plant samples.

GROUP I	**A**	**B**	**C**	**D**
GROUP II	**E**	**F**	**G**	**H**

While all plants of one group have been harvested after incubating for 12 hours in light, all plants of the other group have been harvested after incubating for 12 h in complete darkness. Each group contains **at least** one wild type plant (WT), one starch degradation mutant (sex1) and one starch synthesis mutant (pgm1).

Repeat the following steps for each of the plants A-H to stain them:

1. Transfer the plant specimen into a fresh plastic dish.
2. Remove residual liquid from the dish, with the Pasteur pipette.
3. Stain the plants by covering them with some drops of "Lugol's solution" using the Pasteur pipette.
4. Incubate at room temperature for 1 minute before doing your observations.

Indicate the observed staining pattern of leaves of each plant specimen in Group I (Plants A-D) and Group II (Plants E-H) in the table below with a tick (√). **[4 points]**

Q 6

Group I				Group II			
Plant	**Light orange**	**Dark brown**	**Intense black**	**Plant**	**Light orange**	**Dark brown**	**Intense black**
A				**E**			
B				**F**			
C				**G**			
D				**H**			

Based on the observed plants and staining pattern, indicate the corresponding growth condition for Group I and Group II in the table below with a tick (√). **[1 points]**

Q 7

	Growth condition	
	12 h in light	**12 h in darkness**
Group I		
Group II		

Q 8

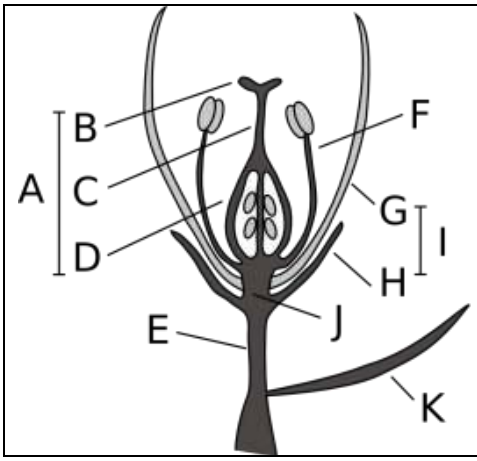
Based on the observed plants and staining pattern, indicate the corresponding strain for each of the plant specimen (A-H) in the table below with a tick (√). **[4 points]**

		Strain		
Group	**Plant**	**WT**	**sex1**	**pgm1**
I	**A**			
	B			
	C			
	D			
II	**E**			
	F			
	G			
	H			

Task 3: Floral morphology and pollination ecology [35.5 points]

Angiosperm flowers show an enormous variety in structure, shape and ecological function. While the number, shape and disposition of different floral parts is often phylogenetically determined, functional characteristics such as the general shape of the flower depend more on ecological factors such as the mode of pollination. In this task, you will study the morphologic traits of five different floral samples (V-Z) and attribute them ecological traits linked with pollination.

To help understand the terminology used below, the following figure gives you an overview of the different parts in a schematic flower.



A: carpel
B: stigma
C: style
D: ovary
E: pedicel
F: stamen
G: petal
H: sepal
I: tepal (when sepals and petals are undifferentiated)
J: floral axis
K: bract

Part 3.1: Floral morphology

Look at the five specimens V-Z preserved in ethanol, containing a flower or an entire inflorescence of plant species commonly found in Switzerland. The flowers are all hermaphrodite, with male and female parts present in the same flowers. The habit, shape and color of the corresponding plants are shown on the color printout.

For each specimen V-Z, you will be asked to make a series of morphological observations. It is recommended to do first all observations for one specimen before going over to the next one.

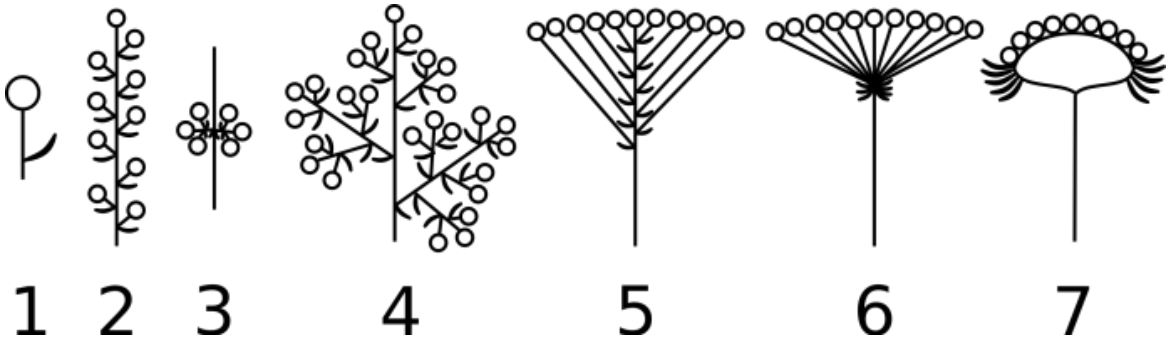
Take the specimen out of the tube and place it in a Petri dish filled with water for easier observation. Please close the tube again to avoid excessive ethanol vapor in the room.

Handle the flower specimens carefully, as you have to get along with the given plant material to do all your observations. In the case a specimen consists of more than one flower, isolate individual flowers to observe specific parts. For such plants you may want to observe a specific part in several flowers as some floral parts (notably stamen and carpels) are easiest observed in flowers in a specific state of maturation.

Observe small details with the dissecting microscope. For a part of the observations you might have to dissect the flower, for which you can use the given material (razor blade, forceps and toothpicks). Make all dissections in the Petri dish.

3.1.1 Type of inflorescence

The figure shows schematic illustrations of different types of inflorescences. Each small circle represents a single flower and is often accompanied by a bract.



Indicate the most appropriate type of inflorescence for each Specimen V-Z with a tick (√).**[2.5 points]**

Q 9

	V	W	X	Y	Z
Inflorescence type 1					
Inflorescence type 2					
Inflorescence type 3					
Inflorescence type 4					
Inflorescence type 5					
Inflorescence type 6					
Inflorescence type 7					

3.1.2 Number of floral parts

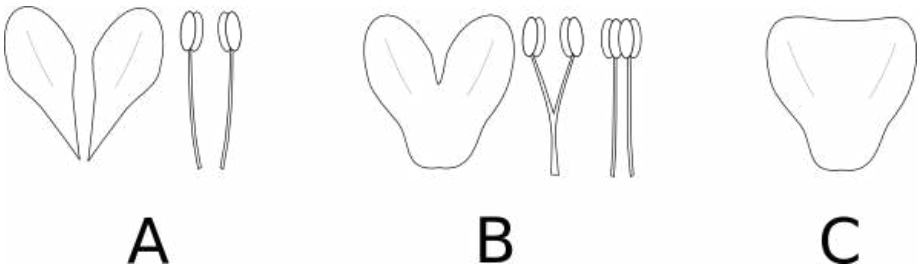
Determine the number of sepals, petals (or tepals), stamen and carpels per flower for each of the Specimens V-Z and write your results in the table below. When more than 10 parts of a specific floral part are present within a single flower, indicate ">10". Omit counting floral parts indicated by a black cell in the table. **[11.5 points]**

Q 10

	V	W	X	Y	Z
number of sepals					
number of petals or tepals					
number of stamens					
number of styles (count branched styles only once)					
number of distinct ovaries					

3.1.3 Fusion of floral parts

As illustrated in the figure with schematic petals and stamen, floral parts can either be free (A), partially fused (B) or completely fused (C). A: free (fused part <10%), B: partially fused (fused part 10-90%), C: completely fused (fused part >90%).



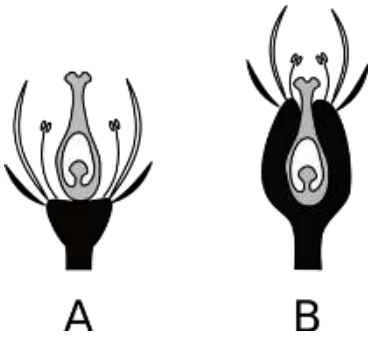
Indicate the correct state of fusion of sepals, petals and stamen for flowers of plant Specimens V-Z with a tick (√) in the table below. **[6.5 points]**

Q 11

		V	W	X	Y	Z
Sepals	Free (fused part <10%),					
	Partially fused (fused part 10-90%)					
	Completely fused (fused part >90%)					
Petals / tepals	Free (fused part <10%)					
	Partially fused (fused part 10-90%)					
	Completely fused (fused part >90%)					
Stamens	Free					
	Partially fused to other stamens at the base or the top (fused part 10-90%)					
	Partially fused with petals / tepals (fused part 10-90%),					
	Partially fused to other stamens AND with petals / tepals (fused part 10-90%)					

3.1.4 Ovary position

The position of the ovary of a flower can be classified as superior (A) or inferior (B), as is shown in the figure below.



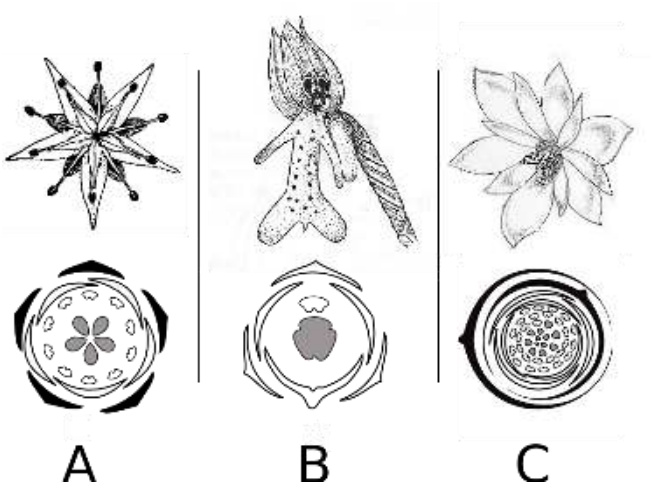
Indicate the ovary position of the flowers for each specimen V-Z with a tick (√) in the table below. **[2.5 points]**

Q 12

	V	W	X	Y	Z
A (superior)					
B (inferior)					

3.1.5 Floral symmetry

The symmetry of a flower can be classified into radial (A), bilateral (B) or asymmetrical (C). The following figure illustrates these symmetries both with example flowers as well as floral diagrams (second row). Floral diagrams show from outside to inside sepals (filled and curved), petals or tepals (open and curved), stamen (open and round) and carpels (grey and round).



Indicate the floral symmetry for flowers of each of the specimens V-Z with a tick (√) in the table below. **[2.5 points]**

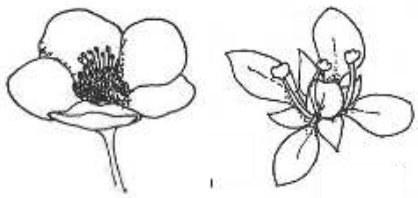
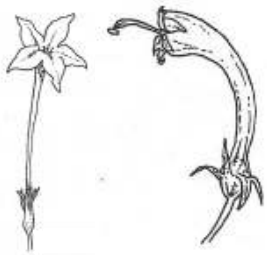
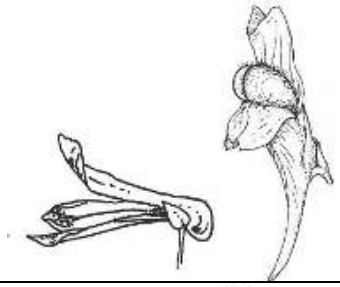
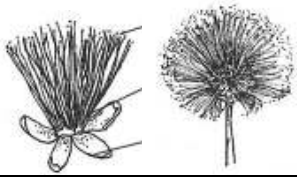
Q 13

	V	W	X	Y	Z
A (radial)					
B (bilateral)					
C (asymmetrical)					

Part 3.2: Pollination ecology

3.2.1 Floral shape

Floral shapes evolved as adaptations to the morphology and behavior of different pollinators. Despite the enormous diversity of flowers and inflorescences, most of them can be classified into one of several broad classes shown in the table below. Note that the relevant unit for attracting pollinators can be a single flower or an entire inflorescence.

n°	Floral shape	Illustration of example flowers or inflorescences	General characteristics
1	Open disk flower		• Pollen and/or nectar freely presented • Flat landing place.
2	Tubular flower with radial symmetry		• Radial symmetry • Pollen and/or nectar partially or completely hidden in a narrow tube • Tube may be built from free or fused floral parts
3	Tubular flower with bilateral symmetry		• Bilateral symmetry • Pollen partially or completely hidden in a narrow tube • Tube may be built from free or fused floral parts
4	Brush flower		• Absent or inconspicuous perianth / petals • Numerous and predominant stamens

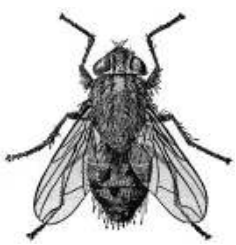
Based on the morphological characteristics you determined above, indicate the most appropriate floral shape class for each specimen V-Z with a tick (√) in the table below. **[5 points]**

Q 14

	V	W	X	Y	Z
Floral shape 1					
Floral shape 2					
Floral shape 3					
Floral shape 4					

3.2.2 Pollinators

As is shown for a subset of common pollinators in the following table, the floral shape and other characteristics of the flowers or inflorescences are often strongly associated with their pollinators.

n°	Pollinator	Characteristics of pollinator	Common shapes of flowers / inflorescences
1	Wind 	• Unspecific • Non-directional	• Inconspicuous • Lack of optically attractive floral parts • Nectar and scent absent • Huge amount of pollen • Anthers and stigma well exposed to the wind
2	Bees/ bumblebees 	• Day-active • Biting mouthparts • Long tongue • Attracted by pollen and nectar • Pollen gathering • Able to hang upside down	Either • Bilateral • Brightly colored (often yellow, violet or blue) • Nectar hidden in a tube • Offering a landing place Or • Flat • Brightly colored (often yellow, violet or blue) • Offering a lot of pollen
3	Moths 	• Night-active • Long proboscis • Do not need a landing platform • Seeking for nectar	• White or nearly white • Fragrant scent • Open during the night
4	Butterfly 	• Day-active • Long proboscis • Do not need a landing platform • Seeking for nectar	• Upright position • Tight tube • Deeply hidden nectar
5	Flies 	• Licking mouthparts • Like bright flat surfaces to sit in the sun	Either • Small and flat • Without scent • Nectar reward freely presented Or • Brown/purple advertisement • Carrion- or excrement-scented

The following table lists additional floral traits of the plant specimen that cannot be observed from the prepared samples (*+: present, ++ : strongly present, -: (nearly) absent*).

	V	W	X	Y	Z
presence of nectar	++	+	+	-	++
scent	++	+	+	-	+

Based on the characteristics of the flowers or inflorescences you observed and given in the table above, indicate with a tick (√) for each of the different plant specimens V-Z its most likely group of pollinators in the table below. Use the numbers indicated for each group in the table above. If several groups are equally likely, pick any one of them. **[5 points]**

Q 15

	V	W	X	Y	Z
Pollinator 1					
Pollinator 2					
Pollinator 3					
Pollinator 4					
Pollinator 5					

End of the Practical Exam

Student Code:

24th International Biology Olympiad

14th-21st July, 2013

Bern, Switzerland

BERN 2013 International Biology Olympiad

Practical Exam 3

Evolutionary Ethology

Total points: **94**

Duration: **90 minutes**

Dear participants,

This test consists of three tasks:

Task 1: Quantifying aggressiveness **4**

Part 1.1: Experimental setup 4
Part 1.2: Aggressive behavior of *Neolamprologus pulcher* 4
Part 1.3: Examples of ramming or biting attacks 5
Part 1.4: Quantify ramming or biting attacks of *N. pulcher* [30 points] 5
Part 1.5: Additional replicates 6
Part 1.6: Statistical analysis of bite or ram attacks [20 points] 6

Task 2: Puffed throat behavior **10**

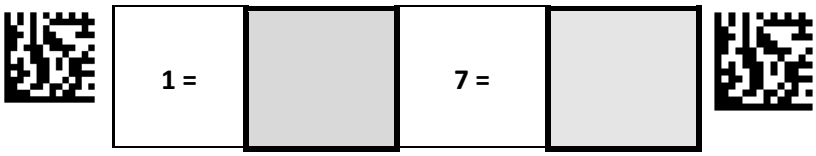
Part 2.1: Puffed throat behavior of *N. pulcher* 10
Part 2.2: Examples of puffed throat behavior 10
Part 2.3: Quantify puffed throat behavior of *N. pulcher* [21 points] 10
Part 2.4: Interpret your results [4 points] 11

Task 3: Social groups **12**

Part 3.1: Quantifying task sharing in social groups of *N. pulcher* [15 points] 12
Part 3.2: Interpret your observations [4 points] 13

Please write your student code into the box on the title page.
There is no separate answer sheet. Please fill in your answers into the specific answers boxes indicated with a gray background. **Only answers given inside these boxes will be evaluated.**

The answers have to be given either with a tick (√) or with Arabic numbers. The numbers "1" and "7" can look very similar in handwriting. To make sure that those two numbers can be well distinguished by the IBO staff, please write them as you normally would into the following box.



Stop answering and **put down your pen IMMEDIATELY** when the bell rings at the end of the exam. Put the entire protocol with all the answers back into the exam envelope. Make sure you wrote your student code into the box on the title page.

Material and equipment

Make sure that you have received all the materials and equipment listed for each task. If any of these items are missing, please raise your hand.

Equipment

- 1 Tablet with preloaded movies
- scratch paper

Introduction

Most organisms are limited in resources such as food, mating partners or shelters. As a result, available resources are often defended by aggressive interactions with conspecific (same species) and heterospecific (different species) competitors. A very common phenomenon to monopolize resources is the establishment of a well-defended territory. But territorial defense is costly as it may expose the territorial resident to an increased risk of injury or predation or reduce the time available for other activities such as foraging, mating or brood care. Given this, a territorial resident that is able to adjust its aggressive response to match the degree of threat posed by an intruder is expected to have a selective advantage.

In this practical you will test if individuals of the territorial African cichlid *Neolamprologus pulcher* are using visual and / or olfactory cues of a competing conspecific to adjust their aggressive response. *N. pulcher* is native to Lake Tanganyika where it forms breeding pairs, which defend a small contiguous territory (mean area = 0.3 m^2) used for feeding, breeding, and shelter from predators.

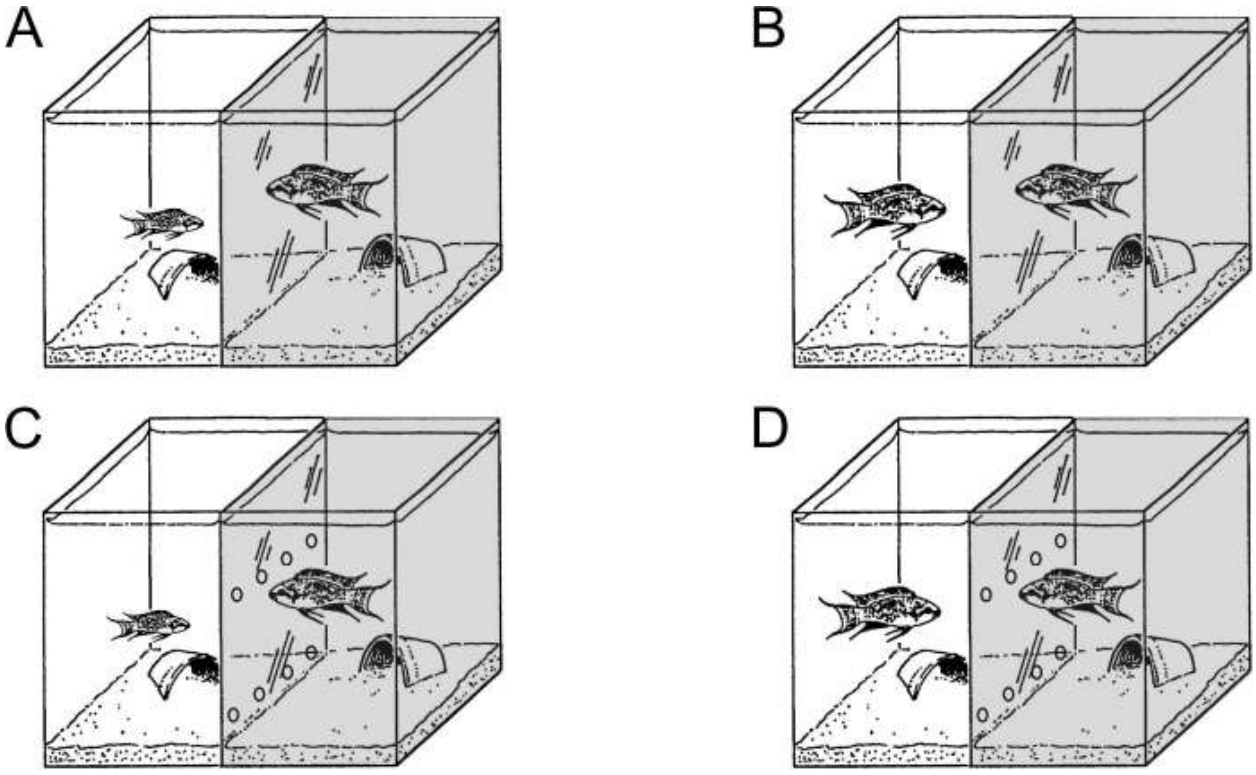
All experiments were conducted under laboratory conditions in aquaria and recorded on video. Your task is now to analyze these recordings by quantifying the behavior of different individuals. All videos are preloaded on a tablet computer and can be played at your convenience (see all videos HERE).

Task 1: Quantifying aggressiveness

In this task, you will quantify the aggressive behavior of *N. pulcher* males by statistically testing if males use visual and / or olfactory cues to adjust their level of aggressiveness to the threat posed by a competing male.

Part 1.1: Experimental setup

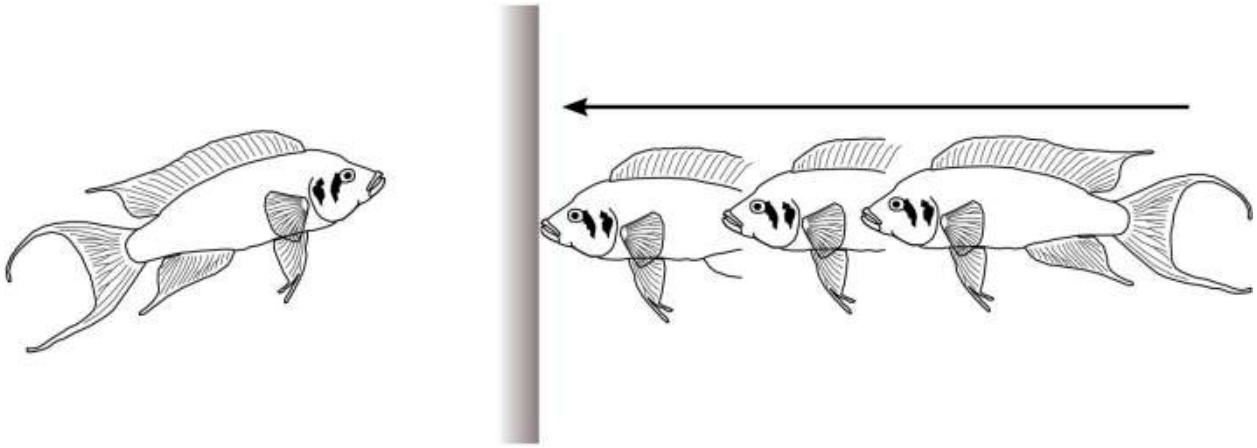
In each experiment, two males are freshly exposed to each other in a previously uninhabited test aquarium that is divided by a glass wall that either seals off the two compartments completely (Situations A and B) or is porous and allows for an exchange of water between the two compartments (Situations C and D). The focal male is always in the right compartment and is exposed to either a considerably smaller male (Situations A and C) or a male of equal size (Situations B and D).



Throughout this task, you will only have to consider the behavioral response of the focal male in the right compartment (indicated in gray).

Part 1.2: Aggressive behavior of *Neolamprologus pulcher*

In this task, you will count the frequency of a particularly conspicuous behavioral response of *N. pulcher* males to conspecifics: ramming or biting attacks. Aggressive behavior is counted as ram or bite (ramming with its mouth open) when the fish is obviously attacking its competitor by swimming towards it and hitting the glass wall separating them. Each hit of the snout against the glass wall is counted as a single event, even if males ram or bite repeatedly within a few seconds. The following figure illustrates this behavior.



Part 1.3: Examples of ramming or biting attacks

Activate your tablet by tapping and sliding with your fingers and open the Folder E_1 (watch all videos HERE). In that folder you will find three movies. Movie I contains a series of obvious biting or ramming attacks. Watch it to get a feeling of what to look for. Next, watch Movies II and III which indicate how these attacks are to be counted. Watch them carefully. Proceed with the protocol as soon as you feel confident with how to count this behavior in *N. pulcher*.

Part 1.4: Quantify ramming or biting attacks of *N. pulcher* [30 points]

Each of the Folders A, B, C and D contain three movies, showing sequences from experiments conducted under the corresponding Situations A, B, C and D indicated in the figure in Part 1.1 and the table below. Count the number of ram or bite attacks of the male in the **right compartment** for each of the twelve movies and report your results in the table below. It is recommended to count by using tally marks on the provided scratch paper.

Q1

Situation	**A**	**B**	**C**	**D**
Separation	sealed	sealed	porous	porous
Size	different	matched	different	matched
Replicate 1				
Replicate 2				
Replicate 3				

Part 1.5: Additional replicates

The table below provides the results of three additional replicates for each experimental setup. These results are to be combined with your results for the statistical analysis below.

Situation	**A**	**B**	**C**	**D**
Separation	sealed	sealed	porous	porous
Size	different	matched	different	matched
Replicate 4	24	45	10	15
Replicate 5	34	41	17	8
Replicate 6	27	38	12	16

Part 1.6: Statistical analysis of bite or ram attacks [20 points]

You will conduct an analysis of variance (or ANOVA) to analyze the obtained results. The goal of this analysis is to infer what part of the variance in your measures is due to stochastic differences between the individuals studied, and what part can be explained by the two factors manipulated in this experiment: 1) the type of separation (sealed vs. porous) and 2) the difference in the size of males (matched vs. different). This is done by partitioning the total variance into different components and testing the relative importance of these partitions

Variance within groups

Compute the mean and variance among the replicates 1 through 6 of each of the four experimental setups A through D. Report your results in the tables below with a precision of one digit after the decimal point. The variance is calculated according to

$$\sigma^2 = \frac{1}{n-1} \cdot \sum_{i=1}^{n} (R_i - M)^2$$

where *i* runs over all replicates 1 through *n* and R_i are the observed counts in replicate *i* and *M* is the mean across all replicates 1 through *n.*

Q2

Situation	**A**	**B**	**C**	**D**
Separation	sealed	sealed	porous	porous
Size	different	matched	different	matched
Mean (*M*)				
sample variance (σ^2)				

Now compute the average sample variance within groups ($\boldsymbol{V_g}$) as the average of the four variances. Report your results in the box below with a precision of one digit after the decimal point.

Q3 average sample variance within groups (V_g)

Variance explained by the type of separation and difference in the size of males

Next compute the variance explained by the type of separation ($V_{separation}$) and the variance explained by the difference in size of males (V_{size})**.** To do so, you will first have to compute the overall mean of bite or ram attacks across all 24 replicates. Report your result in the box below with a precision of one digit after the decimal point.

Q4 overall mean across all replicates (M_{ABCD})

Next, compute the mean number of bite or ram attacks among all replicates for each of the type of separation and the difference in the size of males, independent of the other category. The mean number of bite or ram attacks among all replicates with sealed separations, for instance, is simply given by the average of M_A and M_B, where M_A and M_B refer to the mean number of bite or ram attacks observed in Situations A and B, respectively, which you calculated above. Compute all these means and report your results in the table below with a precision of one digit after the decimal point.

Q5 mean within category

Type of separation		Difference in the size of males	
sealed	porous	different	matched

The variance explained by factor ***x*** (either separation or size) is now computed as

where ***i*** runs over both categories of the factor considered, ***n*** is the number of replicates within each category (12 in your case), M_i is the mean within category ***i*** and M_{ABCD} the overall mean computed in Q4. Compute the variance explained by each factor and report your results in the table below with a precision of one digit after the decimal point.

Q6 variance explained by factor (V_X)

Type of separation	Difference in the size of males

If a factor does not explain any of the observed variance, we expect V_x to be zero. However, due to the stochastic nature of this experiment, deviations from zero are expected. You will now test if the

deviations from zero you observed for both $V_{separation}$ and V_{size} are statistically significant, which would indicate that these factors are significantly explaining part of your observations. This is done by computing the probability of observing deviations as large as or even larger than what you observed. To do so, you will need a F-test, for which you now need to compute the test statistic F_x, which is given by the variance explained by factor x (either separation or size) divided by the average sample variance within groups (V_g) you calculated in Q3.

Calculate F_x for both factors and report your results in the table below with a precision of one digit after the decimal point.

Q7

	Type of separation	Difference in the size of males
test statistic F_x		

You can now use the properties of the F-distribution to translate the test statistic into the probability of observing the V_x values you calculated or even more extreme V_x values if factor *x* does not explain any of the variance observed. This probability is commonly referred to as the p-value. The table below shows the corresponding F-values for a wide range of p-values.

Fx	p-value
0.47	0.5
2.96	0.1
4.32	0.05
8.02	0.01
14.59	10^{-3}
22.89	10^{-4}
33.28	10^{-5}
46.27	10^{-6}
62.46	10^{-7}
82.65	10^{-8}

Use this table to translate your test statistic values into p-values. For each factor, indicate in the table below, the smallest p-value for which the p-value corresponding to the calculated F-value is smaller.

Q8

	Type of separation	Difference in the size of males
p-value <		

Indicate with a tick (√) for each factor if it explains a fraction of the total variance significantly (p-value < 0.05) or not in the table below.

Q9

	Type of separation	**Difference in the size of males**
Explains part of the total variance		
Does **not** explain part of the total variance		

Indicate with a tick (√) if each of the following statements is a valid conclusion from your results or not.

Q10

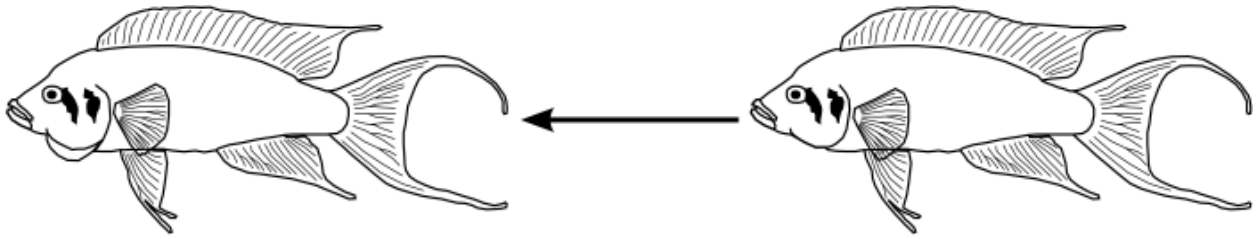
	valid	**not valid**
Males of *N. pulcher* use visual cues of a competing conspecific to adjust their aggressive response.		
Males of *N. pulcher* use olfactory cues of a competing conspecific to adjust their aggressive response.		

Task 2: Puffed throat behavior

In this task, you will check if a second aggressive behavior indicates the same pattern you found for the number of ramming or biting attacks in the previous task by concentrating on the puffed throat behavior.

Part 2.1: Puffed throat behavior of *N. pulcher*

N. pulcher males use puffed throats to impress conspecifics, possibly because it makes them appear larger. A behavior is counted as puffed throat whenever the focal fish flares out its operculum and lower jaw and swims in the direction of his competitor on the other side of the glass wall. Note that males may flare out their operculum and lower jaw cavity for a very short time or an extended period of several seconds. Each flaring out of the operculum and lower jaw is counted as a single event. Be aware that some males may flare out their operculum and lower jaw multiple times within a few seconds, each of which is counted as a single event.



Part 2.2: Examples of puffed throat behavior

Activate your tablet by tapping and sliding with your fingers and open the Folder E_2. In that folder you will find three movies. Movie I contains a series of obvious puffed throat behaviors. Watch it to get a feeling what to look for. Next, watch Movies II and III which indicate how this behavior is to be counted. Watch them carefully. Proceed with the protocol as soon as you feel confident with how to count this behavior in *N. pulcher*.

Part 2.3: Quantify puffed throat behavior of *N. pulcher* [21 points]

Watch the three movies found in each of the Folders A, B, C and D you already analyzed in the first task. Count the number of puffed throat behaviors of the male in the **right compartment** for each of the twelve movies and report your results in the table below. It is recommended to count by using tally marks on the provided scratch paper.

Q11

Situation	A	B	C	D
Separation	sealed	sealed	porous	porous
Size	different	matched	different	matched
Replicate 1				
Replicate 2				
Replicate 3				

Part 2.4: Interpret your results [4 points]

Based on your observations, indicate with a tick (√) if each of the following statements is true or false.

Q12

	true	false
These results are in line with *N. pulcher* males using visual cues of a competing conspecific to adjust their aggressive response.		
The results are in line with *N. pulcher* males using puffed throat behavior to bluff about their size, which is most effective if males are of equal size.		

Task 3: Social groups

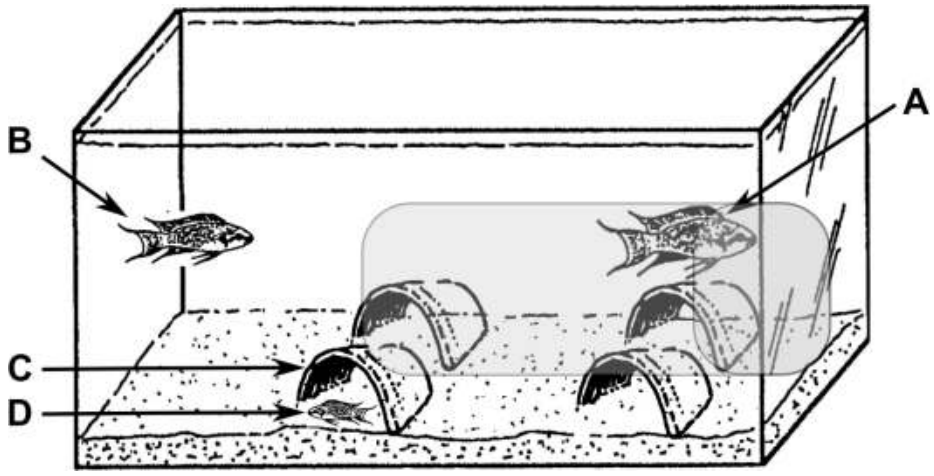
In nature, *N. pulcher* lives almost exclusively in social groups made up of a dominant breeding pair and 1 to 20 smaller subordinates called helpers (average group size = 7 to 9). In each social group, the breeding male is always the largest individual (5.6–7.0 cm in length), the breeding female is usually the next largest fish in the social group (4.8–6.0 cm) and subordinate helpers are generally smaller (1.5–6.4 cm). Both breeders and helpers show three main behaviors:

1. territory defense (against predators and conspecific or heterospecific territory competitors)
2. territory maintenance (by digging and removing debris)
3. brood care (by cleaning and fanning eggs and defending the young)

The goal is to understand task sharing in such groups by observing such a group for several minutes.

Part 3.1: Quantifying task sharing in social groups of *N. pulcher* [15 points]

Activate your tablet by tapping the screen with your fingers and open the Folder X. This folder contains the Movie x with two sequences showing typical territory maintenance and brood care behavior of the simplest social group of *N. pulcher* consisting of a large breeding male, a slightly smaller breeding female and a small helper. You can recognize each individual easily in the very first scene of the sequence (see figure below): the breeding male (A) is standing in the ceramic nesting cave in the background on the right and the breeding female (B) is swimming in open water on the left. The only eggs in the aquarium are attached to the top of the inner wall of the ceramic cave (C) within which the helper (D) hides.



While watching this movie, you will focus on two typical behaviors:

- Digging behavior is a typical territory maintenance behavior in which the fish takes up sand, carries it away in its mouth and spits it out at another location. Count the number of digging sequences performed for each of the three individuals.
- *N. pulcher* individuals perform egg care by cleaning the eggs from small particles such as fungi or biofilms. This behavior is visible by back-and-forth movements in front of the eggs and simultaneous mouth movements. Estimate the total time spent on this behavior by each individual.

Report the total number of digging behavior for each individual in the table below.

Q13

	Breeding male	Breeding female	Helper individual
Total number of digging behavior events displayed			

Indicate with a tick (√) the time spent on egg care for each of the three individuals in the table below.

Q14

	Breeding male	Breeding female	Helper individual
no egg caring or caring for < 5s			
egg caring for > 5s and < 30s			
egg caring for > 30s			

Part 3.2: Interpret your observations [4 points]

Indicate with a tick (√) if each of the following statements is true or false.

Q15

Assuming that the observed social group is a good representation of the majority of social groups in nature, we would conclude that …	true	false
… helpers are likely to have a larger effect on the survival rates of clutches of breeding pairs after an oligotrophic (nutrient-poor) environment was suddenly converted into a highly eutrophic (hypertrophic) environment.		
… large-bodied breeding males are crucial in maintaining breeding caves by digging large quantities of sand.		
... the presence of helpers allows the breeding female to spend most of her time patrolling the territory rather than on territory maintenance.		
... helper individuals accommodate the large breeding male by following it and maintaining the cave currently occupied by it.		

End of practical exam.

Student Code:

24th International Biology Olympiad

14th-21st July, 2013

Bern, Switzerland

BERN 2013 International Biology Olympiad

Practical Exam 4

Comparative and Functional Biosystematics

Total points: **100**

Duration: **90 minutes**

Dear participants,

This test consists of three tasks:

Task 1: Determine presence or absence of characters [42 points] **5**

Part 1.1: Description of morphological characters 5
Part 1.2: Determine all character states [42 points] 5

Task 2: Resolve phylogenetic relationships [24 points] **6**

Part 2.1: How maximum parsimony works 6
Part 2.2: Resolve the phylogenetic relationship using parsimony [10 points] 7
Part 2.3: Identify characters reflecting diet [4 points] 8
Part 2.4: How UPGMA works 9
Part 2.5: Resolve the phylogenetic relationship using UPGMA [10 points] 11

Task 3: Rate of morphological evolution [34 points] **13**

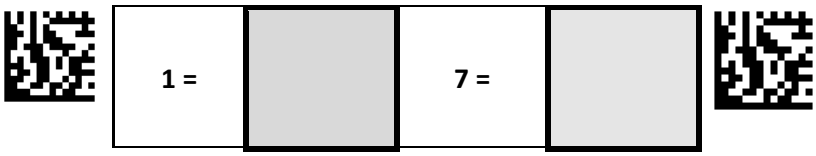
Part 3.1: Obtain necessary skull measurements [15 points] 13
Part 3.2: Calculate the morphological distance between species pairs [15 points] 13
Part 3.3: Plot the phylogenetic versus the morphological distance [2 points] 15
Part 3.4: Interpret the results [2 point] 16

Appendix: Character states (presence or absence) of all specimens **17**

Please write your student code into the box on the title page.

There is no separate answer sheet. Please fill in your answers into the specific answers boxes indicated with a gray background. **Only answers given inside these boxes will be evaluated.**

The answers have to be given either with a tick (√) or with Arabic numbers. The numbers "1" and "7" can look very similar in handwriting. To make sure that those two numbers can be well distinguished by the IBO staff, please write them as you normally would into the following box.



Stop answering and **put down your pen IMMEDIATELY** when the bell rings at the end of the exam. Put the entire protocol with all the answers back into the exam envelope. Make sure you wrote your student code into the box on the title page.

Material and equipment

Make sure that you have received all the materials and equipment listed for each task. If any of these items are missing, please raise your hand.

Equipment

- 1 sliding caliper (150 mm)
- 1 ruler (300 mm)
- 1 calculator
- scratch paper

Skull specimen

Arvicola terrestris	**arte**	European water vole
Capreolous capreoulus	**caca**	European roe deer
Felis catus	**feca**	domestic cat
Lepus europaeus	**leeu**	European/brown hare
Meles meles	**meme**	European badger
Sciurus vulgaris	**scvu**	Eurasian red squirrel
Vulpes vulpes	**vuvu**	red fox

Additional taxa for which data is provided

Ceratotherium simum	**cesi**	white or square-lipped rhinoceros
Didelphis marsupialis	**dima**	common opossum
Equus ferus	**eqfe**	horse
Galago senegalensis	**gase**	Senegal bushbaby
Genetta genetta	**gege**	common genet
Hippopotamus amphibius	**hiam**	hippopotamus
Inia geoffrensis	**inge**	Amazon river dolphin
Manis pentadactyla	**mape**	Chinese pangolin
Macropus rufus	**maru**	red kangaroo
Mustela nivalis	**muni**	least weasel
Pipistrellus pipistrellus	**pipi**	common pipistrelle
Procavia capensis	**prca**	rock or cape hyrax
Pteropus vampyrus	**ptva**	Malaysian large flying fox
Sorex minutus	**somi**	Eurasian pygmy shrew
Tachyglossus aculeatus	**taac**	short-beaked echidna
Tolypeutes matacus	**toma**	southern three-banded armadillo

Print outs

- skull diagrams
- color print outs illustrating the presence (1) and absence (-) of the morphological characters. Note legend on top.

Introduction

Mammals, which consist of more than 5700 extant species, are omnipresent on earth and often play key roles in shaping terrestrial and aquatic ecosystems, both as predators and herbivores. Mammals are morphologically very diverse. The smallest mammals, for instance, weigh only about 2 g, whereas the blue whale, the largest animal that ever existed, is more than 100 million times larger.

In this practical exam, you will attempt to resolve the phylogeny of some representatives of major mammal groups based on morphological characters of the skull (teeth and jaw), as well as molecular data. You will use cladistics, an approach to infer evolutionary relationships, which assumes that members of a taxonomic group have a common evolutionary history. They have therefore inherited a set of derived traits (synapomorphies) from their last common ancestor, which distinguishes members of this group from other groups.

Cladistic analysis consists of identifying specific traits and determining their state in all organisms included in the analysis. Traits can be both molecular (e.g. the specific amino acid at a specific position in a protein) or morphological (e.g. the presence or absence of a particular bone feature). The outcome of a cladistic analysis is a cladogram - a tree-shaped diagram (dendrogram) that represents the inferred phylogenetic relationships between organisms.

Task 1: Determine presence or absence of characters [42 points]

Part 1.1: Description of morphological characters

The following table lists all morphological characters for which **the presence (1) or absence (-)** will be considered in this practical. Examples for each character are found on a color print out (**note the legend on top of each page**). For Characters 7 through 10, only examples of presence (1) are shown. All abbreviations given in brackets (e.g. [P]) refer to positions indicated in the skull diagrams. Note that the positions of incisors **[I]**, canines **[C]**, premolars **[P]** and molars **[M]** are enumerated from the snout to the back of the skull using superscript (upper jaw) or subscript (lower jaw). For example, **[P^3]** refers to the third premolar on the upper jaw.

Number	Description
1	Incisors **[I]** are present on the upper jaw.
2	Elongated incisors **[I]** with abrasive (rubbed) edges indicating continuously growth through life on upper and/or lower jaw.
3	Incisors **[I]** on the front side coated with dental enamel (orange color).
4	Third upper incisors **[I^3]** reduced and positioned behind the second incisors.
5	Diastema on upper jaw. A diastema is defined as a large gap (more than two times the width of the largest tooth adjacent to the gap) between the position of incisors **[I]** or canines **[C]** and the premolars **[P]** or molars **[M].** Some of these teeth may be absent.
6	Canines **[C]** are present on the upper jaw.
7	Carnassial dentition, characterized by **[P^4]** in the upper jaw and **[M_1]** in the lower jaw forming a scissor-like structure which allows cutting and breaking, but not chewing.
8	Selenodont teeth, characterized by low crowns and longitudinal crescent-shaped cusps, when viewed from above. They form a series of triangular cusps when seen from the side.
9	Lophodont teeth, characterized by elongated ridges that run between cusps. Ridges are perpendicular to the jaw (like an old-fashioned washboard).
10	Teeth characterized by a well-developed V- or W-shaped ectoloph (crest).
11	Pointed teeth forming conical tips.
12	The angular extension **[Ang. Pr.]** (*Processus Angularis*) of the lower jaw is bent medial (towards the centre) and therefore more medial than **[Cond. Pr.]** (*Processus condularis*).

Part 1.2: Determine all character states [42 points]

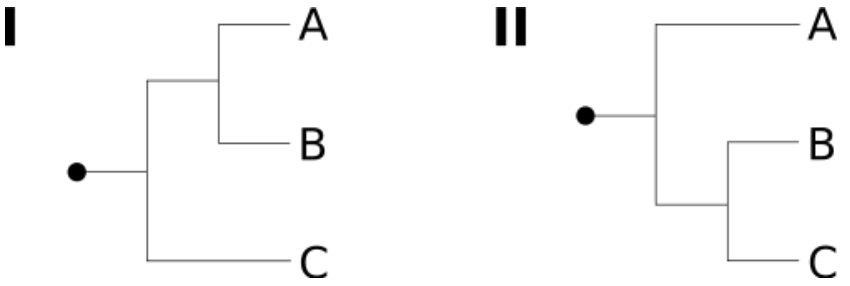
For the later analysis, it is convenient to first determine the presence or absence of each of the morphological characters considered here in each of the species included in the analysis, and to report them in the table provided in **Appendix (last page)**. Indicate presence with a "**1**" and absence with a "**-**". **Please be extra careful when handling the skulls**. All specimens are originals and part of the collection of the Natural History Museum of Bern.

Task 2: Resolve phylogenetic relationships [24 points]

The goal of this task is to resolve the phylogenetic relationship among major mammalian groups. You will use two methods that will be explained in detail. In Part 2.1-2.3, you will apply the concept of maximum parsimony to the presences and absences determined in Task 1 to estimate the phylogenetic relationship between several taxa representing different major groups of mammals. In Part 2.4 – 2.5, you will use the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) approach to resolve the phylogeny among Carnivora to complete a more detailed phylogeny obtained from molecular data.

Part 2.1: How maximum parsimony works

A common approach to resolve phylogenetic relationships between species is to identify the most parsimonious (the simplest) among competing phylogenies. A phylogenetic tree is called the most parsimonious if the fewest number of character state changes are invoked. To illustrate that concept, consider the following two competing phylogenies I and II for three taxa A, B and C.

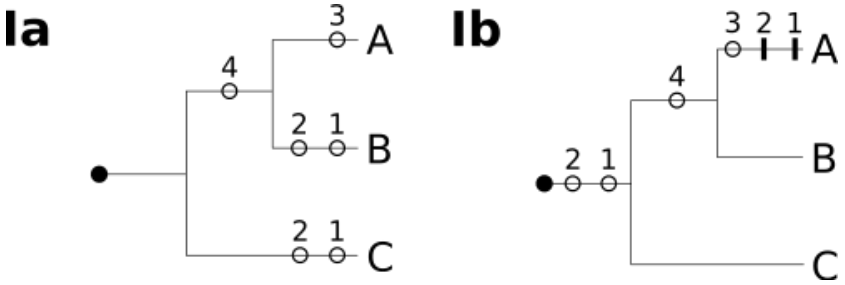


To choose among those phylogenies, the presence or absence of morphological characters 1 through 4 was assessed for each taxa and reported in the table below. All of these characters are known to be absent in closely related taxa, indicating that each character was absent at the root of the phylogenies (indicated by a black dot in the diagrams above).

	1	2	3	4
A	-	-	**1**	**1**
B	**1**	**1**	-	**1**
C	**1**	**1**	-	-

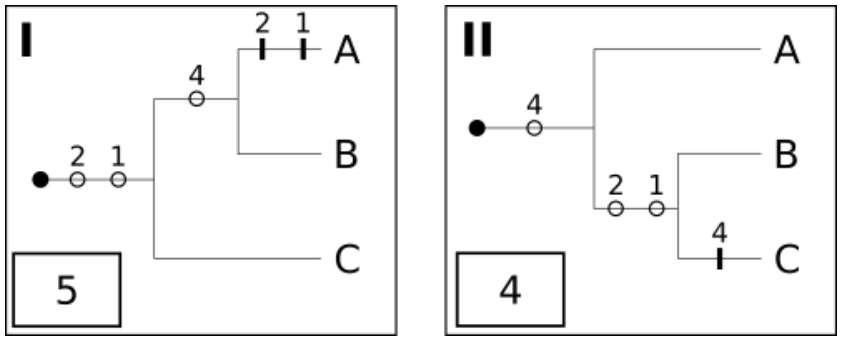
First, the minimal number of state changes has to be determined for each character and phylogeny individually. Trait 4, for instance, is present in Taxa A and B, but not in Taxon C, indicating that the state of this character changed from absent to present along the lineages leading to A and B. While this change could have happened independently on Branches A and B, the explanation leading to the minimal number of state changes (and hence the most parsimonious solution) is a single change on the branch leading to the most recent common ancestor of A and B.

As shown in the following figure, two equally parsimonious Solutions Ia and Ib can be found for Phylogeny I when considering all characters. In Solution Ia, Characters 1 and 2 are assumed to have arisen independently on Branches B and C. Alternatively (Solution Ib), these characters arose on the branch coming from the root of the phylogeny, and were lost on the Branch A. Note that throughout this practical, **open circles indicate gains** (changes from absent → present) and **black bars indicate losses** (state changes from present → absent).



A key observation is that any character for which the state in a single taxon differs from the state at the root (such as Trait 3 in this example) invokes a single character change, regardless of the phylogeny considered. Such characters and those for which the state is identical in all taxa should be regarded as uninformative and discarded prior to the analysis.

The following figure finally shows one of the most parsimonious solutions for each of the competing phylogenies, along with the number of character state changes invoked by these solutions given in the box. Thus, under the concept of maximum parsimony, Phylogeny II is chosen as the best estimate of the true phylogenetic relationship between Taxa A, B and C.



Part 2.2: Resolve the phylogenetic relationship using parsimony [10 points]

You will now use maximum parsimony to resolve the phylogenetic relationship among the following taxa, representing a major mammal clade:

- *Arvicola terrestris* (**arte**)
- *Ceratotherium simum* (**cesi**)
- *Equus ferus* (**eqfe**)
- *Genetta genetta* (**gege**)
- *Lepus europaeus* (**leeu**)
- *Capreolus capreolus* (**caca**)
- *Sorex minutus* (**somi**)

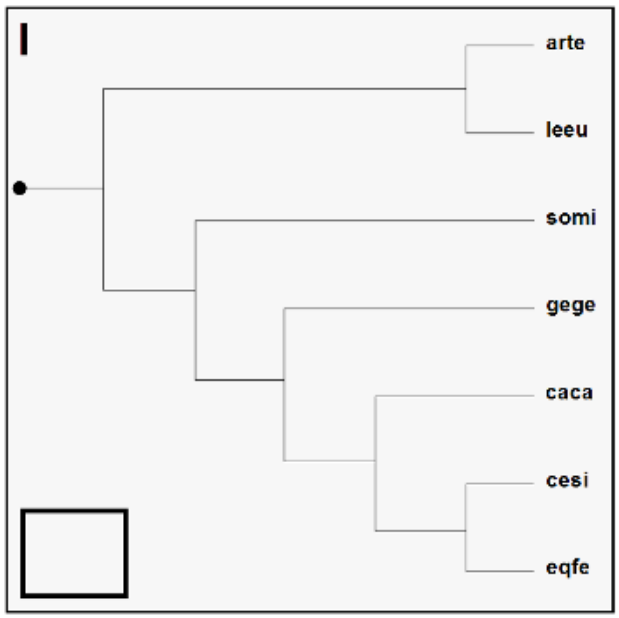
First, indicate with a tick (√) if Characters 1 through 12 (page 5) are informative to resolve the phylogeny of these taxa, or not. Note that all characters are known to be absent at the root of these taxa, with the **exception of 1, 6 and 11, which are present at the root**.

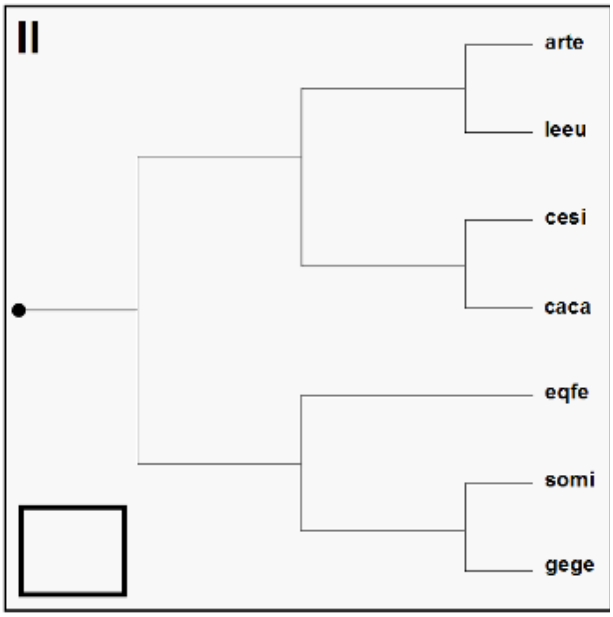
Q2

Character	1	2	3	4	5	6	7	8	9	10	11	12
informative												
not informative												

Next, indicate any of the most parsimonious solutions regarding character state changes for each of the two competing phylogenies I and II directly into the figure. Indicate **gains with empty circles and losses with bars** and write the number of the corresponding characters above it. Finally, indicate the total number of state changes necessary in the box provided.

Q3





Indicate the phylogeny that represents the best estimate of the true phylogenetic relationship among those 7 taxa according to the concept of maximum parsimony with a tick (√) in the table below

Q4

	I	II
Preferred phylogeny according to the concept of maximum parsimony		

Part 2.3: Identify characters reflecting diet [4 points]

Unless a large number of characters are included, maximum parsimony is often too simplistic an approach to correctly disentangle the phylogeny of distantly related taxa. This is particularly true when some character state differences between taxa reflect opposing adaptations to diet rather than a phylogenetic signal. To evaluate this hypothesis, identify the characters that are typical for either an carnivorous or a herbivorous lifestyle.

Among the species included in this practical, the following are very distantly related but are highly carnivorous and prey on living Arthropoda, Mollusca, Annelida and/or Vertebrata:

- *Didelphis marsupialis* (**dima**)
- *Galago senegalensis* (**gase**)
- *Inia geoffrensis* (**inge**)
- *Mustela nivalis* (**muni**)
- *Pipistrellus pipistrellus* (**pipi**)
- *Sorex minutus* (**somi**)
- *Tolypeutes matacus* (**toma**)

The following are very distantly related species but are known to be highly herbivorous and feed on grass, leaves, fruits and roots:

- *Arvicola terrestris* (**arte**)
- *Capreolous capreolus* (**caca**)
- *Equus ferus* (**eqfe**)
- *Lepus europaeus* (**leeu**)
- *Macropus rufus* (**maru)**
- *Procavia capensis* (**prca**)
- *Pteropus vampyrus* (**ptva**)

Indicate with a tick (√) in the table below for each Character 1 through 12 whether its presence or absence is typical for either an carnivorous or herbivorous lifestyle. Here, a typical character state is defined as any character state shared by at least three of the taxa from one of the two groups but absent from all members of the other group.

Q5

Character	1	2	3	4	5	6	7	8	9	10	11	12
typical for carnivores												
typical for herbivores												
not typical for either												

Part 2.4: How UPGMA works

An approach that is purely based on the presence or absence of morphological characters does not lend itself readily for estimating evolutionary distance between taxa quantitatively. This is where molecular data, which provides a large set of characters with similar state change probabilities, becomes very handy. A simple, iterative approach to estimate a phylogeny along with the relative length of each branch from molecular data is UPGMA (Unweighted Pair Group Method with Arithmetic Mean), in which the pair of clusters with the shortest distance is combined into a higher level cluster at each iteration.

To illustrate that concept, consider the molecular distances (e.g. number of base pair differences) between the Taxa A, B, C and D.

	A	**B**	**C**	**D**
A	0			
B	6	0		
C	7	8	0	
D	8	9	4	0

Iteration 1: First, the pair of clusters with the smallest distance is the pair C and D, which is combined into a higher-order cluster (C,D). The relative age of the newly formed cluster is computed as half of the distance between the two clusters combined. In this case, the relative age of the cluster is thus 2.

Next, a new matrix of all distances is generated by computing the distance between clusters as the average distance between all taxa from one cluster to all taxa of the other cluster. The distance between cluster A and cluster (C,D), for instance, is computed as the average between d(A,C) and d(A,D), where d(x,y) is a notation to indicate the distance between clusters x and y.

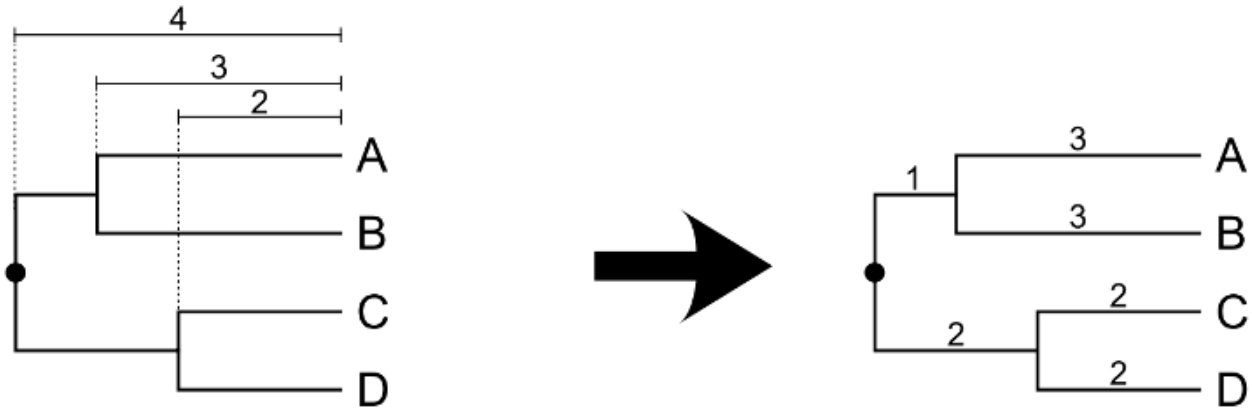
	A		
A	0	**B**	
B	6	0	**(C,D)**
(C,D)	7.5	8.5	0

Iteration 2: The pair of clusters with the smallest distance is now the pair of A and B, which is thus combined into a higher level cluster (A,B) with a relative age of 3. Again, distances are recomputed as indicated above. The distance d((A,B),(C,D)) is thus the average between d(A,C), d(A,D), d(B,C) and d(B,D), which is the same as the average between d(A,(C,D)) and d(B,(C,D)).

	(A,B)	
(A,B)	0	**(C,D)**
(C,D)	8	0

Iteration 3: In the last iteration, the two remaining taxa are combined into the new cluster ((A,B),(C,D)) and the relative age of this cluster is 4.

Computing branch length: After all taxa have been combined into a single cluster, the resulting tree is plotted with the relative age of each cluster. From these relative times, it is then straight forward to compute individual branch length.



Part 2.5: Resolve the phylogenetic relationship using UPGMA [10 points]

Resolve the phylogenetic relationship of all five Carnivora species included in this practical iteratively using the UPGMA method. Standardized molecular distances between all pairs of these taxa are given in the table below.

			A				
vuvu	*Vulpes vulpes*	**A**	0.00	**B**			
muni	*Mustela nivalis*	**B**	4.18	0.00	**C**		
gege	*Genetta genetta*	**C**	4.96	4.96	0.00	**D**	
meme	*Meles meles*	**D**	4.18	0.48	4.96	0.00	**E**
feca	*Felis catus*	**E**	4.96	4.96	2.82	4.96	0.00

Proceed iteratively and always fill in the table with the molecular distances considered. Make sure to always report the code of the cluster in the top and left most row. Use the notation introduced above to denote higher level clusters. The term ((x,y),z), for instance, should refer to a cluster made of the clusters (x,y) and z. Also, report the relative age of the newly formed cluster in each iteration (precision: two digits after the decimal point).

Iteration 1

Q6

	0.00			
		0.00		
			0.00	
				0.00

Relative age of newly formed cluster: _____

Iteration 2

Q7

	0.00		
		0.00	
			0.00

Relative age of newly formed cluster: _____

Iteration 3

Q8

	0.00	
		0.00

Relative age of newly formed cluster: _____

Iteration 4

Q9

Relative age of newly formed cluster: _____

Now draw the resulting phylogeny of the five species to scale and indicate the length of each branch on the top and the species code on the right of it. Note that 5 mm indicates 0.1 unit of relative age of your calculated tree.

Q10



Task 3: Rate of morphological evolution [34 points]

If morphological changes happen at a constant rate, the morphological difference between pairs species measured at multiple characters should be highly correlated with the phylogenetic distance between the species as estimated from molecular data. In this task, you will test this hypothesis by using a series of skull measurements. While the results are provided for many additional species pairs, you will perform the necessary measurements and calculations for the species pair *Arvicola terrestris* (**arte**) and *Lepus europaeus* (**leeu**).

Part 3.1: Obtain necessary skull measurements [15 points]

The table below indicates a series of skull features which you will have to measure for the two species mentioned above. All measurements are also indicated in the skull diagrams.

	Description
M_1	distance in mm between **[I^1]** and **[NF1]**.
M_2	distance in mm between **[Ang. Pr.]** and end of masseter depression (masseter muscle attachment point).
M_3	distance in mm between **[Ang. Pr.]** and **[Cond. Pr.]**.
M_4	distance in mm between **[I]** and **[Cond. Pr.]**.
M_5	distance in mm between end of eyes and end of **[PA]**
M_6	distance in mm between eyes and **[PMX]**.
M_7	distance in mm between **[PMX]** or **[NA]** or **[I]** and **[Cond. Pr.]**.

While results for features M_5 and M_6 will be provided below, measure the five features (M_1 through M_4 and M_7) for both specimens (**arte** and **leeu**). Focus on the right side of the skull (the left side when facing the skull). Report your results in mm in the table to a precision of 0.1 mm

Q11

	M_1	M_2	M_3	M_4	M_5	M_6	M_7
arte *Arvicola terrestris*							
leeu *Lepus europaeus*							

Part 3.2: Calculate the morphological distance between species pairs [15 points]

A major hurdle when studying different skull features is that they are primarily determined by the overall size of the specimen. To study skull shape, begin by correcting for the overall size of the skull by dividing each measurement by the length of the skull (M_7) and taking the logarithm to base 10 from this ratio. For each measurement $M_i = \{M_1, ..., M_4\}$, calculate:

$$\frac{\quad}{\quad}$$

Report your results in the table below with a precision of three digits after the decimal point.

Q12

arte *Arvicola terrestris*					-0.367	-0.478
leeu *Lepus europaeus*					-0.547	-0.382

Next, compute the absolute morphological distance between the two species *x* and *y* for each measurement *i* as

where is the standardized measure *i* of species *x*. The absolute difference is the difference regardless of whether it is negative or positive (indicated by $|\cdot|$). Report your results with a precision of three digits after the decimal point in the table below.

Q13

arte and **leeu**						

When combining the different measures into a single distance, it is important to first standardize the distances in order to give them equal weight. A simple procedure to standardize is to divide the absolute differences by the median difference across all species pairs considered:

The appropriate medians are indicated in the table below.

0.084	0.139	0.067	0.026	0.110	0.082

Standardize all distances and report your results with a precision of three digits after the decimal point in the table below.

Q14

arte and **leeu**						

The combined morphological distance of a pair (*x*,*y*) is defined as the sum across all standardized distances of that pair:

Calculate for arte and leeu and report your results with a precision of three digits after the decimal point in the table below.

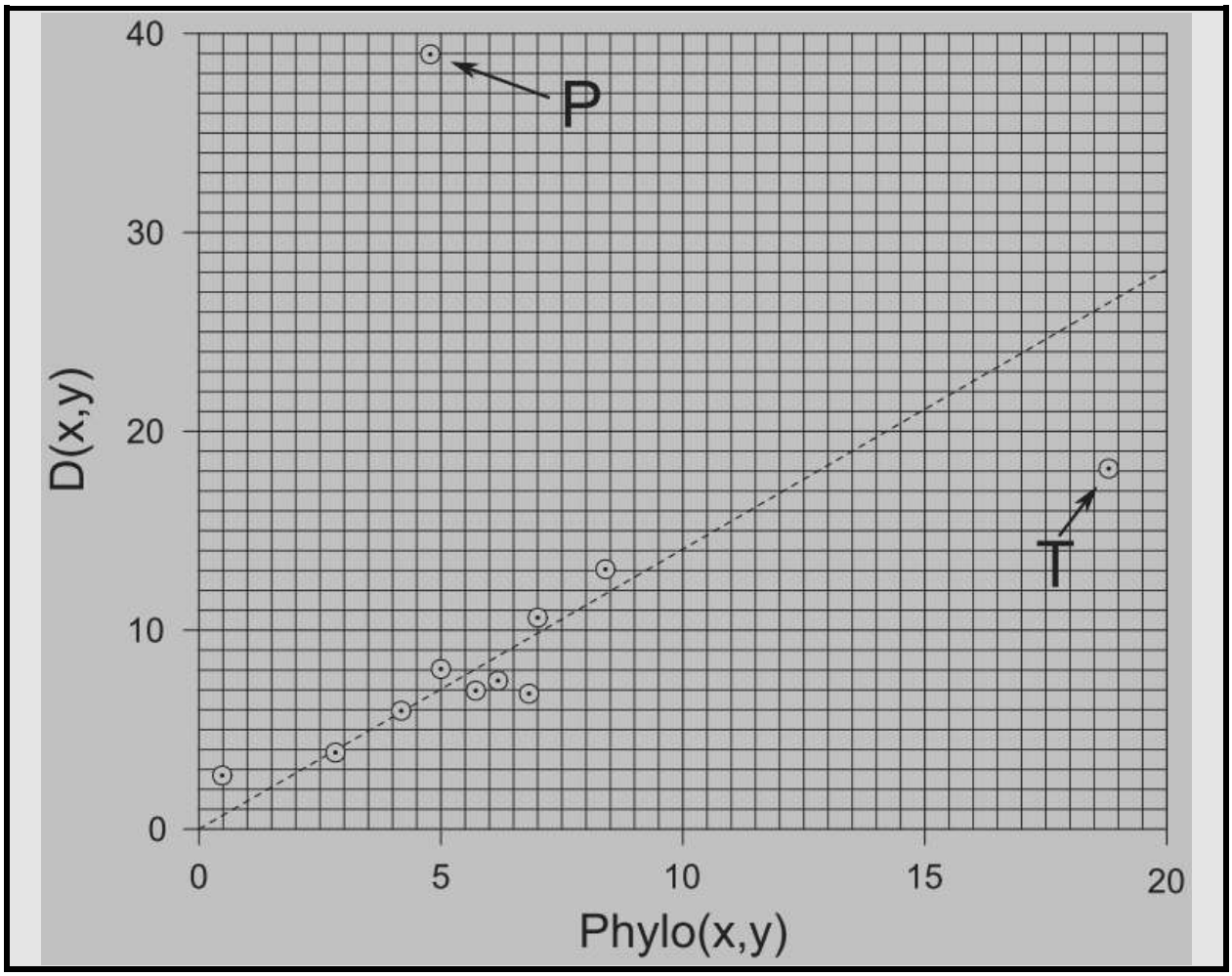
Q15

arte and **leeu**	

Part 3.3: Plot the phylogenetic versus the morphological distance [2 points]

The graph below shows the phylogenetic distance against the morphological distance for pairs of some of the species listed in the Appendix, along with a linear regression best explaining the observed values. Add your calculated distances to this plot by using a dot with a circle around, similar to the other points. The phylogenetic distance between **arte** and **leeu** is 6.80.

Q16



Part 3.4: Interpret the results [2 point]

The plot of the phylogenetic versus the morphological distance shows two obvious outliers, points P and T. Indicate with a tick (√) for each of the following hypotheses whether it could explain these outliers or not.

Q17

	yes	no
Both species of pair P are closely related but were geographically separated and evolved convergent adaptations to very similar habitats.		
The ancestor of one species of pair P colonized a totally different environment with very different selection pressures, changing its skull morphology dramatically.		
Both species of pair T are part of a fast, ecologically driven adaptive radiation.		
While only distantly related, both species of pair T feed on very similar resources, for which their skulls evolved convergent adaptations.		

End of practical exam

Appendix: Character states (presence or absence) of all specimens

Presences are indicated with a "1" and absences with a "-".

Q1

Character		1	2	3	4	5	6	7	8	9	10	11	12
arte	*Arvicola terrestris*												
caca	*Capreolus capreolus*												
feca	*Felis catus*												
leeu	*Lepus europaeus*												
meme	*Meles meles*												
scvu	*Sciurus vulgaris*												
vuvu	*Vulpes vulpes*												
cesi	*Ceratotherium simum*	-	-	-	-	1	-	-	-	1	-	-	-
dima	*Didelphis marsupialis*	1	-	-	-	-	1	-	-	-	-	1	1
eqfe	*Equus ferus*	1	-	-	-	1	1	-	-	1	-	-	-
gase	*Galago senegalensis*	1	-	-	-	-	1	-	-	-	-	1	-
gege	*Genetta genetta*	1	-	-	-	-	1	1	-	-	-	1	-
hiam	*Hippopotamus amphibius*	1	1	-	-	-	1	-	-	1	-	-	-
inge	*Inia geoffrensis*	-	-	-	-	-	-	-	-	-	-	1	-
mape	*Manis pentadactyla*	-	-	-	-	-	-	-	-	-	-	-	-
maru	*Macropus rufus*	1	1	-	-	1	-	-	-	1	-	-	1
muni	*Mustela nivalis*	1	-	-	-	-	1	1	-	-	-	1	-
pipi	*Pipistrellus pipistrellus*	1	-	-	-	-	1	-	-	-	1	1	-
prca	*Procavia capensis*	1	1	-	-	1	-	-	-	1	-	-	-
ptva	*Pteropus vampyrus*	1	-	-	-	-	1	-	-	-	-	-	-
somi	*Sorex minutus*	1	-	-	-	-	1	-	-	-	1	1	-
taac	*Tachyglossus aculeatus*	-	-	-	-	-	-	-	-	-	-	-	-
toma	*Tolypeutes matacus*	-	-	-	-	1	-	-	-	-	-	1	-