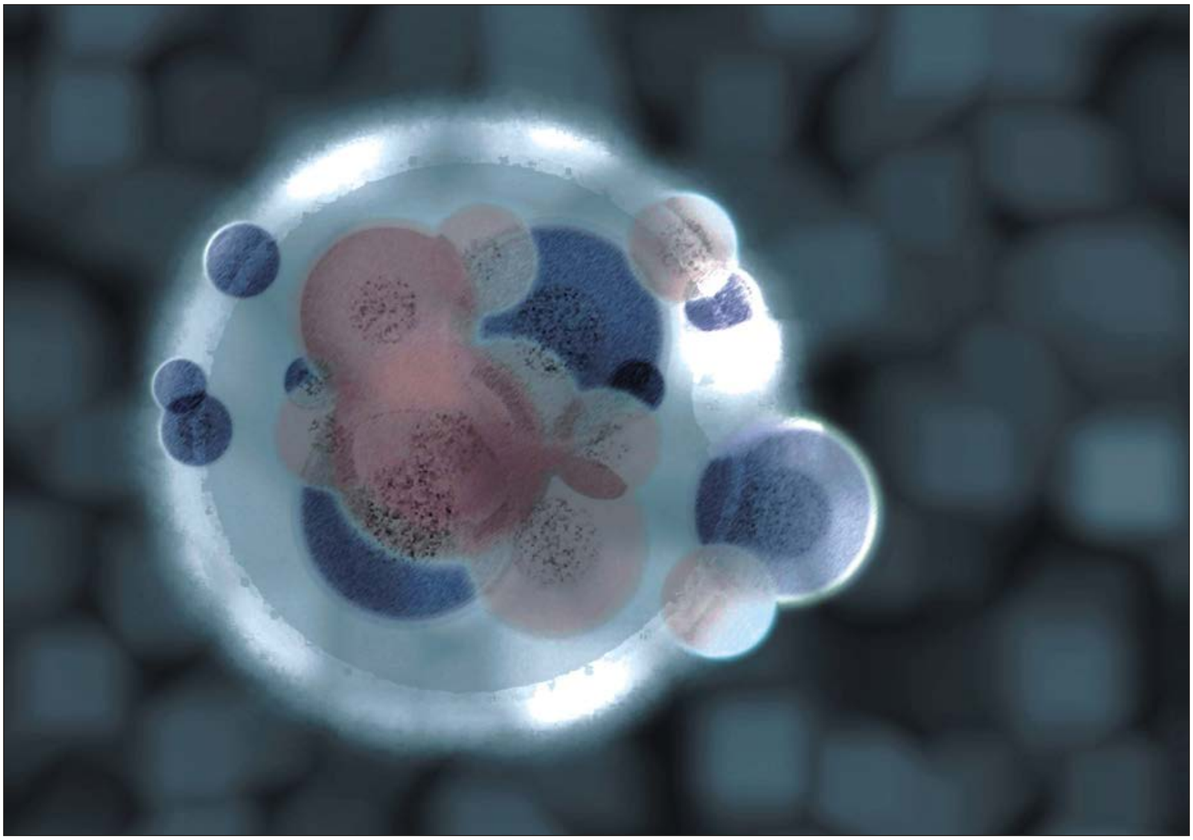

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Skin Characteristics and Organization of the Air-breathing Fish, *Alticus kirkii* (Günther, 1868) along Different Body Regions

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Abstract: *Alticus kirkii* is an air-breathing fish inhabiting the intertidal zone of the Red Sea. The study aimed to determine the role of the skin of this species in air-breathing in terms of histological characteristics of the Epidermis and its Thickness (EPT), blood Capillary Number (CN), Mucous Cell density (MC) and the Diffusion Distances (DD) along ten different body regions of *A. kirkii* and to assess which region of the skin is most effective. The basic statistics of the measured parameters and the patterns of variations were estimated and analyzed using one way analysis of variance. The relationships between skin parameters were revealed by cluster analysis through different skin body regions and vice versa. The principle structure of skin is evident on different body regions with significant variability ($p<0.05$) in epidermal thickness (1.08 to 3.94 μm), diffusion distance (0.89 to 1.67 μm), mucous cell number (0 to 5.55 per 25 μm length) and blood capillary density (0 to 10.55 per 25 μm length). This variability refers to the contribution role of each region in air-breathing; the skin on the ventral side which is easily immersed in water or soft mud during amphibious life has no role in this concern. The epidermal thickness in the different body regions was inversely associated ($p<0.05$) with CN, DD and MC (R = -0.94, -0.55 and -0.62, respectively) and is isolated into a single cluster. CN significantly correlates with MC (R = 0.65) ($p<0.05$) and insignificantly with DD (R = 0.47) ($p<0.05$). These three parameters are classified into one cluster with subcluster of CN and MC. Based on EPT, CN, MC and DD, the ten skin regions are classified into two main clusters, one includes the ventral head and pelvic fin regions and the other cluster includes the rest of skin regions which are more adapted to air-breathing; the crest skin is isolated in a separate subcluster. In conclusion, the high vascularization, numerous mucous cells and the thinness of the epidermis of *A. kirkii* may be ones of the cutaneous adaptations that permit enhanced perfusion during aerial exposure. Accordingly, *A. kirkii* is more adapted to cutaneous respiration as a dual respiratory system.

Key words: Skin, air-breathing, fish, *Alticus kirkii*

INTRODUCTION

Amphibious fishes are those known to utilize both aquatic and terrestrial habitats and spend periods of time out of water, on or above the ground surface, as normal parts of their life histories (Gordon *et al*., 1969). This amphibious behavior is now recorded in at least 16 genera and 60 species of teleosts (Sayer, 2005) distributed in many marine and freshwater habitats including tropical, subtropical and temperate regions (Sayer and Davenport, 1991). The amphibious fishes may stay out of water for periods of a few minutes to a few days and desiccation and thermal imbalance are likely to be the two factors limiting the duration of this behavior (Sayer and Davenport, 1991). In typical natural situations, the physical, chemical and biological variables that change with the tide are numerous, so the number of potential cues that can affect the behavior of fish is also high (Faria and Almada, 2008). Typically, amphibious behavior is correlated with a decline in water quality or quantity and/or biotic factors (e.g., aggression, predation) (Sayer and Davenport, 1991; Martin, 1995; Taylor, 2000). Fishes display diverse adaptations for theses temporary existence on land including species reported to climb trees such as the mangrove killifish (*Rivulus marmoratus*) (Sayer and Davenport, 1991; Taylor *et al*., 2008). The terrestrial activities of amphibious fishes are closely related with feeding, courting and defending territories (Murdy, 1989). The fish are air-breathing when on land and respiration depends largely on gas transfer via the skin (Ikebe and Oishi, 1996; Graham, 1997; Park *et al*., 2006).

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The leaping blenny *Alticus kirkii* is one of the species of Blenniidae which are present in the intertidal zone of the Red Sea. In nature it spends almost their entire lives out of water but near the water's edge (Brown *et al.*, 1992). During the day it emerges from the water to feed upon the algae growing in the splash zone on the rocks (Rozemeijer and Plaut, 1993). Water contact exists only from waves splashing over the rocks. *A. kirkii* exhibits an amphibian lifestyle and meets the most extreme environmental conditions in concern. Many physiological, behavioural, morphological and biological studies were carries out on many amphibious fish species in addition to the structural modifications of the skin as potential air breathing organs (Whitear and Mittal, 1984; Martin and Lighton, 1989; Brown *et al.*, 1992; Rozemeijer and Plaut, 1993; Shephard, 1994; Ikebe and Oishi, 1996; Park and Kim, 1999; Taylor, 2000; Bhikajee and Green, 2002; Park *et al.*, 2003, 2004, 2006; Bhikajee *et al.*, 2006; Regan *et al.*, 2011; Syeda, 2011; Turko *et al.*, 2011). The mechanisms and functional morphology of air-breathing and respiration in intertidal fishes in freshwater and marine species have been discussed in previous reviews (Bridges, 1988; Taylor, 1990; Sayer and Davenport, 1991; Graham, 1997; Martin and Bridges, 1999; Taylor *et al.*, 2003; Sayer, 2005; Taylor *et al.*, 2008; Graham, 2011). The aforementioned authors investigated the amphibious fish species and other species that carry out aerial respiration while they remain immersed (i.e., surface gulpers).

There are no available literatures concerning the morphometrics, meristics or functional structure of air-breathing fish (*A. kirkii*) skin. Therefore, the present study aims to determine the important role of the skin in the life of this air-breathing fish in terms of the morphometric and meristic characteristics of epidermal thickness, vascularization, mucous cell distribution and the diffusion distances along ten body regions of *A. kirkii*. In addition, the respiration effectiveness of each body region was assessed in terms of the above four parameters.

MATERIALS AND METHODS

Specimen collection: Four specimens of *Alticus kirkii* (79-85 mm total length) were collected by using small nets from Jeddah coast at the Red Sea (Saudi Arabia). Specimens were transferred to the laboratory for dissection and measurements.

Histological preparation and examination: For histological preparation, the whole fish were fixed in 10% neutral buffered formaldehyde. Cross sections were taken in the head at the level of the crest, in the trunk region through the dorsal fin and in pectoral, pelvic and caudal fins. For histological skin examination, ten regions of the body were studied (Fig. 1, 2): 1- the Crest (CR), 2- the Dorso-lateral Side of the Head (DLH), 3- Operculum (OP), 4- the Ventral Side of the Head (VH), 5- the Dorsal-lateral Region of the Trunk (LT), 6- the Ventral Region of the Trunk (VT), 7- the Dorsal Fin (DF), 8- the Pectoral Fin (PF), 9- the Caudal Fin (CF) and 10- the Pelvic Fin (PV). The sections were dehydrated through a standard ethanol series to 100%, cleared in xylene and then embedded in wax (Paraplast, Oxford, UK). Sections of 5 μm were deparaffinized and stained with hematoxylin and eosin (Park *et al.*, 2004, 2006). Some sections were stained with Periodic Acid Schiff (PAS) to reveal mucous cells with its secretion (mucopolysaccharides) (Berra and Humphrey, 2002). Morphometric measurements of Diffusion Distance (DD) (the distance between the capillary endothelium of the epidermis and the outer edge of the epidermis) and Thickness of the Epidermis (EPT) were measured in μm. In addition, the number of blood capillaries (Capillary density, CN) and Mucous Cell Density (MC) are estimated per 25 μm length of the epidermis. The counts and measurements was carried out on a research digital microscope (motic China group Co. Ltd.) supplied by motic image software (MIS, 2004).

Statistical analysis: The basic statistics, means, standard deviation and ranges of the measured parameters were estimated. The patterns of variation in the measured parameters were evaluated by one way analysis of variance using SPSS package (SPSS, 1998) at the 0.05 significance level. Levene's test of equality of error variance of the dependent variables was applied and homogeneity of variance was evident. The Tukey-HSD test was considered for multiple comparisons. The relationships between skin parameters were revealed by cluster analysis through skin regions and vice versa using statistical package release 8 (StatSoft, 2007).

RESULTS

Basic structure of skin: The integumentary system of *A. kirkii* is basically composed of two layers, an outer epidermis and an underlying dermis (Fig. 1, 2). The epidermis exhibited a stratified epithelium on a basement membrane. Such principle structure is seen in all 10 body regions studied with variability in the epidermal thickness that range from 1.08 to 3.94 μm. Two to five layers of polygonal epithelial cells were recorded in the epidermis of different body regions. Other components within the epidermis are variably found including unicellular mucous

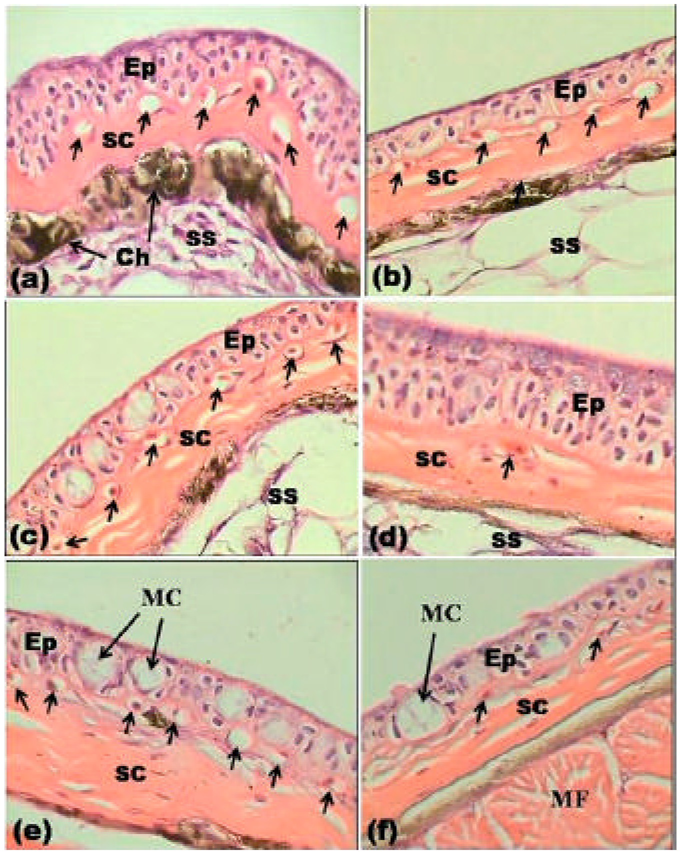

Fig. 1(a-f): Transverse sections of the skin of different 6 body regions of *Alticus kirkii* stained with haematoxylin and eosin (X = 1000), in general the skin consisting of the superficial Epidermis (Ep) and lower dermis which consisting of an outer Stratum Compactum (SC) of collagenous fibers and inner Stratum Spongiosum (SS). (a) crest, (b) lateral side of the head, (c) operculum, (d) ventral side of the head, (e) dorsal-lateral region of the trunk and (f) ventral region of the trunk. Note blood capillaries (small arrows), Mucous cell (MC), Chromatophores (Ch), Muscle Fibers (MF)

glands (Fig. 1-3) and club cells (Alarm cells) (Fig. 3d). The dermis is distinguished into two sub-layers, a stratum compactum consisting of collagenous connective tissue and stratum spongiosum, a loose network of collagen and reticulum fibers. Also pigment cells (melanophores) are often found at the dermal-epidermal boundary (Fig. 1a). The variability in skin of different body regions is reflected by epidermal thickness, diffusion distance, mucous cell number and blood capillary density. This variability refers to the contribution of each region in air-breathing.

Thickness of the epidermis (EPT): The Thickness of the Epidermis (EPT) depends mainly on the size and the number of layers of the polygonal cells that constitute epidermis in different body regions (Fig. 1, 2). EPT of the ten body regions exhibited significant variability (Table 1) ($p<0.05$). Such variability was due to Ventral Head (VH) and Pelvic Fin (PV) regions which have the highest thickness (2.67 and 3.94 μm, respectively). On the other hand, the rest of regions were insignificantly variable ($p>0.05$) with lesser thickness (Table 1) especially the Operculum (OP), Dorso-lateral Head (DLH) and Caudal Fin (CF) regions which are the thinnest (1.31, 1.12, 1.08 μm, respectively).

Number of blood capillaries (CN): A large number of fine blood capillaries in the form of circles containing some erythrocytes, was recorded just below the basement membrane accompanied by dermal collagen (Fig. 1, 2). The number of blood capillaries was generally higher in all body regions except the ventral head (1.63 blood vessels

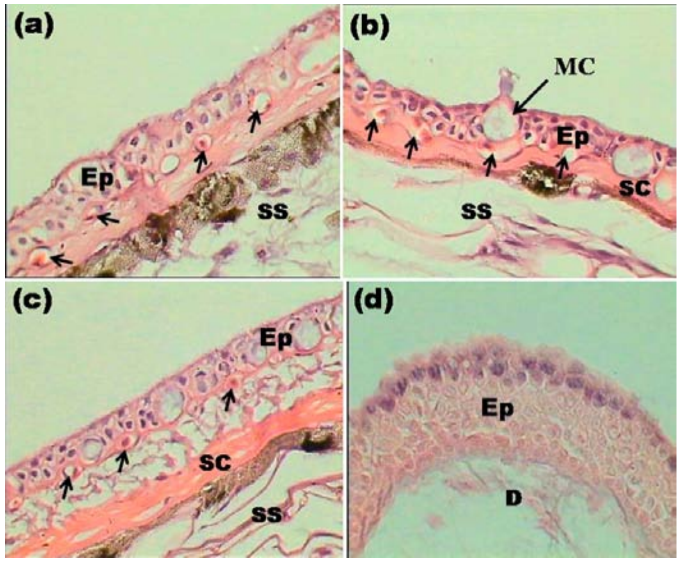

Fig. 2(a-d): Transverse sections of the skin of fins of *Alticus kirkii* stained with haematoxylin and eosin (X = 1000). (a) dorsal fin, (b) pectoral fin, (c) caudal fin and (d) pelvic fin. The Superficial Epidermis (Ep), Stratum Compactum (SC), Stratum Spongiosum (SS) consisting of collagenous fibers, blood capillaries (small arrows), mucous cell expels Mucous (MC), Chromatophores (Ch), Muscle Fibers (MF), Dermis (D)

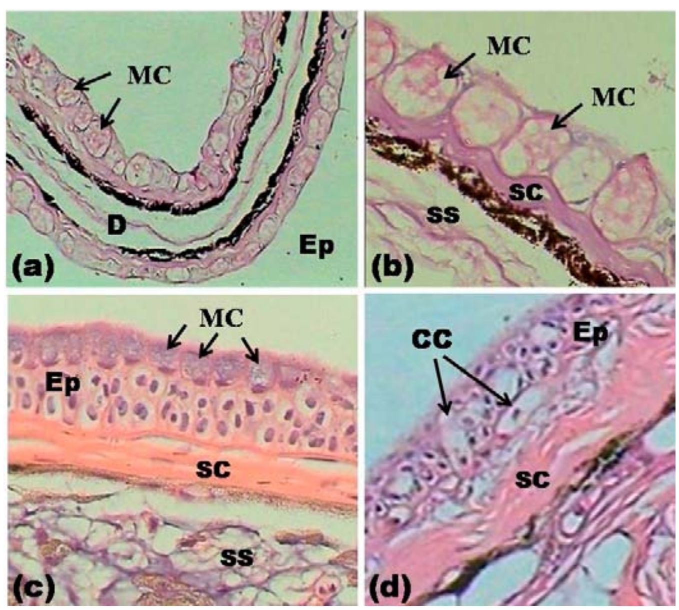

Fig. 3(a-d): Transverse sections of *Alticus kirkii* skin showing epidermal mucous cells in dorsal fin (a, X = 400) and (b, X = 1000), these sections were stained with periodic acid Schiff's reagent (PAS); in ventral head region (c) stained with haematoxylin and eosin (X = 1000) and club cells (d) in lateral region of trunk (X = 1000). MC, mucous cell; CC, club cells; Ep, epidermis; SC, stratum compactum; SS, stratum spongiosum; D, dermis

Table 1: Epidermis thickness in 10 epidermal regions (in µm) of *Alticus kirkii*

Location	N	Mean	SD	Range
1- CR	8	1.54^{a}	0.38	0.94-2.19
2- DLH	43	1.12^{a}	0.52	0.66-2.78
3- OP	16	1.31^{a}	1.02	0.51-2.94
4- VH	19	2.67^{b}	0.68	1.5-4.05
5- LT	20	1.79^{a}	0.54	0.65-2.39
6- VT	9	1.50^{a}	0.23	1.23-1.99
7- DF	8	1.62^{a}	0.48	1.13-2.41
8- PF	12	1.61^{a}	0.57	0.98-2.72
9- CF	11	1.08^{a}	0.14	0.87-1.26
10- PV	9	3.94^{c}	0.43	3.16-4.48

Means followed by different letters are significant different at p<0.05

Table 2: Number of blood capillaries per 25 µm length at 10 epidermal regions of *Alticus kirkii*

Location	N	Mean	SD	Range
1- CR	8	8.13^{a}	2.47	5-13
2- DLH	43	7.58^{a}	1.72	4-12
3- OP	16	9.44^{ab}	2.39	6-15
4- VH	19	1.63^{c}	1.83	0-5
5- LT	20	8.15^{a}	2.23	4-12
6- VT	9	8.00^{a}	1.94	5-12
7- DF	8	8.50^{ab}	2.88	5-13
8- PF	12	7.83^{a}	1.53	5-10
9- CF	11	10.55^{b}	2.46	5-14
10- PV	9	0.00^{c}	0.00	0.00-0.00

Means followed by different letters are significant different at p<0.05

per 25 µm length) and pelvic fin regions (Table 2). In the latter region the blood capillaries are absent from the skin at least in the epidermis and the upper part of the dermis. However, the body regions with higher values of blood capillaries were significantly different (p>0.05). The highest values were recorded in the operculum and caudal fin regions (9.44 and 10.55 blood vessels per 25 µm length respectively).

Diffusion distance (DD): The diffusion distance (µm) between the capillary endothelial cells and the outer edge of the epidermis is variable, ranging from the average of 0.89 to 1.67 µm (Table 3). Apart of the blood capillary free pelvic fin region, there are no significant differences between the average values of the diffusion distance of the rest of body regions (p>0.05). However, the dorso-lateral head, operculum and caudal fin regions show lower values (0.89, 1.11 and 1.00 µm, respectively) but the lateral trunk region shows rather higher value (1.67 µm).

Number of mucous cells (MC): Except for the pelvic fin region, the mucous cells were recorded in the epidermis of all regions studied (Table 4). There are significant differences in the number of the mucous cells in the epidermis of these regions (p<0.05). The lowest numbers are recorded in the crest epidermis (1.0 mucous cell per 25 µm length) whereas the highest value was recorded in the epidermis of the lateral trunk region (5.55 mucous cells per 25 µm length).

The epidermal thickness in the different body regions was inversely associated (p<0.05) with CN, DD and MC

Table 3: Diffusion distance in 10 epidermal regions of *Alticus kirkii*

Location	N	Mean	SD	Range
1- CR	8	1.53^{a}	0.54	0.84-2.26
2- DLH	43	0.89^{a}	0.60	0.19-2.70
3- OP	16	1.11^{a}	1.04	0.28-2.67
4- VH	19	1.52^{a}	1.51	0.00-3.51
5- LT	20	1.67^{a}	0.65	0.22-2.29
6- VT	9	1.38^{a}	0.34	0.96-2.04
7- DF	8	1.23^{a}	0.55	0.62-2.04
8- PF	12	1.45^{a}	0.43	0.92-2.17
9- CF	11	1.00^{a}	0.14	0.79-1.23
10- PV	9	0.00^{b}	0.00	0.00-0.00

Means followed by different letters are significant different at p<0.05

Table 4: Number of mucous cells per 25 µm length at 10 epidermal regions of *Alticus kirkii*

Location	N	Mean	SD	Range
1- CR	8	1.00^{ab}	1.41	0-3
2- DLH	43	2.84^{abc}	2.16	0-8
3- OP	16	4.00^{bc}	3.85	0-11
4- VH	19	2.68^{abc}	3.30	0-10
5- LT	20	5.55^{c}	3.44	0-10
6- VT	9	3.22^{abc}	2.28	0-6
7- DF	8	4.00^{bc}	1.85	2-7
8- PF	12	4.92^{c}	3.34	0-12
9- CF	11	4.45^{bc}	2.66	0-8
10- PV	9	0.00^{a}	0.00	0.00-0.00

Means followed by different letters are significant different at p<0.05

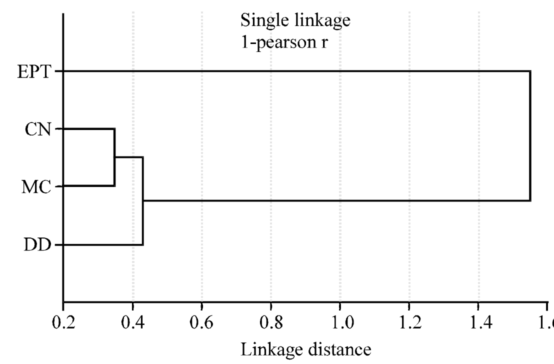

Fig. 4: The relationship between the Epidermal Thickness (EPT), Diffusion Distance (DD), blood Capillary Number (CN) and Mucous Cell Number (MC) through different skin of different body regions of *Alticus kirkii* based on cluster analysis

(R = -0.94, -0.55 and -0.62, respectively) and is isolated into a single cluster (Fig. 4). CN significantly correlates with MC (R = 0.65) (p<0.05) and insignificantly with DD (0.47) (p<0.05). These three parameters are classified into one cluster with subcluster of CN and MC (Fig. 4). Based on EPT, CN, MC and DD, the ten skin regions are classified into two main clusters, the first one includes the ventral head (VH) and pelvic fin (PV) regions and the other cluster includes the rest of skin regions which are more adapted to air-breathing; the crest skin is isolated in a separate subluster (Fig. 5).

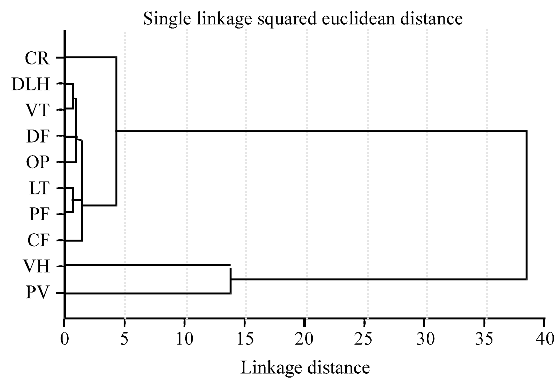

Fig. 5: Clustering of different skin body regions of *Alticus kirkii* (see text for abbreviations) on the basis of the epidermal thickness, diffusion distance, blood capillary number and mucous cell number

DISCUSSION

Within the context of vertebrate evolution, amphibious fishes represent a transitional stage between the totally aquatic habitat and terrestrial colonization (Sayer, 2005). It is believed that the modern amphibious fauna have resulted from numerous terrestrial invasions (Chotkowski *et al*., 1999; Long and Gordon, 2004). The fish species of these fauna offer an idea about the biological adaptations that may have lead to the colonization of land that took place in the early evolution of terrestrial vertebrates (Horn *et al*. 1999). Graham (2011) stated that the long-term view on air-breathing fish emphasizes its importance in the evolutionary transition to vertebrate terrestriality; and the fishes which were the first air-breathing vertebrates played a key role in this process. Accordingly, many studies on amphibious fishes were carried out (Whitear and Mittal, 1984; Martin and Lighton, 1989; Brown *et al*., 1992; Yokoya and Tamura, 1992; Rozemeijer and Plaut, 1993; Ikebe and Oishi, 1996; Luck and Martin, 1999; Park, 2002; Bhikajee and Green, 2002; Sayer, 2005; Park *et al*., 2003, 2004, 2006; Bhikajee *et al*.; 2006; Mazlan *et al*., 2006; Regan *et al*., 2011; Turko *et al*., 2011) especially in concern with skin which is a vascularized tool of cutaneous gas exchanges (Brown *et al*., 1992) and a barrier between the internal and external environment.

Air-breathing *A. kirkii* utilize both aquatic and terrestrial habitats. How does this species face its gills collapse, reducing the area of the respiratory surface and become dry, effectively stopping the diffusion of oxygen into the blood? Generally teleost fish have oxygen-sensitive neuroepithelial cells in the gills that appear to mediate physiological responses to hypoxia but little is known about oxygen sensing in amphibious fish (Regan *et al*., 2011). But, the mangrove rivulus, *Kryptolebias marmoratus*, is an amphibious fish that respires via the gills and/or the skin (Regan *et al*., 2011); their results suggested that oxygen sensing occurs at the branchial and/or cutaneous surfaces and that serotonin and acetylcholine mediate, in part, the emersion response. Moving between media differing in their physicochemical properties especially in the case of extreme environment presents a series of respiratory challenges to bimodal breathing species (Sayer, 2005). Rozemeijer and Plaut (1993) reported that, to survive in such an extreme environment, *A. kirkii* has anatomical, physiological and behavioural adaptations. The body-shape is elongated so that the pressure of the body weight on the skin is reduced, the skin is highly vascularized to improve gas exchange and temperature regulation and the fins are provided with hooks to enhance its grip on the rocks (Zander, 1972). Also, Rozemeijer and Plaut (1993) mentioned that *A. kirkii* is able to withstand desiccation up to 23% of its initial body weight and the periods of immersion and emersion appeared to be regulated by three abiotic factors: relative humidity of the air, rock temperature and the number of waves splashing over the rock where they are situated. Brown *et al*. (1992) recorded that oxygen uptake rates by *A. kirkii* in air were less than half those of fish submerged in seawater over the temperature range 16-24°C. He mentioned that reasons for this difference are unknown but gill and cutaneous gas exchanges probably occur in both environments. In spite of these findings, literature is rarely available on morphometric or merestic data of *A. kirkii.* In the present investigation, the principle structure of skin is evident on different body regions with significant variability in epidermal thickness, diffusion distance, blood capillary number and mucous cell number. Such variability reflects the variability of their role in air-breathing respiration; the skin on the ventral side which is easily immersed in water or soft mud during amphibious life has no role in this concern.

The thickness of the epidermis depends mainly on the size and the number of layers of the cells that constitute the epidermis in different body regions. In the present work, epidermal thickness of the ten body regions of *A. kirkii* exhibited significant variability. The ventral head and pelvic fin regions have the highest thickness (2.67 and 3.94 μm, respectively). On the other hand, the rest of regions were insignificantly variable ($p>0.05$) with lesser thickness especially the operculum, dorso-lateral head and caudal fin regions which are the thinnest (1.31, 1.12 and 1.08 μm, respectively). Such thickness in skin of mudskipper goby *Periophthalmus modestus* was the highest thickness in the pectoral fin (average, 98.6 μm) and the thinnest in 1st and 2nd dorsal fins, averaging 44.4 and 45.3 μm, respectively (Park *et al*., 2004). But in the abdomen of *Periophthalmus magnuspinnature* was ranging from 61.8 to 164.9 μm; mean 107.2 μm (Park *et al*., 2006).

Results of the present investigation revealed that diffusion distance between the closest capillary endothelial cells and the surface of the skin of *A. kirkii* ranged from mean 0.89 to 1.67 μm. Such distance in skin of mudskipper goby *Periophthalmus modestus* ranged from 3.6 to 10.9 μm (Park *et al*., 2004) and in *Periophthalmus magnuspinnature* from 2.6 to 15.4 μm (Park *et al*., 2006).

Desiccation is the main stressor for *A. kirkii* and other amphibious ones (Luck and Martin, 1999; Sayer, 2005; Mazlan *et al*., 2006). The lack of water as a surrounding medium present many physical, physiological and sensory challenges to the fishes, therefore, it must either regulate fluid and thermal balance, effective gaseous exchange and nitrogenous excretion, or be able to tolerate the consequences of non-regulation (Sayer, 2005). The present study revealed a great presence of mucous glands in the epidermis of *A. kirkii* that may offer a huge amount of mucus over the body to maintain the aqueous content of the body and give resistance and protection against the desiccation, physical abrasions and other biotic and abiotic factors. Proposed roles of fish mucus are involved in many biological activities, such as respiration, defense against pathogenic microorganisms, parasites and pollutants, swimming, ionic and osmotic regulation, reproduction, excretion, disease resistance, communication, feeding and nest building (Shephard, 1994; Calabro *et al*., 2005; Sayer, 2005; Mazlan *et al*., 2006). In addition to the factors discussed above, direct solar radiation will also control survival out of water. Whitear and Mittal (1984) explored the potential benefits of hygroscopic mucus production in amphibious fish and the possibilities for volumetric augmentation of mucous secretions in humid conditions.

In the present study, club cells (alarm cells) were detected in the epidermis of *A. kirkii*, they do not open to the surface and require damage to release their contents. Thousands of fish species possess specialized club cells (alarm cells) in their epidermis that release fright substances upon damage and hence reliably indicate the presence of an actively foraging predator (Chivers and Smith, 1998; Brown, 2003). For a long time, it was thought that, the club cells are restricted mainly on the alarm function. But, the evolution of the alarm cells of fishes has intrigued evolutionary ecologists because the release of these alarm chemicals is not voluntary and occurs only when the fish is injured. Recent studies have suggested that the club cells are part of the immune system of the fish; the first evidence that alarm substance cells have an immune function against pathogens, parasites, general injury to the epidermis and the environmental challenges was recorded by Chivers *et al*. (2007) and emphasized by Halbgewachs *et al*. (2009). These authors revealed that the club cells of ostariophysan fishes are part of the innate immune system and that the alarm function of the cells that evoke antipredator responses in nearby shoal mates evolved secondarily. Chivers *et al*. (2007) stated that predation pressure does not influence alarm cell production in fishes but alarm cell production is stimulated by exposure to skin-penetrating pathogens, skin-penetrating parasites and correlated with exposure to UV radiation; also, suppression of the immune system with environmentally relevant levels of Cd inhibits alarm cell production of fishes.

According to the aforementioned findings, one can note that, in regional epidermises of *A. kirkii*, all of the body epidermises can absorb air through their epidermis which is thin, rich with blood capillaries and mucous glands except for the pelvic fin that is thick and empty from blood capillaries. Also, one can conclude that the increase presence of highly vascularization, numerous mucous cells and the thinness of the epidermis of *A. kirkii* may be ones of cutaneous adaptations that permit enhanced perfusion during aerial exposure. Considering the structural features of the skin, it may be considered that *A. kirkiii* is more likely to be related to cutaneous respiration as a dual respiratory system.

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